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Molecular analyses reveal an abundant diversity of ticks and rickettsial agents associated with wild birds in two regions of primary Brazilian Atlantic Rainforest

Hermes Ribeiro Luz*, João Luiz Horacio Faccini, Douglas McIntosh

Departamento de Parasitologia Animal, Universidade Federal Rural do Rio de Janeiro, UFRRJ, Seropédica, Rio de Janeiro, Brazil

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ABSTRACT

Brazilian wild birds are recognized as frequent and important hosts for immature stages of more than half of the 32 recognized species of *Amblyomma* ticks recorded in that country. Several species of *Amblyomma* harbor rickettsial agents, including members of the spotted fever group (SFG). Most studies on this topic relied primarily on morphological characterization and reported large portions of the collected ticks at the genus rather than species level. Clearly, this factor may have contributed to an underestimation of tick diversity and distribution and makes comparisons between studies difficult. The current investigation combined morphological and molecular analyses to assess the diversity of ticks and rickettsial agents associated with wild birds, captured in two regions of native Atlantic rainforest, in the state of Rio de Janeiro, Brazil. A total of 910 birds were captured, representing two orders, 34 families and 106 species, among which 93 specimens (10.2%), were parasitized by 138 immature ticks (60 larvae and 78 nymphs), representing 10 recognized species of the genus *Amblyomma*; together with two reasonably well classified haplotypes (*Amblyomma* sp. haplotype Nazaré and *Amblyomma* sp. strain USNTC 6792). Amplification by PCR and sequencing of rickettsial genes (*htrA*, *gltA*, *ompA* and *ompB*), demonstrated the presence of *Rickettsia* DNA in 48 (34%) of the ticks. Specifically, *Rickettsia bellii* was detected in a single larva and a single nymph of *A. aureolatum*; *R. amblyomatis* was found in 16 of 37 *A. longirostre* and was recorded for the first time in three nymphs of *A. calcaratum*; *R. rhipicephali* was detected in 9 (47%) of 19 *Amblyomma* sp. haplotype Nazaré ticks. The remaining ticks were infected with genetic variants of *R. parkeri*, namely strain ApPR in 12 *A. parkeri* and seven *Amblyomma* sp. haplotype Nazaré ticks, with the strain NOD found in two specimens of *A. nodosum*. Interestingly, a single larvae of *A. ovale* was shown to be infected with the emerging human pathogen *Rickettsia* sp. strain Atlantic rainforest (ARF), suggesting a possible role for birds in the dispersal of ticks infected with this variant of *R. parkeri*. The diversity of ticks and *Rickettsia* recorded in this study is, to our knowledge, the most abundant recorded to date in Brazil and highlighted the value of employing methods capable of providing species level identification of the ixodofauna of wild birds.

1. Introduction

Globally, wild birds are recognized as hosts to an astonishing variety of tick species which, in turn, may serve as vectors or reservoirs for a diverse array of pathogens and parasites of humans and animals (Lachish et al., 2012; Palomar et al., 2012; Parola et al., 2013; Sándor et al., 2014; Scott et al., 2016). As such, wild birds play an important role in the maintenance, amplification and ecology of several recognized and possibly some unrecognized tick-borne diseases.

Brazil serves as home to approximately 2000 species of birds, of which 800 have been registered in the Southeastern state of Rio de Janeiro. The greatest diversity of species was recorded in the mountainous regions of the state, including the Itatiaia National Park (INP) and the Serra dos Orgãos National Park (SONP), that were decreed nature reserves in 1937 and 1939 respectively (Sick, 1997; Mallet-Rodrigues et al., 2008; Piacentini et al., 2015). The establishment of the reserves, was a forward-thinking attitude that reflected a global preoccupation with the degradation of natural environments. As a result, the parks are among the few (less than 6%), regions of primary Atlantic forest that still exist in Brazil (MMA, 2010).

Large scale surveys of the ixodofauna of wild birds, have been undertaken during the last decade, in the major biomes; Amazon, Pantanal, Cerrado, Caatinga and Atlantic rainforest of Brazil (Ogrzewalska et al., 2008, 2009a, 2010; Luz et al., 2012, 2016a,

* Corresponding author.
E-mail addresses: hermesluz@globomail.com (H.R. Luz), jlhfaccini@globo.com (J.L.H. Faccini), pus972@yahoo.co.uk (D. McIntosh).

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2016b; Lugarini et al., 2015; Ramos et al., 2015; Zeringóta et al., 2016). The results of those surveys have been impressive both quantitatively and qualitatively, and have afforded a fascinating insight into the diversity of bird-tick associations, that in turn provided a solid base upon which to develop theories in relation to the bio-ecology of bird ticks in Brazil.

Brazilian wild birds are parasitized predominantly by immature ticks of the genus *Amblyomma*, with only sporadic records of infestations with adult ticks or with hard ticks belonging to the genera *Haemaphysalis, Ixodes* or *Rhipicephalus* (Ogrzewalska et al., 2008, 2010; Luz and Faccini, 2013; Lugarini et al., 2015; Ramos et al., 2015; Ogrzewalska and Pinter, 2016; Zeringóta et al., 2016). The importance of Brazilian birds as hosts for *Amblyomma* ticks should not be underestimated. In this context, a total of 19 (59%) of the recognized 32 species registered in Brazil, have been recorded in association with birds, of which 12 (63%) were identified in diverse regions of the Atlantic forest biome (Luz and Faccini, 2013; Luz et al., 2016a; Ogrzewalska and Pinter, 2016).

Bird ticks are firmly established as sources of a wide range of pathogens including viruses, bacteria and protozoa, which may potentially be transmitted to animals and/or humans during their blood meals (Franke et al., 2010; Hildebrandt et al., 2010; Kazarina et al., 2015; Diakou et al., 2016). Interestingly, there are no confirmed records of direct disease transmission from a wild bird tick to a human. However, it could be hypothesised that pathogen-infected, immature ticks associated with wild birds will develop into infected adults which during their search for new hosts hold the potential to either directly transmit their infections agents, or could infect new host species (e.g. mammals or rodents), that may in turn serve as reservoirs or amplifiers for pathogens that are subsequently transferred to novel ectoparasites, that most likely have no direct contact with birds, but that are true vectors of diseases. In this scenario, bird ticks could be viewed as an incubator for tick-borne diseases.

To date, studies of tick-borne disease agents in Brazilian bird ticks have focussed on bacteria of the genus *Rickettsia* (Ogrzewalska and Pinter, 2016). A total of eight rickettsial agents have been recognized in Brazil (Parola et al., 2013; Nieri-Bastos et al., 2014). Five of them, namely *Rickettsia bellii*, and the following SFG rickettsial agents; '*Candidatus* Rickettsia andeanae', *Rickettsia amblyommatis*- formerly '*Candidatus* Rickettsia amblyommii' (Karpathy et al., 2016), *Rickettsia rhipicephali* and a variety of *Rickettsia parkeri*-like strains (NOD, Paraiba, and ApPR), have been detected in or isolated from *Amblyomma* ticks infesting birds (Ogrzewalska and Pinter, 2016; Zeringóta et al., 2016).

In Brazil, only *Rickettsia rickettsii* and *Rickettsia* sp. strain Atlantic rainforest (ARF), that is considered to represent a genetic variant of *Rickettsia parkeri* (Spolidorio et al., 2010; Silva et al., 2011; Szabó et al., 2013; Krawczak et al., 2016), are confirmed as capable of infecting humans. To date, neither of those agents has been detected in bird ticks. However, the vectors of *R. rickettsii* (=*Amblyomma sculptum* and *Amblyomma aureolatum*), and of strain ARF (=*Amblyomma ovale*) were found parasitizing wild birds in different regions of Brazil (Luz et al., 2012; Luz and Faccini, 2013; Ramos et al., 2015; Ogrzewalska and Pinter, 2016).

Published investigations of bird-tick associations in the state of Rio de Janeiro have been limited to two small scale surveys, conducted in peculiar environments, neither of which assessed the presence of tick-borne pathogens (Santolin et al., 2012; Luz et al., 2016a). The current study aimed to address those shortcomings and was designed to examine the diversity of bird ticks and the rickettsial agents carried by them, using molecular analyses, in two areas of primary/native Atlantic Forest, located within the INP and the SONP nature reserves, over a 26-month period. The collection of data in those locations was considered of relevance given that both are situated in a region with several records of infection by rickettsial agents (Rozental et al., 2006, 2009; Cunha et al., 2009; Gazêta et al., 2009) and because of the perceived role of birds as reservoirs, dispersers and potential amplifiers of spotted fever group rickettsial agents (Hornok et al., 2014; Berthová et al., 2016; Flores et al., 2016; Ogrzewalska and Pinter, 2016).

Table 1
Sampling sites in the Itatiaia National Park (INP) and the Serra dos Órgãos National Park (SONP).

Municipalities	Area	Latitude	Longitude	Altitude (m a.s.l.)
Resende	INP	22° 27" 52′	44° 36" 15′	530
Resende	INP	22° 27" 11′	44° 36" 44′	800
Resende	INP	22° 25" 56′	44° 37" 12′	1600
Resende/Mauá	INP	22° 19" 47′	44° 32" 25′	1052
Teresópolis	SONP	22° 25" 54′	42° 59" 14′	980
Teresópolis	SONP	22° 29" 44′	42° 59" 57′	363

2. Material and methods

2.1. Study site and samples

A total of 28 field trips, two trips each month, each lasting six days, were conducted between May of 2014 through June of 2015 in the Itatiaia National Park (INP) and the Serra dos Órgãos National Park (SONP), both located in the state of Rio de Janeiro, Brazil. The specific locations of the captures are provided in Table 1, together with information in relation to the altitude of the capture points. Birds were caught between 06.00 h and 17.00 h each day, using 10–20 ornithological mist nets (12 m long × 3 m wide, 16 mm and 36 mm mesh) and were photographed and identified following the recommendations of Sigrist (2014), using the nomenclature approved by the Brazilian Committee of Ornithological Records (CBRO, 2014). Each bird was examined for the presence of ticks over the entire body and when present, they were removed using forceps and placed in individual 1.5 mL screw capped micro-centrifuge tubes containing 250 µL of RNA*later*® (Ambion). Samples were initially stored at ambient temperature (for up to 5 days; if captured on the first day of the field trip). Upon arrival in the laboratory, ticks were stored at 4 °C and examined microscopically as detailed below, with subsequent storage at −20 °C in RNA*later*® until processed for molecular analyses.

2.2. Morphological characterization

Larvae were identified morphologically, to the genus level, based upon the dichotomous keys of Clifford et al. (1961). Species level identification of nymphs was performed using the key proposed by Martins et al. (2010). Prevalence, mean intensity and abundance of tick infestations were calculated following the recommendations of Bush et al. (1997).

2.3. Molecular analyses

Total DNA was extracted from individual ticks using the bead-beater/phenol-chloroform method reported by Santolin et al. (2013). Molecular identification of all ticks to species level was attempted by PCR and sequencing of a 460 bp fragment of the mitochondrial sequence encoding 16S rRNA, using the methods reported by Mangold et al. (1998). In cases where no amplicon was obtained for the 16S rDNA, or where identifications were unclear, a second PCR was used to amplify an approximately 380 bp fragment of the gene encoding mitochondrial 12S rRNA (Beati et al., 2012).

To investigate the presence of *Rickettsia*, individual DNA samples were examined by PCR using the primers CS-239 and CS-1069 (generates an 834-bp fragment of the *gltA* gene) and the primers 17k-5 and 17k-3, (generates a 549-bp fragment of the rickettsial *htrA* gene; Labruna et al., 2007). Samples positive for those assays were subjected to additional PCR protocols using the primers Rr190.70p and Rr190.602n (generates a 530-bp fragment of the rickettsial *ompA* gene;

Labruna et al., 2004), and primers 120-M59 and 120–807 (generate an 865-bp fragment of the rickettsial *ompB* gene; Roux and Raoult, 2000). The master-mixes and cycling conditions were as reported by Zeringóta et al. (2016). PCR products were analysed by gel electrophoresis (1.5% agarose), with confirmation of amplicon sizes achieved via comparison to a DNA molecular weight marker (GeneRuler 100 bp DNA Ladder, product # SM024, Thermo Scientific).

PCR-Restriction fragment length polymorphism (PCR-RFLP) analysis of *ompB* amplicons or *htrA* amplicons (in the case of *ompB* negative samples), was used for preliminary, species level identification as reported by Santolin et al. (2013) and Zeringóta et al. (2016).

Nucleotide sequencing of PCR products, in both directions, was performed employing the amplification primers and the BigDye Ready Reaction mix (ABI Corp); reaction products were analysed on a 3500-automated genetic analyzer (ABI Corp). Sequence alignments were performed using Sequencher (Version 5.3, Genecodes Corporation, CA). Aligned sequences were entered into the BLAST search algorithm (Altschul et al., 1990) and the NCBI nucleotide database to determine gene identity.

The study was evaluated and approved by the Animal Experimentation Ethics Committee of the Federal Rural University of Rio de Janeiro and was conducted with the permission of IBAMA; process number 43917/3/2505369.

3. Results

Data in relation to the associations between the birds, ticks and rickettsial agents recorded in this study are detailed in Tables 2 and 3. A total of 910 birds, representing two orders, 34 families and 106 species were captured, among which 93 specimens (10.2%), from 48 species and 25 families, were parasitized by 138 immature ticks of the genus *Amblyomma*; specifically, 60 larvae (LL) and 78 nymphs (NN). The majority of the hosts, 87/93 (92%), belonged to the order Passeriformes (Tables 2 and 3). All ticks were collected from the head, with most located around the ears, nape and crown.

No ticks were recorded on the following birds species (number of individuals in parentheses): Non-passerines – *Accipiter striatus* (2), *Leptotila rufaxilla* (15), *Columbina talpacoti* (16), *Geotrygon montana* (8), *Phaethornis pretrei* (3), *Lophornis magnificus* (7), *Eupetomena macroura* (15), *Thalurania glaucopis* (12), *Clytolaema rubricauda* (8), *Picumnus cirratus* (18), *Veniliornis maculifrons* (9), *Celeus flavescens* (4); Passerines – *Dysithamnus mentalis* (16), *Thamnophilus caerulescens* (13), *Drymophila squamata* (9), *Taraba major* (17), *Lepidocolaptes angustirostris* (9), *Lepidocolaptes squamatus* (19), *Xenops minutus* (23), *Automolus leucophthalmus* (19), *Manacus manacus* (21), *Neopelma chrysolophum* (18), *Thlypopsis sordida* (5), *Tangara desmaresti* (12), *Tangara cayana* (1), *Saltator similis* (2), *Tangara sayaca* (3), *Neothraupis fasciata* (12), *Conirostrum speciosum* (5), *Nemosia pileata* (9), *Coereba flaveola* (11), *Todirostrum poliocephalum* (2), *Mionectes rufiventris* (3), *Platyrinchus mystaceus* (3), *Myiobius atricaudus* (2), *Euphonia chlorotica* (9), *Euphonia violácea* (1), *Turdus flavipes* (8), *Camptostoma obsoletum* (3), *Legatus leucophaius* (7), *Lathrotriccus euleri* (5), *Knipolegus nigerrimus* (11), *Cyclarhis gujanensis* (19), *Vireo chivi* (1), *Basileuterus culicivorus* (22), *Mackenziaena severa* (9), *Conopophaga lineata* (7), *Xiphorhynchus fuscus* (1), *Xiphorhynchus guttatus* (16), *Campylorhamphus falcularius* (22).

Using a combination of morphological characterization and sequencing of 16S and/or 12S rDNA, the following ten recognized species of *Amblyomma* ticks were identified; *Amblyomma longirostre* 5 (LL) and 32 (NN), *Amblyomma brasiliense* 3 (LL) and 23 (NN), *Amblyomma parkeri* 14 (LL) and 2 (NN), *Amblyomma calcaratum* 10 (NN), *Amblyomma nodosum* 4 (LL) and 4 (NN), *Amblyomma sculptum* 4 (LL) and 2 (NN), *Amblyomma naponense* 2 (LL) and 2 (NN), *Amblyomma aureolatum* 1 (LL) and 3 (NN), *Amblyomma ovale* 3 (LL), and *Amblyomma varium* 1 (LL).

The bulk of the ticks demonstrated levels of between 99–100% nucleotide identity to partial sequences of 12S and 16S rDNA deposited in GenBank as derived from 10 of the 11-species cited above, which facilitated their unambiguous identification. The exception, were the 6 ticks, 4 (LL) and 2 (NN), identified as *A. sculptum*. Analysis of the 16S rDNA sequences of the ticks showed them to be identical to each other and with 97% nucleotide identity to sequences deposited as partial 16S rDNA of *A. cajennense* (JX573118; 395/406 bases) and (KJ5571; 391/403 bases) or as *A. sculptum* from Brazil (KT722808; 371/382 bases) and Argentina (KT820361; 394/406 bases). In contrast, sequence analyses of the 12S rDNA amplicons revealed 100% (340/340 bases) and 99.9% nucleotide identity (339/340 bases), to sequences deposited, in 2013, as partial 12S rDNA of *A. cajennense* from Brazil (KF614695 and KF614696). The reasons for the decision to classify these ticks as *A. sculptum*, in apparent contradiction of the results from the BLAST searches, will be discussed below.

A total of 23 larvae could not be identified to the species level. Among them, 19 were shown by sequencing of 16S rDNA to be 100% similar (410/410 bases) to the sequence JN800432, deposited in GenBank as partial 16S rDNA of *Amblyomma* sp. MO-2012 (strain Nazaré B), designated hereafter as "*Amblyomma* sp. haplotype Nazaré", a tick previously collected from wild birds in the Brazilian states of São Paulo (Ogrzewalska et al., 2012) and Minas Gerais (Zeringóta et al., 2016).

The four remaining ticks did not produce amplicon in the PCR targeting 16S rDNA and were therefore examined using the 12S rDNA assay. Three larvae, 2 collected in INP and one from SONP, showed nucleotide identity values ranging from 98.7% (317/321 bases) to 99.4% (311/313 bases), to the sequence AY342275 deposited in GenBank as partial 12S rDNA of *Amblyomma* sp. strain USNTC 6792, a tick collected from the anteater (*Tamandua tetradactyla*) in Brazil. The three larvae were designated *Amblyomma* sp. INP haplotype 1, INP haplotype 2, and SONP haplotype 1. Only one larva, collected in the SONP, failed to produce amplicon in either of the PCR assays and was therefore registered as *Amblyomma* sp.

The novel partial sequences of 16S and 12S mitochondrial rDNA recorded in this study were submitted to GenBank with the following accession numbers; 16S rDNA- *A. sculptum* haplotype INP (KY172626); 12S rDNA- *A. sculptum* haplotype INP (KY172627), *Amblyomma* sp. INP haplotype 1 (KY172629), INP haplotype 2 (KY172630) and SONP haplotype 1 (KY172631).

The family Thraupidae showed the greatest level of infestation, with 50 ticks (25 larvae and 25 nymphs), distributed over 33 specimens of birds. Considering the diversity of the approved tick species (n = 10), a total of eight species were collected from tanagers. The Thraupidae also served as host for almost half of the larvae identified as *Amblyomma* sp. haplotype Nazaré (n = 9/19). Ticks were recorded for the first time on the following bird species *Pulsatrix perspicillata pulsatrix*: (2 NN) *A. calcaratum* and (1 LL) *A. nodosum*; *Arremon semitorquatus*: (1 NN) *A. aureolatum*; *Sporophila frontalis*: (2 NN) *A. calcaratum*; *Chlorophonia cyanea cyanea*: (1 NN) *A. nodosum* and *Cacicus haemorrhous*: (1 NN) *A. longirostre*.

A total of forty-eight ticks, 32 collected in the INP and 16 collected in the SONP, generated amplicons of the expected size using the PCR assays targeting the *gltA* and *htrA* genes (Tables 2 and 3). Subsequently, forty-six of the ticks produced amplicons with the predicted molecular weight in the *ompA* and *ompB* assays. Digestion, using the restriction enzymes *Msp*I and *Rsa*I, of the *htrA* amplicons of the two *ompA/ompB* negative ticks, identified them presumptively as *R. bellii*. This result was confirmed by nucleotide sequencing of the *gltA* amplicons, both of which showed levels of 100% nucleotide identity (792/792 bases), to the sequence JQ906786 deposited in GenBank as partial sequence of the citrate synthase gene of *R. bellii*, strain Peruibe.

Analysis of *ompB* amplicons by PCR-RFLP indicated that 16 *A. longirostre* ticks (2 larvae and 14 nymphs) together with three nymphs of *A. calcaratum* were infected with *R. amblyommatis*; that nine larvae of *Amblyomma* sp. haplotype Nazaré were infected with *R. rhipicephali* and that the remaining ticks; 10 larvae and 2 nymphs of *A. parkeri*, seven

Table 2
Species of birds parasitized by ticks in the Itatiaia National Park (INP), Rio de Janeiro and presence of *Rickettsia* spp., MI: mean intensity, PI: prevalence of infestation, () number of infected ticks.

Birds	N. birds examined	N°. Birds infested	N°. Ticks	PI(%)	MI	N° of nymphs	N° of larvae	Ticks	*Rickettsia* spp.	Altitude (m a.s.l)
NON PASSERIFORMES										
ACCIPITRIFORMES										
ACCIPITRIDAE										
Rupornis magnirostris	1	1	1	100	1.0	1		*Amblyomma parkeri*	*Rickettsia* sp. strain ApPR	800
STRIGIFORMES										
STRIGIDAE										
Pulsatrix perspicillata pulsatrix[a]	1	1	3	100	3.0	2		*Amblyomma calcaratum*		530
							1	*Amblyomma nodosum*	*Rickettsia* sp. strain NOD	530
GALLIFORMES										
CRACIDAE										
Penelope obscura	3	2	16	66.6	8.0	14		*Amblyomma brasiliense*		800
						2		*Amblyomma sculptum*		530
PASSERIFORMES										
THAMNOPHILIDAE										
Pyriglena leucoptera	19	7	7	36.8	1.0	2		*Amblyomma brasiliense*		800
						1		*Amblyomma naponense*		800
							2	*Amblyomma longirostre*	*Rickettsia amblyommatis* (1)	800
							2	*Amblyomma* sp. strain USNTC 6792		800
CONOPOPHAGIDAE										
Conopophaga lineata	14	2	2	14.2	1.0		1	*Amblyomma* sp. haplotype Nazare	*Rickettsia rhipicephali*	1600
							1	*Amblyomma parkeri*	*Rickettsia* sp. strain ApPR	1600
DENDROCOLAPTIDAE										
Dendrocincla fuliginosa	6	1	1	16.6	1.0		1	*Amblyomma* sp. haplotype Nazare	*Rickettsia rhipicephali*	1600
Dendrocincla turdina	17	1	1	6	1.0		1	*Amblyomma parkeri*	*Rickettsia* sp. strain ApPR	1600
Sittasomus griseicapillus	11	1	1	9	1.0	1		*Amblyomma longirostre*	*Rickettsia amblyommatis*	1600
Xiphorhynchus guttatus	11	1	1	9	1.0		1	*Amblyomma* sp. haplotype Nazare	*Rickettsia* sp. strain ApPR	1600
FURNARIIDAE										
Furnarius rufus	4	1	1	25	1.0	1		*Amblyomma calcaratum*	*Rickettsia amblyommatis*	
PIPRIDAE										
Chiroxiphia caudata	18	4	5	22.2	1.25		1	*Amblyomma* sp. haplotype Nazare	*Rickettsia* sp. strain ApPR	1052
							2	*Amblyomma* sp. haplotype Nazare		530
						1		*Amblyomma longirostre*		530
						1		*Amblyomma brasiliense*		1052
THRAUPIDAE										
Lanio melanops	23	10	14	43.4	1.4		4	*Amblyomma* sp. haplotype Nazare	*Rickettsia* sp. strain ApPR (1)	1052
						2		*Amblyomma calcaratum*	*Rickettsia amblyommatis* (1)	1052
						4		*Amblyomma longirostre*	*Rickettsia amblyommatis* (4)	530
							4	*Amblyomma parkeri*	*Rickettsia* sp. strain ApPR (3)	1052
Arremon semitorquatus [a]	3	1	1	33.3	1.0	1		*Amblyomma aureolatum*		1052
Pyrrhocoma ruficeps	4	2	4	50	2.0	3		*Amblyomma longirostre*		530
						1		*Amblyomma calcaratum*		530
Sporophila caerulescens	8	1	1	12.5	1.0	1		*Amblyomma aureolatum*	*Rickettsia bellii*	1052
Sporophila frontalis [a]	2	1	2	50	2.0	2		*Amblyomma calcaratum*		1052
Cyanerpes cyaneus	3	1	1	33.3	1.0	1		*Amblyomma nodosum*	*Rickettsia* sp. strain NOD	800
Tangara ornata	6	1	3	16.6	3.0	2		*Amblyomma longirostre*		800
						1		*Amblyomma nodosum*		800
Tangara seledon	7	1	1	14.2	1.0	1		*Amblyomma longirostre*		800
Tachyphonus coronatus	12	3	8	25	2.6	3		*Amblyomma longirostre*		530
						2		*Amblyomma brasiliense*		800
							2	*Amblyomma naponense*		800
							1	*Amblyomma aureolatum*	*Rickettsia bellii*	800
RHYNCHOCYCLIDAE										
Leptopogon amaurocephalus	15	1	1	6.6	1.0		1	*Amblyomma parkeri*	*Rickettsia* sp. strain ApPR	800

(continued on next page)

Table 2 *(continued)*

Birds	N. birds examined	N°. Birds infested	N°. Ticks	PI(%)	MI	N° of nymphs	N° of larvae	Ticks	*Rickettsia* spp.	Altitude (m a.s.l)
FRIGILLIDAE										
Chlorophonia cyanea cyanea [a]	1	1	1	100	1.0	1		*Amblyomma nodosum*		530
TURDIDAE										
Turdus leucomelas	12	1	1	8.3	1.0		1	*Amblyomma* sp. haplotype Nazare	*Rickettsia rhipicephali*	800
Turdus rufiventris	14	5	7	36	1.4	1		*Amblyomma longirostre*		530
							3	*Amblyomma nodosum*		530
							2	*Amblyomma ovale*	*Rickettsia* sp. strain Atlantic rainforest (1)	530
						1		*Amblyomma calcaratum*		530
Turdus amaurochalinus	5	1	1	20	1.0	1		*Amblyomma aureolatum*		530
TYRANNIDAE										
Myiarchus ferox	9	1	3	11.1	3.0	3		*Amblyomma longirostre*	*Rickettsia amblyommatis* (1)	530
Machetornis rixosa	7	1	2	14.2	2.0	2		*Amblyomma longirostre*	*Rickettsia amblyommatis* (1)	800
Attila rufus	7	2	2	28.6	1.0		1	*Amblyomma* sp. haplotype Nazare	*Rickettsia rhipicephali*	800
						1		*Amblyomma naponense*		800
MOMOTIDAE										
Baryphthengus ruficapillus	2	1	2	50	2.0	2		*Amblyomma longirostre*		530
ICTERIDAE										
Cacicus haemorrhous [a]	1	1	1	100	1.0	1		*Amblyomma longirostre*		530
Molothrus bonariensis	10	2	3	20	1.5	1		*Amblyomma nodosum*		800
							1	*Amblyomma ovale*		530
							1	*Amblyomma varium*		530
Total	**247**	**61**	**99**	**24.6**	**1.6**	**64**	**34**			

[a] New records of ticks on birds.

larvae of *Amblyomma* sp. haplotype Nazaré, one larva and one nymph of *A. nodosum* and a single larva of *A. ovale*, were infected with *R. parkeri*.

Sequencing of the *ompB* amplicons of each rickettsial agent confirmed and extended the PCR-RFLP data. Specifically, the 15 *ompB* amplicons identified as *R. amblyommatis* were identical to each other and showed 100% nucleotide identity (817/817 bases) to the sequence KJ534313, deposited in GenBank as partial sequence of the *ompB* gene of "*Candidatus* Rickettsia amblyommii". The *gltA* and *ompA* amplicons generated from the two (NN) of *A. calcaratum* and those of two (LL) of *A. longirostre* were sequenced and showed 100% similarity to the sequences KJ534310 (*gltA*) and KJ534312 (*ompA*), both derived from "*Candidatus* Rickettsia amblyommii", detected in *A. longirostre* ticks collected from porcupines in the Brazilian state of Bahia.

The nine *ompB* amplicons presumptively identified as *R. rhipicephali*, were identical to each other and showed 100% similarity (817/817 bases) to the sequence KX01805, deposited in GenBank as partial sequence of the *ompB* gene of *R. rhipicephali* strain RrMG, detected in *Amblyomma* sp. haplotype Nazaré ticks in Minas Gerais, Brazil. The *gltA* and *ompA* amplicons from four (LL) were sequenced and displayed 100% nucleotide identity to the sequences KX018048 (*R. rhipicephali* strain RrMG, *gltA* gene; 778/778 bases), and KX018049 (*R. rhipicephali* strain RrMG, *ompA* gene; 509/509 bases).

Sequencing of the *ompB* amplicons identified as *R. parkeri*, by PCR-RFLP, revealed that the gene fragments from 12 *A. parkeri* and seven *Amblyomma* sp. haplotype Nazaré ticks were identical to each other and exhibited 100% nucleotide identity (817/817 bases) to the sequence KX018050, deposited in GenBank as partial sequence *ompB* gene of *R. parkeri* strain ApPR, detected in *Amblyomma* sp. haplotype Nazaré ticks collected from Birds in Minas Gerais, Brazil. Sequence analysis of *gltA* amplicons from four of the 12 *A. parkeri* larvae and three of the seven *Amblyomma* sp. haplotype Nazaré ticks, revealed them as identical to each other and with 100% similarity (706/706 bases) to the sequence JN126320, deposited in GenBank as partial sequence citrate synthase (*gltA*), *R. parkeri* strain ApPR, detected in *A. parkeri* ticks collected in birds from southern Brazil. The *ompA* amplicons of the same seven ticks showed 99.9% nucleotide identity (490/491 bases), to the corresponding sequence (JN126321), of *R. parkeri* strain ApPR.

The *ompB*, amplicons derived from the two *A. nodosum* ticks were identical to each other and showed 99.9% nucleotide identity (775/776 bases) to the sequence EU567179, designated in GenBank as partial *ompB* gene of *Rickettsia* sp. NOD, (a strain considered to be a genetic variant of *R. parkeri*). Sequencing of the *htrA* amplicons revealed 100% nucleotide identity (437/437 bases) to the equivalent sequence derived from *Rickettsia* sp. NOD (EU567178). In addition, the *gltA* amplicons were 100% similar (738/738 bases) to the sequence EU567177, described in GenBank as partial sequence of the citrate synthase (*gltA*) gene, of *Rickettsia* sp. NOD.

Sequence analysis of the remaining *ompB* amplicon, generated from a larva of *A. ovale*, revealed it to be 100% similar (761/761 bases) to GQ855236, deposited in GenBank as *Rickettsia* sp. 'Atlantic rainforest' outer membrane protein B (*ompB*) gene. Levels of 100% nucleotide identity were also recorded, for the *gltA* and *ompA* amplicons produced from the *A. ovale* larva, with the following sequences deposited in GenBank; JQ906783- citrate synthase (*gltA*) of *Rickettsia parkeri* strain Atlantic rainforest (749/749 bases) and JQ906784-outer membrane protein A (*ompA*) gene of *R. parkeri* strain Atlantic rainforest (491/491 bases).

4. Discussion

The diversity of tick species registered in this study (10 recognized species and two reasonably well characterized haplotypes of *Amblyomma* sp.) is, to our knowledge, the most abundant in any survey of bird ticks conducted in Brazil. It was considered likely that the species richness was a consequence of the highly-preserved nature of the sample sites. In this context, a negative correlation between tick diversity and deforestation was previously identified by Brazilian researchers in Atlantic forest biomes (Ogrzewalska et al., 2011a).

Alternatively, the identification of so many species, including low numbers of *A. naponense*, *A. sculptum* and *A. varium*, all rarely reported

Table 3
Species of ticks parasitizing birds captured in the Serra dos Órgãos National Park (SONP), State of Rio de Janeiro and presence of *Rickettsia* spp., MI: mean intensity, PI: prevalence of infestation, () number of infected ticks.

Birds	N° birds examined	N°. Birds infested	N°. Ticks	PI(%)	MI	N° of nymphs	N° of larvae	Ticks	Rickettsia spp.	Altitude (m a.s.l.)
NON PASSERIFORMES										
GRUIFORMES										
RALLIDAE										
Aramides saracura	1	1	2	100	2.0	2		*Amblyomma brasiliense*		980
CORACIIFORMES										
MOMOTIDAE										
Baryphthengus ruficapillus	2	2	2	100	1.0	2		*Amblyomma brasiliense*		980
PASSERIFORMES										
THAMNOPHILIDAE										
Pyriglena leucoptera	13	1	1	7.6	1.0	1		*Amblyomma longirostre*	*Rickettsia amblyommatis*	363
DENDROCOLAPTIDAE										
Dendrocincla turdina	16	3	4	19	1.3		3	*Amblyomma sculptum*		363
							1	*Amblyomma longirostre*		
Sittasomus griseicapillus	10	1	1	10.0	1.0	1		*Amblyomma longirostre*	*Rickettsia amblyommatis*	363
PIPRIDAE										
Chiroxiphia caudata	14	1	1	7	1.0	1		*Amblyomma longirostre*	*Rickettsia amblyommatis*	363
THRAUPIDAE										
Lanio melanops	21	5	8	23.8	1.6	2		*Amblyomma longirostre*	*Rickettsia amblyommatis* (2)	363
							4	*Amblyomma parkeri*	*Rickettsia* sp. strain ApPR (4)	980
							1	*Amblyomma* sp. haplotype Nazare	*Rickettsia* sp. strain ApPR	980
							1	*Amblyomma brasiliense*		980
Ramphocelus bresilius	11	1	1	9	1.0		1	*Amblyomma* sp. haplotype Nazare	*Rickettsia* sp. strain ApPR	980
Tersina viridis	12	1	1	8.3	1.0		1	*Amblyomma longirostre*		
Tachyphonus coronatus	11	3	4	27.2	1.3		1	*Amblyomma* sp. haplotype Nazare	*Rickettsia rhipicephali*	980
							2	*Amblyomma* sp. haplotype Nazare	*Rickettsia* sp. strain ApPR (2)	980
							1	*Amblyomma sculptum*		363
Saltator maximus	6	2	2	33.3	1.0		1	*Amblyomma parkeri*		363
							1	*Amblyomma parkeri*,		363
TURDIDAE										
Turdus albicollis	16	2	3	12.5	1.5		1	*Amblyomma parkeri*		
						2		*Amblyomma longirostre*		363
Turdus rufiventris	11	2	3	18.18	1.5	1	1	*Amblyomma longirostre*		980
							1	*Amblyomma* sp.		980
INCERTAE SEDIS										
Platyrinchus mystaceus	6	1	1	16.6	1.0		1	*Amblyomma* sp. haplotype Nazare		980
TYRANNIDAE										
Myiarchus ferox	8	2	2	25	1.0	1		*Amblyomma parkeri*	*Rickettsia* sp. strain ApPR	363
						1		*Amblyomma calcaratum*		363
Attila rufus	8	1	2	12,5	2.0		1	*Amblyomma* sp. haplotype Nazare	*Rickettsia rhipicephali*	363
							1	*Amblyomma* sp. strain USNTC 6792		363
PASSERELLIDAE										
Zonotrichia capensis	5	2	2	40	1.0		2	*Amblyomma brasiliense*		980
Total	**171**	**31**	**40**	**18.1**	**1.3**	**14**	**26**			

in association with Brazilian birds (Ogrzewalska and Pinter, 2016), may have reflected the fact that almost all ticks (137/138), were identified by nucleotide sequencing to the species level.

Data for three of the tick species collected from birds in this study could be considered as unusual in comparison to previous investigations; firstly, the recovery of 23 nymphs and 3 larvae of *A. brasiliense*, which equated to almost 20% of the total recovered in the current study, was unprecedented and questioned the generally held belief that birds are not important in the life cycle of this tick (Ogrzewalska and Pinter, 2016).

Secondly, the quantities of *A. parkeri* (14 LL) and (2 NN), and *Amblyomma* sp. haplotype Nazaré (19 LL), could be viewed as high, when compared with data from studies conducted in other regions of Atlantic rainforest in Brazil (Ogrzewalska et al., 2009a, 2009b, 2011b, 2012; Pacheco et al., 2012). Alternatively, the level of 14% (19/139) of *Amblyomma* sp. haplotype Nazaré ticks collected in the present survey might be viewed as low in comparison to the value of 40% (77/191), collected from birds in a fragment of Atlantic forest, in the state of Minas Gerais (Zeringóta et al., 2016).

The identification of six ticks as *A. sculptum*, rather than as *A. cajennense*, appeared to disregard the results of BLAST searches performed using the amplified regions of 16S and 12S rDNA. However, our analysis took into consideration the findings of the recent and substantial revision of the *A. cajennense* complex that resulted in the reinstatement of the species *A. sculptum* (Nava et al., 2014). In addition, we considered the robust data of Martins et al. (2016), that showed the geographical distribution of *A. sculptum* to include the Cerrado, Pantanal, and Atlantic forest biomes, while records of *A. cajennense* (sensu stricto) in Brazil were almost exclusively within the Amazon biome. It is clear, that care should be taken when performing

BLAST searches to confirm the locality in which ticks, identified as *A. cajennense* prior to the revision of the complex, were collected.

In the current work, *R. bellii* was detected for the first time, by PCR and identified by PCR-RFLP and sequencing, in one (LL) and one (NN) of *A. aureolatum* recovered from wild birds. The initial observation of *R. bellii* infecting *A. aureolatum*, recovered from dogs in the state of São Paulo, was documented by Pinter and Labruna (2006), with two subsequent reports of this association, also in the State of São Paulo (Sabatini et al., 2010; Ogrzewalska et al., 2012). Information in relation to rickettsial infections of *A. aureolatum*, is considered valuable since it is a recognized vector of *R. rickettsii* (Labruna, 2009) and a potential vector of the Atlantic rainforest (ARF) variant of *R. parkeri* (Barbieri et al., 2014).

Rickettsia amblyommatis was the first member of the SFG of *Rickettsia* reported infecting bird ticks in Brazil (Ogrzewalska et al., 2008), and subsequently emerged as the most frequently detected rickettsial agent in ticks infesting birds in that country (Ogrzewalska et al., 2010; 2011b, 2012, 2015; Pacheco et al., 2012; Santolin et al., 2013; Lugarini et al., 2015; Ramos et al., 2015). On the one hand, the detection of this rickettsial agent in 43% (16/37) of the *A. longirostre* ticks was not entirely unexpected, and adds support to the hypothesis that this may be a symbiotic relationship, as suggested for other tick species and the rickettsial components of their microbiotas (Perlman et al., 2006; Socolovschi et al., 2009; Narasimhan and Fikrig, 2015). On the other hand, it should be mentioned that based on epidemiological, serological and acarological data, other authors considered *Rickettsia amblyommatis*, when carried by *Amblyomma americanum*, to be the pathogen responsible for a growing number of cases of mild, spotted fever group rickettsiosis in the United States of America (Apperson et al., 2008; Jiang et al., 2010; Dahlgren et al., 2016).

In contrast to the data for *A. longirostre*, the finding of *R. amblyommatis* in two nymphs of *A. calcaratum* was unprecedented and increased to seven, the number of species within the genus *Amblyomma* identified as infected with this bacterium in Brazil (Parola et al., 2013; Saraiva et al., 2013; Lugarini et al., 2015). Reports of human parasitism by this tick are rare and, as such, the potential health risks for humans and/or domestic animals could be considered negligible. To date, immature stages of *A. calcaratum* were reported infesting birds in a variety of Brazilian biomes (Luz and Faccini, 2013; Ogrzewalska and Pinter, 2016), while adults were associated almost exclusively with anteaters (Barros-Battesti et al., 2006; Guglielmone et al., 2014; Witter et al., 2015). Prior to the current study, the only rickettsial agent associated with *A. calcaratum* was a *Rickettsia parkeri*-like bacterium (strain NOD), detected in nymphs recovered from Brazilian birds (Ogrzewalska et al., 2013).

The SFG agent *R. rhipicephali* was detected in seven out of 12 *Amblyomma* sp. haplotype Nazaré larvae collected in the INP and in two of the seven ticks collected in the SONP. These finding were considered significant, since they confirmed the association between *Amblyomma* ticks and *R. rhipicephali,* reported for birds captured in a remnant of semi-deciduous forest, embedded in a densely populated urban area of the city of Juiz de Fora in the state of Minas Gerais (Zeringóta et al., 2016), where an infection rate of 40% was observed. In addition, our data extend the geographical distribution of this SFG agent to two regions of primary Atlantic Forest in the State of Rio de Janeiro. To date, no confirmed infections of humans have been attributed to *R. rhipicephali* and as such, it is impossible to determine if the relatively high prevalence of this bacterium in *Amblyomm*a sp. haplotype Nazaré ticks represents a threat to human or animal health, or if this association is primarily symbiotic.

The majority, (3 of 5 from INP) and (4 of 5 from SONP), of the other haplotype Nazaré ticks were found to be infected with the *Rickettsia parkeri*-like agent (ApPR), substantiating and extending the association between these organisms that was initially reported in the State of São Paulo (Ogrzewalska et al., 2012) and subsequently in Minas Gerais (Zeringóta et al., 2016). Interestingly, only three of the 19 haplotype Nazaré ticks were negative for the presence of rickettsial DNA, a proportion similar to that reported by Zeringóta et al. (2016).

Based on the limited molecular data available (partial sequences of 16S and 12S rDNA), the haplotype Nazaré ticks appear to be closely related to *A. parkeri*. In the current study, 75% (12/16) of the *A. parkeri* ticks were shown to be infected with the ApPR strain of *Rickettsia parkeri*. When combined with the findings for haplotype Nazaré ticks, the ApPR strain was the most frequently detected rickettsial agent (19 out of 48 ticks) in the study. This was also the case in the study of Zeringóta et al. (2016), where 42 haplotype Nazaré ticks and a single larva of *A. parkeri* were reported as infected with this bacterium. These findings, raise the status of the ApPR variant from a rarely detected rickettsial agent, to that of the most (at least) numerically abundant *R. parkeri*-like organism reported in bird ticks in Brazil, with records from four states in the South-East and South of the country. Importantly, the detection of this agent in both larvae and nymphs of *A. parkeri* suggest that it may be efficiently perpetuated transstadially. In addition, its presence in larvae of haplotype Nazaré ticks collected over a 12-month period (Zeringóta et al., 2016), could indicate transovarian transmission and maintenance. An alternative explanation would be the existence of a vertebrate reservoir for this agent. Unfortunately, knowledge of the host range of haplotype Nazaré ticks is limited to their association with birds. In contrast, *A. parkeri* is documented as frequently associated with porcupines from the family Erethizontidae (Rodentia) and has been recorded parasitizing the brown howler monkey (*Alouatta guariba*) and a single human being in São Paulo state (Martins et al., 2013; Guglielmone et al., 2014). The latter associations could suggest a predisposition for primates.

Two other variants of *R. parkeri*, were also recorded in this study. The first was strain NOD, an agent of undetermined pathogenicity, previously identified in association with *A. nodosum*, *A. calcaratum* and *A. longirostre* recovered from birds in different regions of Brazil (Ogrzewalska et al., 2009b, 2013; Pacheco et al., 2012; Ramos et al., 2015; Ogrzewalska and Pinter, 2016). The detection of this variant in two *A. nodosum* ticks in the present study, was of limited significance, but served to extend records of its distribution to the state of Rio de Janeiro.

In contrast, the detection of the (ARF) *Rickettsia* sp. in a larva of *A. ovale*, recovered from a specimen of *Turdus rufiventris* could be considered significant for several reasons. Firstly, it represented the first record of this emerging human pathogen in the state of Rio de Janeiro; secondly, it defined another region in Brazil from which infected ticks have been identified and thirdly, it provided evidence of a role for birds in the dispersal of *R. parkeri*-infected *A. ovale* ticks. This potential role could be viewed as the missing piece of our knowledge of the bio-ecology of this important tick-pathogen association in Brazil, where the ARF strain has previously been reported in free living ticks and in *A. ovale* collected from dogs and humans (Szabó et al., 2013; Barbieri et al., 2015; Luz et al., 2016a, 2016b; Nieri-Bastos et al., 2016). A similar role for birds as dispersers of *A. tigrinum* ticks infected with human pathogenic strains of *A. parkeri* was recently proposed by Argentinian researchers (Flores et al., 2016).

Bird ticks represent a valuable resource for studies on the bio-ecology of tick-borne diseases. The current study showed that despite the existence of a substantial body of data on this topic, there is still much to be revealed. To fully disclose the true diversity of the ixodofauna of birds, together with information on their potential role as vectors and/or reservoirs of pathogens, there is a pressing need to develop strategies that will allow large scale, cost effective, species level identification of all the ticks collected during surveys.

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