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Thelazia callipaeda infection in Northwestern Spain: what role does the Iberian wolf play?

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ABSTRACT

Thelazia callipaeda is a vector-borne parasite infecting the eyes of domestic and wild animals, as well as humans, across Europe. In Spain, it is endemic in several regions, with high prevalence reported in dogs and cats, but data on wildlife remain scarce. This study investigated the prevalence of *T. callipaeda* in Iberian wolves (*Canis lupus signatus*) from northwestern Spain and explored epidemiological factors associated with infection. Between 2016 and 2025, 182 wolves from Asturias and Galicia (A Coruña, Lugo, Ourense and Pontevedra) were sampled after necropsy. Eyeworms were collected and identified using morphological and molecular methods, with all specimens corresponding to *T. callipaeda* genotype h1. The overall prevalence was 17 %, with higher rates of infection in Galicia (18.2 %) than Asturias (8.7 %). Infections were restricted to Ourense (37.2 %) and Lugo (26.5 %). Parasite burdens were higher in females and immature wolves (1–3 years) compared to older animals and those in good body condition (BC 4–5). The distribution of infected wolves was associated with the vegetation around Miño and Sil river basins, which may favor the development of the vector *Phortica variegata*. Lower prevalence in pups could be linked to smaller eye size and lower body condition, while sex- and age-related differences may reflect behavioral or immunological factors. These results indicate that Iberian wolves can act as reservoirs of *T. callipaeda* in northwestern Spain, underscoring the need for further research into their role in the epidemiology of this zoonotic parasite.

1. Introduction

Thelaziosis is an arthropod-borne zoonotic disease caused by nematodes of the genus *Thelazia* (order Spirurida) (Otranto and Wall, 2024), which infect the conjunctival sac and nasolacrimal ducts of various animal species worldwide (Beugnet et al., 2018; Otranto et al., 2021; Otranto and Traversa, 2005; Papadopoulos et al., 2022; Shen et al., 2006; Vale et al., 2020). Among the 16 species identified in mammals, only three have been documented to infect humans and carnivores: *Thelazia callipaeda* (Railliet and Henry, 1910), *Thelazia californiensis* (Price, 1930) and *Thelazia gulosa* (Railliet and Henry, 1910; Bradbury et al., 2018; Knierim and Jack, 1975; Otranto and Dutto, 2008).

In Europe, *T. callipaeda*, also known as the "oriental eyeworm" due to its high prevalence throughout Asia and Western European countries (Anderson, 2000), was first reported in dogs from Piedmont, north of Italy (Rossi and Bertaglia, 1989), in the late 20th century, and then in cats and foxes (Otranto et al., 2003a, 2003b). Since then, this parasite has spread across southern and central Europe and is now considered endemic in some countries such as Italy, France, Belgium, Greece and Spain (Diakou, 2017; do Vale et al., 2019; Marino et al., 2020; Papadopoulos et al., 2018; Otranto et al., 2021). In endemic regions of Europe, *T. callipaeda* is primarily transmitted by the drosophilid fruit fly

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Phortica variegata (Fallén, 1823) (Drosophilidae: Steganinae) (Otranto et al., 2006b), although another autochthonous species, *Phortica oldenbergi* (Bezerra-Santos et al., 2022a), has recently been proposed as a potential new vector. These fruit flies acquire the eyeworm's first-stage larvae whilst feeding on lachrymal secretion of a vertebrate host, and they transmit the third-stage larvae to another definitive host after 14–21 days (Otranto et al., 2005a, 2005b).

Thelaziosis caused by *T. callipaeda* can produce different degrees of ocular signs, ranging from mild (lacrimation and/or conjunctivitis) to severe (keratitis, corneal ulcers, blindness, etc.) (Miró et al., 2011; Otranto and Traversa, 2005; Otranto et al., 2021), in a wide range of domestic animals (Otranto et al., 2009), lagomorphs (Gama et al., 2016) and even humans, primarily children and the elderly inhabiting rural areas (Shen et al., 2006). However, in recent years, the increase in the human population and the movement of animals between human-dominated landscapes has facilitated greater contact between the domestic and wildlife cycles (Tsokana et al., 2024), leading to the recent emergence of thelaziosis cases in urban and peri-urban areas, as well as in previously non-endemic regions. These ecological factors may also explain the exclusive presence of haplotype 1 (h1) and another new haplotype 22 (h22) (recently described as a nucleotide mutation of h1 in a human case) of *T. callipaeda* in Europe, suggesting that the parasite has spread across the continent from an original single focus (Otranto et al., 2005a, 2005b) and has even reached North America, where autochthonous cases of thelaziosis have been reported since 2020 (Manoj et al., 2024).

In Spain, *T. callipaeda* has been described infecting dogs (Miró et al., 2011), cats (Hernández et al., 2012; Montoya et al., 2011a), domestic rabbits (*Oryctolagus cuniculus*) (Marino et al., 2018; Marino, 2021a), red foxes (*Vulpes vulpes*) (Calero-Bernal et al., 2013), Iberian wolves (*Canis lupus signatus*) (Nájera et al., 2020) and humans (Deltell et al., 2019; Fuentes et al., 2012; López Medrano et al., 2015). The first autochthonous cases were documented in dogs from La Vera (Cáceres, southwest Spain) in 2010 (Guisado and Sanz, 2010; Miró et al., 2011); thenceforth, the infection has spread all over the Iberian Peninsula, mainly in the western regions (Montoya et al., 2011b), but also reaching the northern (i.e., Ourense), central (i.e., Madrid) and east (i.e., Murcia) provinces (Bello et al., 2012; Marino et al., 2018). In these regions, dogs' prevalence ranges from 26.1 % to 68 %, while only sporadic cases have been reported in cats and humans (Marino et al., 2018). In wild canids, red foxes exhibit a prevalence between 2.2 % and 8.8 % (Marino, 2021b), but no prevalence studies have been conducted in Iberian wolves, up to our knowledge.

Wild canids, due to their crepuscular activity during the summer and their habitat in forests and hills with a variety of vegetation, which are natural environments for the vector *P. variegata* (Otranto et al., 2006b), have been closely linked to the introduction, maintenance and spread of *T. callipaeda* in both endemic and non-endemic areas (Mihalca et al., 2016; Otranto and Deplazes, 2019). In Europe, the red fox seems to be a crucial link between the domestic and wildlife-cycles because of its ecological adaptability (Castañeda et al., 2022; Soe et al., 2017), inhabiting both natural landscapes and urbanized areas where it feeds on garbage (Díaz-Ruiz et al., 2013). This is also suggested by by high prevalence rates (5.6–49.3 %) in regions where the disease is endemic for domestic animals (Otranto et al., 2009), and even in areas where thelaziosis had not been previously described in dogs (27.7 %) (Hodžić et al., 2014). Thus, infected red foxes may play a significant role in spreading *T. callipaeda* into regions where the vector is present but the infection has not yet been established (Hermosilla et al., 2004; Mihalca et al., 2016; Otranto and Dantas-Torres, 2015; Papadopoulos et al., 2018; Vieira et al., 2012).

Eurasian wolves have also been identified as competent hosts of *T. callipaeda* in some European countries (Bezerra-Santos et al., 2022b; Bojan et al., 2019; Nájera et al., 2020; Otranto et al., 2007; Papadopoulos et al., 2022). After their decline during the 19th and 20th centuries, these apex predators have undergone population growth across Europe and are now recolonizing territories where they had been disappeared longtime ago (Herzog, 2018). In Spain, despite being predominantly concentrated in the northwest of the Iberian Peninsula, the Iberian wolf population has expanded its range southward over the past decade, reaching locations such as Madrid and Guadalajara (center Iberian Peninsula) (Blanco, 2017; Viejo and Montesinos, 2013). Wolves are known to cover much greater distances than other wild carnivores (up to 1500 km) (Cortés, 2001; Cortés and Blanco, 2003), potentially spreading the infection to more distant geographical regions. Nonetheless, most reports of *T. callipaeda* infection in wolves are isolated cases (Bezerra-Santos et al., 2022b; Bojan et al., 2019; Nájera et al., 2020; Otranto et al., 2007; Papadopoulos et al., 2022), and the actual impact of this species on the epidemiology of thelaziosis remains unknown.

For all these reasons, the aims of this study were 1) to assess the prevalence of *T. callipaeda* infection in Iberian wolves from the northwestern Iberian Peninsula, 2) to analyze potential epidemiological variables associated with the infection, and 3) to elucidate the role of these wild canids in the spillover of the infection to humans and other domestic and wild animals.

2. Material and methods

2.1. Study area

Study areas included the neighbouring autonomous communities of Galicia and Asturias, both located in the northwest of the Iberian Peninsula, encompassing an overall area of around 40,000 km^2 (29,575 and 10,602 km^2, respectively) (MITECO, 2022). Galicia is composed of four provinces (A Coruña, Lugo, Ourense and Pontevedra), each further subdivided into municipalities or councils (*concellos*), whereas Asturias is a single-province region and is exclusively divided into municipalities (INE, 2023). In both areas, the climate is predominantly oceanic, with limited thermal variation between summer and winter and frequent precipitation year-round (AEMET, 2020). The orography ranges from coastal regions to high mountain ecosystems (0–2172 m a.s.l), resulting in a diverse and dense vegetation cover, with notable presence of oaks (*Quercus* spp.), beeches (*Fagus* spp.) and chestnuts (*Castanea* spp.) (Loidi, 2017; Núñez-Delgado et al., 2023).

2.2. Sample and data collection

Between 2016 and 2025, official agencies from Galicia and Asturias collected the carcasses of 182 Iberian wolves from northwest Spain, which had either died of natural causes or due to vehicle collisions. Prior to necropsy, clinical data were systematically gathered for each animal, including age, sex, weight and body condition score, as well as any other pertinent findings from the physical examination. Age was determined based on body weight, dentition and sexual maturity, classifying animals into three different categories: wolf pups (under 12 months old, lacking full body development), immature or young wolves (1–3 years old, not yet sexually mature) and adults (over 3 years old or of reproductive age). Body condition (BC) was assessed using a five-point scale: 1-sarcopenia, 3-optimal condition and 5-marked overweight (DiGangi, 2018; Reisman, 2012). Then, the wolves underwent necropsy procedures according to standardized protocols (Brownlie and Munro, 2016).

2.3. Eyeworms extraction and morphological identification

Eyeworms were collected from the conjunctival sac using a sterile cotton swab whenever possible, or by performing a flushing technique with physiological saline solution (0.9 % NaCl) (Fig. 1). All nematodes collected from each wolf were preserved in separate tubes containing 70 % ethanol. *Thelazia callipaeda* adults and larvae were identified under light microscope (Nikon ECLIPSE Ei, Japan), based on morphological features described by Otranto et al. (2003a); Otranto et al. (2003b).

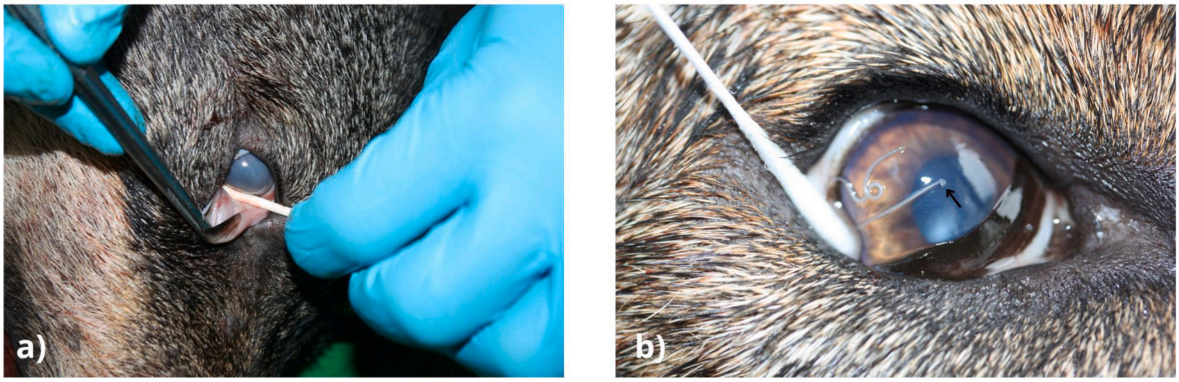

Fig. 1. Swabbing examination of the ocular cavities during the necropsy of an Iberian wolf (a) and *Thelazia callipaeda* adults extracted from the conjunctival sac (b). Note that the eyeworm on the right displays a coiled posterior end (black arrow), a morphological feature characteristic of a male specimen.

2.4. Molecular identification

A pool of 1–4 female worm fragments isolated from sampled wolves found in 3–4 neighbouring municipalities (approx. 200–400 km^2) was used for the molecular analysis. The eyeworms were meticulously washed in distilled water, sectioned with a sterile scalpel blade and then processed using the tissue protocol from the QIAamp® DNA Mini Kit (Qiagen, Germany). Subsequently, PCR amplification targeting a 689 bp partial region of the mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) gene was performed according to Otranto et al. (2005a); Otranto et al. (2005b) procedure. The resulting amplicons were purified using the enzymatic PCR product cleaner ExoSap-IT® (Thermo Fisher Scientific, USA) and sequenced in both directions on the 3730xl DNA Analyzer® (Thermo Fisher Scientific, USA) using the same primers (NTF and NTR) employed for the PCR. The sequences were aligned using the molecular analysis program MEGA 11 (v11.0.13) and transferred to the Basic Local Alignment Search Tool (BLAST®; http://blast.ncbi.nlm.nih.gov/Blast.cgi) in order to compare them with those *cox*1 sequences available in the GenBank, after which the newly obtained sequences from Iberian wolves were submitted to the same database.

2.5. Statistical analysis

The data collected during physical exam and necropsy were employed for conducting descriptive statistical analysis. This included calculating the prevalence (percentage of infected animals) and parasitic load (number of ocular nematodes per infected wolf or NPW). Additionally, the correlarion between *T. callipaeda* infection and the epidemiological factors analyzed (age, sex, weight, body condition and origin of the wolves) was assessed using Pearson's chi-square ($\chi 2$) test with a 95 % confidence interval (significance set at $p \leq 0.05$) for bilateral testing. All statistical analyses were performed using R software (v4.3.0).

3. Results

3.1. Prevalence and identification of ocular nematodes

The overall prevalence of ocular nematode infection in necropsied wolves was 17 % (31/182; 95 % CI: 11.5–22.5). Of the infected individuals, 28 had nematodes in both eyes, while 3 had nematodes exclusively in the right eye. A total of 620 nematodes were collected, with individual counts ranging from 1 to 125 NPW (mean = 22.5). Unfortunately, it was not possible to determine the nematode count for 4 infected wolves, as only fragments of nematodes were recovered due to the poor preservation status of the carcasses.

All nematode specimens were identified as *T. callipaeda* based on their distinct morphological features, including a pale whitish coloration, a small buccal capsule and a striated cuticle that spans the entire length of the body. Females were characterized by the vulvar opening positioned anterior to the esophago-intestinal junction and the presence of first-stage larvae (L1) larvae within the uterus. Males exhibited asymmetric spicules at the posterior end of the body, with the right spicule significantly shorter than the left (Fig. 2). A total of 388 female specimens (ranging from 1 to 84 NPW) and 221 male specimens (ranging from 1 to 41 NPW) were recorded. Only one wolf harboured preadult nematodes (L5 stage), consisting of 11 specimens.

Concerning the molecular analysis, all 12 pools of eye worms from different municipalities yielded successful PCR amplification, with the resulting amplicons showing a 99.8–100 % coincidence match with *T. callipaeda* haplotype 1 (GenBank accession: PV750910).

3.2. Statistical analysis of epidemiological variables

Data for some epidemiological variables could not be determined in certain wolves due to the poor carcass preservation. Most wolves included in this study were from Galicia (87.4 %; 159/182), with a distribution as follows: 49 from Lugo, 49 from A Coruña, 43 from Ourense and 18 from Pontevedra. In contrast, only 12.6 % (23/182) of the wolves analyzed were from Asturias. A total of 104 wolves were males and 77 females. Age categorization identified 33 pups, 81 immature and 66 adults. The body condition scores (out of 5) were distributed as follows: 20.1 % for BC 1–2, 42.1 % for BC 3 and 37.8 % for BC 4–5. The statistical results from each epidemiological variable are presented in Table 1.

Wolves from Galicia exhibited a higher prevalence (18.2 %; 29/159) compared to those from Asturias (8.7 %; 2/23). Within Galicia, positivity was only observed in Ourense (37.2 %; 16/43) and Lugo (26.5 %; 13/49), while no positive individuals were found in A Coruña and Pontevedra (0/49 and 0/18, respectively) (Fig. 3). Statistical analysis revealed significant differences across the four provinces ($p < 0.001$).

Regarding the "sex" variable, both male and female wolves had similar infection prevalences, with 15.4 % and 19.5 %, respectively. However, female wolves exhibited a higher parasitic burden of *T. callipaeda*, with an average of 32.7 NPW, compared to 8.1 NPW in males, a difference that was statistically significant ($p = 0.030$).

Thelazia callipaeda was more prevalent in immature (22.2 %) than in adults wolves (15.2 %) and wolf pups (9.1 %). Following the same trend, immature wolves exhibited more eye worms with 23.9 NPW than adults (16 NPW) and wolf pups (10 NPW). No statistically significant results were obtained for this epidemiological variable ($p = 0.207$).

The prevalence of infection was similar across wolves with different body conditions, with wolves in BC 3 exhibiting a slightly higher infection rate (20.3 %) compared to those in BC 1–2 (18.2 %) and BC 4–5 (17.7 %). Parasitic burden was comparable between wolves in BC 1–2 and BC 3 (22.5 and 26.5 NPW, respectively), while wolves in BC 4–5

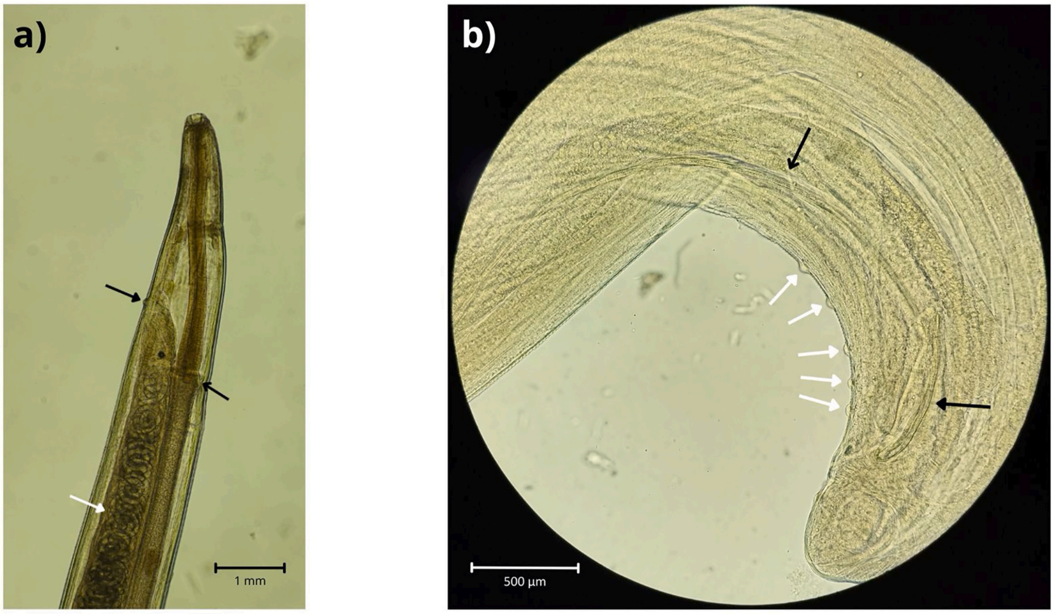

Fig. 2. Main morphological features used for the identification of *Thelazia callipaeda* adults: a) anterior region of a female, showing the vulvar opening (wide-headed black arrow) positioned anterior to the oesophagus-intestinal junction (narrow-headed black arrow), with first-stage larvae (L1) visible within the uterus (white arrow) (x40); b) posterior end of a male, highlighting the long left spicule (narrow-headed black arrow), short right spicule (wide-headed black arrow) and the presence of five precloacal papillae (white arrows) (x400).

Table 1
Epidemiological variables analyzed in the Iberian wolf population from northwestern Spain.

			Prevalence (%)	*n*/N	*p*-value	NPW	*p*-value
Location	Asturias		8.7	2/23	<0.001	2	<0.001
	Galicia		18.5	29/130		23.8	
		Ourense	37.2	16/49		28.7	
		Lugo	26.5	13/49		17.1	
		A Coruña	0	0/49		NA	
		Pontevedra	0	0/18		NA	
Sex	Male		15.4	16/104	0.470	8.1	0.030
	Female		19.5	15/77		32.7	
Age	Pup (<12 months)		9.1	3/33	0.207	10	0.683
	Inmature (1–3 years)		22.2	18/81		23.9	
	Adult (>3 years)		15.2	10/66		16	
Body Condition (BC)	1–2		18.2	6/33	0.927	22.5	0.040
	3		20.3	14/69		26.5	
	4–5		17.7	11/62		10.4	
Year	2016		9.4	3/32	0.450	9.7	0.380
	2017		0	0/11		NA	
	2018		10	2/20		7	
	2019		12.5	3/24		46	
	2020		26.3	5/19		51.5	
	2021		17.9	5/28		13.8	
	2022		22.2	4/18		14.3	
	2023		33.3	4/12		18.3	
	2024		16.7	2/12		23	
	2025		25	1/4		12	

n = number of positive Iberian wolves per group.
N = total number of Iberian wolves per group.
NPW = nematodes per infected wolf.
NA = not applicable.

demonstrated a significantly lower intensity of parasites (10.4 NPW) ($p = 0.040$).

Finally, infection prevalence showed an increasing trend overtime, although no statistically significant differences were observed ($p = 0.450$). Notably, parasite loads peaked in 2019 and 2020, with mean values of 46 NPW and 30.8 NPW, respectively.

4. Discussion

This is, up to date, the largest analysis of *T. callipaeda* infection in wolves. In Europe, *T. callipaeda* infection has been described in wolves from Italy (Otranto et al., 2007), Romania (Mihalca et al., 2016), Greece (Papadopoulos et al., 2018), Serbia (Bojan et al., 2019) and Spain (Nájera et al., 2020). These studies are predominantly case reports or

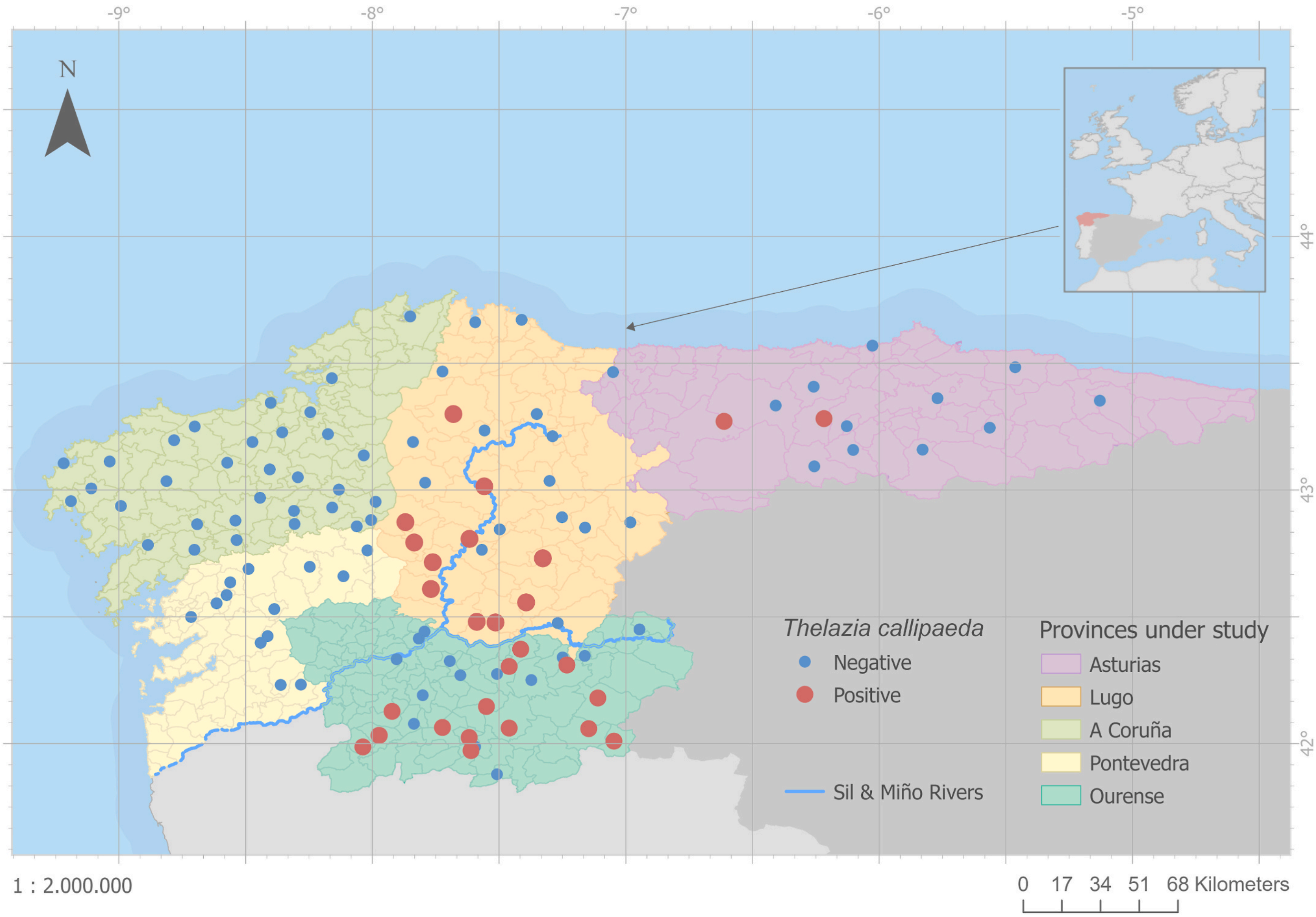

Fig. 3. Distribution map showing Iberian wolves testing positive for *Thelazia callipaeda* in Galicia and Asturias.

involve limited sample sizes (with a maximum of 21 animals), in contrast to the 182 wolves sampled in the present study.

Although *T. callipaeda* was first reported in an Iberian wolf from Guadalajara (central Spain) in February 2020 (Nájera et al., 2020), the first positive animal included in this study was detected in March 2016 in the province of Lugo (unpublished data). This fact indicates that *T. callipaeda* had been circulating in the wolf population from northwestern Spain several years before its first description on southern regions of the country. However, the situation between the first report in dogs (Miró et al., 2011) and red foxes (Calero-Bernal et al., 2013) from Spain and its later detection in Iberian wolves remains unclear, due to the absence of prior testing for *T. callipaeda* before 2016 in this wild carnivore.

The overall prevalence obtained of wolves infected with eye worms in northwestern Spain (17 %) is lower than the rates reported in other European countries (38.1 %–100 %) (Bojan et al., 2019; do Vale et al., 2019; Papadopoulos et al., 2018). However, this apparent discrepancy should be interpreted with caution for two key reasons: first, our considerable large sample size compared to those other articles; and second, the extensive geographic area encompassed, which result in varying prevalence rates when the data is analyzed by autonomous communities or provinces. Thus, the prevalence in Asturias (8.7 %) is lower than in Galicia (18.2 %), a fact that could be explained by the complex orography of that region, with a rugged coastline that abruptly transitions into a high-mountain climate, potentially affecting the phenology of the vector, *P. variegata*. Nevertheless, the sample size from Asturias was small, and further studies involving a larger number of wolves from this region are essential to draw definitive conclusions.

On the other hand, the more moderate orography of Galicia and its climate may favor the life cycle of *P. variegata* and, therefore, the transmission of *T. callipaeda*. Specially, the provinces of Ourense and Lugo are characterized by mountainous areas intercalate with the fluvial valleys of the Miño and Sil rivers. The climate here is wet and temperate year-round, favouring the development of *P. variegata* (Otranto et al., 2006a), as demonstrated by studies conducted in these two provinces (Carles-Tolrá and Lencina Gutiérrez, 2010). This environmental suitability likely contributes to the high prevalence of *T. callipaeda* infection observed in wolves from both Ourense (37.2 %) and Lugo (26.5 %). These rates are similar to those reported in dogs from other highly endemic areas in Spain such as Cáceres and Madrid (19.5–68 %) (González Jiménez et al., 2013; Marino et al., 2018; Miró et al., 2011), as well as to red foxes (49.3 %) and dogs (23.1–41.8 %) from Europe (Otranto et al., 2003a, 2003b).

In contrast, A Coruña and Pontevedra do not seem to provide suitable conditions for the transmission of *T. callipaeda*. Here, the climate and orography are completely different to Ourense and Lugo, with gentle terrain dominated by estuaries and flatter river valleys. These environmental conditions, combined with Atlantic oceanic climate characterized by high rainfall and mild temperatures are less favourable for *Phortica variegata*, which requires warmer and more heterogeneous environments with abundant forest cover to complete its life cycle. The predominance of coastal ecosystems, together with limited areas of suitable vegetation and climatic instability, likely restricts the establishment and density of the vector in these provinces. This assumption is supported by a recent entomological study conducted in Pontevedra, where no specimen of *P. variegata* was detected (González et al., 2025).

On the other hand, the prevalence of infection was slightly higher in female Iberian wolves compared to males (19.5 % and 15.4 %, respectively). However, a more notable finding is the significant difference in the mean number of eyeworms per infected wolf between sexes: females harbour a mean of 32.7 NPW, while males average 8.1 NPW ($p = 0.03$). This disparity may be explained by two potential factors: 1) behavioral differences, as female wolves typically assume the role of caring for the litter, which means they move less than the males, making them more accessible to the vector; and 2) immunological factors, as the females may experience an immunocompromised status during pregnancy and lactation that impair their ability to eliminate the parasite. Nonetheless, this pattern has not been reported in studies conducted on dogs or other wildlife species.

When age is considered, immature wolves exhibited higher prevalence and parasite burden (22.2 %; 23.9 NPW) compared to adults (15.2 %; 16 NPW) and pups (9.1 %; 10 NPW). The lower prevalence and parasite burden in pups aligns with findings from dog studies, which suggest that eye size and individual characteristics may influence the attraction of *P. variegata* to the host. Immature wolves typically assume a more static role, focusing on learning hunting skills and protecting the pack, while adults are in continuous motion due to territorial requirements (Mech and Boitani, 2019); therefore, immatures may be more exposed to the vector than adult wolves. Further research is required to determine whether behavioral factors and/or immune status influence the parasitic burden when regarding sex and age of the wolves.

For instance, *T. callipaeda* infection does not seem to pose a significant threat for Iberian wolves, as the prevalence observed across the three body condition categories was relatively similar. However, animals in the BC 4–5 group exhibited lower infection (10.4 NPW) compared to the other two groups (26.5 NPW for BC 3 and 22.5 NPW for BC 1–2). This finding could be once again attributed to the fact that animals in BC 4–5, which are usually better nourished, or even overweight, may exhibit a stronger immune response to the parasite, resulting in fewer larvae development. An alternative hypothesis is that these animals might have a more synanthropic lifestyle, where *P. variegata* is less likely to find a suitable ecological niche.

Unfortunately, the wolf carcasses found in the wild often exhibited post-mortem autolytic changes, such as ocular globe retraction and corneal opacity, resulting from prolonged exposure to environmental conditions over several days. In the case of animals collected from traffic accidents, pre- and peri-mortem cranial trauma was frequently present, which further complicated the interpretation of clinical signs (Brooks, 2016). These limitations prevented a more detailed evaluation of *T. callipaeda*–associated infection, and the real prevalence in Iberian wolves from northwestern Spain is probably higher than reported, since advanced post-mortem changes and traumatic lesions may have masked ocular alterations and hindered the parasite detection.

The prevalence of wolves parasitized by *T. callipaeda* has seemingly increased since 2016. However, this trend should be interpreted with caution, as the early years of this study involved a limited sample size from Ourense, the region with the highest observed prevalence; as a result, the findings might be underestimated. A similar situation occurred with the high parasite burden intensity observed in 2019 (46 NPW) and 2020 (30.8 NPW), which coincided with a greater focus on sampling female wolves compared to other years.

5. Conclusions

Wolves from northwestern Spain appear to be competent and significant reservoir of *T. callipaeda* infection, particularly those inhabiting the mountainous regions near the Miño and Sil basins, which predominantly flow through the provinces of Ourense and Lugo. Given that the first cases of canine thelaziosis in Spain were reported in Cáceres (central-western Spain), it is likely that this parasite entered through Ourense and subsequently spread up north to Lugo and Asturias, following the courses of the aforementioned rivers, where the vector, *P. variegata*, has an ideal ecological niche. Moreover, due to their ability to cover long distances, wolves could act as effective dispersers of these eyeworms, potentially introducing them into new ecosystems where the vector is endemic, but the infection has not yet been recorded. Further studies are needed to clarify the epidemiological role of wolves in *T. callipaeda* infection. Such studies should not only focus on this wild canid, but also include investigations in domestic animals and even humans from the same geographic areas, in order to better understand transmission dynamics. Additionally, these studies could help to assess the clinical impact of infection on wolves and determine their potential contribution as reservoirs in the local epidemiological cycle of this parasite.

CRediT authorship contribution statement

Efrén Estévez-Sánchez: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Ana M. López-Beceiro:** Writing – review & editing, Resources, Methodology, Investigation, Data curation, Conceptualization. **Clara González-Serrano:** Methodology, Investigation, Formal analysis. **Ana Montoya:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Juan P. Barrera:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Methodology, Investigation, Data curation. **Blanca Fernández:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Valentina Marino:** Writing – review & editing, Methodology, Investigation. **Pablo Moraleda-Berral:** Writing – review & editing, Methodology, Investigation. **Clara Gómez-Velasco:** Writing – review & editing, Methodology, Investigation. **Juliana Sarquis:** Writing – review & editing, Methodology. **Rocío Checa:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Luis E. Fidalgo:** Writing – review & editing, Resources, Methodology, Investigation, Conceptualization. **Guadalupe Miró:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Informed consent statement

Not applicable.

Institutional review board statement

This study was carried out in accordance with the Spanish legislation guidelines (RD 8/2003) and with the international Guiding Principles for Biomedical Research Involving Animals issued by the Council for International Organization of Medical Sciences and the International Council for Laboratory Animal Science (RD 53/2013). Ethical review and approval were waived as the samples for this study were obtained from animals that had died of natural causes.

Data availability statement

Data presented in this study are available on request from the corresponding author.

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Declaration of interest statement

The authors have declared no conflict of interest.

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