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Epidemiology of Brazilian spotted fever in the Atlantic Forest, state of São Paulo, Brazil

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SUMMARY

The tick-borne bacterium *Rickettsia rickettsii* is the aetiological agent of Brazilian spotted fever (BSF). The present study evaluated tick infestations on wild and domestic animals, and the rickettsial infection in these animals and their ticks in 7 forest areas adjacent to human communities in the São Paulo Metropolitan Area (SPMA). The results were compared to ecological traits of each sampled area. Two main tick species, *Amblyomma aureolatum* and *Rhipicephalus sanguineus*, were collected from dogs. The major ticks found on small mammals and birds were *Ixodes loricatus* and *Amblyomma longirostre*, respectively. Both anti-*R. rickettsii* antibodies and *R. rickettsii*-infected ticks were detected on dogs from only 2 areas in the southern part of the SPMA, which were considered to be endemic for BSF; the remaining 5 areas were considered to be non-endemic. Ecologically, the BSF-endemic areas clearly differed from the non-endemic areas by the presence of significantly more degraded forest patches in the former. The present results corroborate historical observations that have indicated that all human cases of BSF in the SPMA were contracted in the southern part of this metropolitan area. However, not all forest patches in the southern part of the SPMA were shown to be associated with BSF endemism.

Key words: *Rickettsia rickettsii*, ticks, *Amblyomma aureolatum*, spotted fever, Atlantic Forest.

INTRODUCTION

Rickettsia rickettsii is a tick-borne intracellular bacterium that causes a febrile illness known as Rocky Mountain spotted fever (RMSF), also known in Brazil as Brazilian Spotted Fever (BSF). The distribution of *R. rickettsii* is restricted to the Americas; confirmed cases of RMSF have been reported in Canada, United States, Mexico, Costa Rica, Panama, Colombia, Brazil and Argentina (Labruna, 2009).

In the State of São Paulo, southeastern Brazil, 2 tick species have been implicated in the transmission of *R. rickettsii* to humans: *Amblyomma cajennense* (Fabricius, 1787) in the central part of the State, and *Amblyomma aureolatum* (Pallas, 1772) in the eastern part, where the humid Atlantic rainforest prevails (Pinter and Labruna, 2006). According to data in the literature, the tick *A. aureolatum* is restricted to the eastern area of South America, from Uruguay to Surinam, including northeastern Argentina, eastern Paraguay, southeastern to southern Brazil, and French Guiana (Guglielmone *et al*. 2003*b*). This tick species is typical of the Atlantic rainforest region, where optimal conditions of high humidity and cool temperatures are provided throughout the year (Pinter *et al*. 2004). In natural, undisturbed forest areas, *A. aureolatum* adult ticks feed mainly on wild carnivore species, such as foxes, *Cerdocyon thous* (Linnaeus, 1766) and *Lycalopex* spp., and raccoons, *Procyon cancrivorus* (Cuvier, 1798) (Guglielmone *et al*. 2003*b*). However, in the rural areas close to rainforest remnants, adult ticks feed mainly on domestic dogs, which play an important role in carrying *A. aureolatum* adult ticks from inside the forest into human settlements. In this case, active ticks can be accidently transferred from dogs to humans (Guglielmone *et al*. 2003*b*, Pinter *et al*. 2004). Only few host records have been reported for the immature stages (larvae and nymphs) of *A. aureolatum*. These records were mostly on passerine birds and a few rodent species (Guglielmone *et al*. 2003*b*).

A recent study demonstrated that *A. aureolatum* is highly susceptible to *R. rickettsii* infection and is highly efficient in maintaining the infection through 100% transstadial perpetuation, transovarial transmission, and filial infection rates under laboratory conditions (Labruna *et al*. 2008, 2011). However, infection rates of *A. aureolatum* by *R. rickettsii* under natural conditions within BSF-endemic areas have

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been reported to be lower than 1% (Pinter and Larbuna, 2006), probably due to the deleterious effects caused by *R. rickettsii* in ticks (Niebylski *et al*. 1999; Labruna *et al*. 2011). Thus, investigations on natural tick populations in order to determine whether a specific area is endemic for BSF could lead to false negative results, since finding a natural infected tick would require examination of a large number of ticks (Sangioni *et al*. 2005; Pinter and Labruna, 2006). In this regard, a feasible way to classify endemic areas for BSF relies on serosurveys for detecting *R. rickettsii*-seropositive sentinel hosts, which are domestic dogs in areas where BSF is transmitted by *A. aureolatum* (Pinter *et al*. 2008; Moraes-Filho *et al*. 2009).

The São Paulo metropolitan area (SPMA) consists of 39 municipalities within 8051 km^2, including the state capital, São Paulo city. A total population of approximately 20 million inhabitants lives in the SPMA, mostly on 2139 km^2 consisting of urban areas. The remaining areas are essentially composed by forest remnants of the original Atlantic rainforest, which provide optimal abiotic conditions for *A. aureolatum*. Although established populations of *A. aureolatum* are widely distributed among the SPMA, historically the BSF cases have been restricted to the southern part of this metropolitan area, encompassing the municipalities of São Paulo, Diadema, Mogi das Cruzes, Santo André, São Bernardo do Campo, Mauá, and Ribeirão Pires (Gomes, 1933; Fonseca, 1935; Pinter and Labruna, 2006). Among these southern municipalities, a total of 70 cases of BSF have been confirmed during the last 14 years, with an overall case fatality of ~50%. All these cases were supposedly transmitted by *A. aureolatum* (Katz *et al*. 2009). Although the overall conditions in the northern part of the SPMA seem to be similar to the southern part (communities close to Atlantic forest remnants, presence of *A. aureolatum*-infested dogs), there has been no evidence of BSF cases in the northern part.

Based on the above statements, the present study aimed to evaluate tick infestations on wild birds, small mammals and dogs, and to evaluate rickettsial infection in dogs, small mammals and ticks from 7 forest areas adjacent to human communities in the SPMA, being 3 areas in the southern part, and 4 areas in the northern part. The results were compared to ecological traits of each sampled area.

MATERIALS AND METHODS

Study areas

This study encompassed 7 localities (Fig. 1, Table 1): 3 small forest areas in the southern part of the SPMA, within the municipalities of Diadema, São Bernardo do Campo, and Santo André, in which BSF cases have occurred during the last years; 3 small forest areas in the northern part of the SPMA, where BSF cases have never been confirmed (municipalities of Arujá, Nazaré Paulista, and Mairiporã); and 1 large forest area (Cantareira State Park) in the northern part of the SPMA, within the São Paulo municipality, considered to be a control area because this Park is a large, preserved continuous forest where recent serological analyses of sentinel animals (dogs, opossums, and capybaras) revealed the absence of *R. rickettsii* circulation in the Park; i.e., all sampled animals were seronegative (M.B.L., *unpublished data*). In all 7 localities, wild birds and small mammals were sampled inside the forest fragment, whereas domestic dogs dwelling in the human communities surrounding the forest were sampled. Sampled areas are located between 765 and 1000 metres above sea level, and have a subtropical climate. Summer is warm and rainy, while winter is mild. The average annual temperature is around 18 °C. The coldest month is July (average 14 °C) and the warmest is February (average 22 °C). The annual pluviometric index is around 1400 mm.

Quantitative analysis of the landscape

In each of the 7 areas, the fragment where wild animals were captured was considered the main patch, while all surrounding forest fragments immediately beside and within a 1000 m radius from the centre of the main fragment were considered secondary patches. Two independent measures were selected for the quantitative landscape analysis: patch size (AREA) and the nearest neighbour distance (NND) from the main to secondary patches. The NND was measured based on the shortest Euclidean edge-to-edge distance between fragments, as described elsewhere (Uezu *et al*. 2005). The landscape database was accessed from System of Forest Information of the State of Sao Paulo (SIFESP). The metrics were calculated in BaseCamp 3·2 (Garmin®).

Capture evenness

In total, 4 visits for collecting samples, at about 3-month intervals, were performed in each of the 7 study areas: (1) Autumn (April 2010–May 2010); (2) Winter (July 2010–August 2010); (3) Spring (September 2010–October 2010); (4) Summer (November 2010–January 2011). In each of the 4 visits in the 7 areas, domestic dogs, wild birds, and small mammals were sampled, as described below.

Domestic dogs

In order to compose cohorts, 30 dogs were selected to be sampled in each locality, based on the following criteria, as suggested elsewhere (Pinter *et al*. 2008):

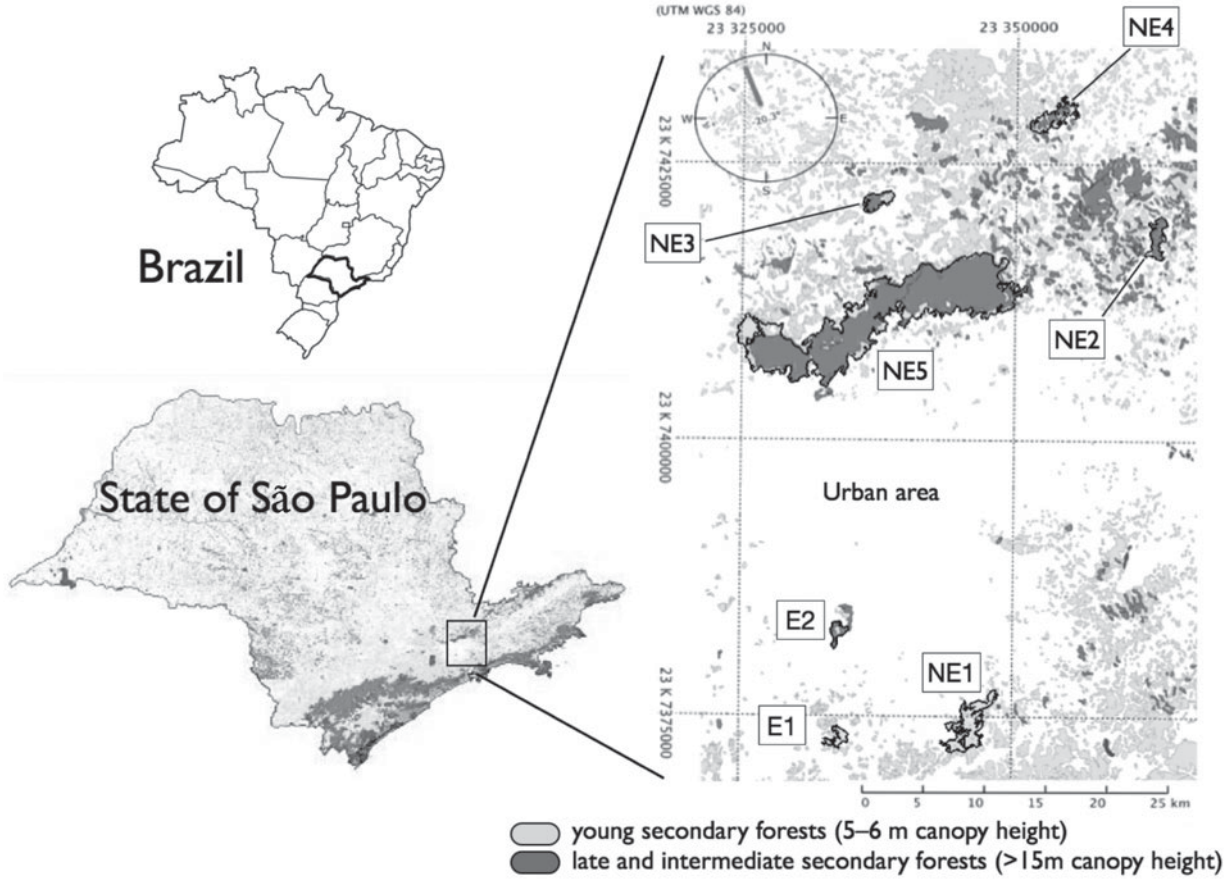

Fig. 1. Location of the study region in the State of Sao Paulo, Brazil. The highlighted rectangle represents the São Paulo metropolitan area, where 7 forest patches (E1, E2, NE1, NE2, NE3, NE4 and NE5) were sampled in the present study.

dogs were born and raised in the area, were at least 1 year old, and without any overt clinical alteration, particularly in vision, proprioception, locomotor and nervous systems; all dogs were reared unrestrained with plenty of access to adjacent forest patches. During each of the 4 visits in each area, the same dogs were checked for the presence of ticks which, when present, were collected and taken to the laboratory. When a dog of a given cohort died or moved away from the area, a new animal was selected based on the same above criteria, in order to maintain the sample size.

Wild birds

Birds were caught using 10 mist nets (12 m long × 2 m wide, 36 mm mesh) displayed along animal trails (2 work-days per area) inside the forest. In each area, mist nets were left open from 6:00 am to 6:00 pm on the first day, and from 6:00 am and 12:00 pm on the second day, resulting in 5040 net-hours for the whole study. Mist nets were checked every 40 min; captured birds were identified to species following Sigrist (2007), examined for the presence of ticks by checking carefully their whole body, banded, and released at the capture site.

Bird categorization

All the accessed birds were categorized into3 groups according to their sensitivity (SI) to environmental degradation inherent to each bird species, as described by Parker *et al*. (1996). Group categories were low, medium and high sensitivity.

Wild mammals

Forest mammals were trapped by using 50 'live-trap' Shermann cages (12 × 14 × 45 cm) during 5 consecutive nights in each visit, resulting in 1400 trap/nights for the whole study. Traps were baited with a mixture of banana, oil from canned sardines, oat flour, peanut butter, and maize, and displayed in the proximity of the trail where birds were caught. Small mammals were identified to species according to Bonvicino *et al*. (2008), Reis *et al*. (2006) and Wilson and Reeder (2005), examined for the presence of ticks, submitted to blood collection (as described below), banded, and released at the capture site.

Tick collection and identification

Adult ticks were identified according to Barros-Battesti *et al*. (2006), whereas nymphal identification followed Marques *et al*. (2004), and Martins

Table 1. Main characteristics of the seven forest areas sampled within the Sao Paulo metropolitan area, Brazil

Code	Municipality	Coordinates		Altitude (m)	Main forest fragment size (ha)	Total secondary forest fragment size (ha)	Mean nearest neighbour distance (m)	Major landscape†	Distance (km) from NE5 (control area)	Status for BSF*
		Latitude (S)	Longitude (W)							
E1	S. B. do Campo	23°44′22″	46°37′29″	788	190	237·5	131·4	Young secondary forests	34	Endemic
E2	Diadema	23°39′18″	46°37′27″	801	350	17	296	Young to late secondary forests	24	Endemic
NE1	Santo André	23°44′46″	46°31′04″	820	1010	329	140	Young secondary forest	37	Non-endemic
NE2	Arujá	23°20′44″	46°20′27″	765	620	2190	24·5	Late secondary forest	32	Non-endemic
NE3	Mairiporã	23°18′41″	46°35′27″	836	550	770	156·7	Young to late secondary forests	15	Non-endemic
NE4	Nazaré Paulista	23°14′52″	46°26′50″	833	480	1780	43·4	Young to late secondary forests	28	Non-endemic
NE5	São Paulo	23°26′15″	46°38′15″	1000	10 100	–	–	Mostly late secondary forest	–	Non-endemic

* The status for Brazilian spotted fever (BSF) was determined in the present study through serological analysis of domestic dogs (see 1st paragraph of Results section in the text).
† See Fig. 1

et al. (2010). Fully engorged larvae of the genus *Amblyomma* were placed in plastic vials containing several grass leaves, and covered by a cork containing several minute holes to keep ticks alive until arriving at the laboratory, where the engorged larvae were placed in an incubator at 25 °C and RH 95% for moulting to nymphs. Either non-engorged or partially engorged larvae were removed with forceps and immediately preserved in absolute isopropanol. For the identification of larvae we used molecular tools as described elsewhere (Ogrzewalska *et al*. 2009). Briefly, each immature specimen was submitted to DNA extraction and PCR using primers that amplify an approximately 460-bp fragment of the tick mitochondrial 16S rRNA gene (Mangold *et al*. 1998). Amplified products were purified and DNA sequenced as previously described (Labruna *et al*. 2004*a*), and compared with NCBI Nucleotide BLAST searches (Altschul *et al*. 1990).

Prevalence of ticks on animals, mean intensity, and mean abundance of the tick infestations on each animal species were calculated following Bush *et al*. (1997). For each sampled site, the following parameters were calculated: bird richness, bird Shannon diversity index, and small mammal richness according to Krebs (1989).

Blood serum samples

In total, 30 canine blood samples were collected from each study site during the first visit (autumn). Sera were separated and stored at −20 °C until tested for serology. A second blood collection was performed during the fourth visit (summer); however, not all 30 dogs per study site were available for blood collection during the fourth visit. All dog sera were tested by the immunofluorescence assay (IFA) following previously described protocols (Horta *et al*. 2004) using Vero cells infected with *R. rickettsii* strain Taiaçu as crude antigen (Pinter and Labruna, 2006). Titres $\geqslant 64$ were considered positive. Positive sera were titrated to the endpoint titres by dilution in 2-fold increments.

Blood samples were taken from the mandibular vein of small rodents and from the tail vein of marsupials. Small mammal sera were tested by IFA against crude antigens of the following *Rickettsia* species known to occur in Brazil: *R. rickettsii* strain Taiaçu, *Rickettsia parkeri* strain At24 (Silveira *et al*. 2007), *Rickettsia felis* strain Pedreira (Horta *et al*. 2006), *Rickettsia bellii* strain Mogi (Pinter and Labruna, 2006), *Rickettsia amblyommii* strain Ac37 (=AcaIII) (Labruna *et al*. 2004*b*), and *Rickettsia rhipicephali* strain HJ#5 (Labruna *et al*. 2007*a*), as described above.

PCR amplification

All tick samples were individually processed by a real-time PCR assay with primers CS-5 and CS-6,

designed to amplify a 147-bp fragment of the citrate synthase gene (*gltA*) of *Rickettsia* spp. (Labruna *et al*. 2004*a*). Once a tick was demonstrated by real-time PCR to contain rickettsial DNA, amplification of a larger fragment of the *gltA* gene was attempted by routine PCR using primers CS-78 and CS-323, which target a 401-bp fragment of the *gltA* gene (Labruna *et al*. 2004*a*), and primers Rr190.70F and Rr190.602R targeting a 532-bp fragment of the *ompA* gene, present only in *Rickettsia* species belonging to the spotted fever group (SFG) (Regnery *et al*. 1991). Routine PCR products were sequenced and submitted to BLAST analysis to determine similarities to other *Rickettsia* species (Altschul *et al*. 1990).

Data analysis

Size areas of the main and secondary forest patches were log (base 10) transformed to present normal distribution characteristics, and thereafter were run against classification of the area (endemic or non-endemic) through *t*-test (Zar, 1996). To test differences between NND against classification of the area (endemic or non-endemic), non-parametric analysis (Mann–Whitney test) was used. Data on mean abundance, and mean intensity of *A. aureolatum* ticks on dogs from each area were compared one to another through the variants of the Mann-Whitney U bootstrap rank Welch test (2000 repetitions) (Reiczigel *et al*. 2005). The Chi-square test was used to compare the prevalence of *A. aureolatum*-infested dogs between endemic or non-endemic areas, and also to compare the number of birds categorized in medium and high SI of all areas. Significant differences were considered for $P < 0{\cdot}05$. We used SOFA 1.1 (Paton-Simpson & Associates Ltd - open source software) for all analyses but the Bootstrap Test, which was processed by Quantitative Parasitology 3.0 (Reiczigel and Rózsa, 2005).

For the present study, we followed the protocol that agrees with Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA), which was approved by the Faculdade de Medicina Veterinária e Zootecnia/USP-Ethical Committee for Animal Research, and to the Brazilian Institute of Natural Resources (IBAMA). Permits and approvals are on file in the office of M.B.L.

RESULTS

Seroprevalence of Rickettsia *spp. in dogs*

Canine blood samples were collected twice (at the first visit between April and May 2010, and at the fourth visit between November 2010 and January 2011). Through IFA analysis, reactive antibodies against *R. rickettsii* (titre $\geqslant 64$) were detected in dogs from only 2 areas in the southern part of the SPMA: 6 (20·0%) out of 30 dogs from the first visit and 4 (25·0%) out of 16 dogs from the fourth visit in São Bernando do Campo; and 15 (50·0%) out of 30 dogs from the first visit and 12 (52·2%) out of 23 dogs from the fourth visit in Diadema (Table 2). Serum endpoint titres ranged from 64 to 2048 in São Bernando do Campo, and from 64 to 8192 in Diadema. The results from all other areas were negative (Table 2). Based on these serological results, we considered that only 2 areas, namely São Bernando do Campo and Diadema, had the status of endemic for BSF, whereas the remaining 5 areas had the non-endemic status. Through the following sections of this manuscript, the 2 BSF-endemic areas were coded as E1 and E2, whereas the non-endemic areas were coded as NE1 to NE5, as described in Tables 1 and 2.

Ticks from dogs

In total, 958 canine examinations were made throughout the study. Two main tick species were observed parasitizing dogs: *A. aureolatum* and *Rhipicephalus sanguineus* (Latreille, 1806). In total, 706 *A. aureolatum* adult ticks were collected from dogs, giving an overall prevalence of 19·3%, mean intensity of 3·8, and mean abundance of 0·7. A total of 450 *R. sanguineus* adult ticks were collected; however, many dogs were also infested by hundreds of larvae and nymphs that were not counted. The overall prevalence of *R. sanguineus* adults was 10·6%, the mean intensity was 19·3, and the mean abundance was 2·0. Overall, prevalence, mean abundance, and mean intensity of ticks infesting dogs tended to be higher in non-endemic areas than in endemic areas, with some statistically significant differences (Table 2). Additionally, 1 female *Haemaphysalis juxtakochi* Cooley, 1946 was found on 1 dog in the area NE3, and 2 dogs from NE4 were heavily infested by *A. cajennense* nymphs (> 500 individuals).

Rickettsial infection in ticks from dogs

Among 526 *A. aureolatum* and 375 *R. sanguineus* adult ticks tested by PCR targeting rickettsial genes, 3 *A. aureolatum* collected on 2 dogs in E1, and 12 *R. sanguineus* from 4 dogs in E3 contained rickettsial DNA, which showed through DNA sequencing to be 100% identical to the corresponding sequences *R. rickettsii* in GenBank (CP000848), for both *gltA* (350/350-bp) and *ompA* (570/570-bp) gene fragments (Table 2). Considering that only 27 *A. aureolatum* ticks from E1 dogs were tested by PCR, the *R. rickettsii* infection rate in *A. aureolatum* from this area was 11·1%. Similarly, the *R. rickettsii* infection rate was 11·2% for the 106 *R. sanguineus* ticks collected from E2 dogs and tested by PCR (Table 2). Among the *A. aureolatum* ticks collected from dogs in NE2 to

Table 2. Rickettsial infection in ticks collected from domestic dogs in seven areas of the São Paulo metropolitan area, Brazil

(*N*, number of canine examinations; I, mean intensity; A, mean abundance. Values followed by different superscript letters in the same column are statistically different ($P<0{\cdot}05$).)

		Amblyomma aureolatum					*Rhipicephalus sanguineus*				
					Rickettsial infection†				Rickettsial infection†	IFA to *R. rickettsii**	
Area	*N*	Prevalence (%)	I	A	*R. rickettsii*	*R. bellii*	Prevalence (%)	I	A	*R. rickettsii*	1st sample	2nd sample
E1	137	15/137 (10·9) ^{a}	1·8 ^{a,b}	0·2 ^{a}	3/27 (11·1)	0/27 (0·0)	18/137 (13·1)	1·9	0·2	0/29 (0·0)	6/30 (20·0)	4/16 (25·0)
E2	138	2/138 (1·4) ^{b}	1·0 ^{a}	0·0 ^{b}	0/2 (0·0)	0/2 (0·0)	26/138 (18·8)	8·5	1·6	12/106 (11·3)	15/30(50·0)	12/23 (52·2)
NE1	130	40/130 (30·7) ^{c}	4·5 ^{b,c}	1·4 ^{c}	0/113 (0·0)	0/113 (0·0)	1/130 (0·8)	5·0	0·03	0/5 (0·0)	0/30 (0·0)	0/20 (0·0)
NE2	132	21/132 (18·9) ^{a}	2·6 ^{b,c}	0·4 ^{a}	0/25 (0·0)	1/25 (4·0)	32/132 (24·2)	46·8	11·3	0/159 (0·0)	0/30 (0·0)	0/23 (0·0)
NE3	132	41/130 (31·5) ^{c}	5·8 ^{c}	1·8 ^{c}	0/200 (0·0)	4/200 (2·0)	2/130 (1·5)	4·0	0·06	0/6 (0·0)	0/30 (0·0)	0/17 (0·0)
NE4	130	44/138 (31·9) ^{c}	3·9 ^{c}	1·3 ^{c}	0/111 (0·0)	7/111 (6·3)	9/138 (6·5)	19·1	1·2	0/46 (0·0)	0/30 (0·0)	0/22 (0·0)
NE5	159	22/159 (13·8) ^{a}	1·6 ^{b}	0·2 ^{a}	0/48 (0·0)	0/48 (0·0)	14/159 (8·8)	2·1	0·2	0/24 (0·0)	0/30 (0·0)	0/19 (0·0)

* No. of canine reactive sera to *R. rickettsii*/No. of tested sera (% reactive sera).
† No. of infected ticks/No. of tested ticks by PCR and DNA sequencing (% infected ticks).

NE4, 2·0 to 6·3% were PCR positive for the rickettsial *gltA* gene, and negative for the *ompA* gene PCR. The sequences of the *gltA*-PCR products of all these ticks were identical to each other, and 100% (350/350) identical to the corresponding sequence of *Rickettsia bellii* (CP000087) (Table 2). Additionally, 1 *A. aureolatum* adult tick was collected from a domestic cat in NE1. This tick yielded positive results by the *gltA*-PCR and, after DNA sequencing, showed 100% identity (332/332) with the corresponding sequence of *Rickettsia felis* (CP000053).

Small mammals

A total of 233 wild small mammals belonging to the following species were captured and examined for ticks: Rodentia: Cricetidae: *Akodon* sp., *Oligoryzomys nigripes* Olfers, 1818, *Nectomys squamipes* (Brants, 1827), *Euryoryzomys russatus* (Wagner, 1848), *Brucepattersonius* sp., *Hylaeamys megacephalus* (Fischer, 1814), *Thaptomys nigrita* (Lichtenstein, 1830); Muridae: *Rattus norvegicus* Berkenhout, 1769; Didelphimorphia: Didelphidae: *Didelphis aurita* Wied-Neuwied, 1826, *Monodelphis theresa* Thomas, 1921, *Monodelphis americana* (Müller, 1776).

Seroprevalence of Rickettsia *spp. in small mammals*

Overall, sera from 3 (13·6%) out of 22 opossums (*D. aurita*) reacted positively to at least 1 *Rickettsia* antigen tested, as follows: 1 *D. aurita* from E1 and 1 from NE1 reacted to *R. rickettsii, R. parkeri, R. amblyommii* and *R. rhipicephali* (endpoint titres ranging from 128 to 512), and 1 *D. aurita* from NE1 reacted to *R. amblyommii* and *R. rhipicephali* (endpoint titres 128 and 64, respectively). Eighty sera from small rodents were tested. Overall, sera from 17 (21·2%) rodents reacted positively (titre $\geqslant$64) to at least 1 *Rickettsia* antigen. From these, all 17 reacted to *R. rickettsii* (endpoint titres ranging from 64 to 8192), 15 reacted to *R. parkeri* (endpoint titres ranging from 128 to 8192), 15 reacted to *R. amblyommii* (endpoint titres ranging from 64 to 8192), 14 reacted to *R. rhipicephali* (endpoint titres ranging from 128 to 8192), 7 reacted to *R. bellii* (endpoint titres ranging from 64 to 256), and 6 reacted to *R. felis* (endpoint titres ranging from 64 to 512). Considering each study area separately, seropositive animals consisted of 7 *Akodon* sp. from E1 (25% of 28 small rodents tested from this area), 4 *Akodon* sp. from E2 (36·4% of 11 small rodents), 1 *Akodon* sp. and 1 *R. norvegicus* from NE1 (13·4% of 15 small rodents), 1 *Akodon* sp. from NE2 (7·7% of 13 small rodents), and 2 *E. russatus* and 1 *O. nigripes* from NE5 (60% of 5 small rodents). Not one animal (0%) out of 3 and 5 small rodents from areas NE3 and NE4, respectively, reacted to any of the 6 *Rickettsia* antigens. Not one seropositive rodent showed an

Table 3. Data for *Ixodes loricatus* ticks collected from small mammals in the São Paulo metropolitan area, Brazil

		Small mammals				No. of ticks		
Order	Family	Species	No. infested/ No. captured	Prevalence (%)	Mean intensity	Larvae	Nymphs	Adults
Rodentia	Cricetidae	*Akodon* sp.	7/138	5·1	1·0	4	3	
		Oligoryzomys nigripes	15/28	53·6	3·8	57		
		Euryoryzomys russatus	2/10	2·0	2·5		5	
	Muridae	*Rattus norvegicus*	1/1	100·0	1·0	1		
Didelphimorphia	Didelphidae	*Didelphis aurita**	30/52	57·7	3·3	1	7	87
		Monodelphis americana	1/2	50·0	1·0	1		

* One *D. aurita* was also found parasitized by 1 nymph of *Haemaphysalis juxtakochi*, and another *D. aurita* was infested by 1 *Amblyomma dubitatum* nymph and 1 *Amblyomma cajennense* nymph.

endpoint titre to a *Rickettsia* species at least 4-fold higher than the titres exhibited to any of the other 5 rickettsial antigens, precluding any inference on which possible *Rickettsia* species infected these animals.

Ticks from small mammals

The predominant tick species found on small mammals was *Ixodes loricatus* Neumann, 1899 (64 larvae, 15 nymphs, 87 adults), followed by *H. juxtakochi* (1 nymph), *Amblyomma dubitatum* Neumann, 1899 (1 nymph), and *Amblyomma cajennense* (1 nymph) (Tables 3 and 4). Individual infestations usually consisted of a few ticks, with mean intensity values <3·8 ticks/host at most of the times. No tick was found on the following animal species (number of examined individuals in parentheses): *N. squamipes* (6), *Brucepattersonius* sp. (1), *H. megacephalus* (2), *T. nigrita* (1), and *M. theresa* (1).

Rickettsial infection in ticks from small mammals

Among 87 adults of *I. loricatus* tested for rickettsial infection, 41 (47·1%) yielded positive results by the *gltA*-PCR, and were negative by the *ompA*-PCR. PCR products from one or two individual ticks from each of the areas E1, E2, NE4, and NE5 were DNA-sequenced and showed to be 100% identical (350/350) to the corresponding sequence of *R. bellii* (CP000087) (Table 8).

Birds

In total, 589 birds were captured, representing 60 species from 4 orders and of these 186 (31·6%) birds from 35 species (orders Passeriformes, Apodiformes and Galbuliformes) were infested with 666 larvae and 70 nymphs from the genera *Amblyomma, Haemaphysalis,* and *Ixodes*. All bird species parasitized by ticks are presented in Table 5. Individual infestations usually consisted of a few ticks, with mean intensity values lower than 4.0 ticks/bird at most of the times. No tick was found on the following bird species (number of examined individuals in parentheses): Piciformes, Picidae: *Celeus flavescens* (Gmelin, 1788) (3), *Picumnus cirratus* Temminck, 182 (1), *Veniliornis spilogaster* (Wagler, 1827) (1); Columbiformes, Columbidae: *Geotrygon violacea* (Temminck, 1809) (1), *Leptotila verreauxi* Bonaparte, 1855 (4); Apodiformes, Trochilidae: *Amazilia versicolor* (Vieillot, 1818) (6), *Phaethornis superciliosus* (Linnaeus, 1766) (14), *Florisuga fusca* (Vieillot, 1817) (1), *Phaethornis eurynome* (Lesson, 1832) (9), *Phaethornis pretrei* (Lesson & Delattre, 1839) (1); Passeriformes, Thamnophilidae: *Myrmotherula unicolor* (Ménétriès, 1835) (1); Dendrocolaptidae: *Xiphocolaptes albicollis* (Vieillot, 1818) (1); Furnariidae: *Synallaxis cinerea* Wied,

Table 4. Data on small mammal richness, prevalence of *Ixodes loricatus* infestations, and rickettsial infection determined by immunofluorescence assay (IFA) performed on sera from small mammals captured in seven areas of the São Paulo metropolitan area, Brazil

Área	No. of examined animals	Mammal richness -no. of species	No. of animals infested with *I. loricatus* (Prevalence%)	No. of sera reactive to *Rickettsia* spp./ No. of sera tested (% reactivity)
E1	72	3	14 (19·5)	8/33 (24·2)
E2	39	3	10 (26·0)	5/16 (31·2)
NE1	41	5	7 (17·1)	3/18 (16·7)
NE2	26	5	1 (3·8)	1/5 (20·0)
NE3	28	3	11 (39·3)	0/13 (0·0)
NE4	14	4	1 (7·1)	0/9 (0·0)
NE5	13	5	4 (30·8)	3/8 (37·5)

1831 (5), *Lochmias nematura* (Lichtenstein, 1823) (4); Tyrannidae: *Cnemotriccus fuscatus* (Wied, 1831) (1), *Hemitriccus orbitatus* (Wied, 1831) (4), *Lathrotriccus euleri* (Cabanis, 1868) (3), *Myiodynastes maculatus* (Statius Muller, 1776) (1), *Poecilotriccus plumbeiceps* (Lafresnaye, 1846) (1); Vireonidae: *Vireo olivaceus* (Linnaeus, 1766) (1); Coerebidae: *Coereba flaveola* (Linnaeus, 1758) (3); Turdidae: *Turdus leucomelas* Vieillot, 1818 (1).

Ticks from birds

Ticks collected on birds were identified as *Amblyomma longirostre* Koch, 1844 (177 larvae, 51 nymphs), *A. aureolatum* (22 larvae, 18 nymphs), *Amblyomma nodosum* Neumann, 1899 (17 larvae), *Amblyomma parkeri* Fonseca and Aragão 1952 (16 larvae), and *Haemaphysalis leporispalustris* (Packard, 1869) (1 larva). The DNA sequences of a mitochondrial 16S rRNA gene fragment generated from 12 *Amblyomma* sp. larvae taken from NE4 birds were identical to each other, but could not be identified to species level with certainty by Blast analysis because the most similar sequences were *A. parkeri* (EU805568) (315/349 bp, 90·2% identity) and *A. longirostre* (FJ424401) (349/395 bp, 88·3% identity) (Table 6). This species was designated as *Amblyomma* sp. haplotype Nazaré.

A total of 288 unengorged larvae could not be identified to species level through molecular analysis, and were identified morphologically as *Amblyomma* spp. In addition, 1 nymph was identified as *Ixodes* sp. All *Amblyomma* nymphs collected from birds, and 58 larvae of *A. longirostre*, 9 *A. aureolatum* and 17 *A. nodosum* larvae that moulted to nymphs were morphologically identified to species level. The remaining ticks were identified by molecular methods (Table 5). The prevalence of different tick species on birds for each area is shown in Table 7.

Rickettsial infection in ticks from birds

Among 127 *A. longirostre* ticks collected from birds and tested by PCR, 53 (41·7%) yielded positive results by *gltA* and *ompA* PCR assays. The *ompA* product from 1 or 2 ticks from each study area were sequenced and showed to be 100% (488/488) identical to the corresponding sequence of *Rickettsia amblyommii* strain Conduru, previously detected in *A. longirostre* from northeastern Brazil (HQ231758).

Among 14 *A. parkeri* and 6 *Amblyomma* sp. haplotype Nazare, 7 (50%) and 3 (50%) ticks, respectively, yielded positive results by *gltA* and *ompA* PCR assays. The *gltA* products were sequenced and shown to be 100% (307/307) identical to the corresponding sequence of *Rickettsia africae* (HQ335126) and *Rickettsia sibirica* (HM050296), and 99% (368/369) identical to a novel strain (ApPR) of *Rickettsia parkeri* recently found in *A. parkeri* ticks collected from birds in southern Brazil (JN126320). The *ompA* products were 99% (488/491) identical to *Rickettsia africae* (CP001612) and 100% identical to *R. parkeri* strain ApPR (JN126321) (Table 8).

Landscape analyses

The total combined patch size between BSF-endemic and non-endemic areas showed that the non-endemic areas are composed of patches significantly larger than the endemic areas ($t = -3{\cdot}217$, D.F. = 39, $P = 0{\cdot}003$). Additionally, the same outcome was observed when only the secondary patch sizes were compared ($t = -3{\cdot}708$, D.F. = 33, $P < 0{\cdot}001$) (Fig. 2). The mean NND did not show a significant difference between endemic and non-endemic areas ($U = 81{\cdot}5$, $P = 0{\cdot}1$); nevertheless, the mean shortest NND distances were found in areas NE2 and NE4; a second group with similar mean NND pattern was found for areas E1, NE1 and NE3, whereas the area E2 showed the largest mean NND (Fig. 3).

Bird sensitivity index (SI)

The control non-endemic area (NE5) was taken as a standard large fragment in order to be compared with the 6 small forest fragments. In NE5 the proportion of the SI was 10·5, 74·0, and 15·8% for low, medium, and high sensitiveness, respectively. The

Table 5. Ticks collected on birds in the Sao Paulo metropolitan area, Brazil

Birds						Ticks		
Order	Family	Species	No. infested/ No. captured	Tick prevalence (%)	Tick mean intensity	Species	No. of larvae	No. of nymphs
Apodiformes	Trochilidae	*Chlorostilbon lucidus*	1/3	33·3	1·0	*A.longirostre*†		1
		Thalurania glaucopis	2/29	6·9	1·0	*A.longirostre*†		1
						Amblyomma sp.	1	
Galbuliformes	Bucconidae	*Malacoptila striata*	1/8	12·5	1·0	*A.longirostre*		1
Passeriformes	Thamnophilidae	*Thamnophilus caerulescens*	4/4	100·0	9·7	*A.longirostre*	9	1
						Amblyomma sp.	29	
		Dysithamnus mentalis	15/28	53·6	4·1	*A.aureolatum*†		1
						A.longirostre	23	4
						A.nodosum	3	
						Amblyomma sp.	31	
		Pyriglena leucoptera	10/18	55·6	10·0	*A.aureolatum*†	11	3
						A.longirostre†	3	2
						Amblyomma sp.	81	
		Myrmeciza squamosa	1/5	20·1	1·0	*A.aureolatum*†		1
	Conopophagidae	*Conopophaga lineata*	9/28	32·1	4·7	*A.aureolatum*	1	
						A.longirostre	6	1
						A.nodosum	11	
						A.parkeri	8	
						Amblyomma sp.	15	
	Scleruridae	*Sclerurus scansor*	1/8	12·5	1	*Amblyomma* sp.*	1	
	Dendrocolaptidae	*Sittasomus griseicapillus*	3/19	15·8	0·3	*A.parkeri*†	1	
						Amblyomma sp.	2	
		Lepidocolaptes squamatus	10/19	52·6	3·4	*A.longirostre*	13	
						A.parkeri†	1	
						A.nodosum†	1	
						Amblyomma sp.	18	
						Amblyomma sp.*	1	
	Furnariidae	*Synallaxis ruficapilla*	8/26	30·8	1·9	*A.aureolatum*†		4
						A.longirostre	1	4
						Amblyomma sp.	6	
		Anabacerthia amaurotis	2/4	50·0	0·5	*A.longirostre*†		1
						Amblyomma sp.	1	
		Philydor atricapillus	1/1	100·0	3·0	*A.parkeri*†	1	
						Amblyomma sp.	2	
		Automolus leucophthalmus	9/18	44·4	5·8	*A.aureolatum*†		3
						A.longirostre	21	2
						A.nodosum†	1	

Table 5. (*Cont.*)

Birds						Ticks		
Order	Family	Species	No. infested/ No. captured	Tick prevalence (%)	Tick mean intensity	Species	No. of larvae	No. of nymphs
						Amblyomma sp.*	6	
						Amblyomma sp.	19	
		Xenops minutus	1/4	25·0	4·0	*A.longirostre*	1	
						Amblyomma sp.*	1	
						Amblyomma sp.	2	
	Tyrannidae	*Mionectes rufiventris*	6/10	60·0	4·3	*A.longirostre*	9	
						Amblyomma sp.	17	
		Leptopogon amaurocephalus	2/12	16·7	1·0	*A.longirostre*	1	1
		Tolmomyias sulphurescens	4/6	66·7	1·5	*A.longirostre*	3	2
						Amblyomma sp.	1	
		Tolmomyias poliocephalus	1/1	100·0	2·0	*A.parkeri*†	1	
						A.longirostre†	1	
		Platyrinchus mystaceus	13/37	35·1	1·2	*A.aureolatum*†	1	
						A.longirostre	10	3
						A.parkeri†	1	
						Amblyomma sp.	8	
	Pipridae	*Chiroxiphia caudata*	9/19	47·4	9·0	*A.longirostre*	16	2
						A.nodosum†	1	
						Amblyomma sp.	62	
	Tityridae	*Schiffornis virescens*	3/16	18·8	9·3	*A.longirostre*†	14	
						Amblyomma sp.*	1	
						Amblyomma sp.	13	
	Vireonidae	*Cyclarhis gujanensis*	2/2	100·0	1·5	*A.longirostre*		3
	Turdidae	*Turdus rufiventris*	8/30	26·7	3·4	*A.aureolatum*	7	2
						A.longirostre	9	1
						Amblyomma sp.	8	
		Turdus amaurochalinus	2/5	40·0	1·0	*A.longirostre*		2
		Turdus albicollis	1/9	11·1	1·0	*A.longirostre*	1	
	Thraupidae	*Trichothraupis melanops*	18/29	62·2	5·8	*A.aureolatum*†	2	2
						A.longirostre	12	9
						A.parkeri	3	
						Amblyomma sp.	75	
						H. leporispalustris†	1	
						Ixodes sp		1
		Habia rubica	7/24	29·2	3·0	*A.longirostre*	9	1
						Amblyomma sp.*	2	
						Amblyomma sp.	9	
		Tachyphonus coronatus	6/10	60·0	2·2	*A.longirostre*	3	3

					Amblyomma sp.	7	
	Pipraeidea melanonota	1/1	100·0	1·0	*A.longirostre*†		1
Emberizidae	*Haplospiza unicolor*	4/11	36·4	1·0	*A.aureolatum*†		2
					A.longirostre†		1
					Amblyomma sp.	1	
	Oryzoborus (Sporophila) angolensis	1/1	100·0	1·0	*A.longirostre*†		1
Parulidae	*Basileuterus culicivorus*	17/59	28·8	1·5	*A.longirostre*	12	2
					Amblyomma sp.	12	
	Basileuterus leucoblepharus	3/25	12·0	0·7	*A.longirostre*†		1
					Amblyomma sp.	1	

* DNA sequence of a fragment of the mitochondrial 16S rDNA was most similar (90%) to *A. parkeri* (EU805568), and 88% identical to *A. longirostre* (FJ424401).
† Reported on this host species for the first time.

presence of bird species within medium and high sensitivity represents more preserved areas, when comparing the number of captured birds within these categories. All non-endemic areas (NE1 to NE4) showed no statistical difference between each other and to the control area ($\chi^2 = 9{\cdot}314$, D.F. = 4, $P = 0{\cdot}054$), while the endemic areas E1 and E2 showed a statistical difference pattern compared to the control NE5 ($\chi^2 = 6{\cdot}755$, D.F. = 2, $P = 0{\cdot}034$) (Fig. 4). There was a marked difference between the BSF-endemic areas and the non-endemic areas, regarding the number of species with medium and high sensitivity; the latter group represented an important part of the accessed avifauna in all non-endemic areas, whereas in endemic areas, this group of birds was significantly less abundant (E2) or not reported (E1).

Diversity and richness of small mammals and birds

It was not possible to calculate diversity for small mammals because there were only few species caught in each locality (maximum 5). On the other hand, it was noticeable that the lowest mammal richness (3 species) was observed in E1, E2, and NE4, and the highest (5 species) richness in NE1, NE3 and NE5 (Table 4). The lowest diversity and richness of birds were observed in the BSF-endemic areas (E1 and E2), whereas the highest diversity was found in the non-endemic areas (NE1-NE5) (Table 7).

Reference numbers

Voucher tick specimens collected during this study have been deposited in the tick collection 'Coleção Nacional de Carrapatos' (CNC) of the Faculty of Veterinary Medicine, University of São Paulo, SP, Brazil (Accession numbers: 1965–2046). GenBank nucleotide sequence Accession numbers for the partial mitochondrial 16S rDNA sequences obtained in the present study are JN800424 - JN800430 (*A. longirostre* larvae), JN800431 (*A. parkeri* larva), JN800432 (*Amblyomma* sp. haplotype Nazare larva), JN800433 (*A. aureolatum* larva), and JN800434 (*H. leporispalustris larva*). GenBank nucleotide sequence Accession numbers for the partial sequences of the rickettsial *ompA* gene are JN800435 (*R. parkeri* strain ApPR from *A. parkeri*), JN800436 (*R. amblyommii* from *A. longirostre*), JN800437 (*R. rickettsii* from *A. aureolatum*), and JN800438 (*R. rickettsii* from *R. sanguineus*). GenBank nucleotide sequence Accession numbers for the partial sequences of the rickettsial *gltA* gene are JN800439 (*R. parkeri* strain ApPR from *A. parkeri*), JN800440 (*R. felis* from *A. aureolatum*), JN800441 (*R. bellii* from *I. loricatus*), JN800442 (*R. bellii* from *A. aureolatum*), JN800443 (*R. rickettsii* from *A. aureolatum*) and JN800444 (*R. rickettsii* from *R. sanguineus*).

Table 6. Larval ticks identified to species level by molecular analysis of a fragment of the tick mitochondrial 16S rDNA gene

No. of larvae	Tick sequence with highest similarity (GenBank Accession number)	% similarity	Taxonomic identification of the larval ticks
132	*Amblyomma longirostre* (FJ424401)	100	*A. longirostre*
2	*A. longirostre* (GQ891951)	99·4	*A. longirostre*
4	*A. longirostre* (FJ424401)	99·7	*A. longirostre*
2	*A. longirostre* (GQ891951)	99·7	*A. longirostre*
1	*A. longirostre* (FJ424401)	99·3	*A. longirostre*
1	*A. longirostre* (GQ891951)	99·4	*A. longirostre*
1	*A. longirostre* (FJ424401)	99·1	*A. longirostre*
13	*Amblyomma parkeri* (EU805568)	100	*A. parkeri*
12	*Amblyomma aureolatum* (AF541254)	97·3	*A. aureolatum*
1	*Haemaphysalis leporispalustris* (L34309)	96·3	*H. leporispalustris*
12	*A. longirostre* (FJ424401)/*A. parkeri* (EU805568)	88·3/90·2	*Amblyomma* sp.

Table 7. Ticks collected on birds in seven forest areas of the São Paulo metropolitan area, Brazil

	Birds			Tick species (% of infested birds)							
Area	No. of examined birds	Richness (No. of species)	Diversity (H')	*A. longirostre*	*A. aureolatum*	*A. nodosum*	*A. parkeri*	*Amblyomma* sp.*	*Amblyomma* sp.	*H. leporispalustris*	*Ixodes* sp.
E1	68	20	2·6	10 (14·7)	3 (4·4)				7 (10·3)		
E2	21	9	1·9	4 (19·0)	4 (19·0)		3 (14·3)		8 (38·1)		
NE1	55	21	2·8	16 (29·1)	3 (5·4)				9 (16·3)		
NE2	142	30	3·1	42 (29·5)	5 (3·5)	6 (4·2)	1 (0·7)	6 (4·2)	26 (18·3)		
NE3	95	25	3·0	7 (7·3)	3 (3·1)				7 (7·4)	1 (1·8)	
NE4	95	30	3·1	37 (38·9)	1 (1·0)		3 (3·1)		22 (23·1)		
NE5	113	26	2·9	8 (7·1)	4 (3·5)		3 (2·6)		11 (9·7)		1 (0·8)

* Designated as *Amblyomma* sp. haplotype Nazare.

DISCUSSION

Tick records

Two tick species, *A. aureolatum* and *R. sanguineus*, were found on dogs in all 7 study areas. These results agree with previous studies that reported these ticks to be commonly found on dogs living in areas of the SPMA, where dogs have access to Atlantic forest fragments (Pinter *et al.* 2004; Moraes-Filho *et al.* 2009, Sabatini *et al.* 2010). As previously discussed, dogs usually become infested by all parasitic stages of *R. sanguineus* while resting in and near the houses (ecological niche of *R. sanguineus*), and/or by adults of *A. aureolatum* while visiting the neighbouring Atlantic forest (ecological niche of *A. aureolatum*) (Moraes-Filho *et al.* 2009).

The major tick species found on small mammals was *I. loricatus,* for which the adult stage was found only on opossums (*D. aurita*), whereas larvae and nymphs were found mostly on Cricetidae rodents. These results agree with literature data from Brazil, Argentina, and Uruguay, where *I. loricatus* adult ticks are usually found on Didelphidae hosts, and immature ticks on both Didelphidae and Cricetidae hosts (Guglielmone and Nava, 2010). At the same time, we provide the first record of *I. loricatus* on *E. russatus* and *R. norvegicus*. In addition, our finding of an opossum *D. aurita* parasitized by *H. juxtakochi* has never been reported.

The predominant tick species infesting birds was *A. longirostre*. This tick is widely distributed in the Neotropical region (Guglielmone *et al.* 2003*a*), where the adult stage feeds primarily on porcupines (*Coendou* spp.) while subadult stages feed primarily on birds, mostly Passeriformes (Aragão, 1936; Labruna *et al.* 2007*b*; Ogrzewalska *et al.* 2008, 2009; Nava *et al.* 2010). Herein, we report for the first time 10 bird species infested by *A. longirostre*. *Amblyomma aureolatum* (larvae and nymphs) was the second most common tick species on birds. Despite that this tick has already been reported on several species of Passeriformes (Arzua *et al.* 2005; Guglielmone

Table 8. Rickettsial infection of ticks collected from wild animals in seven forest areas of the Sao Paulo metropolitan area, Brazil

	Ixodes loricatus			*Amblyomma longirostre*			*Amblyomma parkeri*			*Amblyomma* sp. Nazaré		
Area	*N*	*gltA**	*R. bellii*†	*N*	*gltA**	*R. amblyommii*†	*N*	*gltA**	*R. parkeri*†	*N*	*gltA**	*R. parkeri*†
E1	26	16 (61·5)	2	10	4 (40·0)	2	0			0		
E2	7	1 (14·2)	1	1	3 (33·3)	1	8	4 (50·0)	4	0		
NE1	0			17	4 (23·5)	2	0			0		
NE2	0			35	14 (40·0)	2	0			6	3 (50·0)	3
NE3	0			11	7 (63·6)	2	0			0		
NE4	38	13 (34·2)	2	45	18 (40·0)	2	2	0 (0·0)		0		
NE5	16	11 (68·7)	2	8	3 (37·5)	2	4	3 (75·0)	3	0		

N, number of ticks tested by PCR for rickettsial infection.
* Number of PCR-positive ticks for the rickettsial *gltA* gene (%).
† Refers to the number of ticks that had their PCR products sequenced in order to identify the *Rickettsia* species.

et al. 2003*b*), herein we report 8 bird species (all Passeriformes) infested by *A. aureolatum* for the first time. Immature stages of *A. nodosum* and *A. parkeri* were also found on Passeriformes in the present study, in agreement with previous studies that reported larvae and nymphs of these two tick species on birds (mostly Passeriformes) (Labruna *et al*. 2007*b*; Ogrzewalska *et al*. 2008, 2009, 2010). However, we found these 2 tick species on 7 bird species for the first time.

Interestingly, 12 *Amblyomma* larvae were found on 6 bird species from NE2 that could not be identified to species level. Because the mitochondrial 16S rRNA consensus sequence generated from these 6 larvae was at most 90% similar to any available sequence in Genbank, this species was regarded as *Amblyomma* sp. haplotype Nazaré. Indeed, this new haplotype is closely related to both *A. longirostre* and *A. parkeri*; however, further studies are needed to define the taxonomic position of haplotype Nazaré.

Rickettsial infection in ticks

Five *Rickettsia* species were found infecting ticks in the present study: *R. rickettsii* in *A. aureolatum* and *R. sanguineus; R. bellii* in *A. aureolatum* and *I. loricatus; R. amblyommii* in *A. longirostre; R. parkeri* strain ApPR in *A. parkeri,* and *R. felis* in *A. aureolatum*. All these tick-*Rickettsia* associations have been reported in Brazil (Pinter and Labruna, 2006; Horta *et al*. 2007; Ogrzewalska *et al*. 2008; Moraes-Filho *et al*. 2009; Pacheco *et al*. 2012), except for the latter, which refers to a flea-borne rickettsia (*R. felis*) infecting the tick *A. aureolatum* for the first time.

Rickettsia rickettsii was detected in ticks from only 2 areas (E1 and E2), in agreement with the endemic status of these areas for BSF, as determined by serology of sentinel hosts (dogs). As much as 11% of the *A. aureolatum* and *R. sanguineus* ticks from areas E1 and E2, respectively, were found to be infected by *R. rickettsii*. This infection rate is much higher than 0·9% previously reported for *A. aureolatum* in another endemic area of the SPMA (Pinter and Labruna, 2006) but, at the same time, close to 13·1%, as previously reported for *R. sanguineus* in a BSF-endemic area in the state of Minas Gerais, southeastern Brazil (Pacheco *et al*. 2011). These numbers highlight the high risks of BSF transmission in areas E1 and E2, at least for dogs, which are the primary hosts of *A. aureolatum* and *R. sanguineus*. On the other hand, risks of human disease should be much lower because *R. sanguineus* only rarely bites humans in South America (Guglielmone *et al*. 2006), whereas *A. aureolatum,* a known human biting tick, is present at low densities in the SPMA, as shown by the very low mean intensity and mean abundance values for dogs, especially in the endemic areas E1 and E2. These

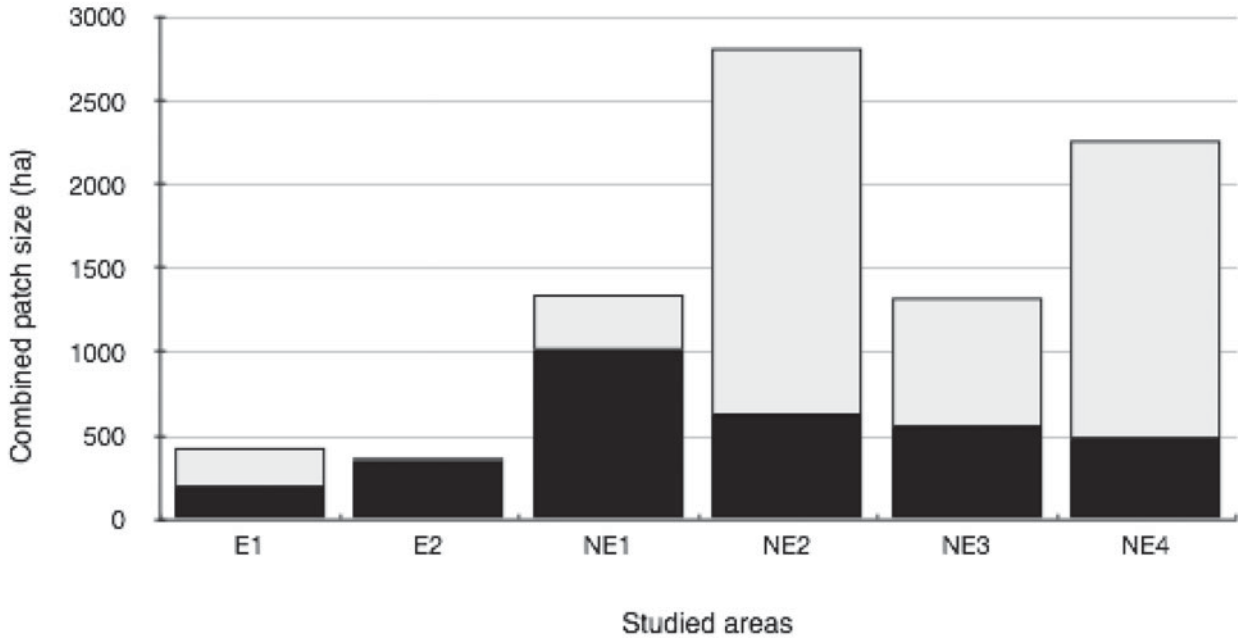

Fig. 2. Combined forest patch size of the 7 areas sampled in the present study. Dark bar represents the main patch and the grey bar represents the secondary patches.

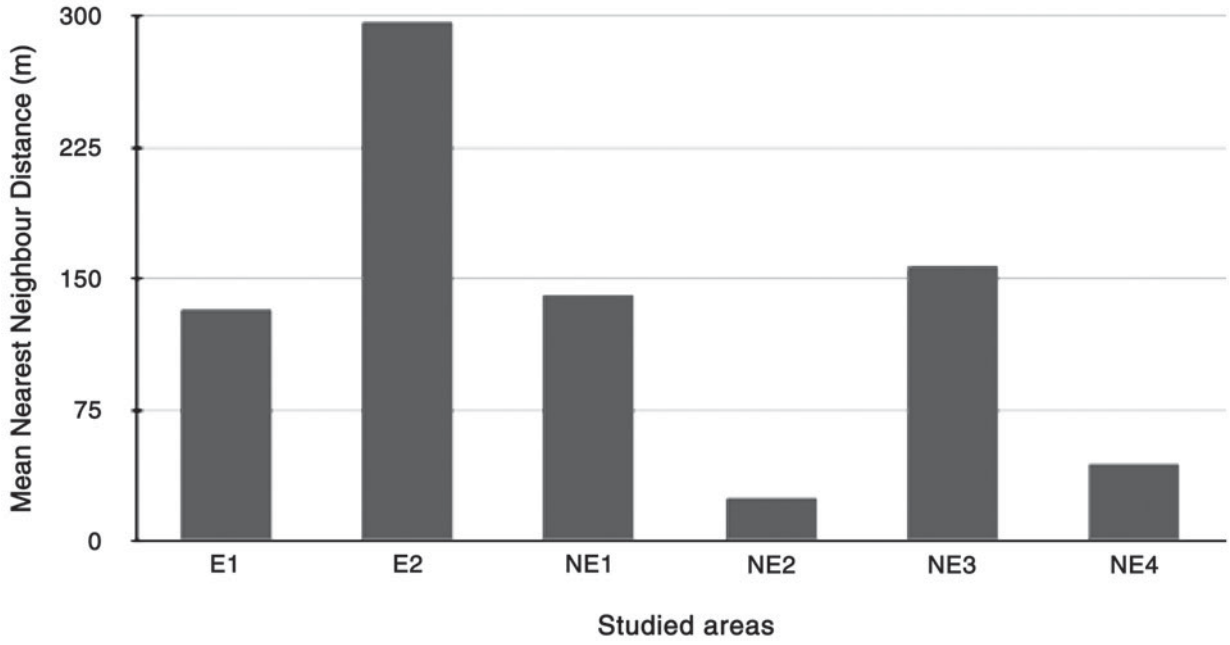

Fig. 3. Mean nearest neighbour distance (NND) from the main forest patch to the secondary patches for each of the 7 areas sampled in the present study.

facts explain the low BSF incidence (0·1 per 100 000 inhabitants) in the SPMA during the last decade (Katz *et al*. 2009), even in the presence of high *R*. *rickettsii*-infection rates in ticks.

Rickettsia bellii was found infecting 2·0 to 6·3% of the *A*. *aureolatum* ticks in 3 non-endemic areas, and around half of the *I*. *loricatus* adult ticks collected from opossums from either endemic or non-endemic areas. This *Rickettsia* has been widely reported infecting different tick species in Brazil, including *A*. *aureolatum* and *I*. *loricatus* in other BSF-endemic and non-endemic areas (Pinter and Labruna, 2006; Horta *et al*. 2007). Currently, *R*. *bellii* is considered non-pathogenic to humans (Labruna, 2009). Both *R*. *amblyommii* and *R*. *parkeri* strain ApPR were detected at relatively high infection rates among *A*. *longirostre* and *A*. *parkeri* ticks collected from birds; the pathogenic role of these two rickettsiae remains unknown (Pacheco *et al*. 2012). Our finding of a single *A*. *aureolatum* tick infected by *R*. *felis* is

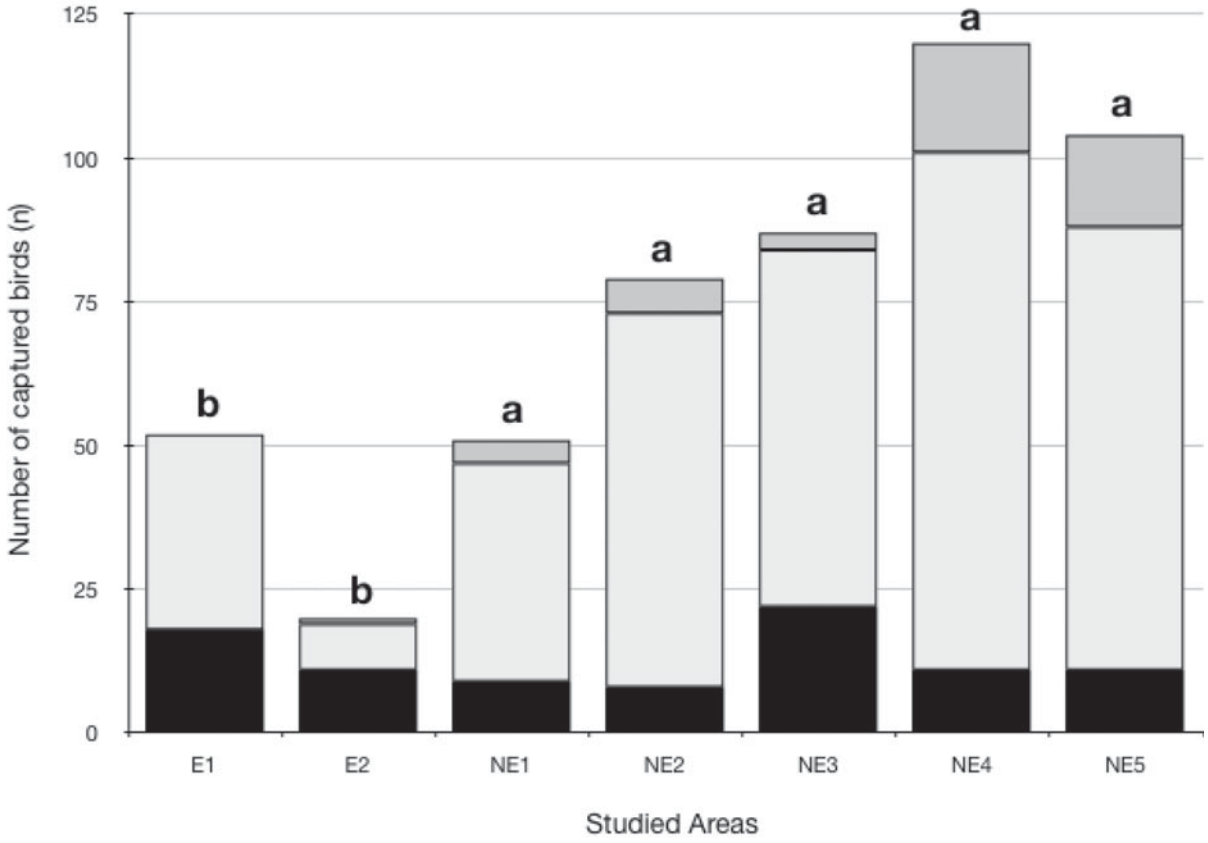

Fig. 4. The proportion of captured bird species in each study area categorized by the sensitivity index (SI). Black, light grey, and dark grey bars represent low, medium, and high sensitivity, respectively, to forest fragmentation.

possibly an accidental event, as a result of simultaneous infestation of the host by both *A. aureolatum* ticks and *R. felis*-infected fleas, since it has been shown that *R. felis*-infected fleas (*Ctenocephalides* spp.) are widespread among cats and dogs in the SPMA (Horta *et al*. 2007).

Rickettsial infection in vertebrate hosts

Vertebrate hosts were indirectly examined for rickettsial infection through serological analysis. About 20–52% of the dogs living in the 2 BSF-endemic areas (E1 and E2) were exposed to SFG rickettsiae, while no dogs from the 5 non-endemic areas were found to be infected by rickettsiae. These canine serological results, in conjunction with our findings of *R. rickettsii*-infected ticks collected from E1 and E2 dogs, allowed us to consider with certainty that these two areas were endemic for BSF, whereas areas NE1 to NE5 were non-endemic. On the other hand, results of the serological analysis of small mammals (rodents and opossums) did not match to this determined endemic status, since 16 to 37% of small mammals from either endemic (E1 and E2) or non-endemic areas (NE1, NE2, and NE5) were shown to be exposed to SFG rickettsiae. Because these animals were not found infested by *A. aureolatum* or *R. sanguineus* ticks, and because their major tick species (*I. loricatus*) was found infected solely by *R. bellii* in both endemic and non-endemic areas, we considered that these small mammals have been exposed to a *Rickettsia* species different from *R. rickettsii*; therefore, they have had no direct role in the epidemiology of *R. rickettsii* in the SPMA. In fact, the species of small rodents and marsupials collected in the present study seem to have no role in the life history of *A. aureolatum* (Guglielmone *et al*. 2003*b*; Horta *et al*. 2007). Interestingly, a recent study in an Atlantic rainforest area in northeastern Brazil (Dantas-Torres *et al*. 2012) reported 68·8% of the small mammals to be seropositive to *Rickettsia* spp., which is a seroprevalence value much higher than those found in the present study. The reasons for such differences remain unknown, as well as the *Rickettsia* species infecting Atlantic rainforest small mammals.

Ecology of BSF-endemic and non-endemic areas

The 2 endemic areas (E1, E2) clearly differed from the 5 non-endemic areas (NE1 to NE5) by significantly smaller combined patch sizes, larger mean NND values, lower bird abundance, richness, and diversity, and lower mammal richness in the endemic areas. In addition, Fig. 1 shows that young secondary forests (< 15 m canopy height) prevail in endemic

areas, whereas late and intermediate secondary forests (>15 m canopy height) prevail in most of the non-endemic areas. These ecological differences might have accounted for the lower prevalence and mean intensity of *A. aureolatum* on dogs in areas E1 and E2, when compared with non-endemic areas. In this case, a combination of factors such as larger patch size, higher host diversity, and higher canopy height (directly related to ground microclimate) could account to more suitable conditions for *A. aureolatum,* namely, higher availability of suitable hosts for active ticks, and better microclimate conditions for off-host tick stages.

Our results also indicate that these ecological differences should be related to the presence of *R. rickettsii*-infected ticks in areas E1 and E2, and the absence of *R. rickettsii* in ticks from areas NE1 to NE5. Because *R. rickettsii* is partially deleterious to *A. aureolatum* engorged females, it seems unlikely that *A. aureolatum* could sustain *R. rickettsii* infection over multiple successive generations solely by vertical transmission because the number of infected ticks would gradually decrease after each generation (Labruna *et al*. 2011). Thus, horizontal transmission through the participation of amplifier vertebrate hosts in the formation of new lineages of infected ticks seems to be crucial for maintenance of *R. rickettsii* in the BSF-endemic areas (Labruna *et al*. 2011). However, competent amplifier hosts of *R. rickettsii* for *A. aureolatum* under natural conditions are not known, in contrast to other endemic areas of Brazil, where capybaras (*Hydrochoerus hydrochaeris*) are major amplifier hosts of *R. rickettsii* for *A. cajennense* ticks (Souza *et al*. 2009; Labruna, 2009), or even in the United States, where several small rodent species are known to be major amplifier hosts of *R. rickettsii* to *Dermacentor* spp. ticks, main vectors of RMSF in North America (Burgdorfer, 1988). Our results suggest that the particular ecological conditions found in areas E1 and E2 facilitates a more frequent contact of *A. aureolatum* immature ticks with a given competent amplifier host, which should have a high reproduction rate in order to maintain a constant introduction of susceptible hosts in the area (Labruna, 2009).

Several studies have shown that an efficient amplifier host for *R. rickettsii* must be used by larvae and/or nymphs of the tick vector. At the same time, a host that is used only by adult ticks is not an efficient amplifier host because transovarial transmission rates are likely to be low or absent when the primary infection of ticks occurs during adult feeding (Parker *et al*. 1933; Burgdorfer and Brinton, 1975; Piranda *et al*. 2011). In this regard, dogs could serve as efficient amplifier hosts for *R. sanguineus*, as shown by Piranda *et al*. (2011), but not for *A. aureolatum* because only the adult stage of this latter tick species primarily feeds on dogs (Labruna *et al*. 2011). Before determining the ideal amplifier hosts of *R. rickettsii* for *A. aureolatum,* it is necessary to know which major vertebrate hosts are used by subadult stages of this tick species in the endemic areas of the SPMA. Unfortunately, the present study provides only a few host records for *A. aureolatum* larvae and nymphs, which were all on passerine birds. Most of the few host records available in the literature were also on passerine birds (Arzua *et al*. 2005), while there are records for only 4 mammal species, including 2 rodents (*Ctenomys* sp. and *Euryzygomatomys spinosus*) (Guglielmone *et al*. 2003*b*). According to Fonseca (1935), the spiny rat *E. spinosus* should be a major host for immature stages of *A. aureolatum* in the SPMA. Although the SPMA is within the distribution area of *E. spinosus* (Woods and Kilpatrick, 2005), not one specimen was trapped in the present study. This result does not indicate absence of this rodent in the study areas because *E. spinosus* is usually not caught by conventional traps (authors' unpublished data). While one study suggested that a few North American bird species have the potential to serve as amplifier hosts for *R. rickettsii* (Lundgren *et al*. 1966), scarce available data suggest that a mammal species such as *E. spinosus* could be an efficient amplifier host of *R. rickettsii* in the SPMA (Pinter *et al*. 2004), a condition yet to be confirmed. Regardless of what animal species is the natural amplifier host for *R. rickettsii* in the SPMA, our results indicate that this animal might be a generalist species that would benefit from forest fragmentation and degradation.

The present results corroborate historical observations that have indicated that all human cases of BSF in the SPMA were contracted in the southern part of this metropolitan area (Gomes, 1933; Fonseca, 1935; Pinter and Labruna, 2006; Moraes-Filho *et al*. 2009). Interestingly, our results show that not all forest patches in the southern part of the SPMA are suitable for BSF endemism. In this regard, the area NE1 showed some ecological characteristics typical of non-endemic areas of the northern part, such as higher patch size, mammal richness and bird diversity. In addition, area NE1 showed a bird sensitivity index statistically similar to NE2 to NE5, and statistically distinct from E1 and E2. Thus, because this irregular distribution of BSF-endemic areas seems to be linked to ecological conditions, namely, forest degradation, it is possible that BSF might emerge in the future into other areas of the SPMA, as long as forest degradation advances to make the area ecologically similar to areas E1 and E2.

It is noteworthy that area NE1 is located only 8 km east of E1, and 4 km west of another endemic area as reported recently (Moraes-filho *et al*. 2009), where 70% of the dogs were seropositive and *R. rickettsii*-infected ticks were found (Moraes-Filho *et al*. 2009). Since *A. aureolatum* larvae and nymphs are known to parasitize passerine birds (Arzua *et al*. 2005; present

study), one could infer that these flying hosts could transport *R. rickettsii*-infected ticks from endemic to non-endemic areas not so distantly separated, such as E1 from NE1. However, our results indicate that even if *R. rickettsii*-infected ticks are introduced into non-endemic areas such as NE1, ecological conditions might preclude the establishment of *R. rickettsii* in the local *A. aureolatum* population. Finally, further population genetic studies are also needed to compare *A. aureolatum* populations between endemic and non-endemic areas, since the establishment of *R. rickettsii* in different *A. aureolatum* populations could also be related to specific genetic traits of local populations.

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