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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

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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,

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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;