Thelazia callipaeda (Spirurida, Thelaziidae) in wild animals: Report of new host species and ecological implications

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ABSTRACT

Thelazia callipaeda infects the eyes of carnivores and humans in Far Eastern Asiatic and European countries. Studies have demonstrated the occurrence of T. callipaeda in foxes from areas where canine thelaziosis is endemic. However, there is little information on the role of wild carnivores as hosts of this nematode. From May 2003 to May 2009, a total of 130 carcasses of red foxes (Vulpes vulpes; n = 75), wolves (Canis lupus; n = 2), beech martens (Martes foina; n = 22), brown hares (Lepus europaeus; n = 13), Eurasian badgers (Meles meles; n = 10), and wild cats (Felis silvestris; n = 8) were examined in an area of southern Italy where canine thelaziosis is highly prevalent. At necropsy, animals were examined and nematodes were collected from the conjunctival sacs of both eyes. All nematodes were morphologically identified and at least five specimens from each of the five host species were molecularly processed by PCR amplification and sequencing of a partial mitochondrial cytochrome c oxidase subunit 1 gene (cox1). Five out of the six wild animal species examined were found to be infected with eyeworms. The overall infection rate, excluding the Eurasian badgers that were all negative, was 39.1%. All the 189 adult nematodes collected (intensity of infection = 4 ± 2.2) were morphologically identified as T. callipaeda. The molecular analysis confirmed that the only haplotype of T. callipaeda circulating in Europe (i.e., haplotype 1) is present in that area. The competence of red foxes, wolves, beech martens, brown hares, and wild cats as definitive hosts for T. callipaeda is discussed in relationship to their ecology and their likely exposure to the vector Phortica variegata in the study area. The role the wild fauna plays in maintaining and spreading eyeworm infection in humans and domestic animals is also discussed.

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1. Introduction

Thelaziosis, also known as eyeworm infection, is caused by nematodes of the genus Thelazia (Spirurida: Thelaziidae), which are transmitted by secretophagous flies into the orbital cavities and surrounding tissues of many species of wild and domestic mammals (reviewed in Otranto and Traversa, 2005). Out of 16 species of Thelazia described so far, Thelazia callipaeda infects carnivores and humans and it has long been referred to as "oriental eyeworm" due to its occurrence in humans and dogs from the Russian Federation and the Far East (i.e., Indonesia, Thailand, China, Korea, Myanmar, India, and Japan) (Bhaibulaya et al., 1970; Kosin et al., 1989; Hong et al., 1995; Shen et al., 2006). This nematode species can also infect cats, foxes, rabbits (Kozlov, 1962; Skrjabin et al.,

1967), and also wolves (Otranto et al., 2007). In affected animals, *T. callipaeda* adult and larval stages may cause mild ocular manifestations (e.g., conjunctivitis, epiphora, and ocular discharge) to severe (e.g., keratitis, and corneal ulcers) (Otranto and Traversa, 2005). In the past two decades, *T. callipaeda* has spurred the attention of the scientific community due to its spread in some European countries (i.e., Italy, France, and Switzerland) (Rossi and Bertaglia, 1989; Otranto et al., 2003; Dorchies et al., 2007; Malacrida et al., 2008) and to imported cases in previously free areas (i.e., Germany; Hermosilla et al., 2004). Furthermore, the first four cases of human thelaziosis in Europe have been diagnosed in northern Italy, in patients with history of travel to north-western Italy and south-eastern France (Otranto and Dutto, 2008).

The highest prevalence of canine thelaziosis has been reported in some areas of southern Italy (Basilicata region), reaching up to 60.14% in some municipalities (Otranto et al., 2003). In the same hyper-endemic area, a number of studies have been carried out to elucidate several aspects of the biology of *T. callipaeda* in definitive and intermediate hosts (Otranto et al., 2004a). *Phortica variegata* (Diptera: Drosophilidae) has been implicated as the vector of *T. callipaeda* under both experimental and field conditions (Otranto et al., 2005a, 2006b). A genetic screening of the hypervariable region of mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox1*) of *T. callipaeda* indicated the existence of only one haplotype (designated as haplotype 1; i.e., h1) in Europe and seven haplotypes in Asia (Otranto et al., 2005b). The presence of a single haplotype of *T. callipaeda* in Europe, irrespective of country of origin (i.e., Italy, France, and Germany) and hosts (e.g., dogs, cats, and foxes) suggested a close association and a likely co-evolution between the nematode and its vector (Otranto et al., 2005b). This knowledge raised questions on the role of wild carnivores in maintaining the life cycle of *T. callipaeda* in nature (Otranto et al., 2006a). Studies have shown the occurrence of *T. callipaeda* in foxes in areas where canine thelaziosis is endemic, indicating that foxes could act as reservoirs of *T. callipaeda* (Rossi et al., 2002; Otranto et al., 2003; Malacrida et al., 2008). However, with the exception of wolves (Otranto et al., 2007), no data is available on the role of wild carnivores as hosts of *T. callipaeda*. Lack of knowledge on the sylvatic life cycle of *T. callipaeda* has constituted a foundation for the present study whose aim was to evaluate the occurrence of *T.*

callipaeda in wildlife species that could be acting as definitive hosts and reservoirs for this nematode.

2. Materials and methods

2.1. Study area

The survey was carried out in the *T. callipaeda*-hyper-endemic area of the Basilicata region in southern Italy (latitude 39° and 41° North, longitude 15° and 16° East) which comprises 12 municipalities (Otranto et al., 2003). The area is situated at an altitude between 800 and 1000 m above sea level (a.s.l.) with an orography characterized by the presence of a ring of sandstone mountains surrounding the examined area. Vegetation characteristics may vary according to altitude, exposure to the sunlight, and relative humidity. In particular, oaks are common, with *Quercus cerris* being the most common species and holly (*Ilex* spp.) found in the undergrowth. The study area is included in the Regional Park "Gallipoli Cognato Piccole Dolomiti Lucane" which hosts many indigenous populations of protected animals including red foxes (*Vulpes vulpes*), wolves (*Canis lupus*), beech martens (*Martes foina*), brown hares (*Lepus europaeus*), Eurasian badgers (*Meles meles*), and European wildcats (*Felis silvestris*). Many dogs usually accompanying sheep and cattle at least once a day during summer are also present in the same area, in which, the population dynamics of *P. variegata* has been monitored demonstrating that these flies are mainly active in July–August at 20–25 °C and 50–75% of relative humidity (Otranto et al., 2006a).

2.2. Post-mortem examination and parasite collection

From May 2003 to May 2009, 130 carcasses of wild animals belonging to different species (Table 1) were examined in the study area. Animals were found within the park territory, killed by trauma (following impact with a motor vehicle) or illegally killed, and thus provided by the local authorities to determine the cause of death. All animals were delivered to the park's resident veterinarian (Dr. Egidio Mallia). Animals were kept frozen until necropsies were carried out based on the number of specimens retrieved in batches of about 10 animals.

At necropsy, animals were examined, their sex recorded and their age estimated. In particular they were categorized as juveniles or adults on the basis of biomorpho-

Table 1
Number (no.) and percentage (%) of different host species found infected by *Thelazia callipaeda* categorized by sex and age. Details about nematodes sex and location and mean intensity of infection are also provided. Data from Eurasian badgers (*n* = 10), which were all negative, have been omitted from the analysis.

Host species (no.)	Host data					Nematode data					
	Male	Female	Juvenile	Adult	Total (%)	Male	Female	Right eye	Left eye	Range (mean intensity ± SD)	Total
Vulpes vulpes (75)	20	17	12	25	37 (49.3)	42	97	82	57	1–13 (3.8 ± 2)	139
Canis lupus (2)	1	–	–	1	1 (50)	–	2	–	2	–	2
Martes foina (22)	1	2	3	–	3 (13.6)	6	9	9	6	3–6 (5 ± 1.7)	15
Lepus europaeus (13)	–	3	–	3	3 (23.1)	3	14	9	8	3–11 (5.7 ± 4.6)	17
Felis silvestris (8)	2	1	1	2	3 (37.5)	9	7	4	12	3–7 (5.3 ± 2.1)	16
Total (120)	24	23	16	31	47 (39.2)	60	129	104	85	1–13 (4 ± 2.2)	189

metrics. The ages of wolves, foxes, wild cats, and beech martens were estimated by the tooth wear technique (Ballard et al., 1995; Zapata et al., 1995; Harris, 1978). The ages of hares were estimated by the characteristics of the coat and skeletal development (Broekhuizen and Maas-kamp, 1979) and body weight (Trocchi and Riga, 2005). The ages of badgers were estimated according to Hancox (1988).

The conjunctival sacs of both eyes were flushed by instillation of 10 ml of saline solution (NaCl 0.9%) and parasites were collected in Petri dishes, labeled and delivered to the Laboratory of Parasitology of the Faculty of Veterinary Medicine of the University of Bari, for identification.

2.3. Parasite identification

All nematodes were morphologically identified according to the keys of Skrjabin et al. (1967) and Otranto et al. (2004b) and then stored in vials containing 70% ethanol. To confirm the morphological identification, at least five specimens from each host species were molecularly characterized. DNA was extracted using a commercial kit (QIAamp Tissue Kit, Qiagen, GmbH, Germany) and then a partial sequence of the *cox1* gene (i.e., 689 bp in length) was amplified by PCR as previously described (Otranto et al., 2005b). Negative (no DNA) and positive (*T. callipaeda* DNA) control reactions were also included. Amplicons were purified using Ultrafree-DA columns (Millipore, Bedford, MA, USA) and sequenced in an ABI-PRISM 377 (Applied Biosystems, Foster City, CA, USA) using the Taq Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequences were determined in both directions (using the same primers individually as for the PCR) and the electropherograms verified by eye. Sequences were aligned using the ClustalX program (Thompson et al., 1997). The alignments were verified by eye and compared with the *cox1* sequences of *T. callipaeda* available in GenBank (accession numbers AM042549, AM04550, AM04551, AM04552, AM04553, AM04554, AM04555, AM04556) (Otranto et al., 2005b).

2.4. Statistical analysis

Statistical analysis was performed using the Epi Info 6.04 program. Prevalence data was compared to sex and age classes of the hosts using the chi square test and differences were considered statistically significant when $p \leq 0.05$. Mean intensity of infection was calculated as described elsewhere (Bush et al., 1997).

3. Results

Five out of the six wild animal species examined were found to be infected by eyeworms (Fig. 1) with different percentages of positivity and an overall infection rate of 39.2% (Table 1). None of the 10 badgers examined were positive for eyeworms. The number and percentage of positive and negative animals, grouped according to sex and age, are reported in Table 1. Overall, 189 adult nematodes (ranging from 1 to 13 per animal) were collected from one or both eyes of positive animals, with a mean intensity (mean ± standard deviation) of infection of 4 ± 2.2. The number of nematodes collected from each animal species is also reported in Table 1.

With regard to statistical analysis, no significant difference was observed between eyeworm infection and either sex or age of the hosts. All nematodes were identified as *T. callipaeda* on the basis of the position of the vulva, located anterior to the oesophago-intestinal junction in all the females and, in the males, on the basis of the presence of five pairs of large post-cloacal papillae in the ventral position and small papillae in sub-ventral position. Sequences obtained were identical to the sequence representing h1 of *T. callipaeda* available in GenBank (accession number: AM042549).

4. Discussion

In this paper, the authors report for the first time the infection of beech martens, wild cats, and brown hares by *T. callipaeda*. In addition, the retrieval of *T. callipaeda* in one of the two wolves examined supports the previous report of nematodes in this animal species (Otranto et al., 2007). The finding of *T. callipaeda* adult specimens in the eyes of these wildlife species stands testimony for their competence as definitive hosts of this nematode, since it implies that third stage larvae, released by the insect intermediate host, developed to adults in their ocular cavity. From a parasitological standpoint, these findings are extremely interesting as *T. callipaeda* displays the broadest spectrum of definitive hosts among all *Thelazia* species. For instance, *Thelazia gulosa* and *Thelazia rhodesi* mainly parasitize cattle and *Thelazia lacrymalis* parasitizes only horses (Anderson, 2000).

In southern Italy the occurrence of *T. callipaeda* in foxes has been found to be much higher (i.e., 49.3%) than that reported in northern Italy (5.1%) (Otranto et al., 2003) and Switzerland (5.7%) (Malacrida et al., 2008). The high prevalence in foxes, along with previous data in domestic dogs (i.e., up to 60.14%; Otranto et al., 2003) indicates that *T. callipaeda* infection is hyper-endemic in the study area. The first retrieval of *T. callipaeda* from beech martens was noteworthy, considering that only *Thelazia depressa* has been so far found to infect a mustelid (i.e., *Mungos fasciatus*) in Africa (Yamaguti, 1961). Similarly, *T. callipaeda* has never been previously recorded in wild cats.

Despite the low number of individuals of some species examined in the present study, it could be possible to relate their behaviour and ecology with their potential exposure to the vector *P. variegata*. Indeed, although the limited amount of information on its biology, ecology, and zoophilic habits, *P. variegata* was captured in the same study area from the first week of May until November 2005, early in the morning and late in the evening, but not at the warmest times of the day (Otranto et al., 2006a). Again, the habitat in which *P. variegata* was collected was an oak wood, situated at about 850 m a.s.l., characterized by high relative humidity and undergrowth constituted mainly of holly. The area surrounding the collection site is characterized by the presence of rivers that further increase the relative humidity.

Fig. 1. Adult specimens of *Thelazia callipaeda* on the eye of different hosts. (A) Red fox; (B) brown hare; (C) wild cat; (D) wolf; (E) beech marten.

In fact, the seasonality and crepuscular activity of *P. variegata* coincide with the activity patterns of those mammal species found to be infected by *T. callipaeda*, but not with the badger. The wildlife species found positive for *T. callipaeda*, even those characterized by predominantly nocturnal habits, show a certain degree of diurnal activity that may vary according to season (summer and winter). The activity patterns of wolves are variable, often determined by their intended prey (Kusak et al., 2005). However, packs in other European montane temperate forests are known to be mainly crepuscular, with peaks of activity at dusk and dawn, and maximum diurnal activity during the summer (Theuerkauf et al., 2003; Eggermann et al., 2009). Although foxes may also have variable activity patterns, they are most active during dawn and dusk (Fedriani et al., 1999). While somewhat more active in the night, wild cats can also be active during the daylight hours and thus are not considered nocturnal, and make preferential use of wooded or forested habitats (Germain et al., 2008). Studies on beech martens have shown that their total diurnal activity can differ significantly through-out the seasons, being maximal during the spring and summer and minimal in autumn and winter (Posillico et al., 1995). The brown hare is characterized by habits prevalently crepuscular and nocturnal, but during the mating season (spring) and in quiet areas, a minimum of activity is observed during the midday hours with a peak in late afternoon (Tottewitz, 1993; Pèpin and Cargnelutti, 1994). Even when inactive during the day, hares remain in nests, simple depressions in the ground in areas of impenetrable vegetation, or in the forest (Trocchi and Riga, 2005), where they may still be exposed to *P. variegata*.

In contrast, almost all recorded activity of the Eurasian badger has been nocturnal (Kowalczyk et al., 2003). Goszczynski et al. (2005) reported no surface activity between 5:00 and 17:00 h, whereas Fedriani et al. (1999) described only minimal activity at dawn and dusk. The badger is also the only species that makes use of underground tunnel systems, or setts, for year-round lodging (Kowalczyk et al., 2003). Predominantly nocturnal activity and underground lodging when inactive may prevent *T. callipaeda* infection by precluding exposure to its vector.

A certain degree of diurnal activity among wild mammals, particularly in the summer period when *P. variegata* is present, would explain the potential exposure of foxes, wolves, wild cats, beech martens, and brown hares to *T. callipaeda*. Likewise, the absence of *T. callipaeda* in the badger could be in relation to an almost complete absence of diurnal activity.

The aforementioned ecological considerations are supported by molecular data that has confirmed that a single haplotype (h1) of *T. callipaeda* is circulating among different host species in the study area. The same h1 was found in other European countries, irrespective of the host species from which they were collected (Otranto et al., 2005b). The molecular findings presented here and studies on *P. variegata* indicate a high degree of specificity of the nematode for its vector (Otranto et al., 2005a,b, 2006b) and a low affinity for its definitive hosts.

Our results support not only the existence of a sylvatic life cycle of *T. callipaeda* but also that it is mainly maintained by foxes and secondarily by a large number of wildlife species. These wild mammal species could play a role in spreading *T. callipaeda* in endemic areas and also account for the increasing number of reports of canine thelaziosis from different parts of Europe in the past few years (Otranto et al., 2003; Hermosilla et al., 2004; Dorchies et al., 2007; Malacrida et al., 2008). Indeed, the migration of wildlife through the Apennines of Italy could explain the spread of the infection. In fact, in other parts of Italy, foxes and mustelids have been identified as possible reservoirs of other helminthes and have been implicated in their dispersal (Di Cerbo et al., 2008). Analysis of the social organization of the red fox presents models based on a dynamic territoriality that determines low population densities due to individual adults of the same sex distancing themselves within their own territories (Nie-wold, 1980; Doncaster and Macdonald, 1991), with a dispersal reaching even more than 100 km (Phillips et al.,

1972). Although wolves are scantly present in the studied area as compared to red foxes, their social structure is more complex. Packs are formed by one reproductive couple, their pups and individuals that are young or of inferior rank position (Mech, 1970). Indeed, wolves occupy vast territories, with a dispersal even greater than 800 km (Mech, 1995). When wolves reach the reproductive age (22–24 months) they leave the pack to search for a new territory where they may be able to reproduce on their own (Mech, 1970). In Italy, limitations of resources (e.g., scarcity of prey and the limited availability of natural habitats) are probably the origin of the fact that wolves live in packs of few (3–5) individuals (Boscagli, 1985) and that the phenomenon of dispersion is perhaps the only strategy that guarantees the survival of the species. The movement of wolf packs in wide territories and the distancing and dispersal of individual wolves and foxes suggest that they could play an important role in the maintenance of *T. callipaeda* in sylvatic environments and in the diffusion of the parasite in other parts of the Apennines of southern Italy, where the competent vector is present.

Finally, the present study increases the already broad range of *T. callipaeda* hosts and provides data for a better understanding of the ecology and epidemiology of eye-worm infection in areas where wild hosts live in close association to human beings.

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