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Angiostrongylus vasorum in foxes (*Vulpes vulpes*) and wolves (*Canis lupus italicus*) from Abruzzo region, Italy

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ABSTRACT

In Europe wildlife animals such as the red fox (*Vulpes vulpes*) are considered the main reservoir for *Angiostrongylus vasorum* as well as a potential threat for domestic dog infection. Though this parasite is endemic in fox populations, data on *A. vasorum* infection in wolves (*Canis lupus italicus*) are still scant, having only recently been described in Northwestern Spain, in Italy, in Croatia and in Slovakia.

Based on the rising number of cases of canine lungworm infection in Central Italy (Abruzzo region), the aim of the present study was to investigate the infection by *A. vasorum* in fox and wolf populations sharing the same geographical area of dogs.

From October 2008 to November 2019, *A. vasorum* specimens were collected, through routine post-mortem examination, from 56 carcasses (44 foxes and 12 wolves). Adult parasites were searched for in the right side of the heart and in pulmonary artery of all carcasses. First stage of larvae (L1) was searched in faeces using the Baermann technique and in lungs by tissue impressions. Overall, 230 adult specimens were collected and identified on a morphological basis. To confirm the morphological identification, 4 adult specimens (n = 3 from fox, n = 1 from wolf) were molecularly identified as *A. vasorum* by amplification of partial fragment of nuclear 18S rRNA (~1700 bp) genes.

The anatomo-pathological and parasitological examinations indicated the presence of *A. vasorum* in 33 foxes (75%) and in 8 wolves (66.7%). The level of prevalence of infested wolves was higher than the previous one reported in other European countries. Interestingly, the prevalence of infection in foxes herein recorded was higher than that described in dogs (8.9%) living in the same geographical area. This result may confirm the hypothesis that the spread of canine angiostrongylosis is linked to fox populations infection.

1. Introduction

Nematodes of the genus *Angiostrongylus* Kamensky, 1905 (Strongylida, Angiostrongylidae) are parasites having a life-threatening potential in several animal species and humans (Spratt, 2015). Among them, *Angiostrongylus vasorum* (Baillet, 1866) is a parasite which may cause severe clinical disease in dogs (*Canis lupus familiaris*), red foxes (*Vulpes vulpes*), wolves (*Canis lupus italicus*) and other carnivores, inhabiting their right-side of the heart and the pulmonary artery (Rosen et al., 1970; Guilhon and Cens, 1973). These final hosts acquire the parasite by swallowing infested terrestrial and aquatic gastropods (Guilhon and Cens, 1973; Ferdushy et al., 2009; Giannelli et al., 2016; Colella et al., 2017). Although the potential role of paratenic hosts, such as frogs (*Rana temporaria*), was described decades ago (Bolt et al., 1993), their

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role in the transmission of *A. vasorum* to dog is still unknown (Morgan and Shaw, 2010). Recently, chickens (*Gallus gallus domesticus*) have also been suggested to be paratenic hosts of *A. vasorum* (Mozzer and Lima, 2015).

Clinical diagnosis of canine angiostrongylosis is challenging since the disease is entirely asymptomatic or just showing clinical symptoms of varying degrees of severity leading to an underestimation of the prevalence of infection (Cavana et al., 2015; Di Cesare et al., 2015; Colella et al., 2016; Olivieri et al., 2017). Nevertheless, the disease could also have a chronic course, characterized by progressive deterioration of the respiratory and cardiac functions, altered blood coagulation and maybe a fatal disease, if not treated (Chapman et al., 2004; Denk et al., 2009; Traversa et al., 2013).

Angiostrongylus vasorum has been reported as being a common parasite in foxes and very frequently in dogs in several endemic countries of Europe (Bolt et al., 1992; Schnyder et al., 2017; Schug et al., 2018), South America (reviewed by Penagos-Tabares et al., 2018), Newfoundland Canada (Jefferies et al., 2010), and it has also been reported from Uganda (Bwangamoi, 1972) and Turkey (Tigin, 1972).

Nevertheless, over the past 20 years, *A. vasorum* has been repeatedly reported in dogs outside the endemic areas (Southwestern France, South of England and Wales, around Copenhagen in Denmark), indicating that this parasite is widely distributed all over Europe (Fig. 1). Serological prevalence of *A. vasorum* infection (from 0.67% in Bulgaria to 6.22% in Slovakia) was recorded in dogs from different European countries (reviewed by Deak et al., 2019), including Italy, where a prevalence of 0.84% was registered.

Reasons for the geographic spread of *A. vasorum* in Europe are currently unclear (Morgan, 2018). The extension of the geographic range of canine angiostrongylosis seems to be related to several conditions, such as climate, temperature and humidity closely associated with the development of intermediate hosts (Simpson and Neal, 1982; Jenkins et al., 2006; Morgan et al., 2009; Willis et al., 2006). Neverthless, no absolute climatic condition seems to be associated with the establishment of *A. vasorum*, since the parasite is present in areas with an average temperature above -4C° (Jeffery et al., 2004) and endemic foci were recorded in the north-eastern region of Slovakia where the average winter air temperature falls below −10 °C (Čabanová et al., 2018).

Furthermore, the urbanization of the red fox (Deplazes et al., 2004; Otranto et al., 2015; Pyšková et al., 2018), the movements of untreated domestic dogs among countries (Deplazes, 2015) and the possible presence of other intermediate and final hosts, still unknown (Morgan and Shaw, 2010), may have played a role in widening the geographic distribution of this parasite.

Indeed, *A. vasorum* is endemic in red fox populations of various European countries, being the main reservoir host for this parasite (Tables 1 and 2).

In Italy *A. vasorum* infection was initially studied in fox populations (Poli et al., 1984, 1991) and subsequently, after the reporting of sporadic cases (Della Santa et al., 2002; Scaramozzino et al., 2007; Traversa et al., 2008), studies confirmed that the parasite is endemic in canine populations in many regions of central and southern Italy as well as in Sardinia (reviewed by Traversa et al., 2019) and coprological surveys attest the prevalence of *A. vasorum* infection between 4.1% (Traversa et al., 2019) to 12.6% (De Liberato et al., 2018) in central Italy.

On the contrary, data on *A. vasorum* infection in wolf are still scarce in Europe and have only recently been reported in Italy (Table 3).

In particular, data of the infection in wolves have been recorded in

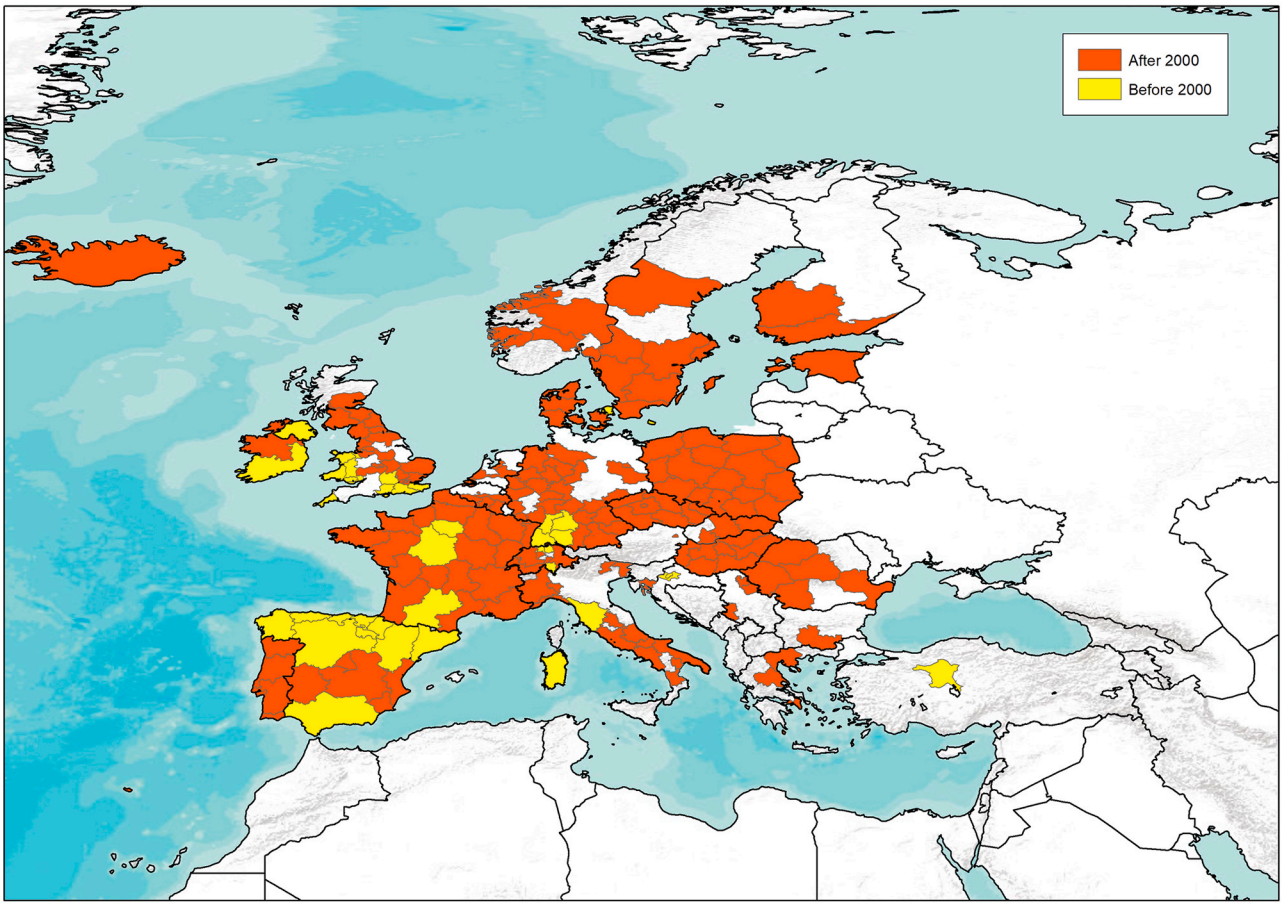

Fig. 1. Map of the geographical areas of Europe where the presence of *Angiostrongylus vasorum* was reported before and after 2000 in definitive and intermediate hosts. This map was created by placing the reports of the literature in Eurostat's nomenclature units for statistics (NUTS).

Table 1
Prevalence of *Angiostrongylus vasorum* infections in foxes in Europe in studies published from 2014 to 2020.

Country	Study period	no. of samples	Specie	Method of analysis	% positive	Reference
The Netherlands (Dutch)	2010–2012	96 carcasses	fox	Necropsy	4.2	Franssen et al. (2014)
Poland (north-eastern)	2013	76 carcasses	fox	Necropsy	5.2	Demiaszkiewicz et al. (2014)
Denmark (Copenhagen; southern Jutland)		70 carcasses (Copenhagen); 48 (Southern Jutland)	fox	Necropsy	80.0 (Copenhagen); 0 (Jutland)	Al-Sabi et al. (2014)
Great Britain	2013–2014	442 carcasses	fox	Necropsy	18.2	Taylor et al. (2015)
Hungary	2013–2014	937 carcasses	fox	Necropsy	17.9	Tolnai et al. (2015)
Spain (north-eastern)	2001–2005	87 carcasses	fox	Necropsy	3.40	Garrido-Castane et al. (2015)
Bosnia and Herzegovina	2015	221 carcasses	fox	Necropsy/PCR	0	Hodžić et al. (2016)
Republic of Ireland	2014–2015	542 carcasses	fox	Necropsy	39.9	McCarthy et al. (2016)
Romania (western)	2016–2017	567 carcasses	fox	Necropsy/PCR	4.2	Deak et al. (2017)
Slovakia	2014–2016	571 faecal samples/frozen	fox	Flotation/PCR	5.43	Čabanová et al., 2018
Serbia (north- central)	2017–2018	83 carcasses	fox	Necropsy	13.2	Gavrilovic et al. (2019)
Spain (north-western)	2008	257 carcasses	fox	Necropsy	43.0	Martinez-Rondan et al. (2019)
Switzerland (north-eastern)	2012–2017	Carcasses: 79 (2012); 87 (2013); 42 (2014); 88 (2015); 83 (2016); 154 (2017)	fox	Necropsy/ Serology	21.5 (2012); 41.4 (2013); 50.0 (2014); 67.0 (2015); 77.1 (2016); 81.8 (2017)	Gillis-Germitsch et al. (2020)
Denmark (whole country)	2017–2018	367 carcasses	fox	Necropsy/PCR	6.8	Lemming et al. (2020)
Norway	2019	300 blood samples	fox	Serology	3.0	Hamnes et al. (2020)

Table 2
Prevalence of *Angiostrongylus vasorum* infections in foxes in Italy in studies published from 1984 to 2019.

Country	Study period	no. of samples	Species	Method of analysis	% positive	Reference
Italy[b] (Tuscany)	1981–1983	180 carcasses	fox	Necropsy/lung impressions	23.8	Poli et al. (1984)
Italy[b] (Tuscany)	1981–1988	509 carcasses	fox	Necrospy/lung impressions	39.09	Poli et al. (1991)
Italy (Sardinia)	1985–1986	85 carcasses	fox	Necrospy/lung impression	15.3	Leoni et al. (1986)
Italy[b] (Tuscany, Grosseto, Cecina, Pisa and Siena provinces)	2004–2005	37 carcasses (Grosseto); 50 (Cecina); 26 (Pisa); 16 (Siena)	fox	Necrospy/lung impression	8.1 (Grosseto); 10.0 (Cecina); 3.8 (Pisa); 0 (Siena)	Magi et al. (2009)
Italy[a] (Liguria, Imperia province)	2004–2005	45 carcasses	fox	Necrospy/lung impression	80.0	Magi et al. (2009)
Italy[a] (Piedmont, Cuneo province)	2004–2005	15 carcasses	fox	Necrospy/lung impression	46.6	Magi et al. (2009)
Italy[b] (Lazio, Roma Province)	2007–2013	62 carcasses	fox	Necrospy/lung impression	43.5	Eleni et al. (2014b)
Italy[a] (Liguria, Imperia province; Pedimont, Cuneo province)	2009–2012	165 carcasses	fox	Necrospy	78.2	Magi et al. (2015)
Italy[c] (Campania)	2012–2014	102 carcasses	fox	Necrospy	33.3	Santoro et al. (2015)
Italy[c] (Apulia)	2011–2013	12 carcasses	fox	Histology/Scanning electronic microscopy of lung	8.3	Passantino et al 2017
Italy[b] (Abruzzo)	2010–2019	222 faecal sample/fresh and frozen	fox	Baermann	16.0	Cocco et al. (2019)

[a] Italy = northern.

[b] Italy = central.

[c] Italy = southern.

the Abruzzo region where, over the last 15 years, the Apennine wolf population has become endemic with an increase in the number of animals (i.e., 38 groups with around 185–249 individuals) (Galaverni et al., 2016).

The aim of the present study was to investigate *A. vasorum* infection in red fox and wolf populations in an area where cases of canine angiostrongylosis have been recorded, to better understand the epidemiology of the disease and to evaluate the possibility of transmission of the parasite in the domestic-wildlife interface.

2. Materials and methods

2.1. Study area and animal's inclusion

From October 2008 to November 2019, foxes and wolves killed by hunters or because of road accidents, for monitoring reasons or simply found dead in different municipalities of the province of Chieti (2600 Km^2, of which 20% of protected areas) in Abruzzo region were included in the study.

Table 3
Prevalence of the presence of *Angiostrongylus vasorum* infections in wolves in Europe in studies published from 2001 to 2019.

Country	Study period	no. of samples/storage	Specie	Method of analysis	% positive	Reference
Spain (northwestern)	1993–1999	47 carcasses	wolf	Necropsy	2.1	Segovia et al. (2001)
Spain (northwestern)	2009–2014	74 carcasses	wolf	Necrospy	21.6	Martinez-Rondan et al. (2019)
Croatia (northwestern)	2002–2011	400 faecal samples/fixed in 90% ethanol	wolf	SAF	3.1	Hermosilla et al. (2017)
Slovakia (Tatra NP, Muranska Planina NP, Polana PLA)	2015–2016	256 faecal samples/frozen	wolf	Flotation/PCR	0.8	Čabanová et al., 2017
Sweden (central-southern)	2010	20 carcasses	wolf	Necrospy/PCR	0	Al-Sabi et al. (2018)
Italia[b] (Rome province)	2011–2012	3 carcasses	wolf	Histology/PCR	100.0 (on 3 carcasses)	Eleni et al. (2014a)
Italy[b] (Lazio region)	2012–2016	25 carcasses	wolf	Histology/PCR	28.0	De Liberato et al. (2017)
Italy[b] (Abruzzo Lazio Molise National Park)	2006–2007	88 Faecal sample/frozen	wolf	SAF	0	Molnar et al. (2019)
France (Mercantour NP)	2006–2007	68 Faecal sample/frozen	wolf	SAF	0	Molnar et al. (2019)
Italy[b] (Abruzzo)	2010–2019	176 faecal sample/fresh and frozen	wolf	Baermann	6.0	Cocco et al. (2019)
Italy[b] (Majella NP in Abruzzo region)	2017	20 faecal sample/frozen	wolf	Flotation	2.0	Di Francesco et al. (2019)

SAF = standard sodium acetate acetic acid formalin; NP= National Park; PLA= Pol'ana protected Landscape; Italy[b] = central.

Abruzzo region is characterized by the class of Mesothermic climates of type C, temperate of the middle latitudes (according to the Köppen-Geiger classification). On the hills and on the side of the Adriatic coast of the province of Chieti, the sub-climate is humid-subtropical, with hot and dry summers and mild and rainy winters; in the hilly and low-mountain Apennine side of the province, the sub-climate is Temperate oceanic with warm and dry summers and cold winters with abundant rain and snow (Aruffo et al., 2018). In the last 40 years, the Abruzzo region has seen an increase in the average daily temperature by 0.6 °C for each decade, clearly above the European average (Aruffo et al., 2018).

In addition, despite selective culling plans put in place within Chieti province (Central Italy) (ISPRA, 2018), the number of foxes remains high from 2013 to 2016 (annual densities ranging from 5.4 to 16.9 foxes/km^2 in the municipality of Orsogna, from 12.2 to 20.82 foxes/km^2 in Ripa Teatina and from 9.1 to 19.7 foxes/km^2 in Casoli) (Demarinis, 2020, personal communication).

The number is high when compared to recently estimated annual densities ranging between 1 and 4 foxes/km^2 in the Tuscany region and in the urban areas of 8 cities in England (Tuscany Region, 2019; Scott et al., 2018).

A total of 44 foxes (23 males and 21 females) and 12 wolves (5 males and 7 females) were delivered at the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise (IZSAM) and subjected to inspection for *A. vasorum*. The carcasses were refrigerated at 4°-6 °C and subjected to necroscopies within 48 h from delivery at the IZSAM. The animals were classified by body size, dentition and extent of tooth wear (Barone, 1981) in two classes of age: juvenile (<1 year of age, 7 foxes and 2 wolves) or adult (>1 year of age, 37 foxes and 10 wolves).

2.2. Wolves genotyping

The skeletal muscle of 6 wolves was sent to the Istituto Zooprofilattico Sperimentale del Lazio e della Toscana to determine their potential hybridization with the dog species, by the genotyping of 18 autosomal microsatellite markers (Lorenzini et al., 2014).

2.3. Parasite collection and Morphological identification

The right-hand area of the heart, pulmonary artery, trachea, bronchi and bronchioles of each animals were cut and examined visually for the presence of adult parasites; then the organs were rinsed thoroughly in water which was placed in a thin layer on large white trays and observed under a light source with the naked eye. Parasites identified were removed and washed in 0.85% saline, fixed in ethyl alcohol 70% and subsequently cleared with glycerin for microscopy studies.

The first larva stage (L1) of *A. vasorum* was instead collected from faecal samples of 35 foxes and 11 wolves by Baermann technique (Traversa et al., 2013), from faecal samples of 29 foxes and 5 wolves by direct microscopic examination and from the lungs of 7 foxes and 2 wolves by tissue impressions (Table 4).

All adult parasites (n = 230) and L1 specimens were identified on a morphological basis using the identification keys previously described (Rosen et al., 1970; Guilhon and Cens, 1973; Costa et al., 2003; McGarry and Morgan, 2009).

Samples were fixed in glutaraldehyde solution and dehydrated through an ethanol series (Allan et al., 2008), critical point dried from carbon dioxide and sputter coated with gold using respectively an Emitech K850 critical point drier and an Emitech K850 sputter coated (Quorum Technologies Ltd, Laughton, United Kingdom). Samples were then observed in a Zeiss DSM 940A SEM (Carl Zeiss, Oberkochen, Germany) operating at 10 kV and images were captured on Carl Zeiss AxioVision Product Suite (Carl Zeiss, Gottingen, Germany).

Carcasses with adult and/or L1 larvae were defined as "cases".

2.4. Post-mortem and histological examination

During necropsies, lung samples were collected from all animals to detect potential disseminated infections. Pulmonary lymph nodes, brain, heart and kidneys were also collected from some carcasses. All tissues were fixed in 10% neutral buffered formalin, embedded in paraffin and routinely processed for histology (Hematoxylin and Eosin stain, H&E).

2.5. Molecular identification

To confirm the morphological identification, 4 adult specimens (n = 3 from fox, n = 1 from wolf) were stored in 70% ethanol and sent to the Parasitology Unit of the Department of Veterinary Medicine (University of Bari, Italy) for molecularly identification. Genomic DNA was extracted using a commercial kit (DNeasy Blood & Tissue Kit, Qiagen, GmbH, Hilden, Germany), in accordance with the manufacturer's instructions. Nematodes were identified by amplification of partial fragment of the nuclear 18S rRNA (~1700 bp) gene using primers and PCR run protocol previously described (Latrofa et al., 2015). Samples without DNA were included as negative controls. PCR products were examined on 2% agarose gels stained with GelRed (VWR International PBI, Milano, Italy) and visualized on a GelLogic 100 gel documentation system (Kodak, New York, USA). The amplicons were purified and sequenced in both directions, using the same primers as for PCR, employing the Taq Dye Deoxy Terminator Cycle Sequencing Kit (v.2, Applied Biosystems), in an automated sequencer (ABI-PRISM 377). The sequences obtained were compared by Basic Local Alignment Search Tool (BLAST – http://blast.

Table 4
Number of animals examined and their positivity (%) for *Angiostrongylus vasorum* obtained by anatomopathological and parasitological examination and number of adult specimens collected.

Identification number of case	Number of adult *A. vasorum*	Stool direct microscopic examination	Baermann technique	Tissue impression from the lung
Foxes				
1+	9	–	+	
2+	9	–	+	
3+	0	–	+	
4+	15	+	+	
5+	1	+	+	
6+	0	–	+	
7	0	–	–	
8	0	–	–	
9+	2			
10	0	–	–	
11+	5	+	+	
12+	15	+	+	
13+	2	–	–	
14	0	–	–	
15+	5	–	–	
16+	9	+	–	
17+	2	–	–	
18+	12	–	+	
19+	2	+	+	
20+	0		+	+
21+	6	+		
22+	15			+
23+	10		+	
24+	2	–	+	
25+	1	+	+	
26+	0	–	+	
27+	25	+		+
28+	0		+	
29	0	–	–	
30	0		–	
31	0		–	
32	0		–	
33+	2			+
34	0		–	
35+	2			
36	0	–	–	
37	0	–	–	
38+	6	+		+
39+	8			+
40+	20		+	
41+	7		+	
42+	4	+	+	–
43+	3			
44+	10	–	–	
***33/44 (75%) (60.5%-85.4%)**	**Total parasites 209 *28/44**	***11/29**	***19/35**	***6/7**
Wolves				
1	0		–	
2	0		–	
3+	0	+	+	
4+	3	–	+	–
5	0	–	–	
6	0			
7+	4		–	
8+	7		+	
9+	4	–	–	–
10+	1	–	+	
11+	1		–	
12+	1		–	
***8/12 (66.7%) (38.6%-86.1%)**	**Total parasites 21 *7/12**	***1/5**	***4/11**	***0/2**

+ = positive; - = negative; Blank space = not examined * = total positive/tested.

ncbi.nlm.nih.gov/Blast.cgi) with those available in the GenBank database.

2.6. Statistics

Data for each type of examinations and in relationship to the species (i.e., foxes and wolves) were analysed by observing the percentage of positives and by the relative 95% confidence intervals (95% CI), calculated using a Bayesian-type approach (using the Beta distribution). A chi-square test analysis was carried out for foxes to verify the difference in the frequencies of positive and negative ones according to sex and age class (in the latter case using Fisher's exact test, due to the frequencies expectations which were less than 5). The non-parametric Mann-Whitney tests were applied to verify differences in the number of parasites found according to sex and age categories. The data related to wolves are reported only from a descriptive point of view (due to their low number).

3. Results

3.1. Animal's inclusion and wolves genotyping

The anatomo-pathological and parasitological examinations indicated the presence of *A. vasorum* in 33 foxes (75%) and in 8 wolves (66.7%) (Table 4). The largest number of foxes came from municipalities lying in the central and southern part of the province (Fig. 2), while as for the wolves from the northern part (Fig. 3).

All six wolves tested by genotyping were pure *Canis lupus italicus* without any mixing genetic material of dog.

3.2. Parasite collection and Morphological identification

Overall, 230 adults and L1 specimens were collected and morphologically identified as *A. vasorum*.

Adult worms have a slender and elongated body that tapers at each end and the oral orifice is small and circular, surrounded by six small papillae (Fig. 4). Males were 14–18 mm in length, with visible copulatory bursa with two symmetrical lateral lobes; spicules were long, strong and subequal (Fig. 4). Females were slightly larger, 18–25 mm in length; the vulva was situated in the posterior region of the body and anteriorly to the anus Fig. 4.

3.3. Post-mortem and histological examination

Light and mild severity pulmonary involvement (Poli et al., 1991) was observed at the post-mortem examination. In the light severity involvement, a presence of scattered slightly raised grey-white encapsulated nodules on the diaphragmatic lobes of the lung and little greyish and firm areas were observed whilst in the mild severity involvement, the ventral part of all lobes showed large wedge-shaped areas of reddish-brown or yellow-brown coloration with an increased consistency of the lung parenchyma (Fig. 5).

Among *A. vasorum* infected foxes and wolves, macroscopic lesions were observed in 28 foxes (84.8%) and in 3 (37.5%) wolves, respectively. More serious lesions with involvement of the pleura, pericardium and mediastinum were not observed. Detailed results are presented in Table 5 and Table 6.

Histopathological investigations revealed parasitic bronchopneumonia in 19 (57.6.%) foxes and in 5 (62.5%) wolves with granulomatous foci in lung parenchyma (Fig. 6 A, B). In addition, parasitic lymphadenitis (2 foxes and 1 wolf) (Fig. 6 C) and parasitic meningoencephalitis (Fig. 6 D, 1 fox) were also observed. At histological examination, parasitic granulomatous foci in lung parenchyma in seven infected foxes were not detected while all infected wolves demonstrated lung and/or pulmonary lymph nodes involvement.

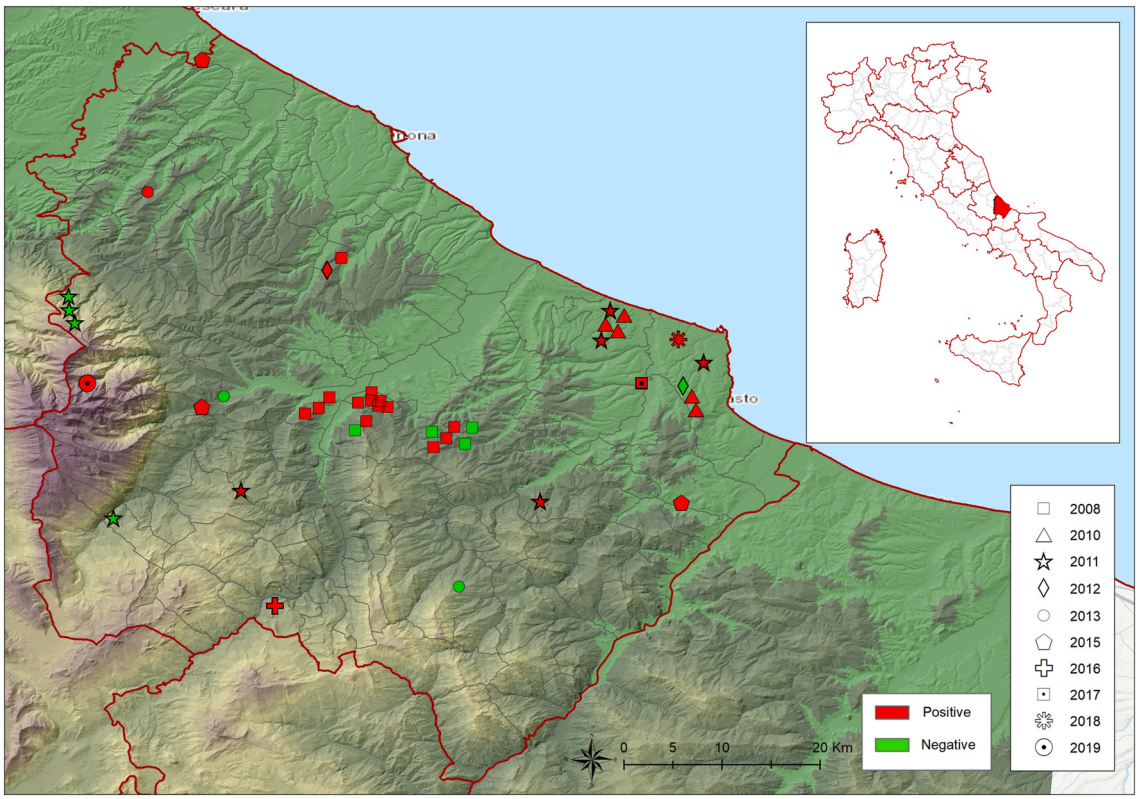

Fig. 2. Province of Chieti, showing the municipalities of origin in which tested foxes resulted positive for *Angiostrongylus vasorum*.

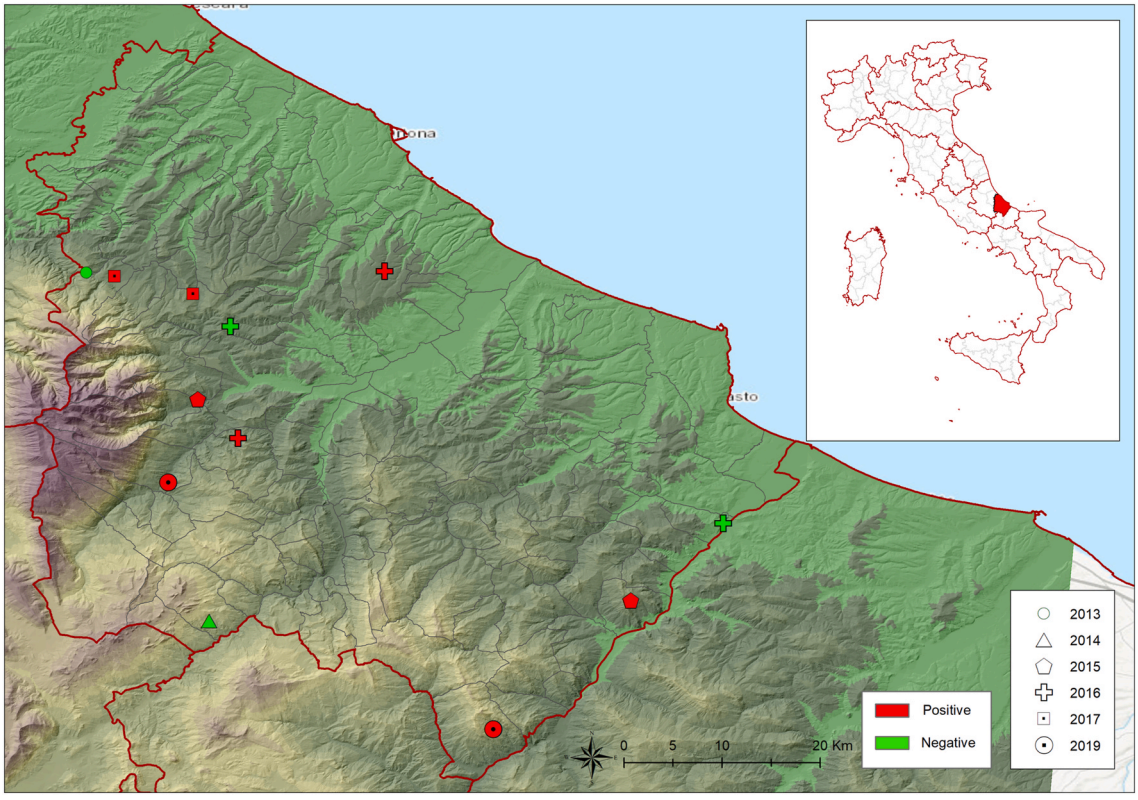

Fig. 3. Province of Chieti, showing the municipalities of origin in which tested wolves resulted positive for *Angiostrongylus vasorum*.

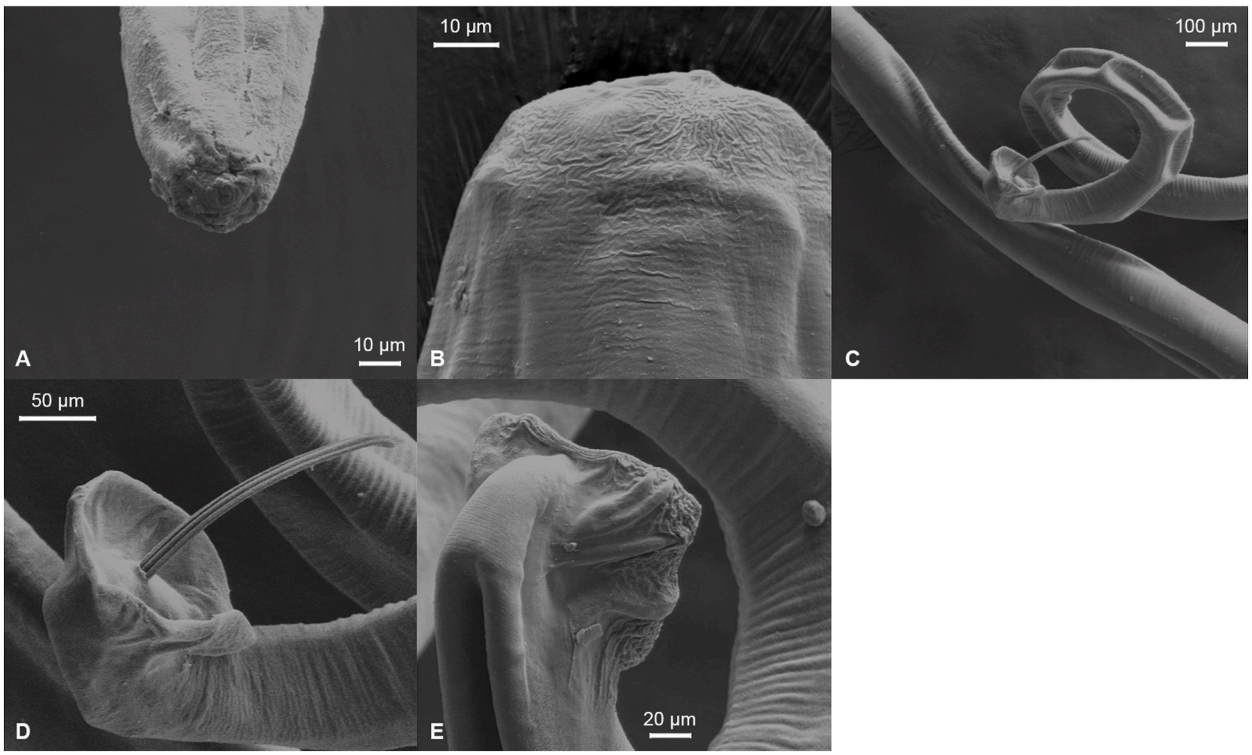

Fig. 4. *Angiostrongylus vasorum*: scanning electron micrographies (SEM). A higher magnification of A) anterior end of adult worm, enface view showing the oral orifice; B) anterior end of adult worm, lateral view; C) posterior end of adult male, enface view showing copulatory bursa and spicules; D) detail of posterior end of adult male at higher magnification; E) posterior end of adult male, showing lateral rays of copulatory bursa.

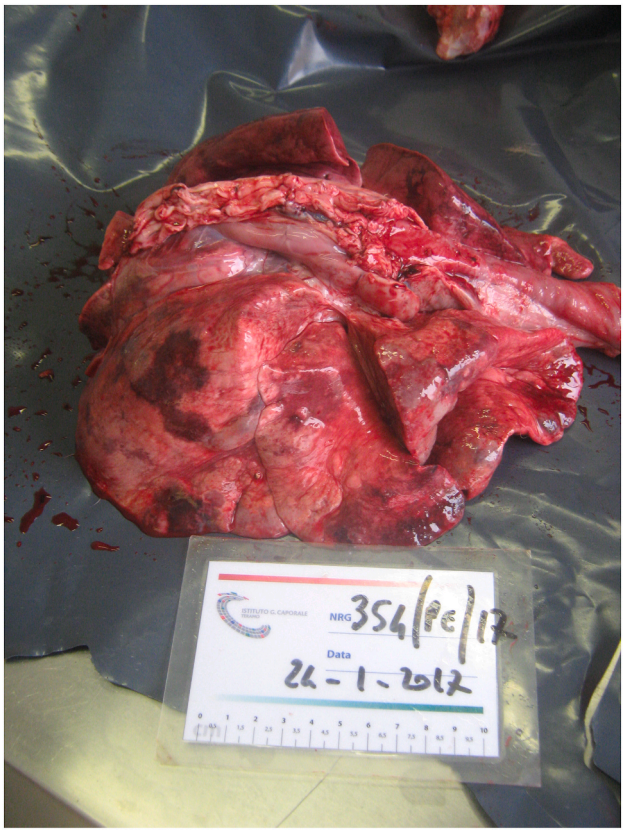

Fig. 5. Lungs of wolf infested with *Angiostrongylus vasorum* in mild severity involvement.

3.4. Molecular identification

PCR amplification of 18S rRNA from individual DNA samples resulted in amplicons of the expected size. The molecular analysis supports the morphological identification, being all the specimens molecularly identified as *A. vasorum*.

BLAST analysis of all sequences obtained showed the nucleotide identity of 100% with those available in GenBank database (EF514916).

3.5. Statistics

Data for each type of examinations and species (distinct by foxes and wolves) are reported (Tables 4 and 5).

In foxes, the chi-squared test found no statistically significant difference in the distributions of positives and negatives by gender (chi-squared = 0.273, p.value > 0.601). The comparison between age categories in the distribution of positive and negative ones did not reveal statistically significant differences (Fisher's exact test p.value = 0.659). There are no statistically significant differences in parasites detected by sex (Mann-Whitney U = 314.5, p.value = 0.082) and by age categories (Mann-Whitney U = 107.5, p.value = 0.482).

No inference can be made on the wolf data due to the low number of observations.

4. Discussion

The results of this study clearly showed that foxes and wolves were infected by *A. vasorum* in Central Italy.

Angiostrongylus vasorum is endemic in Abruzzo region with a higher prevalence in foxes (75%) compared with those recorded in the other regions of central and southern Italy (up to 43.5%, Tuscany, Lazio, Campania and Apulia); the prevalence was the same as that of north-western Italy (up to 78.2%, Liguria and Piedmont) (Table 2). Similarly, the prevalence of *A. vasorum* infection recorded in this study was higher

Table 5
Lung lesions and presence of *Angiostrongylus vasorum* larval/adult on histological examination tissues from foxes and wolves. In positive cases for *A. vasorum* (+), lung injury is reported according to the classification of Poli et al. (1991).

Identification number of case	Macroscopic lung lesion	Lung	Brain	Kidney	Pulmonary lymph nodes
Foxes					
1+	L				
2+	M	–	–	–	
3+	M	+	–	–	–
4+	L	–	–	–	–
5+	M	+	–	–	–
6+	M				
7	Pneumonia	–		–	
8	Pulmonary congestion	–	–	–	
9+	M	+			–
10	N	–			–
11+	L	+	–	–	–
12+	M	–	+	–	+
13+	L	+	–	–	
14	Pneumonia	+ (other parasites)			
15+	L	+			
16+	M	+			
17+	M	+			
18+	M	+			
19+	M	+			
20+	N	–			
21+	N	+		–	
22+	M	+			
23+	M				
24+	M	+			
25+	M				
26+	M	–			
27+	M	+			
28+	N				
29	N	+ (other parasites)			
30	Pulmonary congestion				
31	Pulmonary edema				
32	Pulmonary edema				
33+	M	+	–	–	
34	Pneumonia				
35+	L				
36	Areas of bleeding				
37	Pneumonia	+ (other parasites)			
38+	N	+			
39+	L	–			
40+	L	–	–	–	
41+	M	+	–		
42+	M	+			
43+	N				
44+	M	+			+
Total on 33 positive cases	**M = 20; L = 8; N = 5**	***19/26**	***1/10**	***0/10**	***2/7**
Wolves					
1	N				
2	Areas of bleeding				
3+	N	+			
4+	M	+	–		
5	Areas of bleeding	+ (other parasites)			
6	Areas of bleeding	–			
7+	N				
8+	N	+	–		
9+	M	+	–	–	
10+	N		–		
11+	M	+			–
12+	N				+
Total on 8 positive cases	**M = 3; L = 0; N = 5**	***5/5**	***0/4**	***0/1**	***1/2**

+ = positive; - = negative; Blank space = not examined; L = light; M = mild; N = absence of lesions * = total positive/tested.

Table 6
Distribution of *Angiostrongylus vasorum* positive carcasses according to the severity of macroscopic lung lesions and the age of the foxes and the wolves examined.

Type of lung injury	Adults (n/%)	Juveniles (n/%)	Total (n/%)
In Foxes			
No lesions	5 (15.2)	0	5 (15.2)
Light lesions	8 (24.2)	0	8 (24.2)
Mild	14 (42.4)	6 (18.2)	20 (60.6)
Severe	0	0	0
Total	27 (81.8)	6 (18.2)	33 (100)
In wolves			
No lesions	4 (50)	1 (12.5)	5 (62.5)
Light lesions	0	0	0
Mild	2 (25)	1 (12.5)	3 (37.5)
Severe	0	0	0
Total	6 (75)	2 (25)	8 (100)

than that reported in Europe (up to 43.0%, Great Britain, Hungary, Norway, Poland, Republic of Ireland, Romania, Serbia, Slovakia, Spain, Netherlands), except in Copenhagen (Denmark) and Switzerland (2016–2017) where it is high at the same level (Table 1).

In particular, the high level of prevalence (66.7%) of infested wolves herein described was notably higher than that previously reported in other European countries (Table 3).

Previous studies carried out in Abruzzo region, examining wolf faeces recorded a percentage of infestation lower than 6% (Table 3) probably due to testing frozen samples, which may have led to an underestimated level of infestation (Jeffery et al., 2004).

However, the sampling of *A. vasorum* from carcasses could have affected the results. Indeed, in general the surveys carried out on wildlife animals or carcasses found in the environment may have been affected by biases linked to intrinsic biological factors, to mismatch of sampling scale and processing scale, to diagnostic procedure and to the passive recruitment of the sampled animals, that make data not easily comparable (Lachish and Murray, 2018).

On infested foxes, no significant differences were observed with respect to sex and age as observed in other surveys in Italy (Magi et al., 2015; Santoro et al., 2015; Eleni et al., 2014b).

The prevalence of infection in foxes and in wolves was higher than that in dogs (8.9%) in 2008 from the same geographical area (Tieri et al., 2011). This result was a further confirmation of the hypothesis already formulated, where the spread of canine angiostrongylosis was linked to the presence of the *A. vasorum* infection in foxes, representing an epiphenomena of the parasite's wild cycle (reviewed by Bolt et al., 1994; Morgan et al., 2005). Traversa et al. (2019) have already shown that in central Italy an unlimited outdoor access enhances the possibility of infection for dogs because of higher possibilities of ingestion of intermediate hosts.

In January 2008, immediately after the first post-mortem diagnosis of *A. vasorum* bronchopneumonia in a dog in the province of Chieti, IZSAM provided technical-practical seminars to inform the veterinary practitioners of the area and from neighboring provinces about the

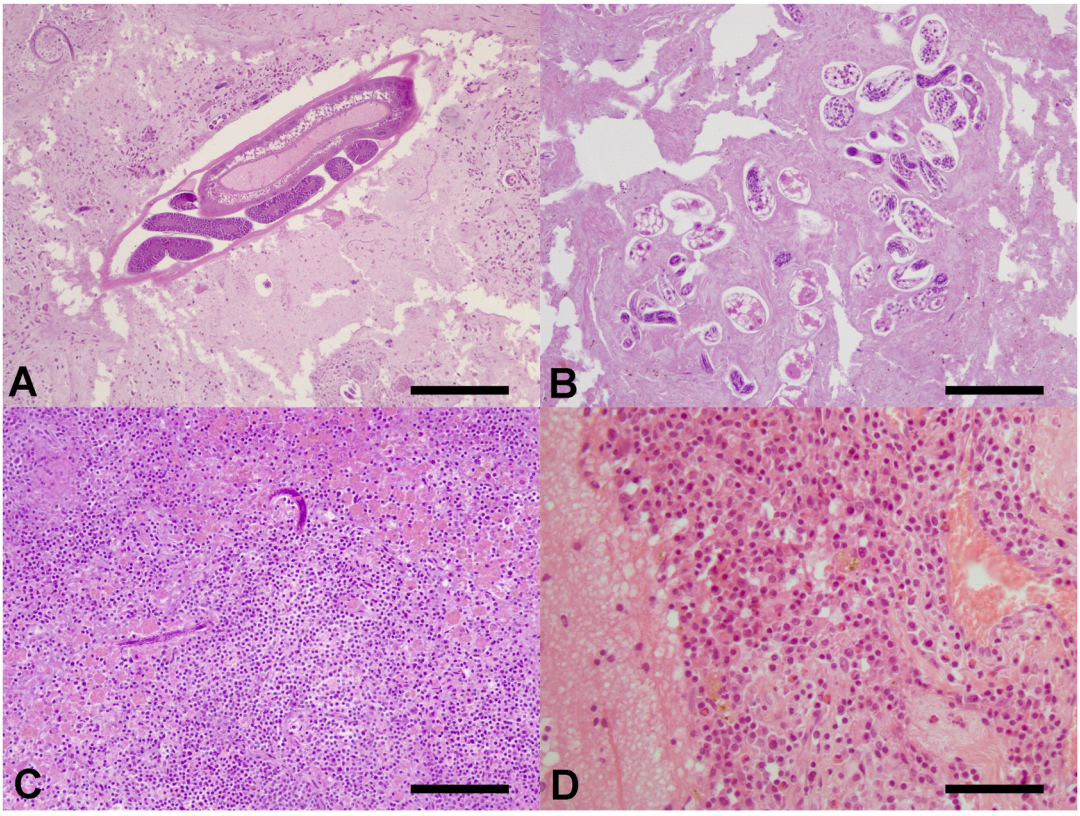

Fig. 6. Lung of red fox: adult nematode within a pulmonary artery (A). Lung of wolf: nematode larvae were observed in alveolar spaces causing a thickening of connective tissue and a fibrotic response (B). Two nematode larvae were visible in the mediastinal lymph node of a red fox (C). Lymphocytic and eosinophilic infiltrates were present in brain tissue of a red fox infected with *A. vasorum* (D). Hematoxylin and Eosin (H&E) stain. Final magnification: × 100 (A–B), ×200 (C), × 400 (D). Scale bar: 200 μm (A–B), 100 μm (C), 50 μm (D). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

presence of the "new" parasite, allowing them to diagnose, prevent and treat the disease in clinics and kennels. Two other cases had been observed in the province of Teramo (Traversa et al., 2008). Therefore a great information activity was carried out throughout the Abruzzo territory by universities and by the local health veterinary services.

Since then, rare cases of canine angiostrongylosis have been observed in the necroscopy rooms of IZSAM (Tieri, 2021, personal communication).

It is impossible to estimate the date of appearance and the origin of the animal's infection, whether it was spread by dogs imported from endemic areas or if local dogs contacted the disease while travelling with their owners (Panarese et al., 2020). This is possible as a result of the introduction of infected hunting dogs and with the subsequent establishment and spread in the local fox population as already established in some specific areas (e.g., Spain) for the eyeworm *Thelazia callipaeda* (Otranto et al., 2021). In addition, the role of imported wildlife from endemic areas should not be disregarded (Bezerra-Santos et al., 2021).

In the last 20 years, the number of hunters has decreased in Italy (Vallini, 2019), but in the province of Chieti there are currently 2985 hunters registered, equivalent to 28% of the Abruzzo region (ISPRA, 2018): it should be checked whether the dog's hunting practice is a risk factor for canine angiostrongylosis as already observed in France (Gossart et al., 2018).

Abruzzo (10,830 Km^2) is the Italian region with more protected areas, known as the "green region of Europe", with its 4 parks and the numerous nature reserves and 53 sites of community importance (SCIs), a large part of the territory (35%) is managed in an ecologically sustainable manner (Di Fabrizio, 2018; Febbo, 2018). After the European "Habitats" Directive 92/43/EEC and "Birds" Directive 2009/147/EC, in the last 20 years the parks have become operational and nature has flourished again (Febbo, 2018; Pace, 2018). Based on population estimates made by Galaverni et al. (2016), the Appennine wolf sub-population seems to be almost the double in size (with 1212–1711 wolves in the period 2009–2013) compared to previous estimates (600–800 wolves between 2006 and 2011). The recovery of the forest in areas previously used for agriculture and pastoralism, the progressive depopulation of vast mountain areas and the consequent decrease in direct persecution, have contributed to the growth of the populations of wild ungulates, including the wild boar, which represents main trophic source of the wolf in this territory (Meriggi and Lovari, 1996).

Based on the results of this study, the province of Chieti appears to be an enzootic focus for canine angiostrongylosis; the increasing populations of foxes and wolves (Galaverni et al., 2016; Demarinis, 2020, personal communication) may play an important role in the maintenance of the parasite in the environment.

Furthermore, the chronic gross and microscopical lesions observed in both animals suggest that the fox and the wolf may have dispersed the larvae in the environment for the entire duration of their life.

The presence of *A. vasorum* in Abruzzo region matches the predicted distribution described by the epidemiological model of Morgan (2009), based on some climatic factors that limit or favour the presence of the parasite, by influencing the life of intermediate hosts, to predict the areas of future expansion of *A. vasorum* in the world.

In 2007, only 2 cases were reported in dogs in the province of Teramo (Traversa et al., 2008); since then there has been the expansion of the disease in all the provinces of Abruzzo.

In view of these results, to ensure protection to the dog population, it is advisable to provide constant monitoring of the spread of *A. vasorum* among sylvatic reservoirs.

Further investigation is needed for a better epidemiological

understanding of the presence of snails and slugs within the dog's environment and to establish whether other wild carnivores, such as badgers (*Meles meles*), whose population is estimated in northern Italy at around 0.93–1.4/Km (Balestieri et al., 2016), may contribute to the spread of *A. vasorum*.

It is also hoped that new studies will provide veterinary practitioners with suitable tools and methods to sensitize dog owners to implement therapy and prevention, which in an endemic area such as the province of Chieti, can probably be defended only with an appropriate chemo-prophylactic regimen.

Declarations of competing interest

None.

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