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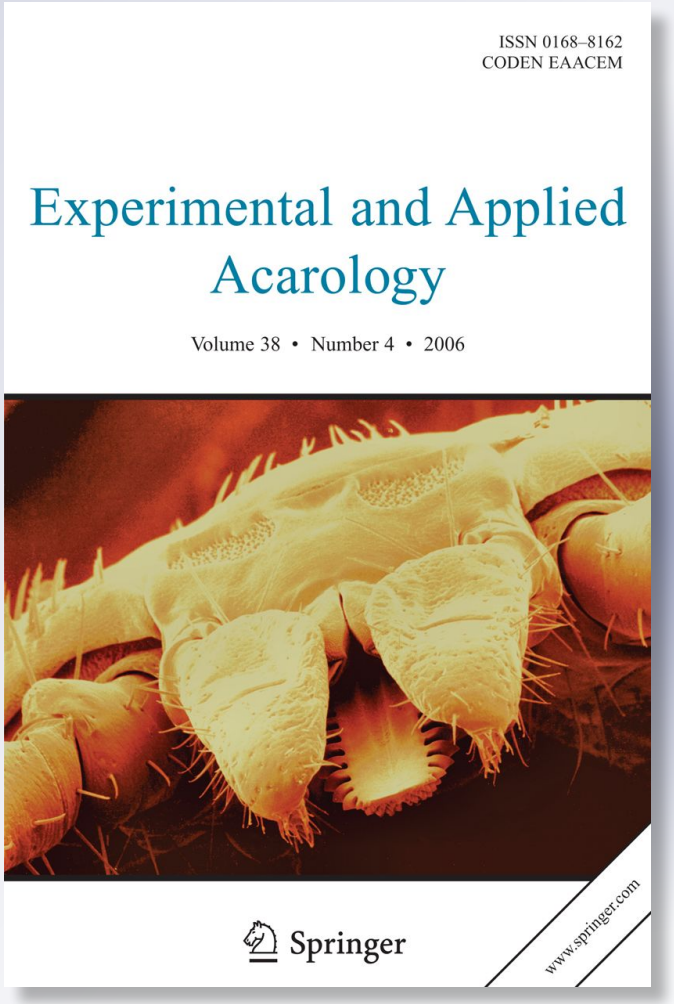

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Abstract The present study aimed to identify ticks collected from road-killed wildlife species retrieved in several localities of southern Italy and to assess the presence of *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum* and *Rickettsia* spp. DNA in ticks. Collections were carried out from January 2000 to December 2009 on wild animals found dead within the territories of 11 municipalities from three regions (i.e., Apulia, Basilicata, and Calabria). In total, 189 carcasses of wild animals belonging to 10 species were checked for tick infestation, and 40 animals belonging to seven species were found parasitized. One hundred and twenty-five ixodid ticks (11 larvae, 14 nymphs, 77 males, and

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23 females) were collected and identified as belonging to nine species, namely *Dermacentor marginatus*, *Haemaphysalis erinacei*, *Hyalomma marginatum*, *Ixodes acuminatus*, *Ixodes canisuga*, *Ixodes hexagonus*, *Ixodes ricinus*, *Rhipicephalus bursa*, and *Rhipicephalus turanicus*. None of the 36 tick specimens tested by PCR was positive for tick-borne pathogens. The results add new information on the tick fauna associated with wild animals in Italy, reporting new tick-host associations. Further field studies are still needed to ascertain the suitability of certain wildlife species as hosts for some tick species, particularly for those implicated in the transmission of pathogens to domestic animals and humans. Finally, from a conservation perspective, it would be interesting to assess whether these wild animals (e.g., *Lepus corsicanus*) are exposed to tick-borne pathogens, investigating the possible implications for their health and behavior.

Keywords Ixodidae · Wildlife · *Borrelia burgdorferi* sensu lato · Lyme Borreliosis

Introduction

Many of the about 700 known species of ixodid ticks (Acari, Ixodidae) act as vectors of pathogens of veterinary and medical concern, including bacteria, protozoa, viruses, and filarial nematodes (de la Fuente et al. 2008). In addition to their impact on livestock production and welfare (Jongejan and Uilenberg 2004), ixodid ticks have a high importance from a conservation standpoint, being able to thwart the fitness of wildlife species (Daszak et al. 2000; Pfäffle et al. 2009). In fact, wild animals may play a crucial role in the epidemiology of tick-borne diseases (TBDs) and in the maintenance of tick populations in certain areas (Bengis et al. 2004). In Italy, several wildlife species have been found to harbor genetic material of various tick-borne pathogens potentially affecting both domestic animals and humans (Cancrini et al. 2007; Iori et al. 2010; Rizzoli et al. 2004; Tampieri et al. 2008). These findings indicate that wild animals are often exposed to tick-borne pathogens, although their role as reservoir or amplifying hosts for such pathogens needs further investigation.

Tick distribution being influenced by the availability of suitable hosts for both immature and adult stages and by environmental (e.g., vegetation patterns) and climatic factors (e.g., temperature and relative humidity) (Oorebeek and Kleindorfer 2008), the study of tick fauna of wild animals can be indicative of ecological changes, especially when unexpected tick-host interactions are found. Furthermore, knowing the tick community infesting wildlife could also help evaluate and, possibly, minimize the impact of these arthropods on the fitness and survival of vulnerable host species (e.g., the Corsican hare, *Lepus corsicanus*) (IUCN 2010). To date, however, research on ticks infesting wildlife in Italy has been patchy, with most of the reports referring to the north and the central parts of the country (Carpi et al. 2008; Curioni et al. 2004; Iori et al. 2010). Therefore, the present study aimed to identify tick species collected from road-killed wildlife species retrieved in several localities of southern Italy and screen them for *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum*, and *Rickettsia* spp. DNA.

Materials and methods

Study area, wild animals, and ticks

The study was carried out from January 2000 to December 2009 on carcasses of wild animals found dead in several localities distributed in three major geographical regions in southern

Table 1 Localities from where road-killed wildlife were retrieved in southern Italy

Municipality	Coordinates	Province	Region
Acquaviva delle Fonti	40°54′N, 16°51′E	Bari	Apulia
Casarano	40°1′N, 18°10′E	Lecce	Apulia
Lecce	40°21′N, 18°10′E	Lecce	Apulia
Leverano	40°17′N, 18°5′E	Lecce	Apulia
Maglie	40°7′N, 18°8′E	Lecce	Apulia
Oliveto Lucano	40°32′N, 16°11′E	Matera	Basilicata
Pignola	40°34′N, 15°47′E	Potenza	Basilicata
Cariati	39°30′N, 16°57′E	Cosenza	Calabria

Italy (Table 1). Climate in all study sites is typical of the Mediterranean biome, characterised by hot and dry summer and moderately cold and rainy winter season (Rundel et al. 1998). During the study period, wild animals found dead in the aforementioned sites were brought to the Unit of Veterinary Parasitology and Parasitic Diseases, Faculty of Veterinary Medicine, University of Bari (Italy), to be checked for tick infestation. All carcasses were collected by the veterinary and para-veterinary personnel employed either in the 'Gallipoli Cognato, Piccole Dolomiti Lucane' natural park (Basilicata) or elsewhere in the study area, and kept frozen until examination. Once in the laboratory, a thorough inspection of skin and hair of each carcass was carried out. Immature and adult ticks were then collected from all animals, and stored in 70% ethanol vials, labeled according to host species, before being morphologically identified using the taxonomic keys of Manilla (1998).

DNA extraction and PCR amplification

Following identification, 36 randomly selected ticks were screened by polymerase chain reaction (PCR) for the presence of *B. burgdorferi* s.l., *A. phagocytophilum* and *Rickettsia* spp. DNA. DNA extraction was performed using All Prep DNA/RNA mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. A multiplex real-time PCR was used for the simultaneous detection of *B. burgdorferi* s.l. and *A. phagocytophilum* using the primers and protocol suggested by Courtney et al. (2004). Real-time PCR was carried out on a Rotor Gene 6,000 real-time PCR system (Corbett, Australia). For *Rickettsia* spp. the common gltA gene was targeted using CS-78 and CS-323 primers as suggested by Labruna et al. (2004). PCRs were performed in an Applied Biosystems Thermocycler (Gene Amp PCR System 9,700) in a final volume of 50 µl containing 5 µl of DNA template, 31.7 µl of molecular-grade water, 5 µl of buffer 10×, 3 µl of $MgCl_2$ 25 mM, 1 µl of dNTPs 10 mM, 2 µl of primers CS-78 (forward) 10 µM and 2 µl of CS-323 (reverse) 10 µM, and 0.3 µl of AmpliTaq Gold 5U. The thermal cycling profile consisted of an initial cycle at 94°C for 10 min, followed by 35 cycles for 15 s at 95°C, 30 s at 55°C, and 30 s at 72°C; and a final cycle at 72°C for 7 min.

Results

A total of 125 ticks (11 larvae, 14 nymphs, 77 males, and 23 females) belonging to nine species (i.e., *Dermacentor marginatus*, *Haemaphysalis erinacei*, *Hyalomma marginatum*,

Ixodes acuminatus, *Ixodes canisuga*, *Ixodes hexagonus*, *Ixodes ricinus*, *Rhipicephalus bursa*, and *Rhipicephalus turanicus*) were collected from 40 wild animals belonging to 7 species (Tables 2, 3). The following tick-host associations (number of occurrences between parentheses) were found: *D. marginatus* on *Canis lupus* (1), *Martes foina* (1), *Vulpes vulpes* (1); *H. erinacei* on *Erinaceus europaeus*, *M. foina*, *Mustela nivalis* (1); *H. marginatum* on *L. corsicanus* (1); *I. acuminatus* on *C. lupus* (1); *I. canisuga* on *V. vulpes* (2); *I. hexagonus* on *E. europaeus* (2), *Felis silvestris* (1), *M. foina* (1); *I. ricinus* on *M. foina* (1), *V. vulpes* (1); *R. bursa* on *M. foina* (2), *V. vulpes* (1); *R. turanicus* on *E. europaeus* (8), *F. silvestris* (1), *M. foina* (4), *V. vulpes* (5). No ticks were found on European hares (*Lepus europaeus*), European badgers (*Meles meles*), and crested porcupines (*Hystrix cristata*). Eight animals were infested by more than one tick species, as follows: one *E. europaeus* by *H. erinacei*, *I. hexagonus*, and *R. turanicus*; three *E. europaeus* by *H. erinacei* and *R. turanicus*; one *M. foina* by *D. marginatus* and *R. turanicus*; one *V. vulpes* by *I. canisuga* and *R. turanicus*; one *V. vulpes* by *D. marginatus* and *R. turanicus*; one *V. vulpes* by *H. erinacei*, *I. ricinus*, *R. bursa*, and *R. turanicus*.

Among the tick species identified, *R. turanicus* was the one with the largest host range ($n = 4$), followed by *D. marginatus*, *H. erinacei*, and *I. hexagonus*¸ retrieved on three different host species each (Table 2). With reference to the hosts, *V. vulpes* and *M. foina* harbored the greatest variety ($n = 6$) of tick species, followed by *E. europaeus* (3 species), *C. lupus* and *F. silvestris* (2 species). *Haemaphysalis erinacei* was the most common tick infesting *E. europaeus* and *M. foina* and the only tick retrieved on *M. nivalis*, whereas *R. turanicus* was the most predominant species on *V. vulpes* (Table 3).

None of the ticks tested by PCR (i.e., 24 *R. turanicus*, 4 *R. bursa*, 2 *I. canisuga*, 4 *I. hexagonus*, 1 *I. ricinus*, 1 *D. marginatus*) was positive for *B. burgdorferi* s.l., *A. phagocytophilum* or *Rickettsia* spp.

Discussion

Ixodid ticks were collected from 40 wild animals belonging to 7 species, whose carcasses were retrieved in different localities in southern Italy, during a 9-year period. Ticks were present on most of the beech marten and hedgehog carcasses examined, suggesting that tick infestation on these two wildlife species in southern Italy is frequent. Worth noting, tick infestation was recently acknowledged as responsible for regenerative anemia and

Table 2 Ticks collected from wildlife carcasses retrieved between January 2000 and December 2009 in southern Italy

Tick species	Larvae	Nymphs	Males	Females	Total	Host richness
Dermacentor marginatus	0	0	4	0	4	3
Haemaphysalis erinacei	0	0	42	10	52	3
Hyalomma marginatum	0	0	0	1	1	1
Ixodes acuminatus	0	1	0	0	1	1
Ixodes canisuga	2	2	0	0	4	1
Ixodes hexagonus	9	10	0	1	20	3
Ixodes ricinus	0	1	1	0	2	2
Rhipicephalus bursa	0	0	4	1	5	2
Rhipicephalus turanicus	0	0	26	10	36	4

Table 3 Wildlife carcasses and associated ticks retrieved between January 2000 and December 2009 in southern Italy

Host	Examined	Infested	Ticks[a]
Canis lupus	3	2	*Dermacentor marginatus* (2 M), *Ixodes acuminatus* (1 N)
Erinaceus europaeus	32	14	*Haemaphysalis erinacei* (3 F, 28 M), *Ixodes hexagonus* (9 L, 1 F), *Rhipicephalus turanicus* (2 F, 13 M)
Felis silvestris	8	2	*I. hexagonus* (8 N), *R. turanicus* (1 F)
Lepus corsicanus	7	1	*Hyalomma marginatum* (1 F)
Martes foina	30	14	*D. marginatus* (1 M), *H. erinacei* (6 F, 13 M), *I. hexagonus* (2 N), *Ixodes ricinus* (1 N), *Rhipicephalus bursa* (4 M), *R. turanicus* (1 F, 5 M)
Mustela nivalis	2	1	*H. erinacei* (1 M)
Vulpes vulpes	81	6	*D. marginatus* (1 F), *H. erinacei* (1 F), *Ixodes canisuga* (2 L, 2 N), *I. ricinus* (1 M), *R. bursa* (1 F), *R. turanicus* (5 F, 9 M)

No ticks were found in the following wildlife species (no. of exemplars examined are in parentheses): *Lepus europaeus* (13), *Meles meles* (11), *Hystrix cristata* (2)

[a] Figures within parentheses represent the number of specimens identified and abbreviations are as follows: *L* larva, *N* nymph, *F* female, *M* male

immunodeficiency, leading to secondary infections, in hedgehogs (Pfäffle et al. 2009). Interestingly, both beech martens and hedgehogs were most frequently infested by *H. erinacei*. Considering the ecology of these two wildlife species and *H. erinacei* (Iori et al. 2006; Manilla 1998), it is likely that both hosts were parasitized by this endophilic triphasic tick while resting in their dens. In particular, the retrieval of a large number of *H. erinacei* adults on beech marten carcasses, as previously reported in the same southern Italian regions (Otranto et al. 2007), suggests the role of mustelids as suitable hosts not only for immature stages of this tick species. Moreover, the finding of *H. erinacei* also on foxes and weasels is consistent with the host associations recorded to date for this tick species, known for feeding on hedgehogs and their predators (Manilla 1998). While the absence of ticks on crested porcupines can be related to the paucity of carcasses examined, the lack of ticks on *M. meles* (11 carcasses) and *L. europaeus* (13 carcasses) was somewhat unexpected, as the former species is usually associated with *Ixodes* ticks (Beichel et al. 1996; Liebisch and Walter 1986; Millán et al. 2007) and the latter with *Ixodes* and *Haemaphysalis* ticks (Gyuranecz et al. 2010).

Not surprisingly, *I. canisuga* was found exclusively on red foxes, which are regarded as the preferential host for such tick species, commonly known as 'the fox tick' (Dantas-Torres et al. 2009; Liebisch and Walter 1986). In particular, the finding of a single larva of *I. acuminatus* on a grey wolf is likely to represent an accidental tick-host association, as immature stages of this tick feed predominately on insectivores and rodents (Iori et al. 2006).

Results from the present study also highlight the broadness of *R. turanicus*' host range. Considered to be the tick species most frequently associated with sheep in Italy (Genchi and Manfredi 1999), *R. turanicus* was frequently found on hedgehogs, foxes and also on beech martens, with the latter species representing, to the best of the authors' knowledge, a new host record for this tick. The finding of *R. turanicus* on hedgehogs is recorded for the first time in Italy and corroborates previous reports from Portugal (Rosalino et al. 2007).

The retrieval of only adult specimens of *R. turanicus* was somewhat expected due to the fact that immature stages are usually associated with several species of rodents (Walker et al. 2000).

Interestingly, out of 20 *I. hexagonus* collected throughout the study period, 8 specimens were retrieved on a wildcat, representing the first record of this tick-host association in Italy. Certainly, a single finding of a wildcat infested by 8 *I. hexagonus* nymphs does not allow us to speculate about the frequency of this tick-host association in nature and whether wildcats are suitable hosts for immature stages of this tick. In any case, further investigations on the suitability of wild carnivores as hosts for *I. hexagonus* would be desirable, since the distribution patterns of this tick species could be more affected by such hosts rather than by hedgehogs or other insectivores, due to the larger home range, predatory and scavenger habits of the former hosts (Hoogstraal and Aeschlimann 1982). Overall, the paucity of *I. ricinus* and *H. marginatum*, often associated with wildlife in Italy (Dantas-Torres et al. 2011), could be due to the fact that carcasses of animals such as wild boars, roe deer, rodents or birds were not examined in this study. In particular, the only *I. ricinus* retrieved was collected from a fox whose carcass was found within the boundaries of the 'Gallipoli Cognato, Piccole Dolomiti Lucane' natural park, representing the first record of such a tick-host association in Basilicata. Noteworthy was the absence of *Ixodes* ticks attached onto the 20 hares examined, considering that lagomorphs are known to be suitable for feeding of all stages of *I. ricinus* (Tälleklint and Jaenson 1993). The only *H. marginatum* specimen collected was retrieved on *L. corsicanus*, in agreement with the results from a recent survey carried out in the 'Gallipoli Cognato, Piccole Dolomiti Lucane' wildlife reserve (Dantas-Torres et al. 2011). The finding of *D. marginatus* on *M. foina* represents a new host record in Italy, but, again, should be interpreted with caution, as further investigations are needed to better understand the validity of this tick-host association. In any case, this finding is not surprising considering the wide host range displayed by *D. marginatus* (Manilla 1998).

In our study, all screened ticks resulted negative for *B. burgdorferi* s.l., *A. phagocytophylum* and *Rickettsia* spp., probably also due to the low number of tested ticks. Indeed, because positivity to pathogens is not only affected by the area of sampling but also by the host species from which ticks were collected, negative PCR results of this study could be due to the fact that ticks were not collected from wild boars, small rodents, or birds, which may act as either reservoirs or carriers of *Borrelia* spirochetes (Comstedt et al. 2006; Gern 2008; Humair 2002; Juricová and Hubálek 2009; Matuschka et al. 1992; Matuschka et al. 1996) as well as *A. phagocytophilum* (Stefanidesova et al. 2007) and spotted fever group rickettsiae (Schex et al. 2011; Stefanidesova et al. 2007). Furthermore, considering the involvement of several of the tick species collected as vectors of pathogens of zoonotic concern (e.g., *Babesia* spp., *Coxiella burnetii*, tick-borne encephalitis and Crimean-Congo haemorragic fever viruses) (Gray et al. 1991; Hoogstraal 1979; Rehácek et al. 1991; Rizzoli et al. 2004), targeting a broader range of bacterial, rickettsial, protozoal and viral zoonotic tick-borne pathogen genes would also be advisable for future studies.

In conclusion, the present study broadens the knowledge on the tick fauna infesting wildlife in Italy, showing a relatively high degree of diversity of tick species associated with 7 different wild hosts. The most abundant tick species was *H. erinacei*, which was found on three different host species, whereas *R. turanicus*, the second most abundant species, was found on four hosts. These findings suggest that adults of these tick species exhibit relatively low host specificity. Remarkably, new tick-host relationships were recorded for the first time in Italy (i.e., *D. marginatus* on beech marten; *I. acuminatus* on grey wolf; *I. hexogonus* on wildcat; *R. turanicus* on beech marten and hedgehog), and in

the Basilicata region (i.e., *I. ricinus* on red fox). Undoubtedly, the significance of these tick-host associations deserves further investigation, as some of these animals might actually be accidental hosts for some of these ticks. Although none of the ticks molecularly tested was positive for tick-borne pathogens, further studies are desirable in order to assess the circulation of pathogens within this tick population. Certainly, this will be valuable for assessing the risk of exposure to tick-borne infections for both animals and humans as well as for planning effective surveillance and prevention initiatives.

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