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Ticks parasitizing wild birds in Portugal: detection of *Rickettsia aeschlimannii, R. helvetica* and *R. massiliae*

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Abstract From January 2002 to December 2004, 152 ticks were collected from 40 wild birds recovered in Santo André Natural Reserve and Monsanto Forestal Park, Portugal mainland. Five ticks species were identified from 22 species of birds, and new host record were provided for some species. In addition, 32 (21%) ticks were screened by PCR to detect infections with agents belonging to order Rickettsiales: *Anaplasma phagocytophilum, Ehrlichia chaffeensis*, and *Rickettsia* spp. PCR amplicons were obtained in 5 (15.6%) tick samples. *Rickettsia* DNA exhibiting *gltA* sequences similar to those of *Rickettsia aeschilimannii, R. helvetica* and *R. massiliae* were identified in *Hyalomma marginatum, Ixodes ventalloi* and in *Rhipicephalus turanicus*, respectively. This is the first report of rickettsiae infections in ticks collected from wild birds in Portugal. Giving the results presented above wild birds play an important role in the maintenance and dissemination of several tick species and associated rickettsiae.

Keywords Birds · Ticks · *Rickettsia* · Portugal

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Introduction

Wild birds are known as important reservoir of some pathogens and disseminators hosts of several arthropods, including ticks. Concerning rickettsial pathogens, birds have been described as reservoirs for *Coxiella burnetii* (Syrucek and Raska 1956) and as hosts for ticks infected with *Rickettsia sibirica* (Somov and Soldatov 1964 in Hubalek 2004) and *Anaplasma phagocytophilum* (Alekseev et al. 2001; Bjoersdorf et al. 2001; Daniels et al. 2002). In Portugal, wild birds were found parasitized by several tick species, namely *Hyalomma lusitanicum, H. marginatum, Ixodes canisuga, I. frontalis, Rhipicephalus pusillus, R. sanguineus, R. turanicus* and *Ornithodoros maritimus* (Dias 1994; Silva et al. 2001). However, there are no references about the occurrence of rickettsial infections neither on birds nor in the ticks they harbour. The aim of this work was to collect more data about tick species that parasitize wild birds in Portugal and to study the occurrence of rickettsial infections in those arthropods.

Material and methods

Between January 2002 and December 2004, wild birds were captured in mist nets at Santo André Natural Reserve, SANR (38 °1′N, 8 °49′W) and in the Bird Rehabilitation Centers of Monsanto Forest Park, MFP (38°44′N, 9°8′W) and Quercus Santo André, QSA (38°1′N, 8°49′W). Bird identification and tick collection were done according to Nature Preservation Institute (ICN) methodologies (CEMPA/ICN 1995; Silva et al. 2001). Ticks were identified by morphological characters using standard taxonomic keys (Cordas et al. 1993; Dias 1994) and separated by species, instars and sex. Ticks that were collected alive and/or in good conditions were preserved at –80°C and processed individually for DNA extraction, as previously described (Schouls et al. 1999). Briefly, each tick was washed in 70% ethanol solution, dried and boiled for 20 min in 100μl of 0.7 M ammonium hydroxide to free the DNA. After cooling, ammonia was evaporated for 20 min at 90°C. To monitor the occurrence of false-positive samples, laboratory ticks not infected were included during DNA extraction. As positive controls we have used *A. phagocytophilum* Webster strain, *E. chaffeensis* Arkansas strain, *R. conorii*. Tick lysate was used directly for PCR. DNA amplifications were performed in a Biometra T-3 thermoblock thermal cycler employing TaqPCR master mix kit (Qiagen) according to manufacturer recommendations. Five sets of primers were used for rickettsial DNA detection according to previous descriptions: (1) Msp465f/Msp980r derived from the highly conserved regions of major surface protein-2 (*msp2*) paralogous genes of *Anaplasma phagocytophilum* (Caspersen et al. 2002); (2) ECC/ECB associated with HE1/HE3 for the amplification of *Ehrlichia chaffeensis* 16S rRNA fragment (Dawson et al. 1996); (3) RpCs.877p/RpCs.1258n and Rr190.70p/Rr190.602n, targeting the rickettsial genes for citrate synthase (*gltA*) and outer membrane protein A (*ompA*), respectively (Regnery et al. 1991). Positive PCR products were sequenced, after DNA purification by a MiniElute PCR Purification Kit (Qiagen), using an ABI automated sequencer (Applied Biosystems) according to manufacturer's instructions. Sequenciation was performed with the forward and reverse primers used for PCR identification. Sequences were identified using the BLAST software (Altschul et al. 1990, 1997).

Nucleotide sequence accession numbers

The GenBank nucleotide sequence accession numbers for partial sequences of *gltA* a gene generated in this study are for PoTiRAv3:DQ459392. For partial sequences of *gltA* and *ompA* gene generated for PoTiRAv26 are DQ459393 and DQ4459388, respectively. For PoTiRAv20 are: DQ459394 and DQ459389, respectively. For PoTiRAv25 are: DQ459395 and DQ459390, respectively. For PoTiRAv27 are: DQ459396 and DQ459391, respectively.

Results

One hundred fifty two ticks were collected from 40 birds, belonging to 22 species. Five species of ticks were found: *Hyalomma marginatum* (111 nymphs, 7 larvae), *Ixodes ventalloi* (11 adults, 3 nymphs), *Ixodes frontalis* (3 adults, 1 nymph), *Rhipicephalus turanicus* (4 adults) and *Haemaphysalis punctata* (3 nymphs). Six ticks (3 adults, 2 nymphs, 1 larva) were grouped as *Ixodes* spp. due to the lack of body parts essential for specific identification. The instars ratio of collected ticks was 22 (14.5%) adults, 122 (80.2%) nymphs, 8 (5.3%) larvae. *H. marginatum* was the most abundant tick, infesting 77.5% (31/40) wild birds, mainly as nymphal stage. Regarding host preferences, this species also showed the widest host range, parasitizing 15 birds species. All these data are summarized in Table 1.

Of thirty-two ticks (21%) screened by PCR we were able to detect rickettsial DNA in 5 (15.6%) specimens (Table 2). Three ticks, identified as *Hyalomma marginatum* contained a rickettsia exhibiting nucleotide sequence of *gltA* 100% (347/347 bp) similar to *R. aeschlimannii* (U59722) and also the same similarity for *ompA* 100% (378/378 bp) (DQ379982). One *Rhipicephalus turanicus* was detected with rickettsial DNA exhibiting nucleotide sequence 100% similar to *R. massiliae* to both *gltA* (347/347 bp) (U59722) and *ompA* (334/334) (U43799). In one *Ixodes ventalloi* was identified a nucleotide sequence of *gltA* 100% (347/347) similar to *R. helvetica* (U59723). All ticks tested negative for the presence of DNA for *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*.

Discussion

This study is the first report of the presence of *Haemaphysalis punctata* and *Ixodes ventalloi* parasitizing wild birds in Portugal. Additionally, is documented for the first time the occurrence of *Haemaphysalis punctata* in *Acrocephalus scirpaceus, Emberiza cirlus* and *Turdus merula; Hyalomma marginatum* in *Athene noctua, Carduelis chloris, Hirundo rustica, Lanius meridionalis, Milvus migrans, Milvus milvus, Parus caeruleus, Parus major, Passer montanus, Tyto alba* and *Turdus merula; I. frontalis* in *Asio otus; Ixodes ventalloi* in *Asio flammeus; Rhipicephalus turanicus* in *Aquila nipalensis* and *Buteo buteo*.

Most of the tick species and instars described here are commonly associated to birds. Immatures of *Hyalomma marginatum*, well known as vectors of pathogens to man and animals, are frequently transported both northward and southward by birds being one of the most recorded ticks on these host (Hoogstraal 1956, 1961; Hoogstraal and Kaiser 1961; Hoogstraal et al. 1961, 1963; Hoogstraal and

Table 1 List of wild birds parasitized by ticks

Bird Species	No of infested birds	Site	Tick species (number and stage)					
			Hyalomma marginatum	*Haemaphysalis punctata*	*Ixodes frontalis*	*Ixodes* spp.[a]	*Ixodes ventalloi*	*Rhipicephalus turanicus*
Asio otus (Long-eared owl)	1	MFP/QSA			3F; 1N			
Asio flammeus (Short-eared owl)	2	MFP/QSA				1F; 2N; 1L	5M; 7F; 5N	
Alcedo atthis (Kingfisher)	2	MFP/QSA	15N					
Athene noctua (Little owl)	2	MFP/QSA	3N					
Acrocephalus scirpaceus (Reed warbler)	3	SANR	2N	1N				
Aquila nipalensis (Steppe eagle)	1	MFP/QSA						1F
Bubo bubo (Eagle owl)	1	MFP/QSA	4N					
Buteo buteo (Common buzzard)	1	MFP/QSA						3F
Carduelis carduelis (Goldfinch)	1	MFP/QSA				1F		
Carduelis chloris (Greenfinch)	1	SANR	1N					
Emberiza cirlus (Cirl bunting)	1	SANR		1N				
Hirundo rustica (Barn swallow)	1	SANR	1N					
Lanius meridionalis (Iberian great grey shrike)	2	SANR	19N					
Milvus migrans (Black kite)	1	SANR	7N					
Milvus milvus (Red kite)	1	MFP/QSA	4L					
Parus caeruleus (Blue tit)	2	SANR	3N					
Parus major (Great tit)	1	SANR	3N					
Passer montanus (Tree sparrow)	2	SANR	2N					
Saxicola communis (Common whitethroat)	1	SANR				1F		
Saxicola torquata (Common stonechat)	6	SANR	28N					
Turdus merula (Black bird)	5	SANR	20N	1N				
Tyto alba (Barn owl)	2	SANR	3N; 3L					
Total (no. ticks/no. birds)	40		118/31	3/9	4/1	6/4	17/2	4/2

M—male; F—female; N—nymph; L—larvae

[a] All the *Ixodes* spp could not be attributed to any species as they were lacking parts necessary for identification

Table 2 List of wild birds parasitized with ticks infected with rickettsial agents

Bird species[a]	Origin	Species of tick	No. positive ticks/ no. tested	Rickettsial detection/tick instars		
				Ehrlichia chaffeensis	*Anaplasma phagocytophilum*	*Rickettsia* spp.
Alcedo atthis (OM/S)	MFP/QSA	*Hyalomma marginatum*	1/7	–	–	*R. aeschlimannii*/1N
Athene noctua (R/F)	MFP/QSA	*Hyalomma marginatum*	1/1	–	–	*R. aeschlimannii*/1N
Asio flammeus (M/F)	MFP/QSA	*Ixodes ventalloi*	1/17	–	–	*R. helvetica*/1M
Buteo buteo (OM/S)	MFP/QSA	*Rhipicephalus turanicus*	1/3	–	–	*R. massiliae*/1F
Bubo bubo (R/S)	MFP/QSA	*Hyalomma marginatum*	1/4	–	–	*R. aeschlimannii*/1N
Total			5/32			

[a] (Bird status: M—migrant, OM—occasional migrant, R—resident; M—male; F—female; N—nymph/season of capture: S—Spring; F—Fall)

Aeschlimann 1982). This study corroborates that finding since *H. marginatum* was the most abundant tick and the species that showed the widest host range. In addition, these tick species might play a role in public health in Portugal, regarding rickettsial diseases. PCR testing has shown that *H. marginatum* was infected with *Rickettsia aeschlimannii*, an agent that has been recently implicated in human disease (Raoult et al. 2002). The genus *Ixodes* includes several species that are commonly or even exclusively associated with birds in Europe (Papadopoulos et al. 2001). *I. frontalis* is known to be exclusively associated with birds, which is in accordance to what we have been observing in our country. Regarding *I. ventalloi*, the bird association is not strict, although we found it on two birds. In fact, the original description of *I. ventalloi* was based on the observation of specimens collected on birds (Gil Collado 1936). The present work also describes *I. ventalloi* infection by *R. helvetica*, associated with human cases of chronic perimyocarditis (Nielsson et al. 1999). *R. helvetica* is commonly associated with *I. ricinus* ticks, which is considered the main vector implicated in human cases. The presence of this rickettsiae in alternative ticks could be attributable to the existence of secondary maintenance cycles, where agents circulate between relatively host-specific, usually non-human biting ticks and their hosts, as described for other tick-borne Rickettsiales in places where *I. ricinus* and other *Ixodes* species co-exist (Bown et al. 2003). Those additional cycles would buffer the agent from local extinction and help to re-establish the primary cycles. This hypothesis might explain our results since it has been observed that the distribution of *I. ricinus* is followed by *I. ventalloi*, in part of the country (Santos et al. 2004). In addition, the potential role of this species in public health might be considered since there are reports of human parasitism by *I. ventalloi* (Gilot and Marjolet 1982). In the literature, immatures of *Haemaphysalis punctata* are also frequently detected on Passeriformes birds, preferentially *Emberiza cirlus* (Osacar-Jimenez et al. 1998). Although rarely collected in Portugal, the presence of *H. punctata* nymphs in wild birds seems to corroborate that finding, but more birds need to be studied (especially *Emberiza cirlus*). It is worth mentioning that we have also found *Rhipicephalus turanicus* that is not commonly associated with birds. This finding could be attributable to bird's health conditions since both birds parasitized by this tick were wounded. This fact could have allowed an unusual contact with the ground which might has favoured their parasitism by *R. turanicus*. PCR assays have shown the infection of this tick species by *Rickettsia massiliae* recently shown to be pathogenic (Giustina et al. 2006).

This is the first report of rickettsiae detection in ticks collected from wild birds in Portugal, although the tick species reported here have been already described in other hosts in different regions of Portugal (Caeiro 1999; Estrada-Peña and Santos-Silva 2005). In a study conduct by Bacellar (1999), the prevalence of infection of Rickettsia like organisms (positive by hemolymph test) in different tick species was: 11.2% (33/294) in *H. marginatum*, 3.6% (13/361) in *R. turanicus*, and 100% (4/4) in *I. ventalloi*. Moreover the successful isolation of rickettsia by shell-vial was achieved in 2 (3.6%) *H. marginatum*, 1 (1.8%) *I. ventalloi* and in 6 (11%) *R. turanicus*. The strain isolates were characterized as *Rickettsia aeschlimannii, R. helvetica* and *R. massiliae*, respectively (Bacellar 1999). Here were found similar rates of rickettsial infection in the same tick species (Table 2). Nevertheless, previous studies were based only on haemocyte test and isolation, comparing with our study that only reports the detection of rickettsial pathogens by PCR. Regarding the migratory

status of birds, the occurrence of *R. helvetica* in *I. ventalloi* collected on migrating *Asio flammeus* potentially reflects the long-distance dispersion of tick-borne pathogens and the settlement of new focus. *Asio flammeus* was captured during fall migration to the south, which means that ticks and their pathogens could have originated in areas of Eastern Europe, where *R. helvetica* is commonly found. Moreover, occasional migrating *Alcedo atthis*, and *Buteo buteo* and resident *Athene noctua* and *Bubo bubo* are more likely to contribute to the maintenance and amplification of local endemic rickettsiae focus. In conclusion, this study corroborates the important role of birds in tick's dissemination and their influence on epidemiology of tick-borne rickettsiae agents in Portugal. Improvement of our knowledge about avian migration patterns and the agents that bird ticks harbour might be useful in helping to predict future outbreaks of infection due to emerging zoonotic tick-borne pathogens.

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