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SERIES XXXV

ARTHROPOD-BORNE PARASITIC DISEASES IN WILD AND DOMESTIC FELIDS IN NORTH-EASTERN ITALY

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Index

Abstract

Cytauxzoonosis, hepatozoonosis and dirofilariosis are vector-borne diseases transmitted by arthropods (i.e., ticks and mosquitos). Felids could be susceptible to blood-feeding vectors and, consequently, to all of these diseases, nevertheless, the awareness of VBDs in these animals is still lacking.

During the past decades, arthropod-borne diseases in felines have stimulated the interest of the Scientific Community and epidemiological studies on *Cytauxzoon* spp., *Hepatozoon* spp. and *Dirofilaria immitis* are increased in Europe. Nevertheless, the presence and distribution of *Cytauxzoon* spp. and *Hepatozoon* spp. in Italy are still scant and mostly limited to a few areas of Northern and Southern regions, respectively. Although canine heartworm (HW) is endemic in North-eastern Italy, very little information has been recorded in felids.

Cytauxzoon and *Hepatozoon* are vector-borne parasites, even if the involved arthropod vector/s are not still known. Recently, *Hepatozoon* DNA was detected only in some species of engorged ticks suggesting that those tick species could act as a vector. Nonetheless, the vector competence of ticks in *Cytauxzoon* spp. and *Hepatozoon* spp. transmission is not yet proved by scientific studies.

Stained blood smears are a not-sensitive method for these haemoprotozoa diagnosis due to the low parasitaemia. Molecular analyses are strongly recommended since they present higher sensitivity (i.e., conventional PCR and real-time PCR).

Dirofilaria immitis has an elusive nature leading to unpredictable effect on cat host. Indeed, the heartworm disease diagnosis is difficult. In addition, no single test is able to detect *D. immitis* in each of its stages in felid hosts and multiple diagnostic methods are necessary to confirm the infection.

The present research project aims to provide new data on the circulation of *Cytauxzoon* spp., *Hepatozoon* spp. and *D. immitis* and to update the current epidemiological scenario in North-eastern Italy (i.e. Veneto, Friuli Venezia Giulia and Trentino Alto Adige regions) *i)* improving the knowledge on the presence and distribution of the considered parasites in different felid species (i.e., domestic cats, wildcats, exotic captive felids); *(ii)* investigating on the possible role of ticks in the transmission of *Hepatozoon* spp. and *Cytauxzoon* spp., and *(iii)* developing diagnostic protocols to provide fast and sensitive screening procedures.

Background

Vector-borne diseases (VBDs) affect various animal species including humans and companion animals. Some VBDs are considered emerging for their rapid expansion in incidence and geographic distribution probably driven by several factors: climate change, in particular, global warming, favouring the increase of arthropod vectors' spread and activity (Michelutti et al., 2021), as well as globalization allowing the invasion and colonization of new territories by invasive arthropod vectors (Silaghi et al., 2017; Montarsi et al., 2019). In addition, the human factor is extremely important in this process. Excessive anthropization leads to the destruction of ecological wildlife niches favouring the approach of wildlife to the urban context and the risk for companion animals coming in contact with new pathogens (Traversa et al., 2014).

In particular, felids are susceptible to a wide range of blood-feeding vectors (i.e., fleas, ticks, mosquitoes), nevertheless, the knowledge relating to VBDs in these animals is still lacking (Otranto et al., 2017; ESCCAP, 2019).

In the last decades, arthropod-borne diseases in felines have stimulated the interest of the scientific community. As a matter of a fact, reports on *Cytauxzoon* and *Hepatozoon* (Díaz-Regañón et al., 2017; Legroux et al., 2017; Nentwig et al., 2018; Panait et al., 2021) and on *Dirofilaria immitis* detection in Europe (Diakou et al., 2019; Genchi and Kramer, 2020; Pană et al., 2020; Kulmer et al., 2021; Schäfer et al., 2021; Tonev et al., 2021; Brianti et al., 2022; Montoya-Alonso et al., 2022) are recently increased. If the epidemiological studies about *Cytauxzoon* spp. and *Hepatozoon* spp. in wild and domestic felids are constantly updated (Hodžić et al., 2017; Panait et al., 2021; Willi et al., 2022), on the other hand, reports of *Dirofilaria immitis* are rarely described in cats compared to dogs (ESCAPP, 2019; Pennisi et al., 2020).

In light of previous considerations, the lack of updated data in North-eastern Italy suggests the need to investigate the presence of these parasites in felids' populations.

Cytauxzoon and *Hepatozoon* are the etiological agents of cytauxzoonosis in felids and hepatozoonosis in a wide range of animals worldwide, whereas *D. immitis* is the causative nematode of heartworm disease in canids and felids.

The genus *Cytauxzoon* was described for the first time in a domestic cat (*Felis silvestris catus*) in 1976 in the US, and due to the host, the species was defined as *Cytauxzoon felis* (Wagner 1976). Since then, cases are limited to the South-eastern, South-central and mid-Atlantic

United States (Reichard et al., 2008; Brown et al., 2010; Shock et al., 2011; Miller et al., 2013; Tarigo et al., 2013).

Since the 2000s, *Cytauxzoon* has been documented in Europe. Interestingly, European species molecularly differ from *C. felis* isolates reported up to that moment; indeed, *Cytauxzoon* is considered as a monophyletic group, characterised by different isolates grouped in separate species (i.e., *C. felis, Cytauxzoon manul*) (Jalovecka et al., 2019). Thanks to recent studies it is possible to characterize even the species circulating in Europe. Among the isolates from European wild felids, three genotypes of *Cytauxzoon* (i.e., major-EU1, minor-EU2, rare-EU3) were defined as three new species: *Cytauxzoon europaeus, Cytauxzoon banethi* and *Cytauxzoon otrantorum* (Panait et al., 2021). The protozoan parasite is widely distributed in Europe, from the Mediterranean basin (Criado-Fornelio et al., 2004; 2009; Alho et al., 2016b; Díaz-Regañón, 2017, Legroux et al., 2017; Nentwig et al., 2018; Panait et al., 2021; Willi et al., 2022) up to Northern (Panait et al., 2020; 2021) and Eastern (Panait et al., 2021) European countries.

Italian cases of cytauxzoonosis are limited to some regions (Carli et al., 2012; 2014; Ebani et al., 2020; Antognoni et al., 2022). An endemic focus was described in the city of Trieste (Friuli Venezia Giulia region) in North-eastern Italy since 2012 (Carli et al., 2012), and some isolated cases are reported in North and Central Italy (Ebani et al. 2020; Carli et., 2014).

Hepatozoon spp. can be found in several host species, i.e., mammals, reptiles, birds, and amphibians (Dahmana et al., 2020).

Among mammals, *Hepatozoon* spp. was reported in domestic cats for the first time in India at the beginning of the 1900s and named *Leucocytozoon felis domestici* (Patton, 1908). Subsequently, it was reclassified in the genus *Hepatozoon* (Wenyon, 1926). Since then, only few reports were published until 1973 due to the description of *Hepatozoon*-like protozoa schizonts in the myocardium of a domestic cat in Israel (Klopfer et al., 1973). *Hepatozoon* has been described in felids worldwide, including Africa (Leeflang and Ilemobade, 1977; Van Amstel, 1979; Pereira et al., 2019), Northern and Southern America (Ewing, 1977; Perez et al., 2004), and Europe (Beaufils et al., 1998; Díaz-Regañón et al., 2017; Criado-Fornelio et al., 2009; Vilhena et al., 2013; Attipa et al., 2017; Kegler et al., 2018; Basso et al., 2019). In Italy, feline hepatozoonosis was reported in the Emilia Romagna region (Ebani et al., 2020) and in Southern regions, i.e., Apulia and Basilicata (Giannelli et al., 2017) and the Aeolian Islands (Otranto et al., 2017).

Cytauxzoon and *Hepatozoon* are apicomplexan protozoa provided, by definition, with an apical complex structure, useful for penetrating the host cell and they are classified in Piroplasmida and Eucoccidiorida order, respectively. Usually, piroplasms carry out an asexual phase in the vertebrate host (i.e., intermediate host) and a sexual phase in the invertebrate one (i.e., definitive host).

The biological cycles of European *Cytauxzoon* and *Hepatozoon* species affecting felids are still not entirely known, nevertheless, there may be similarities with the already-known life cycles of *C. felis* and *Hepatozoon canis,* respectively*. Cytauxzoon* and *Hepatozoon* are vector-borne parasites, even if the involved arthropod vector/s are not still known. In US, *Amblyomma americanum*, the lone star tick, and *Dermacentor variabilis*, the American dog tick, are suitable arthropods for *C. felis* transmission (Wang et al., 2017).

Cytauxzoon felis infection starts when an infected, presumably, tick inoculates sporozoites into a felid host during its blood meal, then sporozoites multiply inside the cells of the myeloid lineage (i.e., macrophages), become schizonts in endothelial cells in lymphatic tissue and organs, i.e., lung, liver, spleen, lymph node (Weiss and Tvedten, 2012), and produce merozoites. Free merozoites infect erythrocytes which a tick takes during its blood meal. Gametogenesis takes place in the gut of the tick and concludes with the production of the zygote and the infectious sporozoites at the level of salivary glands (Wang et al., 2017). In addition, another potential route of transmission is the blood transfusion, thus, it is important to screen blood donor cats, especially in endemic areas (Wang et al., 2017).

The main vector of *H. canis* is *Rhipicephalus sanguineus,* the brown dog tick. Dogs get infected during grooming by the ingestion of infected ticks containing the *Hepatozoon* oocysts. After digestion, the oocyst releases sporozoites that reach the host organs (i.e., liver, spleen, lymph node, muscle and bone marrow) through the lymph-blood route. In organs, the sporozoites generate meronts by asexual replication. Merozoites emerge from meronts, infect circulating neutrophils and develop into gamonts. Ticks become infected by taking parasitized neutrophils during the blood meal. In the tick's gut, sexual reproduction takes place with the production of a zygote or oocyst containing sporozoites.

Further transmission routes are described, such as the vertical transmission from mother to offspring (Schäfer et al., 2022) in *H. canis* life cycle and the predation of an infected paratenic host as reported in *H. americanum* life cycle (Baneth, 2011).

Hitherto, *Hepatozoon* DNA was detected only in different species of engorged ticks suggesting that several tick species could act as a vector, i.e., *Rhipicephalus sanguineus, Ixodes hexagonus,*

Haemaphysalis parva and *Ixodes ricinus* (Bhusri et al., 2017; Duplan et al., 2018; Orkun and Emir, 2020; Hornok et al., 2022). Nevertheless, since they were engorged, there is no certainty that the parasite multiplies in the tick rather than in the host.

The observation of merozoites in red blood cells and gamonts in white blood cells is the expression of cytauxzoonosis and hepatozoonosis, respectively. The stained blood smear may allow the observation of the intraerythrocytic inclusions but unfortunately is not a sensitive diagnostic method. Indeed, cytauxzoonosis is characterized by a low burden of merozoites circulating in the bloodstream both in acute and chronic infection (Brown et al., 2008). Hepatozoonosis in felids is usually asymptomatic and characterized by low parasitaemia, around 1% of infected white blood cells (Baneth et al., 2011). For this reason, stained blood smears are indicative but not the method of choice, and molecular analyses are strongly recommended since they represent the gold standard methods for the diagnosis due to the higher sensitivity (Brown et al., 2008).

Conventional polymerase chain reaction (cPCR) for *Cytauxzoon* and *Hepatozoon* detection, targeting the 18S rRNA gene, is reported and frequently used in recent decades (Criado-Fornelio et al., 2003; Reichard et al., 2005; Bonnet et al., 2007; Meli et al., 2009; Filoni et al., 2012; Hodžić et al., 2015; Kegler et al., 2018; Panait et al., 2020). Nevertheless, mitochondrial protein-coding genes are necessary (i.e., cytochrome B and cytochrome C oxidase subunit I (COI)) to obtain a more specific identification of *Cytauxzoon* species (Panait et al., 2021). Real-time polymerase chain reaction (real-time PCR) may be considered as an alternative sensitive approach. Some protocols targeting the piroplasmid 18S-rRNA gene for the detection of *Cytauxzoon* spp. or *Hepatozoon* spp. one at a time in tissue samples and blood of domestic and wild felids in Europe have been newly described (Basso et al., 2019; Antognoni et al., 2022).

Dirofilaria immitis is the causative nematode of heartworm diseases. The adult stage of the nematode is located in the pulmonary arteries and right heart of definitive hosts such as dogs (*Canis lupus familiaris)* and other canids, i.e., wolves (*Canis lupus*), foxes (*Vulpes vulpes*), and European jackals (*Canis aureus*). Nonetheless, other animals can also be infested, such as the domestic cat (*Felis silvestris catus*), ferret (*Mustela putorius furo*), and coypu (*Myocastor coypus*), although they do not act as reservoirs of the parasite (McCall et al., 2008).

Dirofilaria immitis is reported worldwide. Regarding Europe, the nematode is mostly endemic in Southern countries and islands (Montoya-Alonso et al., 2011; 2015; 2017; Diosdado et al., 2018; Alho et al., 2018) and in the Mediterranean basin (Pantchev et al., 2009; Angelou et al., 2019; Diakou et al., 2019). Nevertheless, the parasite occurs even in Central and Eastern

countries (Mircean et al., 2012; Ciucă et al., 2016; Ionică et al., 2016; ESCCAP, 2019; Pană et al., 2020; Kulmer et al., 2021) in both dogs and cats.

Cats may be definitively at risk wherever infested dogs and competent vectors are present (CAPC, 2020). Feline dirofilariosis has the hypothetical prevalence of 9-18% of dogs' prevalence in an endemic area (Venco et al., 2011).

Northern regions of Italy (i.e., Veneto, Friuli Venezia Giulia, Emilia Romagna, Piedmont and Lombardy regions) are considered hyperendemic due to their location along the Po River Valley, which is considered the largest endemic area in Europe (Genchi et al., 2005).

Felids are potential hosts, but not ideal, due to their innate resistance to this parasite (Genchi et al., 2018). In most cases, microfilaraemia is absent or with a scant burden (Mazzariol et al., 2010; Genchi et al., 2018) due to the fact that heartworms may die before becoming adults and therefore not produce microfilariae. For this reason, unlike the dog, the cat cannot act as a reservoir of the parasite and as a source of infection for the mosquito vectors (Kramer and Venco, 2018).

The parasite rarely reaches the adult stage. Although sometimes it is possible, their number is relatively low and generally, one-third are same-sex nematodes, mostly males (McCall et al., 2008; AHS, 2020). The infection in felids is usually asymptomatic or pauci-symptomatic, and sometimes conducting a sudden death (Alho et al., 2016a; Biasato et al., 2017; Diakou et al., 2019). The elusive nature of the disease and the unpredictable effect on the cat host make heartworm disease difficult to diagnose. No single test is able to detect *D. immitis* in each of its stages (McCall et al., 2008; Nelson, 2008) and multiple diagnostic methods are necessary to confirm the infection (AHS, 2020; Venco et al., 2011).

Although in North-eastern Italy heartworm disease is present since a long time (Birago, 1626) and cytauxzoonosis is reported in stray cats since 2012 (Carli et al., 2012), data on these parasites are not updated. In addition, information on hepatozoonosis in felids is still lacking in the area.

Hence, since the environmental conditions favourable for the survival of the vectors, the presence of the vectors themselves, and the documented cases of diseases in cats, North-eastern Italy represents a scenario that deserves to be investigated in terms of epidemiology, molecular characterization, vector capacity and diagnostic methods.

SECTION 1

Cytauxzoon spp. and *Hepatozoon* spp.

1. Aims of the research and outputs

The research aims are to provide new information on the circulation of the investigated parasites and to update the current epidemiological scenario in North-eastern Italy *i)* improving the knowledge on the presence and distribution of *Cytauxzoon* spp. and *Hepatozoon* spp. in different felid species; *(ii)* investigating on the possible role of ticks in the transmission of *Hepatozoon* spp. and *Cytauxzoon* spp., and *(iii)* developing diagnostic protocols to provide fast and sensitive screening procedures.

Different felid species (i.e., domestic cats, wildcats, exotic captive felids) living in three regions (i.e. Veneto, Friuli Venezia Giulia and Trentino Alto Adige) of North-eastern Italy were recruited to investigate the occurrence of *Cytauxzoon* spp. and *Hepatozoon* spp.

Blood samples were collected from domestic cats (*Felis silvestris catus*) and captive exotic species living in zoological parks located in the investigated areas from 2019 to 2022. In the same period, the chances have been seized to follow and contribute to the first symptomatic case of hepatozoonosis in a domestic cat in Italy, and even to the epidemiological studies performed in Greece.

Moreover, in collaboration with the University of Udine, wildcats (*Felis silvestris silvestris*) found dead in the investigated areas were necropsied and samples of organs and tissues were collected.

The following results show firstly that domestic and wild-cats can be infected by *Cytauxzoon* spp. and *Hepatozoon* spp., highlighting their suitability as hosts for these protozoa. The presence and distribution of both protozoa were confirmed in all investigated territories, and epidemiological data was then updated. In addition, the role of wildcats as sylvatic reservoirs and the possibility of transmission from wild to domestic species and *vice versa*, especially if they share the same habitat and arthropod vectors, were suggested.

The results were collected in the following papers and scientific communications in national and international conferences:

- Grillini M., Frangipane di Regalbono A., Simonato G., Tessarin C., Dotto G. ***Cytauxzoon*** sp. and ***Hepatozoon*** spp. in cats in North-eastern Italy: Preliminary results. Proceeding XXXI National Congress of Italian Society of Parasitology – SoIPa & 2021 European

Society of Dirofilariosis and Angiostrongylosis - ESDA Event, on-line, 16-19 June, *2021*: 84. Oral presentation – Scientific communication 1

- Morelli S, Diakou A, Traversa D, Di Gennaro E, Simonato G, Colombo M, Dimzas D, Grillini M, Frangipane di Regalbono A, Beugnet F, Halos L, Paoletti B, Di Cesare A. First record of *Hepatozoon* spp. in domestic cats in Greece. Ticks Tick Borne Dis. *2021*. 12(1):101580 – PAPER 1

- Grillini M, Simonato G, Tessarin C, Dotto G, Traversa D, Cassini R, Marchiori E, Frangipane di Regalbono A. *Cytauxzoon* sp. and *Hepatozoon* spp. in domestic cats: a preliminary study in North-eastern Italy. Pathogens. *2021*. 10(9):1214 – PAPER 2

- Simonato G., Grillini M., Franco V., Salvatore G., Manzocchi S., Dotto G., Morelli S., Cavicchioli L., Gelain M.E., Zini E. The first clinical case of hepatozoonosis in a domestic cat in Italy. Proceeding XXXII National Congress of Italian Society of Parasitology – SoIPa, Napoli, Italy, 27-30 June, 2022: 150. Poster – Scientific communication 2

- Simonato G, Franco V, Salvatore G, Manzocchi S, Dotto G, Morelli S, Grillini M, Cavicchioli L, Gelain ME, Zini E. First autochthonous clinical case of *Hepatozoon silvestris* in a domestic cat in Italy with unusual presentation. Parasit Vectors. *2022*. 15(1):440 – PAPER 3

- Grillini M., Simonato G., Beraldo P., Modrý D., Hrazdilová K., Dotto G., Marchiori E., Frangipane di Regalbono A. First data on *Cytauxzoon* and *Hepatozoon* in wildcats (*Felis silvestris silvestris*) in North-eastern Italy. Proceeding of the 15th International Congress of Parasitology - ICOPA 15, Copenhagen, Denmark, 21-26 August, *2022*. Poster – Scientific communication 3

- Grillini M., Frangipane di Regalbono A., Beraldo P., Tessarin C., Maurizio A., Cassini R., Marchiori E., Simonato G. *Cytauxzoon* spp. and *Hepatozoon* spp. in questing ticks, wildcats and domestic cats in North-eastern Italy. Proceeding V National Congress of Italian Society of Wildlife Ecopatology - SIEF, Udine, Italy, 14-17 September, *2022*: 9. Oral presentation – Scientific communication 4

In Europe, the arthropod vector of *Cytauxzoon* spp. and *Hepatozoon* spp. is not still known and, as already described in the background, ticks are considered the main involved arthropod. Thus, a further purpose was to investigate the possible role of ticks in the transmission of *Cytauxzoon* spp. and *Hepatozoon* spp. Ticks were collected from April to September 2021 by dragging and flagging in wood areas and public gardens in North-eastern Italy in an area known to be endemic for both protozoa, then morphologically and molecularly identified. The obtained results suggest a potential role of the forest tick *Ixodes ricinus* in protozoa transmission since *Cytauxzoon* and *Hepatozoon* DNA was amplified from questing ticks of this species.
Concurrently, it was possible to participate in a considerable project which aimed to investigate the presence of vector-borne pathogens (VBPs) in ticks and fleas infesting dogs and cats in Cyprus. The outcomes indicate the eventual involvement of *I. gibbosus* and *Rhipicephalus sanguineus* s.l. in *H. felis* transmission. The isolation of protozoa DNA is not sufficient to confirm the role of ticks in their transmission and further investigations on vector capacity would be necessary.

The results were collected in the following paper and scientific communication in international conference:

- Grillini M., Frangipane di Regalbono A., Modrý D., Hrazdilová K., Tessarin C., Dotto G., Maurizio A., Cassini R., Simonato G. ***Cytauxzoon*** **spp. and** ***Hepatozoon*** **spp. in questing ticks in Northeastern Italy**. 10th Tick and Tick-Borne Pathogen Conference - TTP10 Abstract book, Murighiol, Danube Delta, Romania, 29 August-2 September, *2022*: 23 - Oral presentation – Scientific communication 5

- Diakou A., Sofroniou D., Paoletti B., Tamvakis A., Kolencik S., Dimzas D., Morelli S., Grillini M., Traversa D. **Ticks, fleas and harboured pathogens from dogs and cats in Cyprus**. Pathogens. *2022*. 11(12):1403 - PAPER 4

Finally, the last phase of the research involved the development of a new diagnostic molecular protocol in order to screen a large number of samples in a fast and sensitive way, and applicable to different matrixes.
Samples from several feline species (i.e., domestic cats, wildcats and captive exotic felids) were collected and analysed by a real-time assay, able to simultaneously detect *Cytauxzoon* spp. and *Hepatozoon* spp. based on melting temperature curve analysis. The new diagnostic tool aimed to screen a consistent number of samples in a cost- and time-saving approach.

The results of these studies were collected in the following paper and scientific communication at national conference:

- Grillini M., Frangipane di Regalbono A., Tessarin C., Dotto G., Beraldo P., Marchiori E., Simonato G. A qPCR approach for the simultaneous detection of *Cytauxzoon* spp. and *Hepatozoon* spp. in felids. Proceeding XXXII National Congress of Italian Society of Parasitology – SoIPa, Napoli, Italy, 27-30 June, *2022*: 319. Oral presentation – Scientific communication 6

- Grillini M., Beraldo P., Frangipane di Regalbono A., Dotto G., Tessarin C., Franzo G., Marchiori E., Modrý D., Simonato G. Molecular survey on *Cytauxzoon* spp. and *Hepatozoon* spp. in felids by new real time PCR approach. Frontiers. *2022*. *Under review* - PAPER 5 (draft)

2. Paper 1

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First record Hepatozoon spp. in domestic cats in Greece

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Original article

First record of *Hepatozoon* spp. in domestic cats in Greece

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ABSTRACT

Feline hepatozoonosis is an emerging disease of domestic and wild felids though there is limited knowledge of this infection, e.g. regarding geographical distribution and parasite species involved. The present study evaluated microscopically and molecularly the occurrence of *Hepatozoon* spp. in domestic cats from insular (Crete, Mykonos and Skopelos) and continental (prefectures of Attica and Thessaloniki) Greece. Out of 282 cats examined, 72 (25.5 %) scored positive by PCR for *Hepatozoon* spp. and of them, 9 (12.5 %) showed gamonts on the blood smear microscopic examination. Sequences obtained from 35 of the amplicons proved the presence of two haplotypes of *Hepatozoon felis*. One, herein called H1 (34/35 amplicons) resulted 100 % identical with *H. felis* from Italy and isolates from other continents, and ~98 % similar with a *H. felis* isolate causing severe clinical signs in Austria. The haplotype H2, found in a cat in Skopelos, had ~94 % identity with H1, with *H. felis* isolates from Italy, Israel, Spain, a ~92 % identity with the isolate from Austria, and ~94–98 % with isolates from South Africa. These are the first records of *H. felis* in cat populations from Greece and indicate that the infection may be present at high prevalences in different regions of the country. Furthermore, the results of the molecular and phylogenetic analysis support a recent hypothesis indicating the existence of a species-complex classification for *H. felis*. Further studies aiming at elucidating the genetic make-up of *Hepatozoon* populations and possible variations in terms of geographic distribution and clinical relevance are necessary. The importance of a continuous epizootiological monitoring is crucial for the establishment of preventative and control measures protecting the health of cats living in or travelling to enzootic areas.

1. Introduction

Vector-Borne Diseases (VBDs) of companion animals are emerging illnesses in various countries, though there is still a lack of knowledge for such infections in cats (Pereira et al., 2019; Morelli et al., 2019). Among them, infections by *Hepatozoon* spp. in cats are still overlooked and our knowledge needs to be expanded (Basso et al., 2019; Morelli et al., 2019).

Hepatozoon felis is the major hepatozoid identified in felids, though infections caused by *Hepatozoon canis* have also been reported (Smith, 1996; Beugnet and Halos, 2015; Giannelli et al., 2017; Basso et al., 2019). Other mammals, such as foxes, rodents and hyenas, can be infected with *H. felis* (Williams et al., 2014; Kamani et al., 2018; Millán et al., 2018). In addition, a new species infecting both wild and domestic cats, i.e. *Hepatozoon silvestris*, has been recently described (Hodžić et al., 2017) and a different type of *Hepatozoon* (*Hepatozoon* sp. 'Bartozoon' lineage) has been identified in domestic cats in South Africa (Harris et al., 2019). The role of *Hepatozoon* spp. in causing illnesses in cats should also be clarified because although in general feline hepatozoonosis is subclinical or subtle, there are cases of life-threatening clinical signs.

The definitive hosts for *Hepatozoon* spp. are blood-sucking arthropods, usually ticks, in which sexual development and sporogony take place (Baneth et al., 2007). Transmission to the vertebrate host occurs by the ingestion of the arthropod or part of it, containing mature oocysts of the parasite (Smith, 1996). Merogony and gametogony occur in the

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vertebrate host and the gamonts are found in leukocytes, usually neutrophils (Baneth et al., 2007). Although the arthropods involved in feline infections have not yet been identified conclusively, it is likely that ticks are the vectors of *H. felis* and *H. silvestris*. Indeed, *H. felis* DNA was detected in *Rhipicephalus sanguineus* sensu lato (s.l.) (Maia et al., 2014), *Rhipicephalus turanicus* (Karasartova et al., 2018), *Ixodes ricinus* (Aktas, 2014), *Ixodes hexagonus* (Duplan et al., 2018) *Haemaphysalis erinacei* (Diakou et al., 2020) and *Haemaphysalis sulcata* (Aktas, 2014), while *H. silvestris* DNA has been found in *I. ricinus* (Duplan et al., 2018). However, to date, no evidence of *H. felis* or *H. silvestris* oocysts in vectors is available.

In Europe, *H. felis* has been increasingly reported in domestic cats in different countries, such as France (Beaufils et al., 1998), Spain (Díaz-Regañón et al., 2017), Portugal (Vilhena et al., 2013), Cyprus (Attipa et al., 2017), Italy (Giannelli et al., 2017), Austria (Basso et al., 2019) and in a European wildcat (*Felis silvestris*) from Greece (Diakou et al., 2020). From its first detection and molecular characterization in the European wildcat in Bosnia and Herzegovina (Hodžić et al., 2017), *H. silvestris* has now also been described in domestic cats from Southern Italy (Giannelli et al., 2017) and Switzerland (Kegler et al., 2018). In comparison, *H. canis* has been more frequently detected in cats and was reported in Thailand (Jittapalapong et al., 2006), Southern Italy (Giannelli et al., 2017), Spain (Díaz-Regañón et al., 2017) and France (Criado-Fornelio et al., 2009).

Recent large-scale surveys (Diakou et al., 2017, 2019; Latrofa et al., 2017; Hofmann et al., 2019) have shown that vector-borne pathogens can occur at high prevalence rates in dogs and cats in Greece. Given the apparent emergence of this infection in cats, the occurrence of different species probably showing variations in their pathogenetic mechanisms, and the existing limited knowledge of the epizootiology of the disease, the present study aimed at evaluating the occurrence and species identification of *Hepatozoon* spp. in cats, in Greece.

2. Materials and methods

2.1. Animals and study areas

A total of 282 individual blood samples of cats living in insular and continental areas of Greece were collected from the Islands of Crete (n = 27), Mykonos (n. 175) and Skopelos (n = 22) and from continental areas, i.e. prefectures of Attica (n = 31) and Thessaloniki (n = 27). Blood samples were obtained as a convenient dataset, with the consent from the owners and written permission from the Municipality Authorities in the case of stray animals, and during clinical routine examinations or medical checks for other diseases or clinical differentials. Samples were stored in EDTA tubes pending blood smear evaluation and molecular analysis, as follows.

2.2. Microscopic examination

Giemsa-stained blood smears were prepared for the detection of *Hepatozoon* spp. gamonts under the microscope (1000× magnification) and were examined as described previously (Baneth et al., 2013).

2.3. Molecular analyses and phylogenesis

DNA was extracted for each sample using a commercial kit (Exgene Blood extraction kit, GeneAll Biotech) following the manufacturer's instructions. A fragment of ~373 bp of the 18S rRNA gene of *Hepatozoon* spp. was amplified by PCR using specific primers (Tabar et al., 2008). PCR reactions were carried out in a 25 µl reaction mixture containing 2 µl of genomic DNA, 12.5 µl of Ready Mix REDTaq (Sigma, St. Louis, MO), and 0.25 µl of each corresponding primer (50 µM). PCRs were performed in a thermal cycler (2700; Applied Biosystems, Foster City, CA) as previously described (Díaz-Regañón et al., 2017). A convenient dataset (i.e. around 50 % of the amplicons, with high quality and quantity of the PCR products) of PCR-positive samples was purified using a QIAquick® Gel ExtractionKit (Qiagen, GmbH, Hilden, Germany) and sequenced by a commercial laboratory (BMR – Genomics, Padova, Italy). Sequences were determined in both strands, aligned and then compared with each other and with those available in GenBank using the Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST). The phylogenetic analysis was conducted using MEGA_X (Kumar et al., 2018; Stecher et al., 2020). Phylogenetic relationships were inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together was calculated using the bootstrap test (1000 replicates) (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). The tree was rooted using *Karyolysus paradoxa* as an outgroup (KX011039.1) (Cook et al., 2016).

3. Results

3.1. Microscopic examination

Gamonts of *Hepatozoon* spp. were microscopically recovered in 9 out of study cats (3.2 %). In all cases the parasite was found in a neutrophil. Specifically, *Hepatozoon* spp. gamonts were found in 1 out of 27 (3.7 %) cats in Crete, 4 out of 175 (2.3 %) cats in Mykonos, 3 out of 22 (13.6 %) cats in Skopelos, and 1 out of 31 (3.2 %) cats in Attica.

3.2. Molecular analyses and phylogenesis

The DNA of *Hepatozoon* spp. was amplified from the blood of 72/282 (25.5 %) cats examined.

Of the 72 positive cats, 9 (12.5 %) were also positive at the microscopic examination, while no cats that were positive at microscopy scored negative by PCR. Positivity rate at the PCR ranged from 11.1 % (Thessaloniki) to 81.8 % (Skopelos) (Table 1). DNA sequencing was performed for a total of 35 samples, i.e. 8, 12, 11, 1 and 3 for Crete, Mykonos, Skopelos, Attica and Thessaloniki, respectively.

Two haplotypes of *H. felis*, herein called H1 and H2, were recorded for the obtained sequences. H1 was present in 34/35 cats, while H2 was only present in one cat from Skopelos.

Sequences belonging to H1 had an identity percentage of 100 % with *H. felis* GenBank accession number KY649442.1 from Italy (Giannelli et al., 2017), Israel (GenBank Accession Number KC138534.1) (Baneth et al., 2013) and Spain (GenBank Accession Number AY628681.1) (Criado-Fornelio et al., 2006), while H2 was 94.82 % identic with H1 and the other abovementioned isolates. The H1 haplotype showed also a 97.93 % identity with *H. felis* described in a case report found of a symptomatic cat from Austria (Accession Number MK724001) (Basso et al., 2019) while H2 was 92.75 % identic with this latter isolate. The haplotypes found in this study were further compared with those of *Hepatozoon* spp. described by Harris et al. (2019) (Accession Numbers from MK301457 to MK301462) in cats from South Africa and the percentages of identity are shown in Table 2. Moreover, H1 sequences had

Table 1
Hepatozoon spp. infection in cats in various areas of Greece, diagnosed by PCR and microscopy.

Sampling areas	PCR	Blood smear	Sequenced/PCR Positive
	Pos/tot (%)		
Crete	15/27 (55.5)	1/27 (3.7)	8/15
Mykonos	24/175 (13.7)	4/175 (2.3)	12/24
Skopelos	18/22 (81.8)	3/22 (13.6)	11/18
Attica	12/31 (38.7)	1/31 (3.2)	1/12
Thessaloniki	3/27 (11.1)	0/27 (0)	3/3
Total	72/282 (25.5)	9/282 (3.2)	35/72

pos = positive cats; tot = total number of cats examined.

Table 2
Percentage of identity between the haplotypes found in the present study (H1 and H2), those from Harris et al. (2019) (S1, S2, S3), from Basso et al., 2019 (S4) along with *Hepatozoon canis* (HC), *Hepatozoon silvestris* (HS and HS1) and *Hepatozoon americanum* (HA) isolates (Allen et al., 2008; Hodžić et al., 2017; Kegler et al., 2018; Medkour et al., 2020).

	H2	S1	S2	S3	S4	HC	HS	HS1	HA
H1*	94.82	100	98.73	96.61	97.93	96.63	94.71	94.71	91.79
H2		94.82	97.03	94.92	92.75	91.45	89.92	89.92	87.31
S1			98.72	96.62	97.47	95.36	91.94	91.94	90.30
S2				97.02	97.89	95.78	92.28	92.34	88.93
S3					99.16	94.94	92.34	92.34	90.12
S4					98.40	97.61	96.33	96.33	94.92
HC							94.41	94.41	92.73
HS								100	92.64
HS1									92.64

* The 100 % identity with S1 has been calculated replacing an Y present in the S1 sequence, that most probably indicated a C or T: for this reason the identity percentage of H1 and S1 with other sequences variations.

an identity percentage of 94.71 % with *H. silvestris* GenBank Accession Number KX757031.1 from Bosnia and Herzegovina (Hodžić et al., 2017) and MH078194 Switzerland (Kegler et al., 2018) respectively, and of 96.63 % with *H. canis* GenBank Accession Number MK673842.1 from France (Medkour et al., 2020). The sequence H2 showed, instead, a lower identity, i.e. 89.92 % (MH078194 and KX757032.1) and 91.45 % (MK673842.1). In comparison with *Hepatozoon americanum* (GenBank Accession Number EU146062.1, Allen et al., 2008) H1 and H2 showed 91.79 % and 87.31 % identity respectively. The phylogenetic relationships are depicted in Fig. 1.

4. Discussion

To the best of the authors' knowledge, this is the first report of feline *Hepatozoon* spp. infection in domestic cats in Greece. Interestingly, *H. felis* has been recently described in a European wildcat in Xanthi, Northern Greece (Diakou et al., 2020). The present results demonstrate that *H. felis* is enzootic both in insular and continental regions, with high infection rates in most areas and an overall prevalence of 25.5 % (72/282) (Table 1).

Although past studies showed extremely variable *Hepatozoon* spp. infection rates in different countries of the world, the present values fit within ranges recorded until now in Europe (Table 3) and it is similar to the highest rates recorded in Portugal (Vilhena et al., 2013) and Cyprus (Attipa et al., 2017). These results are in accordance with those showing that feline hepatozoonosis is widespread in different kind of environments, e.g. dry as in Mykonos, Cyprus (Attipa et al., 2017) and Cape Verde (Pereira et al., 2019), forested as in Skopelos (present study) and Brazil (Braga et al., 2016), heterogeneous as in Crete, where either dry areas or forests and wetlands are present (present study), and highly urbanized as in Attica and Thessaloniki (present study) and Madrid (Díaz-Reganõn et al., 2017). This biological plasticity is not surprising given that among the ticks wherein *H. felis* DNA has been detected, *R. sanguineus* s.l. is the most widespread tick worldwide due to its high ability of adapting to various environments, including urban settings, continental territories and islands (Dantas-Torres, 2010; Lefkaditis et al., 2015; Diakou et al., 2019). The number of cats sampled in Skopelos, Crete or Attica is relatively low, but it is remarkable that most of them were positive. The high rate of infection in Skopelos may be explained considering the fact that it is a mountainous island with dense

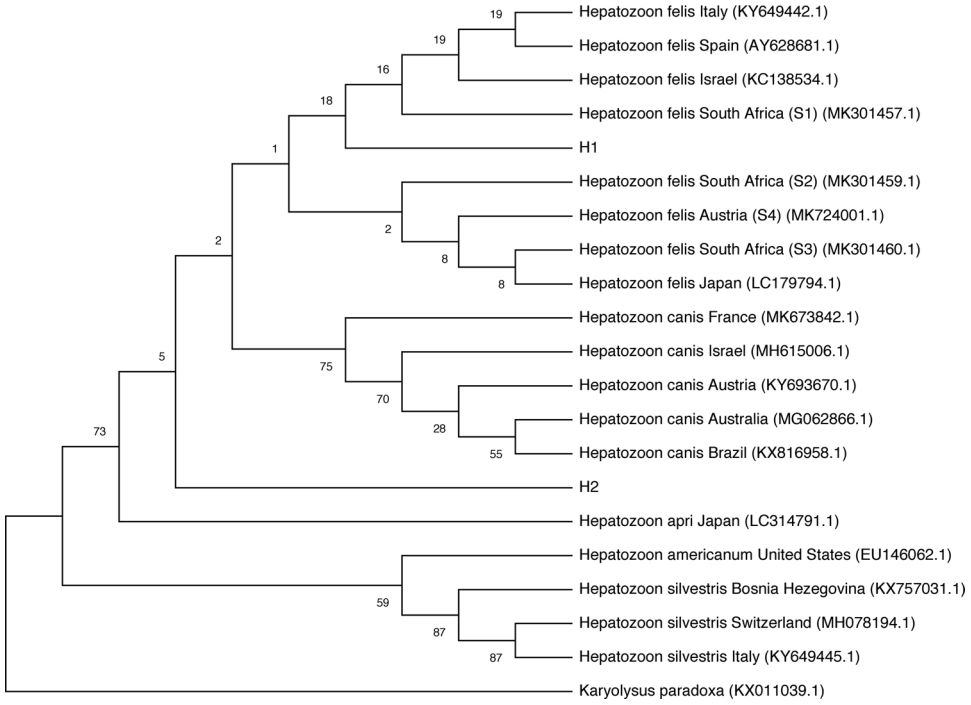

Fig. 1. Phylogenetic tree showing relationships between isolates obtained in the present study and other selected *Hepatozoon* sequences available in GenBank. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

Table 3
Prevalence (n/tot, %) of *Hepatozoon* spp. infection in cats (*Hepatozoon felis*, *Hepatozoon canis*, *Hepatozoon silvestris* when sequencing was performed), detected in previous epizootiological studies, compared to the present study.

	Hepatozoon spp.	*Hepatozoon felis*	*Hepatozoon canis*	*Hepatozoon silvestris*
Asia				
Thailand	–	–	32.3 (97/300)[1]	–
Israel	36.2 (55/152)[2,*]	*	*	–
Iraq	–	39.1 (81/207)[3]	–	–
Africa				
Cape Verde	–	15.0 (12/80)[4]	–	–
Angola	–	2.9(3/102)[5]	–	–
America				
U.S.	2.4 [6]	–	–	–
Brazil	1.6 (3/180)[7]	–	–	–
Europe				
Italy	5.1 (10/196)[9,**]	0.3 (1/330)[8]	**	**
France	–	–	1.7 (2/116)[10]	–
Spain	1.6 (10/644)[12]	4.0 (/100)[11]	–	–
Portugal	–	15.6 (50/320)[13];8.4 (56/649)[14]	–	–
Cyprus	–	37.9 (66/174)[15]	–	–
Greece (Present study)	–	25.5 (72/282)	–	–

[1] Jittapalapong et al., 2006.
[2] Baneth et al., 2013.
[3] Otranto et al., 2019.
[4] Pereira et al., 2019.
[5] Oliveira et al., 2018.
[6] Qurollo, 2019.
[7] Braga et al., 2016.
[8] Otranto et al., 2017.
[9] Giannelli et al., 2017.
[10] Criado-Fornelio et al., 2009.
[11] Tabar et al., 2008.
[12] Díaz-Reganõn et al., 2017.
[13] Vilhena et al., 2013.
[14] Maia et al., 2014.
[15] Attipa et al., 2017.
[*] all *Hepatozoon* spp. positive at PCR samples were positive for *Hepatozoon felis* at sequencincg, except for two cats infected by *Hepatozoon canis*.
[**] all *Hepatozoon* spp. positive samples at PCR were positive for *Hepatozoon felis* at sequencincg, except for one cat infected by *Hepatozoon canis* and one infected by *Hepatozoon silvestris*.

vegetation, representing a suitable habitat for ticks living in forested environments. Some of these tick species could be thus implicated in the transmission of *H. felis* (Aktas, 2014; Latrofa et al., 2017), especially in the case of free-roaming cats, as was the majority of those sampled in this island. Ticks belonging to *Ixodes* spp. and *Rhipicephalus* spp. are present in continental Greece (Pavlidou et al., 2008), both have been reported in Crete (Dimanopoulou et al., 2018) and *R. sanguineus* s.l. has been found in Mykonos (unpublished data), thus representing possible vectors of *H. felis* in these areas.

Hepatozoon felis may also be transmitted vertically from queen to litters (Baneth et al., 2013), and interestingly, the vast majority of the positive cats from Mykonos were from an isolated colony from a confined area of the Island. Most of these cats have been examined for tick infestation in the frame of the present study and none was found positive. Therefore, transplacental transmission probably was a crucial way of infection in these cases.

Vertical transmission could be a mechanism developed by the parasite, ensuring its spreading in environments where definitive and/or intermediate hosts are not abundant. In general, vertical transmission is an important adaptation for many parasites that enhance and in some cases ensure the maintenance of infection in a population and its role in maintaining *Hepatozoon* in felid populations appear crucial as tick infestation rates in cats are generally lower compared to dogs. However, the grooming behavior of cats, during which they mechanically remove ticks from their body (Day, 2016; Little et al., 2018) could be responsible of the vector ingestion, implementing the transmission of *Hepatozoon* spp. infection in cats. The high presence of *H. felis* in various regions may also rely on another source of infection, i.e., predation. In fact, some *Hepatozoon* species are transmitted to their vertebrate hosts through their preys, or through the ticks attached to the preys (Baneth, 2011) although this has not been proven yet for *H. felis*. Previous studies reported a significant association between hepatozoonosis and outdoor lifestyle in cats, suggesting that easier contact with ectoparasites and/or predatory activities are relevant predisposing factors (Baneth et al., 2013; Lloret et al., 2015; Basso et al., 2019). Further studies are warranted to confirm these biological and epizootiological features.

Studies have been evoked for a definitive clarification of *H. felis* taxonomical status. The high genetic diversity of *H. felis* led to the proposal of a species-complex where the different genotypes are classified (Harris et al., 2019). Accordingly, the H2 haplotype herein found in a single cat from Skopelos island could support the species-complex hypothesis (Harris et al., 2019) especially indicating that rare isolates may be present in small and confined areas. Genetically different *H. felis* haplotypes could have different characteristics in terms of pathogenicity and it seems that in the *H. felis* species-complex some genotypes are more widespread than others. Indeed, the 100 % identity between H1 and the haplotypes described in Italy, Israel, Spain (Criado-Fornelio et al., 2006; Baneth et al., 2013; Giannelli et al., 2017) and one of the haplotypes recorded in South Africa (Harris et al., 2019) indicate that this *H. felis* genotype circulates worldwide (Table 2). Similarly, in South Africa three haplotypes (herein identified aligning the sequences deposited in GenBank and using Clustalw) were recorded (Harris et al., 2019). The phylogenetic analysis (Fig. 1) fits with the recent results, which have supported the existence of a *H. felis* species-complex (Harris et al., 2019). The sequence H2 showed less identity with H1 than most closely related *H. canis* sequence in GenBank (MK673842.1). Therefore, H2 could be an undescribed species of *Hepatozoon*, as suggested by the phylogenetic tree, showing that this sequence is more distant from H1 if compared to *H. canis* (Fig. 1). Further studies are necessary to confirm the existence of a novel *Hepatozoon* species infecting cats.

Overall, H1 sequence and the related ones had a ~98 % identity with a haplotype described recently in Austria causing a severe clinical disease in a domestic cat suffering from acute hepatic and renal impairment, without other comorbidity detection (Basso et al., 2019). Given that in general feline hepatozoonosis is considered a subclinical or subtle disease (Baneth et al., 2013) it cannot be excluded that some isolates / haplotypes may have a different pathogenic potential. Indeed, none of the cats included in this study showed clinical signs. The genetic variability among *H. felis* isolates, along with the distance degrees between *H. felis* isolates and *H. silvestris*, *H. canis* and *H. americanum* could reflect differences in terms of biology and pathogenetic potential, further supporting the complex-species taxonomic classification proposed by Harris et al. (2019). For instance, the higher identity of H2 with *Hepatozoon* isolates from dogs rather than with other isolates from felids would require further studies and characterizations to elucidate the taxonomical status of this haplotypes and its biological and epizootiological significance. Moreover, it could be possible that *H. felis* isolates with a higher degree of identity with *H. silvestris* or *H. americanum*, as in the case of KM435071.4 and MK724001 (Table 2), have the same tropism for some anatomical sites (e.g. muscles, myocardium) and higher chances to induce a clinical disease in the infected animals. Therefore, further investigations are necessary to understand the role (if any) of various genetic haplotypes in causing clinical signs. It would be of utmost importance to ultimately identify at the molecular level the

H. felis isolates that are able to cause clinical disease. This is also important if one considers that, as expected, PCR is able to unveil infections that cannot be detected by microscopic examination of blood smears. Of 72 that were PCR-positive for *Hepatozoon* spp., 9 showed gamonts at the blood smear examination. These results fits with previous studies, as due to the low level of parasitemia in cats, less of 1 % of neutrophils and monocytes contain gamonts (Baneth, 2011; Basso et al., 2019; Pereira et al., 2019).

An improved knowledge on the areas endemic for VBDs in general, and for hepatozoonosis of cats in particular, is of great importance under an epizootiological point of view. Companion animals travelling with their owners may introduce new pathogens that are enzootic from their country of origin, or at the same time, may acquire infections when visiting enzootic areas and bring diseases when they go home. This is particularly true for the Mediterranean basin, that is considered a major epizootiological hub for feline and canine VBDs (Otranto and Dantas-Torres, 2010; Diakou et al., 2015, 2019). Thus, the present data extend the knowledge on VBDs occurring in feline populations of Europe. Appropriate prevention measures based on the use or repellents and ectoparasiticides are crucial to protect animals that live or visit areas enzootic for VBDs, including hepatozoonosis (Basso et al., 2019; Kegler et al., 2018). Furthermore, an epizootiological surveillance should be encouraged where stray and free-roaming cats are widespread or where these animals live in colonies or catteries where prevention and treatment measures are not regularly performed.

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CRediT authorship contribution statement

Simone Morelli: Conceptualization, Investigation, Writing - original draft, Writing - review & editing. **Anastasia Diakou:** Conceptualization, Investigation, Writing - original draft, Writing - review & editing. **Donato Traversa:** Conceptualization, Investigation, Writing - review & editing, Supervision. **Elisa Di Gennaro:** Investigation. **Giulia Simonato:** Investigation. **Mariasole Colombo:** Investigation. **Dimitris Dimzas:** Investigation. **Marika Grillini:** Investigation. **Antonio Frangipane di Regalbono:** Conceptualization. **Frederic Beugnet:** Conceptualization, Funding acquisition. **Lenaig Halos:** Conceptualization, Funding acquisition. **Barbara Paoletti:** Investigation. **Angela Di Cesare:** Conceptualization, Investigation, Writing - review & editing, Supervision.

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3. Paper 2

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Cytauxzoon sp. and *Hepatozoon* spp. in domestic cats: a preliminary study in North-Eastern Italy

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Cytauxzoon sp. and *Hepatozoon* spp. in Domestic Cats: A Preliminary Study in North-Eastern Italy

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Abstract: Knowledge on the presence of *Cytauxzoon* sp. and *Hepatozoon* spp. in Italy is scant and mostly limited to a few areas of Northern and Southern regions, respectively. The present study updated the current epidemiological scenario by investigating the occurrence of these protozoa in domestic cats from three broad regions of North-Eastern Italy. Blood samples from cats at risk of vector-borne diseases were processed by PCR to detect *Cytauxzoon* and *Hepatozoon* DNA. Blood smears were observed for haemoparasite inclusions. The influence of cat individual data (e.g., provenance, management, indoor/outdoor lifestyle) on the prevalence of haemoprotozoan infections was statistically evaluated. Among 158 cats, *Cytauxzoon* and *Hepatozoon* DNA were detected in 6 (3.8%) and 26 (16.5%) animals, respectively. No *Hepatozoon* gamonts were detected in blood smears, whereas all *Cytauxzoon* PCR-positive samples were microscopically positive, though with low levels of parasitaemia. Two species of *Hepatozoon* were identified, *Hepatozoon felis* (n = 10) and *Hepatozoon silvestris* (n = 16). *Hepatozoon silvestris* prevalence values were significantly ($p < 0.05$) higher in the region Friuli Venezia Giulia and in stray cats. *Cytauxzoon* sp. was detected in 6/39 (15.4%) stray cats from Friuli Venezia Giulia (Trieste province). These data add new information on the occurrence of these neglected protozoa in domestic cats' populations.

Keywords: *Cytauxzoon* sp.; *Hepatozoon felis*; *Hepatozoon silvestris*; cat; Italy

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1. Introduction

Cytauxzoon sp. and *Hepatozoon* spp. are two apicomplexan protozoa belonging to Orders Piroplasmida and Eucoccidiorida, respectively [1]. The genus *Cytauxzoon* was reported for the first time in a domestic cat (*Felis silvestris catus*) in 1976 in the US, and the species was named *Cytauxzoon felis* [2]. Then, reports of *Cytauxzoon* in cats were described only in some US regions [3,4], until the 2000s, when cases were also reported in Europe. More recently, cats positive for *Cytauxzoon* have been recorded in Spain [5,6], France [7,8], Portugal [9], Switzerland [10], and Germany [11]. In Italy, cases were limited to an area in the North-Eastern region of Friuli Venezia Giulia, where an endemic focus was described in the city of Trieste with a prevalence rate of 23% among owned and stray cats [12]. Subsequently, clinical cases were then recorded in other Italian regions, i.e., Veneto, Tuscany, and Latium [13]. Molecular analyses showed that isolates of *Cytauxzoon* in Europe are different from *C. felis* affecting felid populations in the USA. Indeed, *Cytauxzoon* is a monophyletic group, characterised by different isolates grouped in separate species (i.e., *C. felis*, *Cytauxzoon manul*) [14]. In addition, among the isolates from European wild felids, three genotypes of *Cytauxzoon* (i.e., major-EU1, minor-EU2, rare-EU3), defined as three new species, were recently detected [15].

Hepatozoon spp. was reported in domestic cats in India at the beginning of the 1900s [16], then only a few reports were published until 1973, when schizonts of *Hepatozoon*-like protozoa were described in the myocardium of a domestic cat in Israel [17]. Since then, *Hepatozoon* has been described worldwide, including in Africa [18–20], the US and South America [21,22], and Europe [6,7,23–28]. In Italy, hepatozoonosis was described in the Emilia Romagna region [29] and in Southern regions, i.e., Apulia and Basilicata [30] and the Aeolian Islands [31]. Three species of *Hepatozoon* infect cats (i.e., *Hepatozoon felis*, *Hepatozoon silvestris*, and *Hepatozoon canis*) [27,30].

Bridging parasite infections between wild felids and domestic cats occur frequently in areas of sympatry with relevant clinical and epizootiological impacts, as recently described for nematodes [32–35]. Different species of wild felids are reservoirs for *Cytauxzoon* sp. and *Hepatozoon* spp.: bobcat (*Lynx rufus*) in North America [36], Pallas' cat (*Otocolobus manul*) in Asia [37], and Iberian lynx (*Lynx pardinus*) [38], Eurasian lynx (*Lynx lynx*), and European wildcat (*Felis silvestris silvestris*) [39] in Europe. In particular, both *Cytauxzoon* sp. and *Hepatozoon* spp. occur frequently in European wildcats [15,33,40–42]. The recent rise of reports of cytauxzoonosis and hepatozoonosis in domestic cats of Europe [6,8,10,15,28] indicates the merit to further investigate the presence of these protozoa in populations of domestic cats at risk of infection for the occurrence of arthropod vectors and/or local presence of wild reservoirs.

Due to the merit in improving knowledge on the occurrence of cat cytauxzoonosis and hepatozoonosis in populations of domestic cats, the aim of this work was to investigate the presence and distribution of *Cytauxzoon* sp. and *Hepatozoon* spp. in domestic cats in North-Eastern Italy, aiming towards an update of the current epidemiological scenario.

2. Results

2.1. Feline Population

Overall, 158 domestic cats were included in the study, both owned (n = 103, 65.2%) and stray cats (n = 55, 34.8%), living in Veneto—Site 1 (n = 99, 62.7%), Friuli Venezia Giulia—Site 2 (n = 39, 24.7%), and Trentino Alto Adige—Site 3 (n = 20, 12.7%) regions. Regarding their habits, recruited cats had mostly an outdoor lifestyle (n = 112, 70.9%). Descriptions of individual data regarding the region of provenance (Sites 1, 2, and 3), sex, age classes (<12 months, 12–35 months, ≥36 months), management (owned, stray cats), lifestyle (indoor, outdoor), immunosuppressive infections (FIV, FeLV), clinical signs, and ectoparasites infestations are reported in Table 1.

Table 1. Description of individual data of the feline population distributed among the three investigated sites.

		Site 1 n (%)	Site 2 n (%)	Site 3 n (%)	Total n (%)
Sex	M	49 (49.5)	13 (33.3)	12 (60.0)	74 (46.8)
	F	50 (50,5)	26 (66.7)	8 (40.0)	84 (53.2)
Age classes	<12 months	38 (38.4)	9 (23.1)	5 (25.0)	52 (32.9)
	12–35 months	23 (23.2)	15 (38.5)	8 (40.0)	46 (29.1)
	≥36 months	37 (37.4)	13 (33.3)	7 (35.0)	57 (36.1)
	NR [a]	1 (1.0)	2 (5.1)	0 (0.0)	3 (1.9)
Management	Owned cats	64 (64.6)	19 (48.7)	20 (100.0)	103 (65.2)
	Stray cats	35 (35.4)	20 (51.3)	0 (0.0)	55 (34.8)
Lifestyle	Indoor	28 (28.3)	11 (28.2)	7 (35.0)	46 (29.1)
	Outdoor	71 (71.7)	28 (71.8)	13 (65.0)	112 (70.9)
Immunosuppressive infections (FIV and/or FeLV)	Positive	15 (15.2)	9 (23.1)	1 (5.0)	25 (15.8)
	Negative	84 (84.8)	30 (76.9)	19 (95.0)	133 (84.2)
Clinical signs (gastro-intestinal and respiratory signs)	Presence	10 (10.1)	1 (2.6)	1 (5.0)	12 (7.6)
	Absence	89 (89.9)	38 (97.4)	19 (95.0)	146 (92.4)
Ectoparasites infestations	Presence	15 (15.2)	11 (28.2)	3 (15.0)	29 (18.4)
	Absence	84 (84.8)	28 (71.8)	17 (85.0)	129 (81.6)
Total		99	39	20	158

[a] Age not reported.

A total of 29 cats (18.4%) were reported to be infested with ectoparasites (21 with only fleas, 2 with only ticks, 5 with fleas and ticks, and 1 with fleas and lice).

2.2. Laboratory Analysis and Geographical Distribution

From the microscope observation, 6/158 blood smears evidenced mild parasitaemia (1–5 erythrocytes with parasitic inclusions) attributable to *Cytauxzoon*, whereas no samples showed circulating *Hepatozoon* gamonts.

Out of 158 sera, 25 (15.8%) were positive for the immunosuppressive infections FIV and/or FeLV (8 of which for FIV, 14 for FeLV, and 3 co-infected).

PCR amplified *Cytauxzoon* sp. and *Hepatozoon* spp. DNA in 6/158 (3.8%) and 26/158 (16.5%) blood samples, respectively. Among *Hepatozoon*-positive samples, *H. felis* (10/26, 38.5%) and *H. silvestris* (16/26, 61.5%) were identified, comparing the obtained nucleotide sequences to those deposited in GenBank® using BLAST software (https://blast.ncbi.nlm.nih.gov/Blast) (accessed date: 2 August 2021).

All *Cytauxzoon* blood smear samples were also positive using the molecular assay.

All sequences of *Cytauxzoon* sp. (from MZ227613 to MZ227618), *H. felis* (from MZ227585 to MZ227594), and *H. silvestris* (from MZ227596 to MZ22611) were deposited in GenBank.

The BLAST analysis retrieved 99.68–100% homology with sequences depositedas *Cytauxzoon* sp. isolated from domestic cats in France [8], in Portugal [9], in Switzerland [10], and in Germany [11], together with isolates from the European wildcat in Romania and Bosnia and Herzegovina [39,42].

Regarding *H. felis*, the same analysis retrieved 97.92–100% identity from domestic cats in Southern Italy [30], Spain [43,44], and Israel [45]. For *H. silvestris,* BLAST analysis retrieved 96.28–97.71% identity from domestic cats in Southern Italy [30] and in Switzerland [26], and in addition, from European wildcat in Bosnia and Herzegovina [41,42].

Regarding geographical distribution, *Cytauxzoon* sp. was found only in Site 2, in particular in one province (Trieste). Contrariwise, *Hepatozoon* spp. was distributed in all investigated regions (Figure 1).

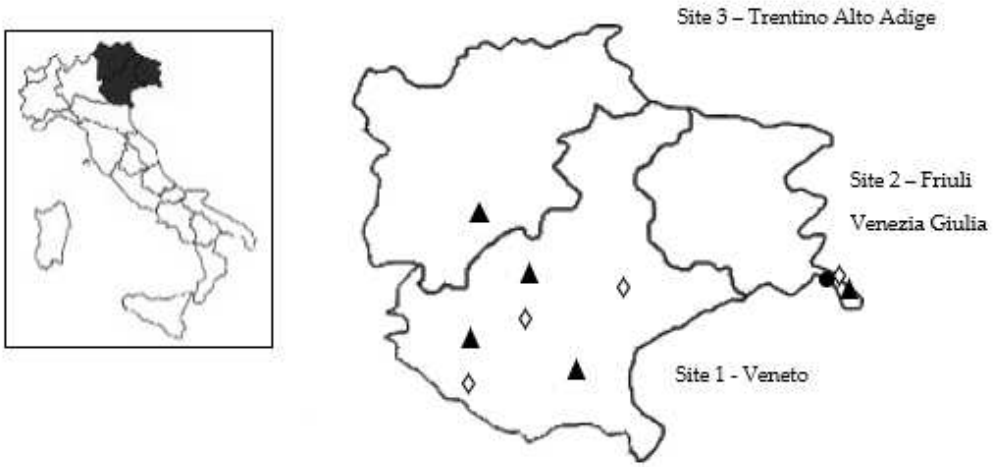

Figure 1. Map depicting Site 1, Site 2, and Site 3, showing the areas resulted positive to *Cytauxzoon* sp. (●), *Hepatozoon felis* (▲), and *Hepatozoon silvestris* (◊).

Individual data of cats positive for *Cytauxzoon* sp. and *Hepatozoon* spp. are reported in Table 2.

Table 2. Distribution of positivity according to individual factors in investigated cats.

			Haemoparasite						
Factors	**Variables**	**Tested**	***Cytauxzoon* sp. n (%)**		***Hepatozoon* spp. n (%)**		***Hepatozoon felis* n (%)**	***Hepatozoon silvestris* n (%)**	
Sex	M	74	1 (1.4)		13 (17.6)		5 (6.8)	8 (10.8)	
	F	84	5 (6.0)		13 (15.5)		5 (6.0)	8 (9.5)	
Age Class	<12 months	52	0 (0.0)		9 (17.3)		5 (9.6)	4 (7.7)	
	12–35 months	46	1 (2.2)		7 (15.2)		0 (0.0)	7 (15.2)	
	≥36 months	57	4 (7.0)		9 (15.8)		5 (8.8)	4 (7.0)	
	NR [a]	3	1 (33.3)		1 (33.3)		0 (0.0)	1 (33.3)	
Region	Site 1	99	0 (0.0)		12 (12.1)		5 (5.1)	7 (7.1)	
	Site 2	39	6 (15.4)	*	11 (28.2)		2 (5.1)	9 (23.1)	*
	Site 3	20	0 (0.0)		3 (15.0)		3 (15.0)	0 (0.0)	
Management	Owned cats	103	0 (0.0)	*	12 (11.7)		9 (8.7)	3 (2.9)	*
	Stray cats	55	6 (10.9)		14 (25.5)		1 (1.8)	13 (23.6)	
Lifestyle	Indoor	46	0 (0.0)		5 (10.9)		4 (8.7)	1 (2.2)	
	Outdoor	112	6 (5.4)		21 (18.8)		6 (5.4)	15 (13.4)	
Immunosuppressive infections (FIV and/or FeLV)	Positive	25	3 (12.0)		5 (20.0)		2 (8.0)	3 (12.0)	
	Negative	133	3 (2.3)		21 (15.8)		8 (6.0)	13 (9.8)	
Clinical signs (gastro-intestinal and respiratory signs)	Presence	12	0 (0.0)		1 (3.8)		0 (0.0)	1 (8.3)	
	Absence	148	6 (4.1)		25 (16.9)		10 (6.8)	15 (10.1)	
Ectoparasites infestation	Presence	29	2 (6.9)		8 (27.6)		2 (6.9)	6 (20.7)	
	Absence	129	4 (3.1)		18 (14.0)		8 (6.2)	10 (7.8)	
Total		**158**	**6 (3.8)**		**26 (16.5)**		**10 (6.3)**	**16 (10.1)**	

Note: significant differences ($p < 0.05$) based on the Pearson Chi-Square test or the Fisher exact test are evidenced by *. [a] Age not reported.

2.3. Statistical Evaluation

Differences in the infection rate among sub-groups of animals were found by the Pearson Chi-Square test for *Cytauxzoon* sp. and *H. silvestris* for two factors: a significantly higher prevalence ($p < 0.05$) was found in stray cats compared to owned animals, and in the cats living in Trieste province (Site 2) compared to the other two sites. Moreover, cats infected with immunosuppressive viruses seem to be at higher risk of positivity of *Cytauxzoon* sp. ($p = 0.051$), and cats with ectoparasites had a higher prevalence of *H. silvestris* ($p = 0.080$). However, in both cases, the Fisher exact test showed a *p*-value

slightly higher than the 0.05 threshold. No significant differences were observed for *H. felis*, nor for the other factors in general.

3. Discussion

To date, cytauxzoonosis and hepatozoonosis are neglected diseases in feline populations. Data on the *Cytauxzoon* species circulating among European cats are still limited [6,46,47] and information on *Hepatozoon* spp. in felids is also poor [48,49].

The present study confirmed that Trieste (Site 2) is an endemic site for the presence of *Cytauxzoon* sp. in domestic cats. As in a previous study, these results are supported both by blood smear examinations and molecular analysis, with a prevalence value similar to that reported (23%) almost ten years ago in 2012 [12].

Site 2 is the only region in which different wild felids acting as reservoirs for cytauxzoonosis are endemic, i.e., the Eurasian lynx [50] and the European wildcat [39,51]. Moreover, Site 2 is a border region, and wildlife movements from the nearby Slovenia are extensively described [52].

The significant difference in the prevalence between the type of management (owned vs. stray cats) highlights how stray cats that live mostly outdoors are more exposed to cytauxzoonosis than owned cats (10.9% vs. 0.0%). This is probably due to the sharing of the same environment with wild felids and the presence of infected vectors. Indeed, the continuous reduction of wildlife habitat due to anthropization favours the sympatric occurrence of wild and domestic cats in many areas [53], and this has the implication of sharing parasites with high pathogenic potential, as recently investigated for nematodes affecting the cardio-respiratory system [32–35,54].

Two species of *Hepatozoon* spp. have been found in North-Eastern Italy (i.e., *H. felis* and *H. silvestris*). The finding of *H. silvestris* in Northern Italy is especially noteworthy. Indeed, this species has been reported mainly in wild felids in Europe, and rarely in domestic cats. Nevertheless, it was recently described in a domestic cat in Southern Italy during an epidemiological survey [30] and in another one in Switzerland associated with a fatal myocarditis [26].

In agreement with Baneth et al. [49], who described an extremely low level of parasitaemia in felids, no sample showed *Hepatozoon* gamonts in blood smear examinations.

Positive cats were mostly sub-clinically infected, in apparently good physical condition, and only in one case were diarrhoea and rhinitis present (Table 2), thus evoking the infections as well-tolerated in most cases. No correlation between *Hepatozoon* positivity and potentially immunosuppressive infectious diseases (i.e., FIV and FeLV) was statistically found, as already reported by Baneth et al. [45]. Instead, cats positive for immunosuppressive viruses showed a higher prevalence of *Cytauxzoon* sp., indicating a tendency of being more at risk to becoming infected with haemoprotozoa, as previously suggested [8,12].

This result underlines the importance of investigating subclinical infections, and in parallel highlights the diagnostic limitations posed by stand-alone cytology. Differently, all *Cytauxzoon*-positive cats presented mild parasitaemia. Although few parasitised erythrocytes per monolayer were observed, the positivity suggests a potential epidemiological role of clinically healthy animals as carriers and sources of infection for potential vectors.

No significant differences between individual variables (i.e., provenance, management, and lifestyle) and *H. felis* prevalence were found. However, the high prevalence value obtained in indoor and owned cats, that are commonly less exposed to vectors' activity due to their lifestyle, suggests that alternative ways of transmission are possible, as already predicted (e.g., vertical transmission, predation of infected preys) [45]. Indeed, *H. canis* may also be spread through intra-uterine transmission from the mother to the offspring, and *Hepatozoon americanum* may be transmitted by ingestion of infected preys [49].

On the contrary, *H. silvestris* showed a significant difference in its distribution between regions, especially in Site 2, achieving a prevalence rate of 23.1%, most likely for a habitat/vector sharing between domestic and wild felids, as previously mentioned for *Cytauxzoon* sp.

The presence of *H. silvestris* in Site 1, where the Eurasian lynx and the European wildcats are absent, indicates that the role of wildlife as reservoirs could be unnecessary. This supports new considerations, as the possibility that *H. silvestris* might have another route of transmission related to the predatory instinct of cats and the carnivorism of potential paratenic hosts such as small rodents could be supported, as already described for *H. americanum* in the US [49]. As *H. silvestris* was found mainly in stray cats, this hypothesis is even more appropriate due to their predatory and hunting activities.

In conclusion, this study demonstrated that *Cytauxzoon* sp. and *Hepatozoon* spp. circulate in the feline population of North-Eastern Italy involving both owned and stray cats, focusing on the risk of exposure that some individual attitude or lifestyle factors might encourage.

Information about these haemoprotozoa is still lacking, and further studies are needed to obtain important data about their lifecycles with the evaluation of their pathogenicity and their impact on cat health as well as the potential ways of transmission, including wildlife as possible reservoirs and the involved arthropod vector, to carry out adequate control measures.

4. Materials and Methods

4.1. Blood Collection, Blood Analysis, and DNA Extraction

K3EDTA blood and blood smears were collected in collaboration with veterinary practitioners working in the investigated regions of North-Eastern Italy, during routine clinical examinations, from cats of all ages, exposed to at least one season at risk of arthropod vectors' activity, preferably without any regular treatments against ectoparasites.

For each sampled cat region of provenance (i.e., Veneto—Site 1, Friuli Venezia Giulia—Site 2, and Trentino Alto Adige—Site 3, Figure 1), sex, age classes (<12 months, 12–35 months, ≥36 months), management (owned, stray cats), lifestyle (indoor, outdoor), clinical signs, and ectoparasite infestations were reported. Moreover, all the involved owners or veterinary health authorities for colony/stray cats signed an informed consent form for participating in the study. Recruited animals were submitted to routine veterinary procedures not depending on this research project.

Blood smears were stained using Hemacolor® (Merck KGaA, Darmstadt, Germany) and then observed by microscope at 100× magnification with immersion oil to evaluate the presence of *Hepatozoon* gamonts and *Cytauxzoon* merozoites according to an existing key [12,40,48]. The parasitaemia level for *Cytauxzoon* sp. was graded as reported by Carli et al. [12], observing how many erythrocytes presented parasitic inclusions per the entire monolayer and defining based on the following scale: mild ($n \leq 5$ parasitised red blood cells), moderate ($n \leq 20$), marked ($n \leq 50$), and very marked ($n > 50$). Serum obtained from each blood sample was also analysed by the SNAP® Combo Plus FeLV Ag/FIV Ab test (IDEXX Laboratories Inc., Westbrook, ME, USA) following the manufacturer's instructions.

DNA extraction was performed starting from 200 µL of k_3EDTA blood by the NucleoSpin® Tissue kit (Macherey-Nagel, Düren, Germany), in accordance with the manufacturer's protocol.

4.2. Molecular Analysis and Sequencing

DNA was processed by conventional PCR targeting the 18S-rRNA gene using the Piroplasmid primers pair 5′-CCAGCAGCCGCGGTAATTC-3′ and 5′-CTTTCGCAGTAGTTYGTCTTTAACAAATCT-3′, as already described by Tabar et al. [55]. Positive (i.e., DNA of sequenced field sample) and negative (no DNA added) controls were included in each PCR reaction. Amplicons were sequenced following Sanger technology (Macrogen Spain, Madrid, Spain) and the obtained nucleotide sequences were compared to those deposited in GenBank® using BLAST software (https://blast.ncbi.nlm.nih.gov/Blast) (accessed date: 2 August 2021)..

4.3. Data Analysis

In order to evaluate the presence of differences in infection rates among subgroups of the investigated cat population, a statistical evaluation was performed by means of the Pearson Chi-Square test or the Fisher exact test, if appropriate, using SPSS for Windows, version 27.0. The factors taken into consideration were: sex (i.e., males, females), age classes (i.e., <12 months, 12–35 months, ≥36 months), region of provenance (i.e., Site 1, Site 2, Site 3), lifestyle (i.e., indoor, outdoor), management (i.e., owned, stray cat), infection with immunosuppressive virus (i.e., positive for FIV and/or FeLV, or negative), presence of clinical signs (i.e., gastro-intestinal and respiratory signs), and ectoparasite infestation.

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Informed Consent Statement: Informed consent for participating to the study was obtained from all the involved owners or veterinary health authorities for colony cats.

Data Availability Statement: The authors declare that data are available upon request to the corresponding author, by email.

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4. Paper 3

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First autochthonous clinical case of *Hepatozoon silvestris* in a domestic cat in Italy with unusual presentation

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Parasites & Vectors

BRIEF REPORT

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First autochthonous clinical case of *Hepatozoon silvestris* in a domestic cat in Italy with unusual presentation

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Abstract

Hepatozoon spp. is the causative agent of a vector-borne parasitic disease in many animal species. In felids, *Hepatozoon felis, Hepatozoon canis* and *Hepatozoon silvestris* have been molecularly isolated. Hepatozoonosis usually causes asymptomatic infections in domestic cats, but clinical cases have recently been reported in Europe. We describe the first Italian case of hepatozoonosis in a cat with an unusual presentation. An 11-year-old neutered European shorthair cat was urgently hospitalized for intestinal intussusception. Hematology, biochemistry, FIV-FeLV tests, blood smears and molecular investigation targeting the 18S rRNA gene of *Hepatozoon* spp. were performed on blood samples; in addition, histological and molecular investigations were performed to analyze surgical samples to identify *Hepatozoon* infection. *Hepatozoon* gamonts were detected in granulocytes in the blood smear, and *Hepatozoon* spp. DNA was confirmed by PCR on blood. The intussusception was caused by a sessile endoluminal nodule that was surgically removed. Histologically, many elements referring to parasitic tissue forms were identified in the intestinal cells, and then the specimens were molecularly confirmed to harbor *H. silvestris*. This is the first description of symptomatic hepatozoonosis in a domestic cat in Italy. *Hepatozoon silvestris* has been described in wild felids, which are usually resilient to the infection, whereas the domestic cat seems to be more susceptible. Indeed, *H. silvestris* in cats usually presents tropism for skeletal muscle and myocardium with subsequent clinical manifestations. This is the first description of a domestic cat with *H. silvestris* localized in the intestinal epithelium and associated with intussusception.

Keywords: Domestic cat, Infection, Intestinal nodule, *Hepatozoon silvestris*, Italy

Hepatozoonosis is a vector-borne disease affecting many of animals, including reptiles, birds and mammals; it is caused by an apicomplexan parasite, of which almost 340 species are currently described [1–3]. Almost 50 species are recognized in mammals [1], but comprehensive information regarding their life cycle is known for only a few of them. Usually, the *Hepatozoon* life cycle involves an intermediate and a definitive host represented by a vertebrate animal and an arthropod vector, respectively [4]. In contrast to other vector-borne protozoa (e.g. *Babesia* spp., *Leishmania infantum*) transmitted to humans and animals bitten by infected arthropods, in hepatozoonosis, the vertebrate host becomes infected through the ingestion of infected arthropods [1, 2]. In the vertebrate host, the asexual replication of *Hepatozoon* takes place, generating intracellular gamonts that circulate in the bloodstream. The vectors, mostly represented by ticks, ingest *Hepatozoon* gamonts through blood-feeding from infected animals, and sexual replication takes place within the ticks, ending in the production of mature oocysts that are ready to infect a new vertebrate host

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and complete the life cycle when the arthropod will be ingested [2]. Interestingly, other transmission routes have been reported, e.g. in the *Hepatozoon canis* and *Hepatozoon felis* life cycles, vertical intrauterine transmission is described, and in the *Hepatozoon americanum* life cycle, predation (i.e. the ingestion of infected prey) has been proven to be an additional transmission route [2, 5, 6].

In felids, hepatozoonosis is still mostly unknown worldwide, but recent epidemiological studies and case reports are raising attention about this parasite and its pathogenicity [7, 8]. Currently, three species of *Hepatozoon* are recognized in wild and domestic felids in Europe, i.e. *Hepatozoon felis*, *Hepatozoon canis* and *Hepatozoon silvestris* [3, 9–11], but no information about the involved arthropod vectors are available, even if ticks seem to be the most likely arthropod vector [12–15]. Usually, hepatozoonosis in domestic and wild felids is considered subclinical, despite replicative forms (i.e. meronts) being found in skeletal muscles and in the myocardium of infected felids [2, 16, 17]. Recently, two cats with clinical signs were described in Central Europe; the first cat was presented with severe and fatal myocarditis and *H. silvestris* infection [7], and the second cat was in poor general condition and exhibited lethargy, anorexia, icterus, a painful abdomen, fever, ruffled hair and *H. felis* infection [8]. This study describes, for the first time in Italy to our knowledge, a clinical and survival case of hepatozoonosis with an unusual presentation in a domestic cat.

An 11-year-old neutered European shorthair cat living in a cat household with > 30 individuals in a pre-alpine area at 700 masl (45°15′98”N, 7°40′56”E) and having free outdoor access was examined for its yearly routine veterinary check-up (day -7). A tick was found attached to the cat's neck and was removed; then, the cat was treated with fipronil. The blood count presented some alterations in red blood cell, reticulocyte and monocyte total counts, and serum albumin was low (IDEXX Laboratories, Italy); no other remarkable findings were observed (Table 1). Five days later, the cat presented mild depression and loss of appetite; in a few days, a worsening of the clinical signs was observed, with anorexia, severe depression and vomiting. The cat was admitted to the hospital (day 0), and no particular findings were revealed during the physical examination such as fever, abdominal mass or pain. A complete hemato-biochemical profile (IDEXX Laboratories, Italy) and an abdominal ultrasound were performed.

The blood count showed an inflammatory leukogram with left shift and monocytosis, and the biochemical profile presented several alterations (i.e. electrolytes, CPK, AST, fructosamine) reported in Table 1. Microscopy of May Grunwald Giemsa-stained blood smear revealed single ovoid inclusions in neutrophils attributable to *Hepatozoon* gamonts (11.2 × 5.1 µm, with an ovoid central nucleus, Fig. 1); subsequently, the protozoan parasite was confirmed at the genera level (i.e. *Hepatozoon* spp.) on a blood sample by PCR targeting the 18S-rRNA gene with a cycle threshold (Ct) of 36.3 (IDEXX Laboratories, Germany).

Additionally, the cat tested negative for feline immunodeficiency virus (FIV) antibodies and feline leukemia virus (FeLV) antigens (SNAP FIV-FeLV Combo test, IDEXX Laboratories, Inc.). The results were also confirmed by molecular investigations targeting the FeLV proviral DNA (IDEXX Laboratories, Germany). The abdominal ultrasound identified an intestinal intussusception; thus, the cat was urgently admitted to the operating room for a laparotomy.

At surgery, a jejunal endoluminal pedunculated nodule of approximately 1.5 cm, almost occluding the intestinal lumen and causing intussusception, was found. The biochemical parameters improved and normalized within 13 days after surgery (Table 1). The cat was administered a 30-day regimen of oral doxycycline at 5 mg/kg bid. Blood PCR was performed 10 days after the end of doxycycline administration (day + 40), and the result was positive (Ct = 36.9) for the targeted *Hepatozoon* DNA (IDEXX Laboratories, Germany); 1 month later (day + 70), it finally became negative. The time line of the events and their brief descriptions are reported in Fig. 2.

No other vector-borne pathogens were tested because the hemato-biochemical profile and the rapid worsening of clinical conditions were suggestive of acute and severe disease; it was decided to wait for the clinical recovery of the cat before testing for other pathogens; then, since the cat recovered quickly, no other investigations for VBDs were done.

The intestinal nodule was submitted to histopathological and molecular investigations (University of Padova). Tissue sections revealed a severe inflammatory reaction characterized by chronic ulcerative enteritis with polypoid proliferation and severe lymphangiectasia. Many protozoal inclusions were revealed within the enterocytes of the intestinal villi and near the lumen (Fig. 3). The protozoa were roundish, of variable size (with an average size of 15 to 25 µm) and characterized by dark basophilic-staining small nuclei. These forms were referred to as parasitic inclusions of *Hepatozoon* spp. Away from the nodule, along the surgical section in the healthy intestinal tissue, no protozoal inclusions in the enterocytes were observed. Subsequently, to identify the protozoa affecting the intestinal tissue and causing the local host reaction and the nodule, conventional PCR was performed, targeting the 18S-rRNA of *Hepatozoon* spp. with primers described by Tabar et al. [18]. A positive (sequenced DNA of naturally infected cat) and negative (no DNA

Table 1 Hemato-biochemical profiles from the first check-up (1 week before the surgery) to the recovery of the *Hepatozoon*-infected cat

	Reference ranges	Days			
		−7	0	+4	+13
Blood count					
RBC	7.1–11.5 M/µl	6.9	7.0	5.3	5.0
Hct	28.2–52.7%	30.4	28.8	20.9	19.5
Hb	10.3–16.2 g/dl	10.8	10.3	7.9	7.3
MCV	39–56 fl	44.4	41.4	39.3	39.3
MCH	12.6–16.5 pg	15.8	14.8	14.8	14.7
MCHC	28.5–37.8 g/dl	35.5	35.8	37.8	37.4
Reticulocytes (total count)	K/µl	115.1	25	10.1	32.2
WBC	3.9–19 K/µl	15.7	20	15.6	14.2
Neutrophils (total count)	2.62–15.17 K/µl	13.368	14.763	13.706	12.581
Band neutrophils (total count)	0–300/µl	0	2195	0	0
Lymphocytes (total count)	0.85–5.85 K/µl	1.443	1.795	0.749	0.895
Monocytes (total count)	0.04–0.53 K/µl	0.706	1.197	1.124	0.653
Eosinophils (total count)	0.09–2.18 K/µl	0.157	0	0.03	0.07
Basophils (total count)	0–0.1 K/µl	0	0	0	0
PLT	155–641 K/µl	259	266	211	183
Notes			Rouleaux (+++) Burr cells (++) Anisocytosis (+) *Hepatozoon* gamont in neutrophils Platelet aggregates	Rouleaux (+) Heinz bodies (+) Doehle bodies Neutrophils: foamy (+) and basophilic (++) cytoplasm	Rouleaux (+) Platelet aggregates
Instrument: Sysmex XT2000iV, Sysmex, Kobe, Japan					
Biochemical profile					
Glucose	63–140 mg/dl	85	80	84	97
SDMA	0–14 µg/dl	12	12	19	11
Creatinine	0.9–2.3 mg/dl	1.5	1.6	1.0	0.8
BUN	16–38 mg/dl	23	67	31	18
Phosphates	2.48–6.81 mg/dl	4.34	5.26	4.02	4.02
Calcium	8.82–11.62 mg/dl	8.82	8.42	8.0	8.82
Magnesium	1.46–2.67 mg/dl	2.19	3.4[a]	2.19	1.7
Sodium	147–159 mmol/l	149	140	149	154
Potassium	3.3–5.8 mmol/l	4.8	4.8	5.0	4.5
Chloride	109–129 mmol/l	115	94	116	122
Total protein	5.9–8.7 g/dl	6.2	6.7	5.8	6.8
Albumin	2.7–4.4 g/dl	2.4	2.5	1.9	2.2
Globulins	2.9–5.4 g/dl	3.8	4.2	3.9	4.6
Albumin/globulins	> 0.57	0.64	0.60	0.48	0.49
ALT	27–175 U/l	38	31	51	34
AST	14–71 U/l	29	83	49	26
ALP	12–73 U/l	35	23	60	37
GGT	0–5 U/l	< 1	2	< 1	< 1
Bilirubin (total)	0–0.4 mg/dl	0.2	< 0.1	0.3	0.2
Cholesterol	86–329 mg/dl	123	196	199	149
Triglycerides	21–432 mg/dl	33	60	45	34
Lipase	0.1–45 U/l	nd	21	nd	14
CPK	52–542 U/l	111	2371	1156	313

Table 1 (continued)

	Reference ranges	Days			
		− 7	0	+4	+13
Urinary creatinine	mg/dl	373	224	nd	nd
Urinary total protein	mg/dl	61.6	42.4	nd	nd
PU/CU	< 0.33	0.2	0.2	nd	nd
Fructosamine	137–286 µmol/l	148	305	150	150
General notes			Vomiting, anorexia, stress, severe depression		Good general conditions

Instrument: Beckman-Coulter, Brea, CA, USA

RBC red blood cell count, *Hct* hematocrit, *Hb* hemoglobin, *MCV* mean corpuscular volume, *MCH* mean corpuscular hemoglobin, *MCHC* mean corpuscular hemoglobin concentration, *WBC* white blood cell count, *PLT* platelet count, *SDMA* symmetric dimethylarginine, *BUN* blood urea nitrogen, *ALT* alanine transferase, *AST* aspartate transferase, *ALP* alkaline phosphatase, *GGT* gamma-glutamyl transferase, CPK creatine phosphokinase, *PU/CU* urine protein to creatinine ratio, nd Not done

[a] Hemolytic serum

added) control was added to each PCR. The PCR products were sequenced (Macrogen, Spain) in both directions with the same primers used in PCR. The sequences were compared with those already deposited in GenBank by BLAST software (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence analysis revealed the presence of *Hepatozoon silvestris* (100% homology, accession number: KY649445.1).

Epidemiological studies reported the detection of *H. silvestris* DNA in wildcats from Bosnia and Herzegovina [3, 21] and in domestic cats from northeastern [10], southern Italy [9] and central Europe [7, 11]. This study describes the case of a cat coming from a hilly area in northwestern Italy close to the Swiss borders, where a fatal case of myocarditis caused by *H. silvestris* in a cat was recently reported [7] and where the presence of wild felids such as the European lynx is frequently described [22, 23]. European lynx and wildcats are already reported as potential reservoirs of several parasites for domestic cat populations sharing the same context of living [10, 21, 24]. Even though in the northwestern Italian regions the presence of lynx and wildcats is rarely observed, the possibility that these animals cross the Alps from highly endemic territories such as neighboring France and Switzerland is probable, as already demonstrated in recent years [23]. The pre-alpine area where the case report took place is close to the French border, and the outdoor lifestyle of the cat suggests exposure to the risk of sharing parasites and arthropod vectors with the sylvatic environment.

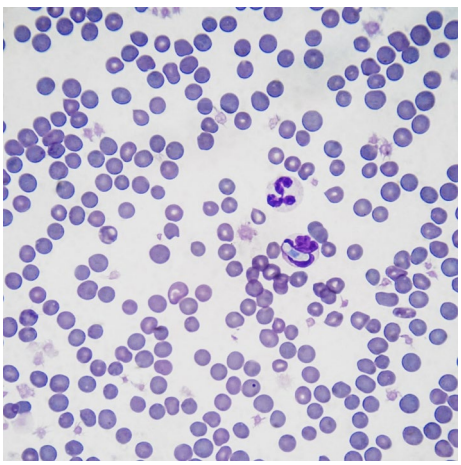

Fig. 1 A gamont of *Hepatozoon* sp. in a granulocyte. The blood smear was stained with May-Grünwald Giemsa

Recent studies reported the identification of *H. felis* DNA in *Rhipicephalus sanguineus* sensu lato and *Rhipicephalus turanicus* ticks [12, 14, 25] and *H. silvestris* DNA in *Ixodes ricinus* ticks [15]. This is not sufficient to define the competence role of these ticks as a biological vector. Since *I. ricinus*, already known as the forest tick or castor bean tick, is the most widespread tick in European wild areas, it might be considered to have a potential role in the transmission of *H. silvestris* [7, 13, 26]. The presence of the tick on the cat's neck during the yearly routine examination (day -7) suggests that exposure to arthropod activity and the ingestion of infected ticks by the cat during fur grooming are possible. Unfortunately, the tick was removed and not conserved; thus, morphological identification and molecular investigations for detecting *Hepatozoon* DNA were not possible; in addition, no other ticks were found after the cat was treated with fipronil. The predation of infected prey, e.g. mostly rodents, was considered another possible route of *Hepatozoon* transmission [2, 5].

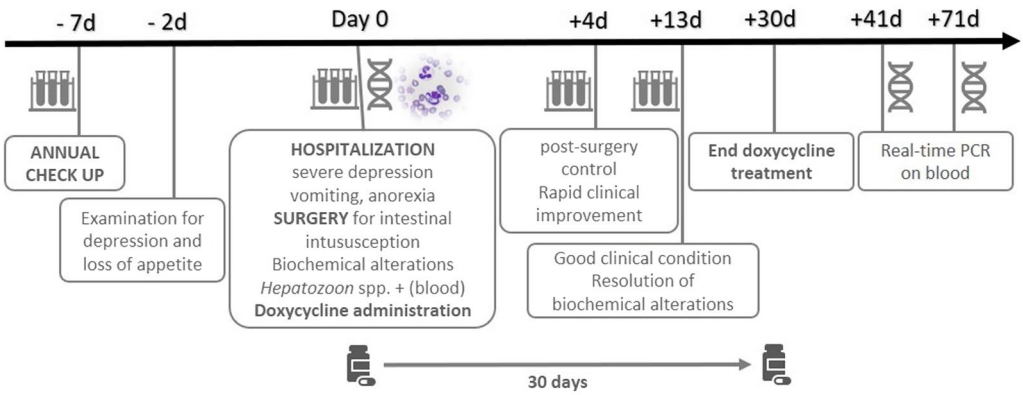

Fig. 2 Descriptive time line of clinical conditions, laboratory analyses and treatment of *Hepatozoon*-infected cat

In domestic and wild cats, *Hepatozoon* causes a generally subclinical inflammation of skeletal muscles and myocardium [2, 19, 20] as well as elevated values of CPK enzyme in the majority of affected subjects [2, 16, 17]. This finding was observed in our case study, suggesting a potential involvement of skeletal muscles. In cats, the level of parasitemia is generally low and not correlated with the infection burden and the presence of meronts in muscle tissues, and the reason is not yet clear [16, 19]. Neutrophils containing gamonts are usually < 1% [2, 16], as observed in our blood smear evaluation. Specifically, rare gamonts were identified only in the blood smear performed during hospitalization; in all subsequent blood smears, not one gamont was observed.

The intestinal intussusception was generated by a sessile endoluminal nodule, which could have been due to (1) the inflammatory local response to the parasite's penetration through the intestinal mucosa and/or (2) an inflammatory process that was already present where the *Hepatozoon* found a good substrate for replicating. Considering the *H. canis* life cycle already described by Baneth et al. [4], the parasitic inclusions (15–25 µm) found in the histological sections of the sessile nodule, even if smaller than those reported in the literature, could be referred to as protozoan replicative forms such as meronts of *H. silvestris*, suggesting that the nodule was probably the first site of protozoan replication. In addition, the altered values of CPK and AST at day 0 suggested light skeletal muscle damage (i.e. subclinical myositis), as previously reported in cats with hepatozoonosis [16, 17], even if, in this particular case, inflammation of the intestinal muscle layer could be hypothesized. At day 0 circulating sodium and chloride concentrations were low probably because of vomiting. In addition, fructosamine concentration was increased and normalized a few days later; although unproven, it is possible that longstanding stress of the disease temporarily and mildly increased glucose levels. The

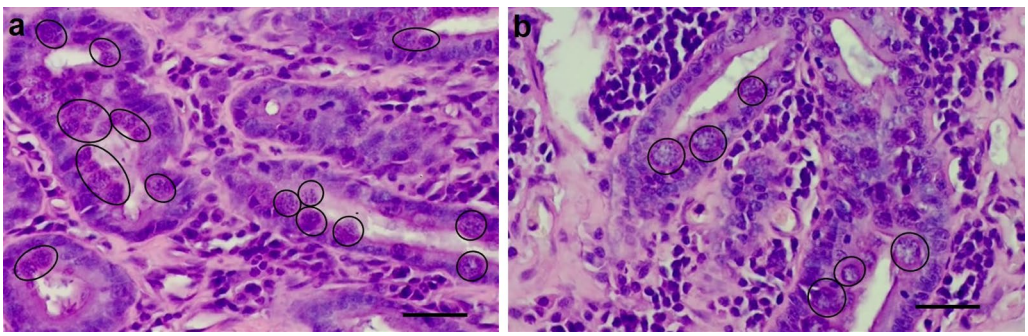

Fig. 3 **a**–**b** Histological sections of cat intestinal nodule: protozoan inclusions in the enterocytes (black circles). Hematoxylin-eosin staining. Bar 40 µm

improvement of CPK immediately after surgery supports the hypothesis that the nodule was the source of the clinical signs. Finally, doxycycline therapy seemed to be helpful for the complete recovery of the cat. Other treatment protocols are reported in cats in the literature such as the combination of doxycycline with primaquine, oxytetracycline with primaquine and imidocarb dipropionate with doxycycline [8, 17]. Actually, there were no controlled studies on the treatment of feline hepatozoonosis, and all information is anecdotal with debatable results. The choice to adopt only doxycycline was due to the difficulties of (i) the off-label use of imidocarb dipropionate in Italy and (ii) finding primaquine on the market easily and/or quickly.

Unfortunately, further histological investigations from other muscle sections and organs before and after treatment would have been useful to evaluate the skeletal muscle involvement, infection burden and efficacy of the treatment protocol but would have been unethical. In addition, the cat was treated to ensure its complete recovery without considering the novelty of *Hepatozoon* infection and the scientific publication.

Further investigations are needed to improve the scientific knowledge on *Hepatozoon* infections in felids, particularly in domestic cats, to prevent severe and potentially fatal clinical cases. Increased knowledge regarding the *Hepatozoon* life cycle in wild and domestic felids as long as arthropod vectors are involved would surely be useful for the adoption of adequate preventative measures in cats.

In conclusion, contrary to the other European case [7] in which *H. silvestris* caused fatal myocarditis in a domestic cat, in this report, the patient recovered completely after surgical removal of the "parasitic" nodule and monthly doxycycline therapy. The intestinal intussusception caused the sudden worsening of the clinical conditions, and surgical resolution was necessary to save the cat. However, the intestinal nodule was probably the result of a local inflammatory reaction to limit the *Hepatozoon* penetration, and it became the first site of protozoan replication; its surgical removal helped the cat to rapidly recover. Despite the unusual clinical presentation of this case, surgery should not to be the treatment of choice in every hepatozoonotic infections with intestinal signs and/or ultrasonographic abnormalities. Feline hepatozoonosis is an emerging vector-borne disease, and considering the recent reports of symptomatic cases, monitoring in cat populations is strongly advised.

Abbreviations

CPK: Creatine phosphokinase; AST: Aspartate transferase; Ct: Cycle threshold; FIV: Feline immunodeficiency virus; FeLV: Feline leukemia virus; VBDs: Vector-borne diseases.

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Author contributions

VF and GSa followed the clinical case and provided all data regarding the cat from anamnesis to recovery; SMa performed hemato-biochemical analyses; LC and MEG performed histopathological evaluations; SMo, MG and GD performed DNA extraction and molecular analyses on nodule sections; GSi and EZ conceived and revised the manuscript; GSi wrote the manuscript draft. All authors read and approved the final manuscript.

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Availability of data and materials

All data analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

All procedures were performed within the routine veterinary activity.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Ticks, fleas, and harboured pathogens from dogs and cats in Cyprus

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Ticks, Fleas, and Harboured Pathogens from Dogs and Cats in Cyprus

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Abstract: Ticks and fleas are blood-sucking ectoparasites that cause irritation and anaemia to their hosts and act as vectors of pathogens (vector-borne pathogens, VBPs) of relevance for animal and human health. In the present study, tick and flea species in dogs and cats from Cyprus were recorded and VBPs were detected in the collected specimens. Ectoparasites were collected from 220 animals (161 dogs and 59 cats), and a questionnaire including demographic, clinical, and other information was filled out for each animal. The ectoparasites were morphologically identified and the detection of VBPs was performed by PCR-coupled sequencing. *Rhipicephalus sanguineus* sensu lato was found on 108 dogs and 13 cats, and *Ixodes gibbosus* on 2 dogs. *Ctenocephalides felis* was the predominant flea species (on 62 dogs and 45 cats), while one dog and one cat were infested by *Ctenocephalides canis* and *Echidnophaga gallinacea*, respectively. The VBPs in ticks were *Anaplasma platys, Rickettsia massiliae, Rickettsia conorii, Rickettsia felis*, *Hepatozoon felis* and *Hepatozoon canis*, while *Rickettsia felis*, *Rickettsia* sp., *Bartonella koehlerae*, *Bartonella clarridgeiae*, and *Bartonella henselae* were recorded in fleas. Statistical analysis (chi-square test and multiple univariate generalized linear model) showed that animals up to 6 months of age were less likely to be infested with ticks than older animals, but more likely to be infested with fleas. Ticks were more prevalent in sheltered than in owned animals, while the odds ratio of flea presence was higher in owned animals than those living in shelters. The present study is the first investigation on the occurrence of ticks and fleas in dogs and cats from Cyprus, showing the presence of different VBPs in these important ectoparasites. The results point out the importance of systematic ectoparasite control in dogs and cats.

Keywords: ectoparasites; epidemiology; pet animals; vector-borne pathogens

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1. Introduction

Ticks and fleas are blood-sucking arthropods, infesting several vertebrates, among them dogs and cats. They have been extensively studied because of their direct clinical impact on animals, the pathogens they transmit, and their relevance in human health [1,2]. These ectoparasites can cause discomfort and may severely impact the health and well-being of dogs and cats. Ticks cause nuisance, anaemia, irritation, cutaneous lesions with inflammation and eosinophilic aggregation, secondary infections occasionally leading to abscesses or even pyaemia, and toxicosis (tick paralysis). Fleas cause severe irritation, pruritus and self-wound formation, blood loss and anaemia, and flea-associated allergic dermatitis [3–5]. Ticks and fleas may also transmit various vector-borne pathogens (VBPs)

to their hosts, many of which are zoonotic. Pathogens transmitted by ticks to dogs and cats include mostly protozoa (e.g. *Babesia* spp., *Hepatozoon* spp., *Cytauxzoon* spp.) and bacteria (*Rickettsia* spp., *Ehrlichia* spp., *Anaplasma* spp., *Coxiella* spp., *Borrelia* spp.). Fleas are vectors of *Bartonella* spp., *Rickettsia felis*, and *Yersinia pestis*, and are also an intermediate host of the cestodes *Dipylidium caninum* and *Hymenolepis diminuta*, and the nematode *Acanthocheilonema reconditum* [3,6–8].

Specific drivers, e.g., climate change and global warming, destruction of wild habitats for agriculture intensification, landscape modification, poor ecosystem protection, and increase in pet travel have a significant impact on the epidemiology and the increasing occurrence of ectoparasites [6]. Consequently, the affiliated VBPs and associated diseases are expected to expand, emerge, or re-emerge in many areas [9]. Knowledge of the current epidemiology of ticks, fleas, and transmitted pathogens is still scant in many areas of Europe and their distribution and occurrence are constantly changing over time [10].

In Cyprus, some investigations on ticks and tick-borne pathogens have been conducted in the past [11–16], while data on fleas and flea-borne pathogens are limited to only rats, foxes, and hares [14,17,18]. Therefore, the aim of the present study was (i) to investigate the infestation by ticks and fleas in dogs and cats from Cyprus; (ii) to detect the presence of VBPs in these ectoparasites; and (iii) to associate findings with different possible risk factors, in order to update and enrich knowledge about the epidemiology of these important ectoparasites.

2. Materials and Methods

2.1. Animals and Ectoparasite Collection

The survey was conducted on 220 animals (161 dogs and 59 cats), living in five districts of Cyprus, i.e., Ammochostos, Larnaca, Lemesos, Lefkosia, and Paphos (Figure 1), and presented to a private veterinary clinic in Limassol for routine clinical examinations (e.g., vaccination, castration, investigation of clinical condition, injury). Ectoparasites were detected by fur and skin inspection and by combing with a stainless-steel flea comb. The ectoparasites were collected by entomological forceps, stored in Eppendorf tubes containing 70° ethanol, and tagged with an individual code. For each animal included in the survey, a questionnaire was filled out, with information about age, sex, country district, lifestyle, last ectoparasiticide administration, the reason for the visit, and clinical and laboratory findings.

Figure 1. The map of Cyprus and the districts from which the sampled animals originated.

2.2. Identification of Ectoparasites

The collected ectoparasites were transferred to the Laboratory of Parasitology and Parasitic Diseases, School of Veterinary Medicine of the Aristotle University of Thessaloniki. The ectoparasites were examined under a stereomicroscope (8×–64×) and a light microscope (100×, 400×) for identification based on their morphological characteristics [19–21].

2.3. Detection of VBPs

After identification, ectoparasite specimens were transferred to the Laboratory of Parasitology of the Faculty of Veterinary Medicine, University of Teramo, for the detection of VPBs by molecular methods.

Ticks and fleas were examined in pooled samples per animal, into groups of one to five individuals. Overall, 122 pooled tick samples and 111 pooled flea samples were examined, excluding highly engorged tick specimens to avoid excess nucleic acids of vertebrate host origin. The ectoparasite pools were homogenized before DNA extraction. Briefly, the specimens were taken from the 70° ethanol solution, air-dried, and mechanically crushed in a 1.5 ml safe-lock tube with sterile pestles. The homogenates were incubated with proteinase K solution overnight at 56 °C and total nucleic acids were extracted from these homogenates in accordance with the manufacturer's instructions (Exgene Tissue SV, Gene All, South Korea). In ticks, *Anaplasma* spp./*Ehrlichia* spp., *Babesia* spp., *Bartonella* spp., *Rickettsia* spp., and *Hepatozoon* spp., and in fleas, *Bartonella* spp. and *Rickettsia* spp., were detected by polymerase chain reaction (PCR). A fragment of the 18S rRNA gene of *Anaplasma*/*Hepatozoon* spp. and *Babesia* spp., a partial sequence of the 16S–23S rRNA intergenic species region (ITS) of *Bartonella* spp., and a fragment of the rickettsial outer membrane protein A (ompA) gene were amplified using primers and protocols described previously [22–25]. The primers used for the amplification of the targeted DNA are shown in Table 1. All amplifications included a positive control containing genomic target DNA and a negative control without DNA. PCR products were visualized under UV illumination after electrophoresis migration on a 1.8% agarose gel. PCR products were sequenced in one direction, using the same primers as those used for DNA amplification. Sequences were compared for similarity to sequences in GenBank, using the BLAST program hosted by NCBI, National Institutes of Health, USA (http://www.ncbi.nlm.nih.gov, accessed on 1 August 2022).

Table 1. Primers used for the detection of VBPs in ectoparasites of dogs and cats from Cyprus and corresponding references (Ref).

Primer	Pathogen	Target gene	Nucleotide Sequences (5′-3′)	Product Size (bp)	Ref
Rrl9O.70p	*Rickettsia*	190 kDa antigen	ATGGCGAATATTTCTCCAAAA	~532	[22]
Rrl9O.602n			AGTGCAGCATTCGCTCCCCCT		
325s	*Bartonella*	16S-23S rRNA ITS	CTTCAGATGATGATCCCAAGCCTTYTGGCG	~600	[23]
1100as			GAACCGACGACCCCCTGCTTGCAAAGCA		
Piro A	*Babesia*	18S rRNA	AATACCCAATCCTGACACAGGG	400	[24]
Piro B			TTAAATACGAATGCCCCCAAC		
EHR16SD	*Anaplasma/ Ehrlichia*	18S rRNA	GGTACCYACAGAAGAAGTCC	345	[24]
EHR16SR			TAGCACTCATCGTTTACAGC		
Tabar F	*Hepatozoon*	18S rRNA	CCAGCAGCCGCGGTAATTC	373	[25]
Tabar R			CTTTCGCAGTAGTTYGTCTTTAACAAATCT		

2.4. Statistical Analysis

The occurrence of fleas and ticks on dogs and cats was evaluated in relation to factors expressing demographic details (gender, age), status (owned or sheltered), and previous

treatments (time passed since the last dosing). Moreover, the existence of VBPs in the ectoparasites was associated with additional factors: geographic region, the status of the animal (owned or sheltered), and clinical examination or laboratory findings associated with disease (e.g., anorexia, weight loss, eye lesions, neurological signs, positive in-clinic diagnostic test for infectious diseases). The chi-square test of independence was used to assess the effect of the above factors on the occurrence of ectoparasites and the existence of VBPs, respectively. The significant factors defined by the chi-square test were then entered into a multiple univariate generalized linear model (GLM) for determining their combined effect on the occurrence of ectoparasites [26]. The odds ratios with their corresponding confidence intervals (C.I.) were used to compare the proportion of the occurrence of each ectoparasite among the factor groups. The information collected through the questionnaires, about the veterinary product used on some of the animals, was not included in the statistical analysis owing to missing or unreliable data. The statistical analysis was implemented using the R package version [27] and the Rcmdr package [28].

3. Results

3.1. Study Animals

The demographics and other details of the examined animals are shown in Table 2.

Table 2. Recorded data for the dogs and cats (n = 220) with ectoparasites examined in Cyprus.

	Factor	Dogs (n = 161)	Cats (n = 59)
Status	Owner/Shelter	134/27	51/8
Region	Lefkosia	32	3
	Lemesos	91	42
	Larnaca	28	6
	Paphos	8	4
	Amochostos	2	4
Sex	Male/Female	81/80	23/36
Age	<6 months	23	17
	6–≤12 months	13	14
	>1–≤7 years	93	25
	>7 years	32	3
Last treatment for ectoparasites	≤1 month	17	2
	1–≤3 months	17	6
	>3–≤6 months	11	3
	>6–≤12 months	22	0
	>12 months	94	48
Reason for visit or findings	Disease/Other	88/73	34/25

3.2. Ectoparasites

From a total of 161 dogs with ectoparasites, 98 and 51 had ticks or fleas only, respectively, while 12 had mixed tick and flea infestation. Accordingly, in total, 110 (68.3%) dogs were infested with ticks and 63 (39.1%) had fleas, including both single and mixed infections. From a total of the 59 cats infested with ectoparasites, 9 had only ticks; 45 had only fleas; 3 had ticks and fleas; 1 had fleas and lice; and 1 had a mixed infestation with ticks, fleas, and lice. In total, 13 (22%) cats were infested with ticks, 50 (84.7%) with fleas, and 2 (3.4%) with lice, including both single and mixed infections (Tables 3 and 4).

Table 3. Number (n) of animals (dogs or cats) in Cyprus, infested with different types of ectoparasites, with the corresponding confidence interval (C.I.) of the occurrence percentage.

Animal Species (Sample Size)	Ticks n (%C.I.)	Fleas n (%C.I.)	Ticks and Fleas n (%C.I.)	Fleas and Lice n (%C.I.)	Ticks, Fleas, and Lice n (%C.I.)
Dogs (n = 161)	98 (60.8 ± 7.7)	51 (31.7 ± 6.7)	12 (7.5 ± 3.1)	0	0
Cats (n = 59)	9 (15.3 ± 7.0)	45 (76.3 ± 12.2)	3 (5.1 ± 3.3)	1 (1.7 ± 1.4)	1 (1.7 ± 1.4)

Two different tick species were identified: *Rhipicephalus sanguineus* sensu lato (s.l.) on 108 dogs and 13 cats, and *Ixodes gibbosus* on 2 dogs. The most abundant flea species was *Ctenocephalides felis*, found on 62 dogs and 45 cats, while *Ctenocephalides canis* and *Echidnophaga gallinacea* were found on one dog and one cat, respectively. The mixed infestations included 10 dogs and 4 cats with *R. sanguineus* s.l. and *C. felis*; two dogs with *I. gibbosus* and *C. felis*; one cat with *R. sanguineus* s.l., *C. felis*, and the louse *Felicola subrostratus*; and one cat infested with *E. gallinacea* and *F. subrostratus* (Table 4).

Table 4. Species identification of ticks and fleas and mixed infections in dogs and cats from Cyprus.

Animal Species (Sample Size)	*Rhipicephalus sanguineus* s.l.	*Ixodes gibbosus*	*Ctenocephalides felis*	*Ctenocephalides canis*	*Echidnophaga gallinacea*
Dogs (n = 161)	108 [1]	2 [2]	62 [1,2]	1	0
Cats (n = 59)	13 [3,4]	0	45 [3,4]	0	1 [5]

[1] Ten dogs with mixed infestation by *R. sanguineus* and *C. felis;* [2] 2 dogs with mixed infestation by *I. gibbosus* and *C. felis;* [3] 4 cats with mixed infestation by *R. sanguineus* and *C. felis;* [4] a cat with a mixed infestation by *R. sanguineus*, *C. felis*, and *Felicola subrostratus;* [5] a cat with a mixed infestation by *E. gallinacea* and the louse *F. subrostratus.*

3.3. Detection of VBPs

In total, 233 ectoparasite samples (122 tick and 111 flea samples) were examined for the detection of VBPs. In the case of multiple ticks or flea specimens per animal, a pooled sample (per ectoparasite type and per animal) was prepared. VBPs' detection by PCR was not possible for one tick and two flea samples owing to an insufficient or not suitable DNA sample. Overall, 32 (14.5%) animals were infested with ectoparasites that harboured one or more VBPs, whereas 35 (15%) ectoparasite pool samples were positive for VBPs, because, in three cases (two dogs and one cat) with a mixed infestation by *R. sanguineus* s.l. and *C. felis*, VBPs were found in both ticks and fleas.

The DNA of six different pathogens was detected in ticks, i.e., *Anaplasma platys*, *Rickettsia massiliae*, *Rickettsia conorii*, *Rickettsia felis*, *Hepatozoon felis*, and *Hepatozoon canis,* while no *Babesia* spp. was found in the examined specimens. The DNA of five different VBPs was detected in fleas, i.e., *Rickettsia felis*, *Rickettsia* sp., *Bartonella koehlerae*, *Bartonella clarridgeiae,* and *Bartonella henselae*. Details about the species and number of animals in the ectoparasites of which these VBPs were detected are shown in Table 5.

Table 5. Vector-borne pathogens (VBPs) detected in 122 tick and 111 flea pooled samples (per ectoparasite type and per animal) collected from dogs and cats in Cyprus.

Animal Species	VBPs in Ticks						VBPs in Fleas				
	A. p	*R. m*	*R. c*	*R. f*	*H. c*	*H. f*	*R. f*	*R.* sp.	*B. k*	*B. c*	*B. h*
Dogs (n)	3	10	1	-	3	1	3	4	1	2	-
Cats (n)		2	-	1	-	1	5	-	-		1
Total	3	12	1	1	3	2	8	4	1	2	1

n = number of animals in the ectoparasites of which the pathogen was found, A. p. = *Anaplasma platys*; R. m = *Rickettsia massiliae*; *R. c = Rickettsia conorii; R. f = Rickettsia felis; H. f = Hepatozoon felis; H.c = Hepatozoon canis; R.* sp. = *Rickettsia* sp.*; B. k = Bartonella koehlerae; B. c = Bartonella clarridgeiae; B. h = Bartonella henselae.*

Sequencing of PCR products and BLAST analysis revealed similarities of the herein detected VBPs with DNA sequences published in GenBank, as shown in Table 6.

Table 6. Vector-borne pathogens (VBPs) detected in ticks and fleas from dogs and cats in Cyprus, and their similarity with GenBank entries.

VBP (n of Sequences Analyzed)	GenBank Accession Number	Similarity
Anaplasma platys (n = 3)	JX392984.1	99%
Rickettsia massiliae (n = 12)	MW026209.1	97–99%
Rickettsia felis (n = 9)	KP318094.1	96–99%
Hepatozoon felis (n = 2)	KY649442.1	100%
Hepatozoon canis (n = 3)	MK645969.1	97–100%
Rickettsia conorii (n = 1)	AE006914.1	97%
Rickettsia sp. (n = 4)	MF134884.1	96–99%
Bartonella koehlerae (n = 1)	MT095046.1	98%
Bartonella clarridgeiae (n = 2)	EU589237.1	96%
Bartonella henselae (n = 1)	KT314216.1	100%

3.4. Statistical Analysis

Chi-square test of independence showed that neither tick nor flea presence was related to the time passed since the last ectoparasitic treatment ($\chi^2 = 3.68$, df = 4, $p > 0.05$ for ticks and $\chi^2 = 3.54$, df = 4, $p > 0.05$ for fleas) or the animal's sex ($\chi^2 = 0.60$, df = 1, $p > 0.05$ for ticks and $\chi^2 = 0.02$, df = 1, $p > 0.05$ for fleas). On the other hand, the occurrence of ectoparasites was associated with the age of the host ($\chi^2 = 27.19$, df = 3, $p < 0.001$ for ticks and $\chi^2 = 20.90$, df = 3, $p < 0.001$ for fleas) and their "owned or sheltered" status ($\chi^2 = 14.99$, df = 1, $p < 0.001$ for ticks and $\chi^2 = 16.34$, df = 1, $p < 0.001$ for fleas) (Table 7).

Table 7. Chi-square test of independence showing associations between the occurrence of ectoparasites and various factors recorded for each animal.

	Ticks			Fleas		
Variable	**Positive**	**Negative**	***p*-value**	**Positive**	**Negative**	***p*-value**
Last treatment			0.451			0.471
≤1 month	14 (73.7%)	5 (26.3%)		6 (31.6%)	13 (68.4%)	
>1–3 months	12 (52.2%)	11 (47.8%)		13 (56.5%)	10 (43.5%)	
>3–6 months	6 (42.9%)	8 (57.1%)		8 (57.1%)	6 (42.9%)	
>6–12 months	13 (59.1%)	9 (40.9%)		11 (50.0%)	11 (50.0%)	
>12 months	78 (54.9%)	64 (45.1%)		75 (52.8%)	67 (47.2%)	
Sex			0.438			0.875
Male	61 (58.7%)	43 (41.3%)		54 (51.9%)	50 (48.1%)	
Female	62 (53.4%)	54 (46.6%)		59 (50.9%)	57 (49.1%)	
Age category			**0.000 ***			**0.000 ***
<6 months	13 (32.5%)	27 (67.5%)		30 (75.0%)	10 (25.0%)	
6–12 months	7 (25.9%)	20 (74.1%)		20 (74.1%)	7 (25.9%)	
>1–7 years	81 (68.6%)	37 (31.4%)		49 (41.5%)	69 (58.5%)	
>7 years	22 (62.9%)	13 (37.1%)		14 (40.0%)	21 (60.0%)	
Status			**0.000 ***			**0.000 ***
Owned	93 (50.3%)	92 (49.7%)		106 (57.3%)	79 (42.7%)	
Sheltered	30 (85.7%)	5 (14.3%)		7 (20.0%)	28 (80.0%)	

* Statistically significant factor, $p < 0.001$.

The investigation of the association between VBPs' occurrence and various factors showed that VBPs' detection was not associated with clinical signs or findings of disease

(χ^2 = 2.42, df = 1, p > 0.05), the animals' "owned or sheltered" status (χ^2 = 1.06, df = 1, p > 0.05), or the region of living (χ^2 = 3.62, df = 4, p > 0.05) (Table 8).

Table 8. Contingency tables with chi-square test results between VBPs' existence and other factors.

Variable	VBPs		*p*-Value
	Positive	Negative	
Signs/findings			0.120
Disease	21 (17.8%)	97 (82.2%)	
Other	10 (10.3%)	87 (89.7%)	
Status			0.304
Owned	24 (13.3%)	156 (86.7%)	
Sheltered	7 (20.0%)	28 (80.0%)	
Region			0.460
Ammochostos	2 (40.0%)	3 (60.0%)	
Larnaca	3 (9.4%)	29 (90.6%)	
Lemesos	18 (13.7%)	113 (86.3%)	
Lefkosia	6 (17.1%)	29 (82.9%)	
Paphos	2 (16.7%)	10 (83.3%)	

The age category and the "owned or sheltered" status were further analysed for their combined effect on the occurrence of ticks or fleas using multiple GLM (Table 9). The analysis showed that animals up to 6 months and those between 6 and 12 months had the same likelihood to be infested by ticks or fleas (multiple GLM p-values > 0.05). However, young animals had a higher likelihood of being infested with fleas, whereas older animals had a higher likelihood of being infested with ticks. Indeed, animals up to 6 months were 0.26 and 0.27 times less likely to be infested with ticks than animals 1 to 7 years or older, respectively. Animals up to 6 months were 3.59 and 4.88 times more likely to be infested with fleas than animals from 1 to 7 years and those older than 7 years, respectively (multiple GLM p-value < 0.01). The status (owned or sheltered) of the animal was also found to be related to the presence of ectoparasites (multiple GLM p-value < 0.01). Ticks were five times (i.e. the inverse of 0.2 odds ratio shown in Table 9) more likely to be found on sheltered animals than owned animals, while the odds ratio of flea presence was 4.84 times higher in owned animals than in those living in shelters.

Table 9. Assessment of risk factors of ectoparasites' occurrence including the results of the multiple generalized linear model (GLM).

Variable	Ticks			Fleas		
	Odds Ratio	95% CI	GLM *p*-value	Odds Ratio	95% CI	GLM *p*-Value
Age category						
<6 m vs. 6–12 m	1.40	(0.47, 4.43)	0.557	1.05	(0.32, 3.30)	0.928
vs. 1–7 years	0.26	(0.11, 0.55)	**0.001** *	3.59	(1.61, 8.54)	**0.002** *
vs. >7 years	0.27	(0.10, 0.67)	**0.007** *	4.88	(1.83, 13.82)	**0.001** *
Status						
Owned vs. Sheltered	0.20	(0.06, 0.52)	**0.002** *	4.84	(2.04, 12.91)	**0.001** *

* Multiple GLM p-value < 0.01, identifying a risk factor.

4. Discussion

Cyprus, an island country in the Eastern Mediterranean Sea, is a cosmopolitan hub and a centre of tourism, market, education, and other activities, which receives a great number of visitors throughout the year. On the other hand, Cyprus has a large number of

dogs and cats, living as owned pets, free-roaming, or strays. A significant number of animal shelters in the country are actively facilitating adoption of stray animals, which, in many cases, travel abroad, to their new home, in different areas of the world. In this context, investigating and monitoring pathogens that may be transmitted locally or in remote countries via ticks and fleas is of great epidemiological importance.

The subtropical–Mediterranean climate of Cyprus with mild winters and warm to hot summers is favourable to ticks and fleas, because their development, especially the rate of transition from one development stage to the next, which in most cases takes place in the environment, is temperature-dependent [29,30]. The present results are in line with the fact that tick parasitism is more common in dogs than in cats, while the opposite is true for flea infestations, probably because of the different behaviour of dogs and cats and the different biology of these ectoparasites [1,31].

The ectoparasite species identified herein have a worldwide distribution and are prevalent in Southern Europe [32]. The predominant tick species, *R. sanguineus* s.l. [33], also made up the majority (89–92%) of the ticks collected from dogs in earlier surveys in Cyprus, showing limited affiliation to other host species (mouflons, foxes, hares, goats, sheep, and bovines) [13,15]. It is a three-host tick, a fact that facilitates the transmission of VBPs from animal to animal and is the vector of many VBPs [4,34]. Accordingly, 6 different VBPs were detected in 22 out of 120 *R. sanguineus* s.l. samples examined in the present study.

The prevalence of *A. platys*, the agent of canine cyclic thrombocytopenia (CCT), varies between 0.4% and 87.5% in different areas of the world [35]. In Cyprus, this bacterium has been detected only once in a dog [16]. *Anaplasma platys* is a recognized zoonotic agent [35], and enriching information on its occurrence in areas where data are lacking is important. The present results confirm that this VBP is circulating among ticks and dogs in Cyprus.

Even though seropositive dogs to *R. conorii* are highly prevalent in southern Europe [9,36–39], usually, only a small number of the examined ticks score positively in PCR [40–42], which is consistent with the present results. The infection in dogs is usually subclinical, but in humans, *R. conorii* is the agent of Mediterranean spotted fever [43], thus creation of epidemiological information is essential. Interestingly, *R. massiliae* was the most prevalent VBP in the present study. It is considered an emerging pathogen in Africa, Europe, and the USA, incriminated for several human cases with clinical signs similar to Mediterranean spotted fever [44]. On the basis of the present findings, *R. massiliae* is a possible emerging public health threat in Cyprus and the awareness towards this bacterium should be increased.

Both *H. canis* and *H. felis* were found in ticks, albeit at a low prevalence. In Cyprus, *H. canis* has been previously reported in dogs [45], while *H. felis* has been detected with a high prevalence (37.9%) in cats [46]. Similarly, *H. felis* occurs with a high prevalence in cats in other European enzootic areas, reaching 25.5% in Greece [47]. *Hepatozoon* species circulating in Europe, i.e., *H. canis* in dogs and *H. canis*, *H. felis*, and *Hepatozoon silvestris* in cats, have diverse pathogenic potentials. Although infections are often subclinical, animals may develop severe disease depending on the species or haplotype involved [47–50].

To the best of the authors' knowledge, this is the first record of *I. gibbosus* on dogs in Cyprus. It is one of the most common *Ixodes* species on the island [15,31] and it was previously reported on mouflons [51]. *Ixodes gibbosus* is adapted to warm and dry climates, replacing *Ixodes ricinus* in the eastern Mediterranean, which is less resistant to such conditions [31]. Further investigations into the prevalence and vectorial capacity of *I. gibbosus*, as the dominant *Ixodes* species in the area, would be of merit.

Ctenocephalides felis is the vector of important pathogens, including *B. henselae* detected herein and previously reported in rats and cats of Cyprus [6,46,52]. On the other hand, to the best of the authors' knowledge, the present report of *B. koehlerae* and *B. clarridgeiae* is the first in the country. *Bartonella* spp. are agents of disease in both animals and humans; for example, *B. henselae* is the agent of cat-scratch disease [53], thus constant surveillance of the presence of these VBPs in dogs, cats, and ectoparasites is pivotal.

Rickettsia felis is the agent of human flea-borne spotted fever and an emerging VBP [6]. In Cyprus, it has been detected previously in *C. felis* from rats [17]. The cat flea is the primary vector of *R. felis*, but it is probably also transmitted by other flea species, ticks, and other blood-sucking arthropods [6,54] and it was also detected in *R. sanguineus s.l.* in the present study.

Ctenocephalides canis, the dog flea, is less common in dogs than *C. felis* [6]. Accordingly, this flea species was found only on one dog in the present study. Nevertheless, in some areas, *C. canis* is reported to be more prevalent than *C. felis* [55]. The dog flea may also transmit pathogens including *R. felis* and *B. henselae*; however, because of its limited abundance compared with the cat flea, its vectorial role is considered inferior [56].

The flea *E. gallinacea*, also known as the "sticktight flea", was found on one cat. This species is common on fowl, but it also infests mammals, most frequently cats, probably owing to bird hunting [57]. It is a flea species of both veterinary and medical importance, transmitting fowl viruses, *Y. pestis*, *R. typhi*, and *D. caninum* [58], which renders it an important target for study and control, despite its low frequency.

An incidental finding in the examination for ticks and fleas was the cat louse *F. subrostratus* on two cats. Cat louse has a worldwide distribution and infestation is often an indication of a poor general health condition and lack of care [59]. Even if out of the scope of the present article, this finding is important as the cat louse has been identified as a potential intermediate host of a *Dipylidium* species, genetically distant from *D. caninum*, infecting hyenas, dogs, and cats [60].

The use of ompA gene appears to be specific and discriminating for the spotted fever group *Rickettsiae*, but some authors recommend that multiple gene targets should be used to gain an accurate identification [61]. This could be the reason that, for a few isolates, identification only to the genus level was feasible (Table 5). The remainder of VBPs identified in the present study showed a varying level of similarity with GeneBank deposits, isolated from different hosts and areas of the world (Table 6). It is worth noting that the detection of *Rickettsia* spp. DNA, mainly in *R. sanguineus*, provides evidence that this tick may be among the main vectors of *Rickettsia* spp. in Cyprus, according to previous studies [40,62].

The finding that young animals (<6 months) were significantly less likely to be infested with ticks, but more likely to be infested with fleas, may be attributed to the fact that young animals will spend most of their time in a restricted environment near their home, in close proximity to their mother and siblings, a condition that favours host-to-host flea transmission [1]. This contrasts with older animals that spend more time roaming a wider area outdoors. As such, older animals are more likely to come into contact with ticks, explaining the finding that older animals were significantly more likely to harbour ticks than young animals.

The activity within a wider or restricted environment may also be the reason ticks were more prevalent on sheltered animals, especially considering that some of them were introduced recently and were previously roaming in a wider area of their region. Accordingly, the occurrence of fleas was more frequent in owned animals, living in a confined/restricted environment (indoors for most cats, indoors or/and in the garden for dogs), which can often maintain flea infestation, compared with those living in shelters.

Interestingly, the infestation was not statistically associated with the time that had passed since the last ectoparasitic application. Thus, animals with a recent ectoparasite treatment were at the same risk of infestation as the rest of the animals. Although drug resistance development in ectoparasites is a known problem [29,63], the lack of specific investigation into the products used and the application practices does not allow further evaluation of this finding.

5. Conclusions

Ticks and fleas are a major concern for pet owners, veterinarians, and medical doctors because of their clinical impact on dogs and cats and the VBPs they transmit. The results of the present study provide new knowledge about the occurrence of ticks and fleas in

dogs and cats from Cyprus, and the pathogens that these ectoparasites may transmit, covering a relevant gap in knowledge. Companion animals travelling for adoption (commonly sheltered animals) or with their owners for vacations may facilitate the spreading of VPBs [45]. This risk is lurking, particularly in animal movements from and to Mediterranean areas, including Cyprus, as this part of Europe is considered a major epidemiological hub for VBPs [47]. Systematic ectoparasite control is pivotal and a plethora of veterinary products are available for this purpose. Furthermore, the research into new animal and environment-friendly tools for control is ongoing, and effective biological or botanical-based compounds and vaccines may also be available in the future [64,65].

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6. Paper 5

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Molecular survey on *Cytauxzoon* spp. and *Hepatozoon* spp. in felids by new real time PCR approach

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Molecular survey on *Cytauxzoon* spp. and *Hepatozoon* spp. in felids by new real time PCR approach

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Abstract

Tick-transmitted apicomplexans of genera *Cytauxzoon* and *Hepatozoon* affect a wide range of felids worldwide but knowledge on them is still scant. Recently, several studies addressed the species circulating in Europe, their distribution and hosts. Molecular assays are the method of choice for their detection. Unfortunately, conventional PCRs already described are time and cost-consuming and specific either for *Hepatozoon* or *Cytauxzoon* detection. This survey was developed in order to evaluate i) the occurrence of *Cytauxzoon* and *Hepatozoon* spp. in felids using a fast and cost-saving real-time PCR able to detect simultaneously both protozoa, ii) the distribution of *Cytauxzoon* and *Hepatozoon* species in Europe, and iii) the involvement of other susceptible felid hosts in the same area.

A SYBR® Green based real-time PCR with primers targeting the 18S-rRNA was validated and applied to 237 felid samples, i.e. whole blood from 206 domestic cats and 12 captive exotic felids, and tissues from 19 wildcats. Positive results were achieved through the melting temperature curve analysis thank to the specific melting peak of both protozoa. Positive samples were submitted to conventional PCR followed by Sanger sequencing. Phylogenetic analyses were performed to assess relatedness among European isolates. Data on domestic cats (age class, sex, provenance, management, lifestyle) were recorded and statistical analyses were performed to identify potential risk factors. Thirty-one/206 (15%) domestic cats were positive for *Hepatozoon* spp. (i.e., 11 *H. felis,* 20 *H. silvestris*) and 6/206 (2.9%) for *C. europaeus*.

H. felis prevalence was significantly ($p < 0.05$) higher in owned cats, while *H. silvestris* in stray cats and animals coming from Friuli Venezia Giulia region. *C. europaeus* was detected in stray cats only from Friuli Venezia Giulia (Trieste province). Among captive felids, a tiger was infected by *H. felis* and another by *H. silvestris*; 8/19 (42%) wildcats were positive for *Hepatozoon* spp. (i.e., 6 *H. felis*, 2 *H. silvestris*) and 4/19 (21%) for *Cytauxzoon europaeus*. No co-infections were detected.

Introduction

Cytauxzoon spp. and *Hepatozoon* spp. are the etiological agents of cytauxzoonosis in felids and hepatozoonosis in a wide range of animals worldwide. The first description of the *Cytauxzoon* genus was recorded in a domestic cat in the USA and it was named *Cytauxzoon felis* (1). In Europe, a different species of *Cytauxzoon* was recorded in Spain (2, 3), France (4-6), Portugal (7), Switzerland (6, 8), Germany (9, 10), Luxembourg (10) Romania (10), Czech Republic (10) and in Italy (11-15).

Recently, three species affecting wild and domestic European felids were described: *Cytauxzoon europaeus, Cytauxzoon banethi* and *Cytauxzoon otrantorum* (10).

Hepatozoon spp. has been described in several host species, i.e., mammals, reptiles, birds, amphibians (16). In particular, three species of *Hepatozoon* are described and reported in wild and domestic felids in Europe: *H. felis*, *H. silvestris* and *H. canis*. *Hepatozoon* infections are widespread, being reported in Spain (3) France (4, 17), Portugal (18), Cyprus (19), Germany (20), Austria (21), Greece (22) and Italy (14, 23, 24).

The observation of specific parasitic inclusions (i.e., merozoites) in red blood cells is suggestive of piroplasm infection and cytauxzoonosis. Unfortunately, the stained blood smear is not sufficient to achieve a diagnosis and molecular analysis is strongly recommended. Moreover, since cytauxzoonosis is characterzed by a low burden of merozoites circulating in the bloodstream in both acute and chronic phases of the infection, the molecular analysis represents the gold standard for the diagnosis due to its high sensitivity and the possibility to identify the involved *Cytauxzoon* species (25).

Although the detection of *Hepatozoon* gamonts could be sufficient for hepatozoonosis diagnosis, the molecular detection is strongly suggested, since hepatozoonosis in felids is usually asymptomatic and characterized by low parasitaemia around 1% of infected white blood cells (26).

In the literature, conventional polymerase chain reaction (cPCR) assays for *Cytauxzoon* and *Hepatozoon* detection are described usually targeting the 18S rRNA, a highly conserved gene for both protozoa (8, 19, 27-32) and cytochrome B and cytochrome C oxidase subunit I (COI), mitochondrial protein-coding genes, more specific for *Cytauxzoon* species identification (10).

Real-time polymerase chain reactions (real-time PCR) may be a reasonable alternative to quickly screen a large number of samples. This procedure is time-saving because the electrophoresis gel analysis is not required and the fluorescence analysis allows the operator to collect data during the real-time PCR running. In 2007, Criado-Fornelio et al. (33) developed a real-time PCR assay for the detection of *Hepatozoon* spp. in canine and feline blood samples, and the procedure seemed more sensitive than cPCR in feline samples. Recently, real-time PCR

assays targeting the piroplasmid 18S-rRNA gene were applied to individually detect *Cytauxzoon* spp. and *Hepatozoon* spp. in tissue samples and blood of domestic and wild felids in Europe (6, 15). However, to our knowledge, no assay that simultaneously detects *Cytauxzoon* spp. and *Hepatozoon* spp. DNA was developed yet.

In the light of previous considerations, the study aimed to evaluate i) the occurrence of *Hepatozoon* spp. and *Cytauxzoon* in felids using a real-time PCR for the simultaneous detection of both pathogens ii) to assess the distribution of *Cytauxzoon* and *Hepatozoon* species and their genetic diversity in felids, 3) to study the involvement of felines other than domestic cats.

Materials and methods

Study area

The molecular survey was developed in North-eastern Italy where *Cytauxzoon* and *Hepatozoon* circulation in domestic cats was already proven (11,14). In particular, Veneto (Site 1), Friuli Venezia Giulia (Site 2), and Trentino Alto Adige (Site 3) regions were investigated. Blood samples collected from captive felids living in zoological parks of other regions (i.e., Latium, Piedmont) have also been included.

Felid sampling and individual data collection

Different species of felids were sampled: domestic cats (*Felis silvestris catus*) from the Site 1, 2 and 3, European wildcats (*Felis silvestris silvestris*) from Site 2 and captive exotic felids, i.e., tigers (*Panthera tigris*), leopards (*Panthera pardus*), lions (*Panthera leo*), and caracals (*Caracal caracal*), living in zoological parks located in Site 1, except for 2 animals coming from zoological parks of Latium and Piedmont regions. Whole blood was collected from domestic cats and captive exotic felids during routine clinical visits and/or surgical procedures (not depending on this research study) thanks to the collaboration of some veterinary private practices/clinics operating in the investigated territory. Tissues (i.e., heart, lung, spleen, liver, lymph node, and blood clots) were collected from European wildcats found dead in the monitored areas and stored at -20°C until post-mortem examination. Individual data were recorded for each recruited animal. In particular, provenance (i.e., Site 1, Site 2, Site 3), sex, age classes (i.e., <12 months, from 12 to 36 months, >36 months), management (i.e., owned, stray cats) and lifestyle (i.e., indoor, outdoor) were registered for domestic cats; provenance, sex, and age classes (i.e., <12 months = sub-adults; ≥12 months = adults) were recorded for captive exotic felids and European wildcats.

DNA extraction, molecular analysis and sequencing

DNA was extracted using the NucleoSpin® Tissue kit (Macherey-Nagel, Düren, Germany) and starting from 200 µL of whole blood or 25 mg of organs/clot, according to the manufacturer's instructions.

DNA extracts were analysed by real-time PCR using the QuantiNova SYBR® Green PCR Kit (QIAGEN Group, Hilden, DE) with primers targeting a 373 bp fragment of piroplasmid 18S-rRNA gene (Table 1).

The assay was performed in the Roche LightCycler®96 thermocycler with the following amplification cycle: incubation at 95 °C for 2 min, followed by 45 cycles of amplification steps at 95 °C for 5 sec and 60 °C for 10 sec, concluding at 95°C for 10 sec, 65 °C for 1 min and 97 °C

for 1 sec. The melting curve analysis was performed by continuously monitoring the fluorescence while decreasing the temperature from 95°C to 65°C. Positive (i.e. DNA of sequenced field samples) and negative (no DNA added) controls were added in each PCR reaction.

Fluorescence specificity and genus identification were achieved through the melting temperature (T_m) curve analysis (i.e., *Cytauxzoon* spp. T_m = 81 °C; *Hepatozoon* spp. T_m = 78-78.5 °C) (Figure 1) (34). Amplicons of *Hepatozoon* spp. positive samples were submitted directly to sequencing, whereas *Cytauxzoon* spp. positive samples were processed by a nested PCR targeting the cytochrome B gene using primers (Table 1) and protocols already described (10). Amplicons of real-time PCR and nested PCR were Sanger sequenced (Macrogen Spain, Madrid, ES) and the obtained nucleotide sequences were compared to those deposited in GenBank® using BLAST software (https://blast.ncbi.nlm.nih.gov/Blast) (accessed date:15 October 2022).

Phylogenetic analysis

The obtained sequences were submitted to BLAST® analysis and a collection of closely related *H. felis* and *H. silvestris* sequences were identified and downloaded from Genbank®. Moreover, a representative sample of *H. canis* sequences was also downloaded to be used as an outgroup in the phylogenetic analysis. Selected sequences were aligned with the ones obtained in the present study using MAFFT (37). A neighbour-joining tree was reconstructed using MEGA X (38) selecting as the substitution model the one with the lowest Akaike information criterion (AIC), calculated with JModeltest (39). The reliability of inferred clades was inferred by performing 1000 bootstrap replicates.

A comparable approach was applied to the analysis of *C. europaeus* sequences. The obtained sequences were submitted to BLAST® analysis and a collection of closely related *C. europaeus* sequences were identified and downloaded. Thereafter, the alignment and phylogenetic analysis were performed as previously described for Hepatozoon. *Cytauxzoon felis* sequences were also downloaded to be used as an outgroup.

Data Analysis

The differences in infection rates among domestic cat populations in relation to the individual factors was statistically evaluated through the Pearson Chi-Square test or the Fisher exact test, if appropriate, using R software 4.1.2. The considered factors were sex (i.e., male, female), age classes (i.e., <12 months, from 12 to 36 months, >36 months), provenance (i.e., Site 1, Site 2, Site 3), lifestyle (i.e., indoor, outdoor), management (i.e., owned, stray cat). Captive felids and wildcats were not included in the statistical analysis due to the low number of samples.

Results

Felid populations description

A total of 237 felid samples were collected and analysed by real-time PCR: 206 domestic cats, 12 captive exotic felids, and 19 wildcats.

Among the domestic cat population, 70.9% (n = 146/206) came from Site 1, followed by 19.4% (n = 40/206) from Site 2 and 9.7% (n = 20/206) from Site 3. Most of them were owned cats (n = 131, 63.6%), and the rest were stray cats from street colonies (n = 75, 36.4%). Domestic cats

had mostly an outdoor lifestyle (n = 139, 67.5%) and were equally distributed among sex and age classes. Individual data of recruited domestic cats are shown in Table 2.
The 12 captive exotic felids included 4 tigers (*Panthera tigris*), 2 lions (*Panthera leo*), 3 leopards (*Panthera pardus*), and 1 caracal *(Caracal caracal*) from different zoological parks located in Site 1 and 2 tigers coming from zoological parks of Latium and Piedmont regions.
The wildcats, road-killed or found dead in Site 2, were 10 (52.6%) males and 9 (47.4%) females and their age was estimated on teeth evaluation classifying them in adults (n = 17/19, 89.5%) or sub-adults (n = 2/19, 10.5%).

Analysis results

Real-time PCR detected *Hepatozoon* spp. infection in 41/237 (17.3%) felids and *Cytauxzoon* spp. in 10/237 (4.2%). Among *Hepatozoon*-positive felids, the sequencing confirmed the dirfo and revealed the involved species: 18/41 were *H. felis* and 23/41 *H. silvestris*. The 10 *Cytauxzoon*-positive samples were confirmed by nested PCR and amplicons submitted to sequencing were all identified as *C. europeus.* No co-infections were detected. Results in detail are summarized in Table 3.
Cytauxzoon-positive cats were all stray animals from Trieste province in Site 2, whereas *Hepatozoon* spp.-positive cats were distributed in all investigated sites. Individual data concerning infected domestic cats were reported in Table 4.
A significantly higher prevalence ($p<0.05$) of *C. europeus* and *H. silvestris* infection was found in stray cats and animals living in Site 2. On the other hand, owned cats were statistically more infected by *H. felis* than stray cats as well as kittens younger than 1 year or adults older than 3 years (Table 4). Among captive felids, only two tigers resulted positive for *Hepatozoon* spp., one for *H. felis* and one for *H. silvestris*. Both were from a zoological park located in Veneto region (Site 1). No exotic felid species was positive for *Cytauxzoon* spp. All positive wildcats were adults (older than 1 year), mostly females from several provinces of Site 2 (i.e., Trieste, Udine, Pordenone). The distribution of *C. europeus*, *H. felis* and *H. silvestris* in domestic cats, European wildcats and captive exotic felids is shown in Figure 2.
All sequences isolated from domestic cats, wildcats and tigers of *C. europaeus* (from OP757647 to OP757655), *H. felis* (from MZ227585 to MZ227594 and OP693639 to OP693646), and *H. silvestris* (from MZ227596 to MZ22611 and to OP694164 to OP694169) were deposited in GenBank®.

Phylogenetic analysis

Cytauxzoon europaeus phylogenetic tree revealed that the obtained sequences were different and sparse along the tree, whereas *Hepatozoon* species formed well-separated clades. *Hepatozoon felis* sequences obtained in the present study were part of 2 clusters including, besides other Italian strains, also sequences from Spain, Hungary and Germany. Similarly, *H. silvestris* strains were part of 2 clades, one including sequences from Italy, Switzerland and Bosnia Herzegovina, and the other comprising Italian and Turkish strains (Figure 3 and 4).

Discussion

Vector-borne diseases have stimulated the interest of the scientific community in the last decades, indeed the epidemiological data for *Cytauxzoon* spp. and *Hepatozoon* spp. in wild and

domestic felids is continuously updated (6, 10, 40). In Italy, *Cytauxzoon* spp. and *Hepatozoon* spp. circulation was reported in both domestic and wildcats (11, 14, 23, 41), highlighting the need to better investigate in their circulating species and genotypes. A recent survey on *Cytauxzoon* spp. and *Hepatozoon* spp. infections in asymptomatic domestic cats confirmed the presence and the establishment of a domestic cycle for both protozoa in North-eastern Italy. Hepatozoonosis was quite equally distributed in all investigated regions while cytauxzoonosis was strictly limited around Trieste province in Friuli Venezia Giulia region (14). The present survey (i) updates the epidemiological data on domestic cats in the same areas using a new, fast and sensitive molecular procedure (real-time PCR) able to detect and differentiate simultaneously both protozoa, (ii) adds information on protozoa species, their distribution, and (iii) investigates other potential felid hosts in same areas and their potential role in the transmission of both protozoa.

Among 237 animals, 4.2% were infected by *Cytauxzoon*. In particular, the 10 positive animals were 6 stray cats and 4 wildcats coming from the same site (Site 2 – Friuli Venezia Giulia region) where the first Italian *Cytauxzoon* report was identified in 2012 (11) and recognized endemic around ten years later (14). Trieste province is close to Slovenia and is considered an ecological corridor for wildlife movements (42). In addition, the province is characterized by a wide peri-urban area overlapping with sylvatic environment inhabited by wildcats, suggesting possible role of these wild felids as a reservoir. Moreover, Eurasian lynx is present in the region (43, 44) and can be involved in the parasite circulation, as already reported (45).

Indeed, the stray cats in Site 2, including mostly street colony cats, were statistically more infected than owned cats and this supports the hypothesis that animals with an exclusively outdoor lifestyle, virtually living in sympatry with sylvatic species and without regular controls and treatments are more exposed to the risk of infection. No captive exotic felid presented *Cytauxzoon* spp. infection. Considering that all tested animals lived in zoological parks of Site 1 (Veneto region) this could be related to the absence cytauxzoonosis in domestic or wildcat in the considered area. On the other hand, exotic felids are a susceptible host for other *Cytauxzoon* species such as *C. felis* and *C. manul* (46).

In North-eastern Italy, *Cytauxzoon* spp. was firstly reported in domestic cat in 2012 (11) and in wildcats in 2016 (41); nevertheless, species identification was not achieved until recently when a new molecular approach targeting more variable genes (i.e., cytochrome B and cytochrome C oxidase subunit I - COI) allowed phylogenetic analyses (10). In this survey all sequences had a percentage identity of 99-100% with reference *C. europeus* sequences, the most frequent species isolated in felids in Europe (10).

The obtained *Cytauxzoon* phylogenetic analysis showed a scenario that partially contrasts with what was observed in Germany, where most of the sequences were identical, with few exceptions Unfortunately, the limited sequence availability, and the lack of proper knowledge about the epidemiology of this parasite, prevent any definitive conclusion and only speculative hypothesis may be advocated, therefore, more extensive, and systematic studies should be performed to formally evaluate the infection prevalence, and to investigate on the spreading patterns and the involved countries.

The wildcat presence in Friuli Venezia Giulia region is known, even thanks to the potential movement of these animals across the Alps from near Slovenia (42). Recently European wildcat distribution has been updated in Italy (47) and collected data shows that the wildcat is expanding its territory towards the northern part of the Veneto region and the southern areas of the Trentino Alto Adige region (48, 49).

A significantly higher prevalence ($p<0.05$) of *H. silvestris* infection in stray cats and animals living in Site 2 was observed, suggesting that a free-ranging habit increases the exposure to vectors. *H. silvestris* was isolated also in domestic cats of Site 1, most of them coming from Verona province, followed by Vicenza, Treviso and Belluno provinces. These positives are particularly interesting because seemed to follow the recent wildcats' movements in the Italian territory. Even if the infected cats of Site 1 were equally divided between stray and owned animals, all of them had an outdoor lifestyle. The major risk of exposure is thus related to living outdoor and coming in contact with ticks, although it must be considered that other ways of transmission could be possible. In *H. canis* and in *H. felis* life cycle the vertical transmission from mother to offspring during pregnancy was proven (26, 50) and the predation of infected prey is demonstrated to be a way of transmission in *H. americanum* life cycle (26). In felids no information is available and it can be only assumed that vertical and/or horizontal transmission through the ingestion of infected prey can be possible. These considerations justify *H. felis* results. Firstly, *H. felis* is quite equally distributed in all investigated territories and affects both domestic and wild felids. In particular, *H. felis* was found more frequently in owned cats and animals younger than 1 year and older than 3 years with prevalence values statistically significant. If outdoor habit was a significant risk factor for the domestic cat in *C. europeus* and *H. silvestris* infection, for *H. felis* seems to be not relevant; indeed, positive cats were equally divided between animals living in-home and animals with outdoor access and some were infected in areas where wild felids are not yet reported (e.g. southern areas of Site 1), suggesting a domestic cycle of *H. felis*. In addition, younger animals could be more probably infected through the vertical route (50) and older ones because they had more time to be exposed to the infection through the ingestion of infected arthropods and/or infected prey. The same considerations could justify the isolation of *H. felis* from wildcats; indeed, all the animals were adults and came from different provinces of Site 2 (i.e., Trieste, Udine, Pordenone). Among captive wild felids, two tigers with hepatozoonosis came from the same zoological park located in Venezia province in Site 1, where both *H. felis* and *H. silvestris* were isolated in domestic cats. To our knowledge, this is the first description of *H. felis* and *H. silvestris* in captive tigers in Italy, since three tigers from a zoological park located in Southern Italy were found positive for *H. canis* (51).

The *H. felis* sequence analysis revealed 2 distinct clusters, whose features support the above-mentioned scenario. One includes sequences mainly from domestic cats, with only 3 exceptions (i.e. the tiger and 2 wildcats, one from Italy and one from Spain), while the other comprises strains from wildcats only, originating from Italy and Eastern Europe, with the only exception of one strain detected in the present study from a wildcat. Therefore, despite the separation between the domestic and wild cycles, a certain strain exchange, in both directions, likely occurs. Finally, the relationship between sequences obtained from captive animals and

domestic ones supports the involvement of a domestic cycle, although the precise contact path remains obscure. Similar evidence emerged from *H. silvestris*, where a close relationship between Italian strains obtained from *Panthera tigris* and a domestic cat occurred. Moreover, also, in this case, two clusters were observed, one including sequences mainly, but not only, from wildcats, linked to strains collected from Eastern Europe countries, and another group comprising strains of domestic cats only (being the tiger one the only exception). In the latter, the clustering with Turkish strains can hardly be explained supporting the speculative hypothesis about the role of humans and their pets' travels in parasite dispersal.

The observation of merozoites in red blood cells and *Hepatozoon* gamonts in white blood cells in stained blood smears of felines is strongly suggestive of these protozoan infections. However, since cytauxzoonosis and hepatozoonosis usually present a low burden of parasitaemia in feline hosts (8, 14, 26), the blood smear is not a sensitive method and molecular procedures can be considered the method of choice for the diagnosis. Several studies support the higher sensitivity of molecular procedures compared to stained blood smears in hepatozoonosis diagnosis. In 2006, a study reported that 32% of cats resulted positive for *Hepatozoon* spp. by PCR, while only 0.7% showed gamonts in blood smears (52). Similarly, Pereira et al. (53) described that 12.5% of cats in Cape Verde were PCR-positive for *H. felis* with no gamonts observed in blood smears. Conventional PCR is currently the most common method applied for the single detection of *Hepatozoon* spp. and *Cytauxzoon* spp.; nevertheless, it has some limits (e.g., time- and cost-consuming) which can be overcome by alternative solutions, such as a real-time PCR protocol.

The real-time PCR procedure adopted in this survey was developed to guarantee a high-sensitivity protocol able to simultaneously detect and differentiate both *Cytauxzoon* sp. and *Hepatozoon* spp. DNA through melting curve analysis in different matrixes and quickly screen a consistent number of samples. Conventional PCR was successively adopted to further characterize the involved species and strains in positive samples by Sanger sequencing. In conclusion, this study, through a new, fast and sensitive real-time PCR, updated the epidemiological data on cytauxzoonosis and hepatozoonosis in feline hosts achieving new information regarding involved species and strains, their genetic correlation with European isolates, susceptible feline hosts and their distribution in North-eastern Italy.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Conceptualization, M.G., G.S. and A.F.d.R; methodology, M.G., G.D., C.T., G.F. and K.H.; validation, G.D. and C.T.; investigation, M.G., G.S., E.M. and P.B.; software, GF; resources, G.S; data curation, M.G.; writing—original draft preparation, M.G.; writing—review and editing, G.S., A.F.d.R. and D.M.; supervision, A.F.d.R. and G.S.; project administration, G.S. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

The authors declare that data are available upon request to the corresponding author, by email.

Informed Consent Statement:

Informed consent for participating to the study was obtained from all the involved owners or veterinary health authorities for colony cats.

Institutional Review Board Statement:

Ethical review and approval were waived for this study, due to the involved animals being submitted to routine veterinary procedures not depending on this research project.

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Tables

Table 1. Primers used for *Hepatozoon* spp. and *Cytauxzoon* spp. molecular detection.

Gene	Primer	Sequence	Amplicon size (bp)	Reference
18S-rRNA	PIROPLASMID-F	CCAGCAGCCGCGGTAATTC	373	(35)
	PIROPLASMID-R	CTTTCGCAGTAGTTYGTCTTTAACAAATCT		
cytochrome B	Cytaux_cytb_F1	CTTAACCCAACTCACGTACC	1434	(36)
	Cytaux_cytb_R3	GGTTAATCTTTCCTATTCCTTACG		
	Cytaux_cytb_Finn	ACCTACTAAACCTTATTCAAGCRTT	1333	(10)
	Cytaux_cytb_Rinn	AGACTCTTAGATGYAAACTTCCC		

Table 2. Description of individual data of the domestic cat population distributed among the three investigated sites.

		Site 1 n (%)	Site 2 n (%)	Site 3 n (%)	Total n (%)
Sex	M	74 (50.7)	14 (35.0)	12 (60.0)	100 (48.5)
	F	70 (47.9)	26 (65.0)	8 (40.0)	104 (50.5)
	NR[1]	2 (1.4)	0 (0.0)	0 (0.0)	2 (1.0)
Age classes (months)	< 12	51 (34.9)	9 (22.5)	5 (25.0)	65 (31.6)
	12-36	41 (28.1)	17 (42.5)	8 (40.0)	66 (3 2.0)
	> 36	43 (29.5)	12 (30)	7 (35.0)	62 (30.1)
	NR[1]	11 (7.5)	2 (5.0)	0 (0.0)	13 (6.3)
Management	Owned	91 (62.3)	20 (50.0)	20 (100.0)	131 (63.6)
	Stray	55 (37.7)	20 (50.0)	0 (0.0)	75 (36.4)
Lifestyle	Indoor	42 (28.8)	18 (45.0)	7 (35.0)	67 (32.5)
	Outdoor	104 (71.2)	22 (55.0)	13 (65.0)	139 (67.5)
Total		146 (70.9)	40 (19.4)	20 (9.7)	206 (100.0)

[1] Not reported.

Table 3. Distribution of positivity according to felid species, melting temperatures and sequencing.

Felids	n/tot (%)	T_m (°C)	Real-time PCR	n/tot	Sequencing	% Identity
Domestic cats	31/206 (15)	78/78.5	*Hepatozoon* spp.	11/31	*H. felis*	99.7-100%
				20/31	*H. silvestris*	97-100%
	6/206 (2.9)	81	*Cytauxzoon* spp.	6/6	*C. europeus*	100%
Wildcats	8/19 (42.1)	78/78.5	*Hepatozoon* spp.	6/8	*H. felis*	97.3-99.7%
				2/8	*H. silvestris*	99.7%
	4/19 (21)	81	*Cytauxzoon* spp.	4/4	*C. europeus*	98.8-100%
Exotic felids	2/12 (16.7)	78/78.5	*Hepatozoon* spp.	1/2	*H. felis*	99.7%
				1/2	*H. silvestris*	99.7%

Table 4. Distribution of positivity according to individual factors in the cat population.

	Variables	Tested	*C. europaeus* n (%)		*H. felis* n (%)		*H. silvestris* n (%)	
Sex	M	100	1 (1.0)		5 (5.0)		10 (10.0)	
	F	104	5 (4.8)		7(6.7)		9 (8.6)	
	NR[1]	2	0 (0.0)		0 (0.0)		0 (0.0)	
Age classes (months)	< 12	65	0 (0.0)		5 (7.7)		5 (7.7)	
	12-36	66	2 (3.0)		0 (0.0)	*	8 (12.1)	
	> 36	62	3 (4.8)		6 (9.7)		5 (8.1)	
	NR[1]	13	1 (7.7)		1 (7.7)		1 (7.7)	
Region	Site 1	146	0 (0.0)		7(4.8)		10 (6.8)	
	Site 2	40	6 (15.0)	*	2 (5.0)		9 (22.5)	*
	Site 3	20	0 (0.0)		3 (15.0)		0 (0.0)	
Management	Owned	131	0 (0.0)	*	11 (8.4)	*	6 (4.6)	*
	Stray	75	6 (8.0)		1 (1.3)		13 (17.3)	
Lifestyle	Indoor	67	0 (0.0)		4 (6.0)		3 (4.5)	
	Outdoor	139	6 (4.3)		8 (5.8)		16 (11.5)	
Total		206	6 (2.9)		12 (5.8)		19 (9.2)	

[1] Not reported.

*significant differences ($p < 0.05$) based on the Pearson Chi-Square test or the Fisher exact test

Figures caption

Figure 1. Specific melting temperature of *Cytauxzoon* spp. (T_m=81 °C; blue line) and *Hepatozoon* spp. (T_m=78-78.5 °C, red line).

Figure 2. *Cytauxzoon europeus*, *Hepatozoon felis* and *Hepatozoon silvestris* distribution among different felid hosts in North-eastern Italy represented by regions and provinces (Green - Veneto region - Site 1: BL, Belluno; PD, Padova; RO, Rovigo; TV, Treviso; VE, Venezia; VI, Vicenza; VR, Verona; Purple - Friuli Venezia Giulia region - Site2: GO, Gorizia; PN, Pordenone; TS, Trieste; UD, Udine; Orange - Trentino Alto Adige region - Site 3: BZ, Bolzano; TN, Trento).

Figure 3. Neighbour-joining phylogenetic tree reconstructed based on a region of cytochrome B gene (870 bp) of *C. europaeus* using the HKY+G substitution model. The bootstrap support is reported nearby the corresponding node. Isolates from this study are identified with a red dot,

Cytauxzoon species, country and region of provenance and host species. For graphical reasons, the *C. felis* outgroup is not shown. Collection country and host were annotated in the sequence name when available.

Figure 4. Neighbour joining phylogenetic tree reconstructed based on a region of the 18S-rRNA gene (350 bp) of *Hepatozoon* spp. using the T92+G substitution model. The bootstrap support is reported nearby the corresponding node. Isolates of *H. felis* and *H. silvestris* from this study are identified with a red and a blue dot, respectively, *Hepatozoon* species, country and region of provenance and host species. Collection country and host were annotated in the sequence name when available.

7. Scientific communication

Scientific communication 1

Proceeding XXXI National Congress of Italian Society of Parasitology– SoIPa & 2021 European Society of Dirofilariosis and Angiostrongylosis - ESDA Event, on-line, 16-19 June, 2021: 84.
Oral presentation

Cytauxzoon sp. and *Hepatozoon* spp. in cats in North-eastern Italy: preliminary results.

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Keywords: *Cytauxzoon*, *Hepatozoon*, Cat, North-eastern Italy

INTRODUCTION. In Italy, data on presence and distribution of tick-borne protozoa, such as *Cytauxzoon* sp. and *Hepatozoon* spp., are scarce and limited to single areas, e.g. in Trieste province in North-eastern (Carli et al., 2012 Vet Parasitol. 183: 343-52) and Southern regions (Giannelli et al., 2017 Ticks Tick Borne Dis. 8: 721-24), respectively. In the present study, we investigated the occurrence these protozoa in domestic cats from North- Eastern (NE) Italy.

MATERIALS AND METHODS. This study was carried out in Veneto (V), Friuli-Venezia Giulia (FVG) and Trentino Alto-Adige (TAA) regions. K3EDTA blood samples were collected from cats of all age-classes, exposed to at least one season at risk for VBDs, without clinical signs and any treatment against ectoparasites. Blood smears were observed for haemoparasites according to existing key (Hodžić et al., 2017 Parasitology 144(5): 650-61; Baneth et al., 2013 Parasit Vectors. 6: 102). A conventional PCR was performed to detect *Hepatozoon* and *Cytauxzoon* DNA targeting 18S-rRNA gene (Tabar et al., 2008 Vet Parasitol. 151: 338-36). Nucleotide sequences were compared in GenBank® dataset. A preliminary evaluation of potential risk factors associated with haemoprotozoan infection in relation to epidemiological data (provenance, owned/free-ranging cats, in/outdoor lifestyle) was done by Chi-square test (SPSS for Windows, version 27.0).

RESULTS AND CONCLUSIONS. A total of 158 cats (103 owned, 55 free-ranging) was recruited. *Cytauxzoon* and *Hepatozoon* DNA was detected in 6 (3.8%) and 26 (16.5%) cats, respectively. No *Hepatozoon* gamonts were detected in blood smears, while all *Cytauxzoon* PCR-positive

samples evidenced parasitaemia. No co-infections were detected. Two species of Hepatozoon were found: *Hepatozoon felis* (n=10) and *Hepatozoon silvestris* (n=16). No significant differences were showed between *H. felis* prevalence and epidemiological data, whereas *H. silvestris* prevalence was significantly ($p<0.05$) higher in FVG and in free-ranging cats. Cytauxzoon sp. was detected only in free-ranging cats from FVG, with prevalence value (6/39, 15.4%) close to that previously reported in the same area (Carli et al., 2012 Vet Parasitol. 183: 343-52). In conclusion, this study indicates that two species of *Hepatozoon* (i.e. *H. felis, H. silvestris*) can infect domestic cats in NE Italy and *Cytauxzoon* sp. is still present in FVG region.

Scientific communication 2

Proceeding XXXII National Congress of Italian Society of Parasitology– SoIPa, Napoli, Italy, 27-30 June, 2022: 150.
Poster

The first clinical case of hepatozoonosis in a domestic cat in Italy.

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Keywords: Cat, *Hepatozoon silvestris*, Italy

INTRODUCTION: *Hepatozoon* spp. is a vector-borne protozoa affecting several animal species all over the world. hepatozoonosis in felids is almost unknown, but recently three species (i.e. *Hepatozoon felis*, *Hepatozoon canis* and *Hepatozoon silvestris*) were molecularly isolated from european domestic and wild felids (Giannelli et al., 2017. Ticks Tick borne dis, 8:721–24; Hodžić et al., 2017. Parasitology, 144:650-61). Infected felids are usually asymptomatic, and some clinical cases have been newly reported in domestic cats from Central europe (Kegler et al., 2018. Parasit vectors, 11: 428; Basso et al., 2019. Parasitol Int, 72:101945). We describe the first clinical case in Italy of hepatozoonosis in a domestic cat with a peculiar clinical picture.

MATERIALS AND METHODS**:** An 11-years old European short-hair cat, living in a hilly area of the Piedmont region, was hospitalized for a severe intestinal intussusception caused by a sessile endoluminal nodule in the jejunum. blood samples were collected for haematology and clinical biochemistry; the intestinal nodule was surgically removed and histologically evaluated. In addition, molecular investigations targeting *Hepatozoon* SSu-rdna were performed on surgical samples. haematology was normal and the biochemical profile showed increased creatine phosphokinase (CPk: 2371 u/l; reference range: 52-542 u/l). rare *Hepatozoon* gamonts were observed in granulocytes in the blood smear, then molecularly confirmed. histological sections of the intestinal nodule revealed a severe inflammatory reaction characterized by chronic ulcerative enteritis with a polypoid proliferation and severe lymphangiectasia. many inclusions similar to protozoan replicative forms were observed in enterocytes near the lumen with a high burden of infection in all histological sections. Molecular investigations in tissue samples

confirmed *Hepatozoon silvestris* infection. after surgery, the patient was treated with doxycycline at 5 mg/kg/q24h for 30 days. The cat progressively improved and was fully recovered after two weeks with normalization of CPk.

RESULTS AND CONCLUSIONS**:** This is the first case of hepatozoonosis in a domestic cat in Italy. The unique manifestation of the infection makes this cat particularly interesting. Clinical signs are usually related to the tropism of *H. silvestris* for skeletal muscles and myocardium. In this case, the intestinal nodule was probably due to the inflammatory local reaction of the host around the site of protozoa penetration; the increased CPk might suggest subclinical myositis. excision of the intestinal nodule and resolution of the intussusception was life-saving in this cat. doxycycline treatment might have contributed to clearing the *Hepatozoon* infection.

Scientific communication 3

Proceeding 15th International Congress of Parasitology - ICOPA 15, Copenhagen, Denmark, 21-26 August,2022.
Poster

First data on *Cytauxzoon* and *Hepatozoon* in wildcats (*Felis silvestris silvestris*) in North-eastern Italy

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INTRODUCTION. The vector-borne protozoa *Cytauxzoon* spp. and *Hepatozoon* spp. affect wild felids worldwide. In Europe, both protozoa are recently reported in European wildcats (*Felis silvestris silvestris*) but data on epidemiology, life-cycle and pathogenicity are still fragmentary. In this study, both protozoa were investigated in wildcats in North-eastern Italy and potential target organs were evaluated.

METHODS. Wildcats found dead in road accidents were collected. DNA was isolated from blood-clot, lung, liver, lymph-nodes, heart and spleen. A conventional PCR (18S-rRNA) was performed to detect both protozoa. Then, a nested PCR (cytochrome B gene) was run to determine *Cytauxzoon* species and to consider potential coinfection. Amplicons were sequenced and compared to those deposited in GenBank ®. Fisher exact test (R software, version 4.1.2) was performed to evaluate potential correlations between protozoan infections and positive tissue in order to identify target organ/s.

RESULTS. Among 19 wildcats, 4 (21.05%) animals were infected by *Cytauxzoon europaeus* and 8 (42.11%) by *Hepatozoon* spp. (i.e., *Hepatozoon felis*, n=6; *Hepatozoon silvestris,* n=2). Only 1

co-infection by *C. europaeus* and *H. silvestris* was detected. Liver and spleen were target tissue for *H. silvestris* and *C. europaeus,* respectively.

CONCLUSION. In North-eastern Italy wildcats are infected by both protozoa with high prevalence rates, suggesting their potential role as reservoir. Since in the North-eastern Italy domestic and wild cats share the same habitat, the isolation of *C. europaeus, H. felis* and *H. silvestris* in wildcats highlights the potential health risk for domestic ones. The identification of target organs simplifies and accelerate *Cytauxzoon* and *Hepatozoon* diagnosis processes.

Scientific communication 4

Proceeding V National Congress of Italian Society of Wildlife Ecopatology – SIEF, Udine, Italy, 14-17 September2022: 9.
Oral presentation

Cytauxzoon spp. and *Hepatozoon* spp. in questing ticks, wildcats and domestic cats in North-eastern Italy.

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Introduction. *Cytauxzoon* spp. and *Hepatozoon* spp. are tick-borne pathogens infecting a wide range of mammals worldwide. Data on epidemiology, life-cycle and transmission between wild and domestic felids in Europe are still scant. To date, no arthropod vectors were found positive for *Cytauxzoon* spp., whereas feline *Hepatozoon* DNA was already reported in engorged Ixodes ticks, but their competence in transmission has not yet been proven.
In this study, *Cytauxzoon* and *Hepatozoon* were investigated in ticks, European wildcats (*Felis silvestris silvestris*) and domestic cats (*Felis silvestris catus*) from North-eastern Italy, known to be an endemic region for both protozoa.

Material and Methods. Nineteen European wildcats found dead from 2013, due to road accidents, in Trieste, Udine and Pordenone provinces (Friuli-Venezia Giulia region, North-eastern Italy) were included in the study and 40 domestic cats from the same provinces were sampled from 2019 to 2021 thanks to the aid of local veterinarian practitioners. DNA was isolated from wildcats blood or clot, lung, liver, lymph node, heart and spleen and from domestic cats K3EDTA blood samples. A conventional PCR (18S-rRNA) was performed to detect both protozoa. Then, a nested PCR (Cytochrome B gene) was run to determine Cytauxzoon species. Ticks were collected in public gardens and wooded areas in Trieste province (where sampled domestic cats were located). Sampling took place from April to September 2021 by dragging and flagging. Ticks were morphologically identified then stored at -20 until DNA isolation. Nymphs were grouped according to species and sampling date/site in pools (up to 10 individuals per pool), whereas adult ticks were examined individually.

DNA from ticks was isolated and submitted to conventional PCR (16S- and 12S-rRNA) to confirm morphological identification. Then, the same PCR protocol for 18S-rRNA gene was performed for the protozoa detection. Amplicons were sequenced and sequences were compared to those in GenBank® dataset. The infection rate for pools was obtained using generalized linear modelling to calculate maximum-likelihood estimates of prevalence with Epitool (https://epitools.ausvet.com.au/ppvariablepoolsize).

Results and Discussion. Overall, *Cytauxzoon europaeus* was found in 4/19 (21.1%) wildcats spleens, whereas all other tested tissues and organs resulted negative. Besides, 6/40 (15%) blood samples of domestic cats were found positive to the same species.

Hepatozoon DNA was isolated in at least one organ of 8/19 (42.1%) wildcats: in particular, 2/8 hearts were found positive for *Hepatozoon silvestris* and 6/8 wildcats were positive for *Hepatozoon felis*, respectively in blood/clot (n=2), lung (n=1), liver (n=1), lymph-node (n=1) and heart (n=1). Eleven/40 (27.5%) domestic cats were infected by *Hepatozoon* spp. (i.e. *Hepatozoon felis*, n=2; *Hepatozoon silvestris*, n=9). A total of 582 questing ticks were collected and identified as follows: 547 *Ixodes ricinus* (42 males, 25 females, 480 nymphs) and 35 *Haemaphysalis punctata* (1 male, 4 females, 30 nymphs). *Hepatozoon felis* was sequenced in 6/54 *I. ricinus* nymph pools, *H. silvestris* in 1, Cytauxzoon spp. in 5, corresponding to an estimated pooled prevalence of 1.3%, 0.2%, and 0.9%, respectively. Besides, *H. felis* was detected in 2 males, while *H. silvestris* in 4 males and 1 female. Adults and nymph pools of H. puntacta were found all negative to both protozoa.

The results show that in Friuli-Venezia Giulia region, ticks, wild and domestic felids are infected by the same tick-borne parasites targeted by the present study (i.e., *Cytauxzoon* spp. and *Hepatozoon* spp.) suggesting a possible transmissibility, although the roles of both host species need to be clarified with further studies.

The detection of these protozoa in *I. ricinus* supports the hypothesis that these parasites are maintained during the moult from larvae to nymphs and from nymphs to adult, suggesting their potential role in transmission to mammals and their involvement in maintaining protozoa in the sylvatic cycle. On the contrary, the tick species *H. puntacta* doesn't seem to play any role in these pathogens life cycle.

Scientific communication 5

Abstract Book 10th Tick and Tick-Borne Pathogen Conference - TTP10. Murighiol, Danube Delta, Romania, 29 August-2 September, 2022: 23.
Oral communication

Cytauxzoon spp. and *Hepatozoon* spp. in questing ticks in North-eastern Italy

Grillini M[1], Frangipane di Regalbono A.[1], Modrý D.[2,3,4], Hrazdilová K.[5,6], Tessarin C.[1], Dotto G.[1], Maurizio A.[1], Cassini R.[1], Simonato G[1].

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Cytauxzoon spp. and *Hepatozoon* spp. are tick-borne pathogens infecting a wide range of felids and canids all over the world. Information about their transmission to wild and domestic felids in Europe is still scant. To date, no arthropod vectors were found positive for *Cytauxzoon* spp., whereas *Hepatozoon* DNA was already reported in engorged ticks, but their competence in transmission was not yet proved. A survey on *Cytauxzoon* and *Hepatozoon* detection in ticks was conducted in North-eastern Italy in areas known to be endemic for *Cytauxzoon* and *Hepatozoon* in domestic and wild cats.

Ticks were collected from April to September 2021 by dragging and flagging, then morphologically identified. DNA from ticks was isolated and submitted to conventional PCR (16S- and 12S-rRNA) to confirm morphological identification. Then, a PCR targeting piroplasms 18S-rRNA was performed (Tabar et al. 2008). Amplicons were sequenced and sequences were compared to those in GenBank® dataset. A total of 582 questing ticks were collected and identified as follows: 547 *Ixodes ricinus* (42 adult males, 25 adult females, 480 nymphs) and 35 *Haemaphysalis punctata* (1 adult male, 4 adult females, 30 nymphs). Nymphs were grouped according to species and sampling date/site in pools (up to 10 individuals per pool), whereas adult ticks were examined individually. The infection rate for pools was obtained using generalised linear modelling to calculate maximum-likelihood estimates of prevalence. Among 54 *I. ricinus* nymph pools, *H. felis* was sequenced in 6, *H. silvestris* in 1, *Cytauxzoon* spp. in 5,

and *Babesia venatorum* in 3, corresponding to an estimated pooled prevalence of 1.3%, 0.2%, 0.9%, and 0.6%, respectively. Besides, *H. felis* was detected in 2 males, *H. silvestris* in 4 males and 1 female, and *B. venatorum* in 1 adult male. Adults and nymph pools of *H. punctata* were found all negative to piroplasms. This study describes the molecular detection of *Cytauxzoon* and *Hepatozoon* feline species in questing ticks in an endemic area of North-eastern Italy. The obtained results suggest a potential role of *I. ricinus* in protozoa transmission, since the detection of *Cytauxzoon* and *Hepatozoon* in questing ticks supports the hypothesis that these parasites are maintained during the moult from larvae to nymphs and from nymphs to adult. However, further studies are needed to clarify the vectorial competence of *I. ricinus*. Worthy of note, the *B. venatorum* isolation in the study area for its zoonotic potential and consequently the risk exposure for humans.

Scientific communication 6

Proceeding XXXII National Congress of Italian Society of Parasitology– SoIPa. Napoli, Italy, 27-30 June, 2022: 319.
Oral communication

A new qPCR approach for the simultaneous detection of *Cytauxzoon* spp. *Hepatozoon* spp. in felids

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Keywords: *Cytauxzoon*, *Hepatozoon*, qPCR

INTRODUCTION: *Cytauxzoon* spp. and *Hepatozoon* spp. are protozoa responsible of cytauxzoonosis and hepatozoonosis in a wide range of mammals worldwide. Nevertheless, they are still little studied in felids. molecular assays reported in literature (usually conventional PCR protocols and among them nested-PCrs) are often time- and cost-consuming with different sensitivity/specificity. Real-time quantitative polymerase chain reactions (qPCRs) to detect some piroplasms' species such as *Theileria anulata* in cattle and buffalo (Ros-Garcia et al., 2012. Parasit vectors, 5:171; Kundave et al., 2014. Trop biomed, 31:728-35), *Theileria equii* and *Babesia caballi* in horses (Lobanov et al., 2018. Parasit vectors, 11:125) are reported. Since qPCr protocols targeting simultaneously *Hepatozoon* and *Cytauxzoon* have never been set up, the aim of this study was to develop a new qPCR assay to quickly screen a large number of samples.

MATERIALS AND METHODS: Primers designed by Tabar et al., 2008 (Vet Parasitol, 151: 332-6) were used to amplify a 373 bp region of 18S-rrna gene of the order Piroplasmida by Sybr green qPCR. Standard curves and limit of detection of the assay were determined by using 5 (i.e. 1, 10, 10^2, 10^3, 10^4 copies/µl) fold dilution series of DNA of *Babesia microti* ATCC isolate, and the specificity tested on a panel of different species of protozoa (ATCC isolates of *B. microti* and *Toxoplasma gondii*, sequenced field samples of *Cytauxzoon europaeus*, *Hepatozoon felis*, *Hepatozoon silvestris*, *Babesia venatorum*, *Babesia caballi*, *Babesia bigemina*, *Leishmania infantum*).

The assay was tested on experimental samples, i.e. whole blood from 206 owned/stray cats and 12 captive exotic felids (i.e. tiger, lion, leopard, caracal), and organs and blood clots of 19 wild cats. Each assay was performed in duplicate. results were achieved through the melting curve temperature (Tm) analysis.

RESULTS AND CONCLUSIONS: This assay showed high specificity for piroplasms and high sensitivity (limit < than 10 copies/µl). based on Tm is possible to quickly distinguish *Cytauxzoon* spp. infection from *Hepatozoon* spp. as the results of species-specific temperature peak (i.e. 81°C *C. europaeus*, 78°C *H. felis*, 78.5°C *H. silvestris*).In addition, the qPCR was able to detect and differentiate some other piroplasms such as *T. gondii* (75°C), *B. venatorum* (79°C), *B. caballi* (80°C), *B. bigemina* (80.5°C), and *B. microti* (81°C). The limit of the study is represented by the same Tm of *C. europaeus* and *B. microti*. This case unavoidably requires a further step of sequencing for the distinction.
Overall, 12 cats were positive to *H. felis*, 19 to *H. silvestris* and 6 to *C. europaeus*, 1 tiger to *H. felis* and 1 to *H. silvestris*, 6 wild cats to *H. felis*, 2 to *H. silvestris* and 3 to *C. europaeus*. all confirmed by conventional PCR and subsequent sequencing. This procedure could represent a useful method to confirm *Cytauxzoon* spp. and *Hepatozoon* spp. infection in felids, to evaluate other potential piroplasms infection, and to quickly screen a large number of samples.

SECTION 2

Dirofilaria immitis

1. Aims of the research and outputs

Domestic cat, wildcats and captive exotic felids were investigated in order to update the occurrence of the heartworm disease (HW) in North-eastern Italy. Since the diagnosis of HW in felid is complicated due to the particular features of *D. immitis* life-cycle and host immunity response, antigens and antibodies were firstly evaluated, then molecular procedures were performed on positive serological samples.

Wildcats blood-clots were collected during necropsy, whereas cats and captive exotic felids blood samples during routinely clinical examinations.

Several provinces proved to be positive for the circulation of *D. immitis* in domestic cats. Interestingly, anti-*D. immitis* antibodies were revealed in cats living in a mountainous area recently colonized by new mosquito species.

Concerning wild felids, this is the first report on wildcats' heartworm disease in Italy.

The results of these studies were collected in the following paper and scientific communication at national conferences:

- Grillini M., Zanotelli G., Frangipane di Regalbono A., Simonato G. **Serological survey on cat heartworm disease in North-eastern Italy: Preliminary results**. Proceeding 74° Congress of Italian Society of Veterinary Sciences - SISVET, on-line, 23-25 June, 2021: 386. Poster – Scientific communication 7

- Grillini M., Frangipane di Regalbono A., Tessarin C., Beraldo P., Cassini R., Marchiori E., Simonato G. **Evidence of *Dirofilaria immitis* in felids in North-eastern Italy**. Pathogens, 2022, 11(10): 1216. PAPER 6

1. Paper 6

Published in *Pathogens* 2022 11(10): 1216. doi: 10.3390/pathogens11101216.

Evidence of *Dirofilaria immitis* in felids in North-eastern Italy.

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pathogens

MDPI

Article

Evidence of *Dirofilaria immitis* in Felids in North-Eastern Italy

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Abstract: *Dirofilaria immitis* is a mosquito-borne nematode, causing heartworm (HW) disease in wild and domestic canids. HW can also affect felids with different clinical patterns from asymptomatic pictures to sudden death, making the monitoring and diagnosis complicated. Canine HW is endemic in North-eastern Italy; however, very little information has been recorded for felids. This study aims to provide new information on HW in felids in North-eastern Italy. Two hundred and six domestic cats from Veneto, Friuli-Venezia Giulia, Trentino Alto-Adige regions (North-eastern Italy), nine captive felids from zoological parks from Veneto, and nineteen European wildcats from Friuli Venezia Giulia were recruited. Sera/plasma was analysed for the detection of anti-HW antibodies (Ab) and HW antigens (Ag); positive blood samples were molecularly analysed, targeting the HW DNA (5S-rRNA gene). Twelve out of two hundred and six (5.8%) cats presented with Ab, and three out of two hundred and six (1.5%) presented with Ag, mainly those from the Veneto region, already known as a canine HW-endemic area. Among Ab-positive cats, two were from Belluno, a mountain province previously considered free, suggesting the expansion of HW into the northern areas. No cats were positive for both Ab and Ag. Three out of nineteen (15.8%) wildcats were Ag-positive, constituting the first HW report in Italy. No captive felids were positive. *Dirofilaria immitis* DNA was not amplified in positive samples, suggesting the low sensitivity of PCR on blood. This study provides new data on the occurrence of HW in domestic cats and wildcats in North-eastern Italy.

Keywords: *Dirofilaria immitis*; heartworm; cat; wildcat; felid; North-Eastern Italy

Citation: Grillini, M.; Frangipane di Regalbono, A.; Tessarin, C.; Beraldo, P.; Cassini, R.; Marchiori, E.; Simonato, G. Evidence of *Dirofilaria immitis* in Felids in North-Eastern Italy. *Pathogens* **2022**, *11*, 1216. https://doi.org/10.3390/pathogens11101216

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1. Introduction

Dirofilaria immitis is a nematode endemic present in many parts of the world, from European countries to the northern states of America and in South-east Asia. Additionally, an increasing frequency has been reported in the African regions [1].

In Europe, *D. immitis* is mostly endemic in the southern countries such as Spain and the Canary Islands [2–5], Portugal [6], France [7], and Greece [8,9]; as for central and eastern countries, the nematode has been detected in both dogs and cats from Romania [10–13], Czech Republic, Slovenia, Bulgaria [14], and Austria [15].

In Italy, northern regions, such as, for instance, Veneto, Friuli Venezia Giulia, Emilia Romagna, and Piedmont e Lombardy, are reported as hyperendemic. Indeed, the largest endemic area in Europe is along the Po River Valley [16]. *Dirofilaria immitis* is generally reported as being considerably distributed in the northern and central Italian regions [17].

Nevertheless, in Italy and in other European regions, the nematode is expanding its geographical range, affecting previously free areas [11,16,18].

Recently, some authors described an increase in *D. immitis* in Central and Southern Italy, throughout the Tuscany and Umbria regions [19,20] and in Sardinia and the Sicily

islands [21]. Finally, *D. immitis* has been recently detected in the Calabria and Apulia regions [19,22,23].

The definitive hosts for this heartworm (HW) are dogs (*Canis lupus familiaris)* and other canids such as wolf (*Canis lupus*), fox (*Vulpes vulpes)*, and European jackal (*Canis aureus*). Nevertheless, a large percentage of other species could be infected by this species, such as, for instance, the domestic cat (*Felis silvestris catus)*, ferret (*Mustela putorius furo*), and coypu (*Myocastor coypus*); however, these, unlike canids, do not act as a reservoir for this parasite [1].

Focusing on felids, *D. immitis* infestation is reported with much more complexity than in dogs due to the fact that felines are vulnerable hosts but not the HWs' favourite ones [24]. Indeed, the prepatent period in felines is extended up to 9 months compared to canids [25]. Moreover, adult worms are lower in number, with a shorter lifecycle, sometimes reaching more ectopic locations than the target ones, and microfilaremia is rarely present, with a scant burden [24,26].

Genchi et al. [17] recently reported data focusing on HW disease in domestic cats, collected through a national questionnaire sent to Italian veterinarian practitioners. Between 2017 and 2018, one or two clinical cases of HW disease/year were diagnosed in the provinces of Veneto, Emilia Romagna, Lombardy, Piedmont, Tuscany, and Sardinia, and more than two clinical cases were diagnosed in the Lombardy and Sardinia provinces.

Even if data on the prevalence of HW in dogs are not fully updated in North-eastern Italy, the circulation of the parasite is well known, and the adoption of preventative measures in dogs is widespread during the period of mosquito activity (i.e., from late spring to late autumn). On the other hand, feline HW disease is not fully understood, and vet awareness in endemic areas is still scant and data on HW prevalence rates in felids are still lacking.

Nevertheless, it is important to consider that the HW disease in cats may potentially occur wherever infested dogs and competent vectors are present in the same context [27]. The hypothetical prevalence of *D. immitis* in cats is around 9–18% of that in dogs in the same area [28]. Although North-eastern Italy has experienced the presence of *D. immitis* for a long time [29], information on HW in felids is still lacking. Some invasive competent diurnal mosquitoes were recently introduced [30,31], exposing dogs and cats to a major risk of infestation. The aim of this study is to provide new information on *D. immitis* circulation in different species of felines.

2. Results

2.1. Feline Population

Overall, 234 felids (i.e., 206 domestic cats, 9 captive exotic felids, and 19 European wildcats) were included in the study. Among the cats, 146 (70.9%) were from Veneto (Site 1), 40 (19.4%) were from Friuli Venezia Giulia (Site 2), and 20 (9.7%) were from Trentino Alto-Adige (Site 3); 131 (63.6%) and 75 (36.4%) were owned and stray cats, respectively. Most of the cats (n = 145, 70.4%) were recorded as having an outdoor lifestyle, and the rest (n = 61, 29.6%) had an indoor lifestyle. Recruited subjects are almost equally distributed among the sex, provenance, and age classes. Individual data are detailed in Table 1.

Table 1. Descriptions of individual data of domestic cats.

		Site 1 n (%)	Site 2 n (%)	Site 3 n (%)	Total n (%)
Sex	M	74 (50.7)	14 (35.0)	12 (60.0)	100 (48.5)
	F	70 (47.9)	26 (65.0)	8 (40.0)	104 (50.5)
	NR [1]	2 (1.4)	0	0	2 (1.0)
Age classes (months)	< 12	51 (34.9)	9 (22.5)	5 (25.0)	65 (31.6)
	12–36	41 (28.1)	17 (42.5)	8 (40.0)	66 (32.0)
	>36	43 (29.5)	12 (30.0)	7 (35.0)	62 (30.1)
	NR [1]	11 (7.5)	2 (5.0)	0	13 (6.3)
Management	Owned	91 (62.3)	20 (50.0)	20 (100.0)	131 (63.6)
	Stray	55 (37.7)	20 (50.0)	0	75 (36.4)
Lifestyle	Indoor	42 (28.8)	12 (30.0)	7 (35.0)	61 (29.6)
	Outdoor	104 (71.2)	28 (70.0)	13 (65.0)	145 (70.4)
Cardio-respiratory Signs	Presence	8 (5.5)	1 (2.5)	1 (5.0)	10 (4.9)
	Absence	138 (94.5)	39 (97.5)	19 (95.0)	196 (95.1)
Dirofilaria immitis preventative measures	Presence	27 (18.5)	6 (15.0)	1 (5.0)	34 (16.5)
	Absence	108 (74.0)	33 (82.5)	19 (95.0)	160 (77.7)
	NR [1]	11 (7.5)	1 (2.5)	0	12 (5.8)
Total		146 (70.9)	40 (19.4)	20 (9.7)	206 (100.0)

[1] Not reported.

The exotic felids included three tigers (*Panthera tigris*), two lions (*Panthera leo*), three leopards (*Panthera pardus*), and one caracal *(Caracal caracal*) from zoological parks located in Site 1. Among them, five were male and four were female, and all were aged between 4 and 20 years.

The collected European wildcats (*Felis silvestris silvestris)* were road-killed in Site 2 (i.e., the Trieste (TS), Udine (UD), and Pordenone (PN) provinces). The wild felids included 10 (52.6%) males and 9 (47.4%) females. An estimated age based on teeth evaluation classified the animals as adults if older than one year (17/19, 89.5%) or sub-adults if younger (2/19, 10.5%).

2.2. Laboratory Analysis and Geographical Distribution

Twelve out of two hundred and six (5.8%) domestic cats presented positive for anti-HW Ab, and three out of two hundred and six (1.5%) presented positive for HW Ag. None of them presented positive for both tests. Among the three Ag-positive animals, one was positive only after the heat treatment of the serum. Positive cats (n = 15/206, 7.3%) were mostly asymptomatic. Nevertheless, 3/15 (20.0%) positive cats presented cardio-respiratory signs: one Ab-positive cat showed a light cardiac murmur, one Ag-positive cat a mistral regurgitation, and another Ag-positive one wheezing and shallow breathing.

Eleven out of twelve Ab- and three out of three Ag-positive cats came from Site 1 and only one Ab-positive cat was from the Trieste (TS) province in Site 2.

Three out of nineteen (15.8%) wildcats, including one adult and two sub-adults, revealed slight positivity to antigens, whereas none were positive for Ab. Among them, two came from Udine (UD) and another from Trieste (TS).

No exotic felids tested positive at serological tests.

Serological positive samples were all negative after the molecular analysis.

Dirofilaria immitis is widely distributed in Site 1 and sporadically present in Site 2 (Figure 1).

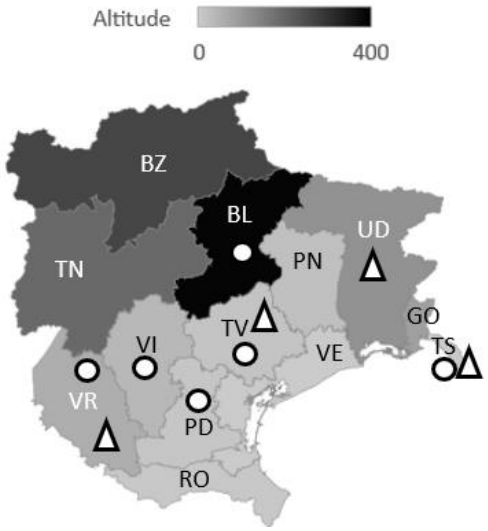

Figure 1. Altitude map showing Italian provinces with felines positive to anti-*D. immitis* antibodies (○) and to *D. immitis* antigens (Δ); Site 1 provinces: BL, Belluno; PD, Padova; RO, Rovigo; TN, Trento; TV, Treviso; VE, Venezia; VI, Vicenza; VR, Verona; Site 2 provinces: GO, Gorizia; PN, Pordenone; TS, Trieste; UD, Udine; Site 3 provinces: BZ, Bolzano; TN, Trento.

The Ab-positive cats from the Belluno (BL) province (Site 1) were asymptomatic and came from a street colony without a history of travelling. No cats were recruited from the Rovigo (RO, Site 1), Gorizia (GO, Site 2), and Bolzano (BZ, Site 3) provinces; thus, no information regarding the presence of *D. immitis* is available for these areas. On the contrary, the Trento (TN, Site 3) province did not record any positivity.

Individual data of Ab- and Ag-positive cats are reported in Table 2.

Table 2. Individual data of domestic cats with anti-HW antibodies and HW antigens.

Factors	**Variables**	**Tested N**	***Dirofilaria immitis***	
			Ab+ n (%)	**Ag+ n (%)**
Provenance	Site 1	146	11 (7.5)	3 (2.1)
	Site 2	40	1 (2.5)	0 (0.0)
	Site 3	20	0 (0.0)	0 (0.0)
Sex	M	100	7 (7.0)	1 (1.0)
	F	104	5 (4.8)	2 (1.9)
	NR [1]	2	0 (0.0)	0 (0.0)
Age classes (months)	<12	65	2 (3.1)	2 (3.1)
	12–36	66	5 (7.6)	1 (1.5)
	>36	62	4 (6.5)	0 (0.0)
	NR[1]	13	1 (7.7)	0 (0.0)
Management	Owned	131	7 (5.3)	2 (1.5)
	Stray	75	5 (6.7)	1 (1.3)
Lifestyle	Indoor	61	2 (3.3)	1 (1.6)
	Outdoor	145	10 (6.9)	2 (1.4)
Cardiorespiratory signs	Presence	10	1 (10.0)	2 (20.0)
	Absence	196	11 (5.6)	1 (0.5)
Dirofilaria immitis preventative measures	Presence	34	3 (8.8)	2 (5.9)
	Absence	160	8 (5.0)	1 (0.6)
	NR[1]	12	1 (8.3)	0 (0.0)
Total		206	12 (5.8)	3 (1.5)

[1] Not reported.

No significant difference in prevalence was observed among the groups, even if stray cats showed a higher rate of positivity for Ab anti-*D. immitis* as well as cats with an outdoor lifestyle and younger cats with an age between 12 and 36 months, whereas positivity rates for Ag seem to proportionally decrease with an increase in age.

3. Discussion

In the last years, several drivers have contributed to modifying the epidemiological distribution of parasites in Europe. For example, climate change is facilitating an increase in vectors' spread and activity [32], and globalization in terms of animal and goods movement across the world is allowing the introduction of arthropod vectors and pathogens in new territories [33,34]. Moreover, the decimation of ecological wildlife niches is responsible for the approaching of wild animals to urban contexts, exposing domestic animals to new pathogens [35], and the pets' movement is facilitating the spread of pathogens in new areas [36]. These factors have expanded the influence of parasites including cardio-pulmonary nematodes from areas already endemic to regions previously described as free [35].

Specifically, environmental factors (e.g., temperature, humidity, vegetation, etc.), the density of competent mosquito populations, the new introduction of invasive competent mosquitoes [37,38], the presence of the main reservoirs of *D. immitis* (i.e., wild and domestic canids), together with the movement of microfilaremic individuals, plays an important role in the increased risk of exposure, even for the feline population.

Canids remain the favourite definitive hosts of *D. immitis* and contribute to the maintenance of the parasite in the domestic and sylvatic cycles. In addition, in some mosquito species (i.e., *Culex* spp. and *Aedes* spp.), the main competent vectors of *D. immitis* are very common in urban areas and feed on both dogs and cats with no preferences [1].

Generally, cats living in endemic and hyperendemic areas for canine HW disease should be considered at risk [39], and the prevalence rates in the feline species are considered to fluctuate from 9 to 18% of that in the canine population in the same area [28]. In this study, the provenance of stray cats from neighbouring areas and the data collected from cats' owners allow us to rule out with a good level of certainty the introduction of domestic cats from endemic regions.

Unfortunately, HW disease is difficult to diagnose in felines due to the fact that it is much more elusive and because infection leads to unpredictable effects in this host. As previously reported, felines are usually asymptomatic or paucisymptomatic, and infection can also lead to sudden death [9,40,41]. Moreover, no single test is able to detect the presence of *D. immitis* in all its stages [1,42]. Indeed, usually, microfilaremia is not frequent and, if present, has a scant burden [24,26] due to the fact that heartworms tend to die before reaching adult stage.

Consequently, more diagnostic methods should be combined to confirm the exposure and/or the infestation, always considering the limits of available serological tests [25,28].

In this study, the use of two different tests was planned in order to detect the presence of anti-*D. immitis* Ab and/or *D. immitis* Ag. No cats tested positive for both tests, and this could be due to the production of Ab anti-*D. immitis* in the first stage of parasitosis when the humoral immune response reacts to larval stages developing in the host's tissues, whereas antigens are present in the final stage only when the nematodes become sexually mature adults. Antibodies are early detectable at 3–4 months post-infection, whereas Ags are present for around 6–8 months post-infection [25]. Our findings (5.8% prevalence for Ab, 1.5% for Ag) confirm that the Ab prevalence rates are usually higher than the Ag values because, in cats, *D. immitis* immature larvae have more difficulty reaching the adult stage, and because circulating immune complexes, often present in cats, act to mask the Ag.

The presence of circulating anti-*D. immitis* Ab in felines is considered a useful indication in the diagnostic process. Indeed, it provides early information of dirofilariosis onset, considering that Abs develop within 4 months of the infestation. This condition may permit the identification of animals potentially infested and can be proceeded with further diagnostic tests to confirm the risk of the subsequent onset of the disease. However, we should be aware that the Ab presence may be simply an indication that cats came in contact with the parasite; alternatively, it may mean that, although infected, they will not necessarily develop the disease. In fact, felines seem to tolerate the infestation well, sometimes with no clinical signs or with signs that occur only transiently [25]. Additionally, the HW disease often has a self-limiting course in felids with a spontaneous resolution due to the natural death of the parasites [1,28]. In general, the specificity of antibody tests can drop significantly due to possible cross-reactivity with other parasites. Regardless, it can be assumed that the tests commercially available to detect *D. immitis* antibodies rarely cross-react with gastro-intestinal parasites [43]. Moreover, no autochthonous cases of *D. repens* infection have been reported in the investigated regions [17].

Dirofilaria immitis DNA could not be molecularly detected in any positive samples, suggesting the low sensitivity of PCR on the blood matrix. Indeed, molecular procedures are frequently used for nematode identification in dog blood samples [44–47], whereas, in cats, they are just marginally applied [48,49].

Most of the Ab-positive cats had an outdoor lifestyle (6.9%), and this condition definitely exposes cats to vectors' action night and day, as well as to wildcats. Nonetheless, a lower percentage (3.3%) of Ab-positive cats were recorded as having an indoor lifestyle. An indoor lifestyle can only partially protect them from vectors' bites since some mosquito species are attracted inside human dwellings [50] and are active both night and day.

In Europe, few reports of feline HW clinical cases have been described in southern Romania [13] and in Austria [15], and only a few serosurveys in Spain [3,4] and in Greece [9] were reported. Seroprevalence data registered in our study agrees with previous studies in other endemic regions of Spain; indeed, Ab and Ag seropositivity rates in Northeastern Italy were 5.8% and 1.5%, respectively, compared to those reported in Madrid (7.30% and 0.20%) [4] and Barcelona (11.47% and 0.26%) [3].

In Italy, the Po Valley is a hyperendemic area for canine HW disease and thus for cardio-pulmonary dirofilariosis in cats [16], as confirmed by the number of Ab- (11/146, 7.5%) and Ag-positive (3/146, 2.0%) samples registered in cats from the Veneto region, followed by cats from Friuli Venezia Giulia, where 1/40 (2.5%) was Ab-positive and 3/19 (15.8%) wildcats were Ag-positive. No cat from the Trentino Alto-Adige region was Ab- or Ag-positive, as has been already described in another study [17], suggesting that cats are not exposed to the risk of infestation, probably due to the climatic conditions that are not yet suitable for the establishment of a *D. immitis* lifecycle.

Feline dirofilariosis was diagnosed in 4.8% of Italian facilities in 2018 [17]. In Northeastern Italy, only a few cases of infested cats were described in the Treviso (TV) province, and no information was reported from the Belluno (BL) province.

In this study, several provinces proved to be positive for the circulation of *D. immitis* in cats. Particularly, two cats showed positivity to anti-*D. immitis* antibodies in Belluno, which represents an area recently colonized by a new mosquito species [30,31,33]. This is worthy of note due to the fact that, to the best of the authors' knowledge, this is the first description in this province that has the highest mean altitude (390 masl) among the other provinces included in the study. This altitude is commonly not suitable for mosquitoes, even if, since 2011, a new species (i.e., *Aedes koreicus*)-competent vector for *D. immitis* with a particular resistance to colder environmental temperatures was described [30]. It may suggest that, hitherto, *Dirofilaria*-free areas are to be considered at potential risk of spread.

Focusing on wildcats, to the best of the authors' knowledge, it is the first time that wildcats have been investigated for heartworm in Italy.

Felines do not properly act as reservoirs, since the parasites rarely reach the sexually mature adult stage that produces microfilariae. Regardless, in this study, the presence of

D. immitis Ag in some felids supports the presence of mature adult worms, in contrast with the necropsy not showing their presence. This result could be due to the very few adult worms (as usual in felines) reaching the target organs investigated at the necropsy, and/or to the deteriorating effects on worms' tissues due to the long freezing storage (more than 1 year).

Exotic felids were negative on both tests. Indeed, these felines come from zoological gardens that are used to adopt prevention programmes based on oral ivermectin administration. This could reinforce our findings and confirm the efficacy of chemoprophylaxis in captive felids which are housed outdoors in endemic HW areas since they were all found to be negative; however, the number of sampled animals was low.

The antiparasitic treatment is crucial in cats as well as in dogs. As documented by Genchi et al. [17], more than half of veterinary practitioners do not recommend HW prophylaxis for cats. This aspect agrees with our data regarding the preventative measures adopted by cat owners. In this study, it is not possible to affirm that cats with preventive measures were protected for their whole life until blood sampling. Only 16.5% of cat owners adopted formulations for endo and/or ectoparasites treatment, not aware that the molecules were also effective against the larval stages of *D. immitis*. Unfortunately, the irregular regimen of treatments or the administration only when necessary for other parasitosis makes the preventative measures against dirofilariosis partially ineffective.

A registered adulticide molecule is not available for cats, and it is considered to be a last-resort medical treatment for those with uncontrolled clinical signs after empirical corticosteroid therapy [25]. To date, data on melarsomine dihydrochloride in cats are scant and its use is not recommended [25]. Moreover, preliminary studies suggest that melarsomine at a regimen of 3.5 mg/kg is toxic for cats [51,52]. Ivermectin in monthly doses seems to reduce the number of adult worms by 65% compared to untreated cats; on the other hand, its use could lead to anaphylactic reactions due to the death of heartworms. Currently, there are no studies showing that adulticide therapy is competent enough to increase the rate of survival of cats infected by adult worms [25].

The adoption of several topical and/or oral formulations available on the market is the only suggested way to protect cats from mosquitoes' bites and guarantee cat health [17]. Since mosquitoes are anthropophilic, this treatment should also be provided for cats which have an indoor habit. Indeed, an indoor lifestyle can partially limit their exposure but cannot avoid the risk of infestation [49,53].

This study proved the circulation of *D. immitis* in domestic cats and, for the first time, in wildcats from several provinces of North-eastern Italy. As a consequence, it has highlighted the importance of taking regular preventative measures for cats living in endemic or hyper-endemic areas, along the need to include HW disease in the differential diagnosis. In clinical practice, the elusive picture and the unpredictable effects of HW infection in cats leads to the need for a combination of more diagnostic methods to confirm the exposure and/or the infestation. Considering the limits of available serological tests, the possibility to employ imaging diagnostics to determine the presence of heartworms is recommended.

4. Materials and Methods

4.1. Blood Collection, Analysis, and DNA Extraction

Blood samples from different felid species from North-eastern Italy were sampled from October 2019 to March 2022. Among felid species, domestic cats, captive exotic felids (i.e., tigers, lions, leopards, caracals) from zoological parks, and wild European wildcats were included. All felids were exposed to at least one season at risk of arthropod vectors' activity.

The investigated areas included the Veneto (Site 1), Friuli Venezia Giulia (Site 2), and Trentino Alto-Adige (Site 3) regions (Figure 2). Mean altitudes of the different provinces are represented in Figure 1.

Blood samples (i.e., entire blood and blood in k3EDTA) were collected from cats and exotic captive felids during routine clinical visits and/or following surgical procedures (not depending on this research study) in veterinary clinics. Individual data upon provenance (Site 1, Site 2, and Site 3), sex, and age classes (<12 months, 12–36 months, >36 months), management (owned, stray cats, captive, wild), lifestyle (indoor, outdoor, mixed), and clinical signs (cardio-respiratory alterations) were recorded.

The included European wildcats were found road-killed in the territory and kept frozen until post-mortem examination. During necropsy, organs were observed to reveal the presence of *D. immitis* pre-adults and adults. Heart clots and/or uncoagulated blood (when present) were sampled for serological and molecular investigations.

Sera were analysed for the detection of antibodies (Ab) anti-*D. immitis* by one-step lateral flow immunoassay Solo Step® FH (HESKA® Corporation, Loveland, Colorado, USA) and antigens (Ag) of *D. immitis* by enzyme immunoassay PetChek® HTWM PF, (IDEXX Laboratories, Westbrook, Maine, USA) according to manufacturer's instructions.

For the Ag test, the domestic cats' sera were tested before and after heat treatment (104°C for 10 min, then centrifuged at 13,000 rpm) to avoid the potential interference of the antigen–antibody complex and to guarantee more accurate results in the antigen test [54]. The serum of 12 domestic cats was insufficient and was not analysed for antigens. For the same reason, the serum of exotic felids and wildcats was tested only at room temperature.

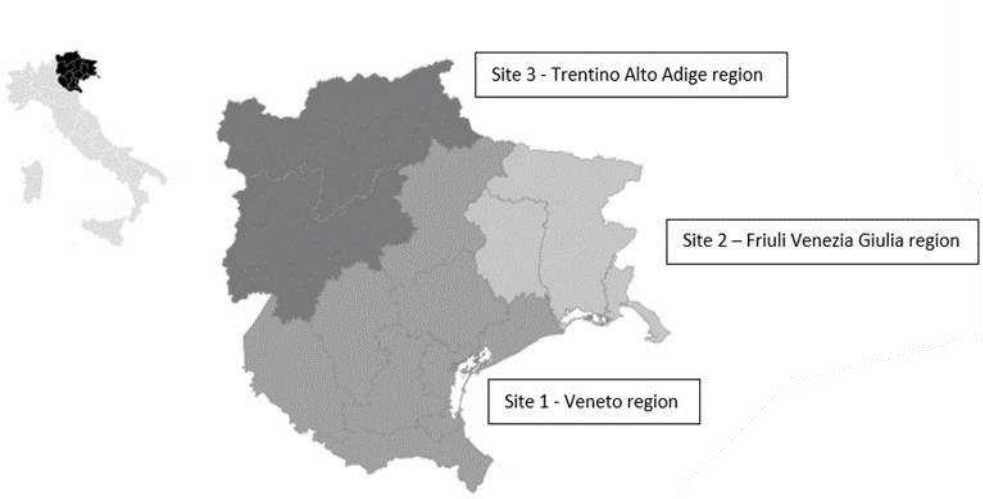

Figure 2. Map depicting investigated regions of North-eastern Italy.

4.2. Molecular Analysis and Sequencing

Samples tested positive for serological investigations (i.e., Ab and/or Ag positive) were subsequently analysed by molecular method to detect the DNA of *D. immitis*.

The DNA extraction kit NucleoSpin®Tissue (Macherey-Nagel, Düren, Germany) was used on 200 µL of whole blood or clot. All procedures were performed according to the manufacturer's instructions.

DNA was extracted from 200 µL of whole blood or blood clots with the NucleoSpin®Tissue kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

PCR amplification of the ribosomal large subunit (5S) was carried out using the specific primers S2 (5′-GTTAAGCAACGTTGGGCCTGG-3′) and S16 (5′-TTGACAGATCGGACGAGATG-3′) [55] according to Favia et al. [56] with slight modifications: initial step at 95 °C for 5 min, followed by 35 cycles of denaturation step at 95 °C for 30 sec, annealing

step at 56.5 °C for 30 sec, extension step at 72 °C for 30 sec, and final extension cycle at 72 °C for 2 min. Positive (i.e., DNA of *D. immitis*) and negative controls were included in each PCR reaction.

4.3. Data Analysis

In order to evaluate differences in infection rates among the subgroups of the investigated domestic cat population, a statistical evaluation was performed by means of the Pearson chi-square test or the Fisher exact test, if appropriate, using SPSS for Windows, version 27.0. The factors taken into consideration were sex (i.e., males, females), age classes (i.e., <12 months, 12–36 months, >36 months), region and province of provenance (i.e., Site 1, Site 2, Site 3), lifestyle (i.e., indoor, outdoor), management (i.e., owned, stray cat), and presence of clinical signs (i.e., cardio-respiratory signs).

Due to the low number of samples, captive and wild felids were not included in this analysis.

Author Contributions: Conceptualization, M.G., G.S. and A.F.d.R.; methodology, M.G. and C.T.; software, R.C.; validation, C.T.; formal analysis, M.G. and R.C.; investigation, M.G., G.S., E.M. and P.B.; resources, G.S.; data curation, M.G.; writing—original draft preparation, M.G.; writing—review and editing, G.S. and A.F.d.R.; supervision, A.F.d.R. and G.S.; project administration, G.S.; funding acquisition, G.S. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Ethical review and approval were waived for this study, due to the involved animals being submitted to routine veterinary procedures not depending on this research project.

Informed Consent Statement: Informed consent for participating to the study was obtained from all the involved owners (or the person who acts in their stead) or veterinary health authorities for colony cats.

Data Availability Statement: The authors declare that data are available upon request to the corresponding author, by email.

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2. Scientific communication

Scientific communication 7

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Poster

Serological survey on cat Heartworm disease in North-eastern Italy: preliminary results

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The prevalence of feline heartworm (HW) disease in an area can be estimated as 10% of the known prevalence of the infected dogs as reported in previous studies [1]. In Italy, data on *Dirofilaria immitis* distribution in cats are limited [2] even in the endemic North-eastern (NE). In cats, diagnosis is complicated by mostly sub-clinical pictures and their natural inborne resistance [3], and combined use of serological tests could be a useful support [1]. This study aims to investigate the presence and distribution of *D. immitis* in cats from NE Italy, evaluating potential risk factors. Preliminary results are herein reported, while statistics will be performed at the end of the study. Serum samples were collected by veterinarians during routine clinical examinations from cats exposed to at least one season at risk for mosquito bites, collecting information on antiparasitic treatment and lifestyle (outdoor/indoor). *Dirofilaria immitis* antibodies (Ab) were detected by Solo Step®, HESKA-USA, and antigens (Ag) by Pet-Check® HTWM antigen, IDEXX Lab.-USA before and after heat treatment to avoid Ag-Ab complex [4]. A total of 151 cats (97 owned, 54 free-ranging) were recruited. *D. immitis* Ab and Ag were detected in 11 (7.3%) and 3 (2%) samples, respectively. No *D. immitis* Ag were detected in Ab positive samples. One/3 Ag-positive samples were detected after heat treatment. Out of 11 Ab- and 3 Ag-positive cats, 9 and 2 had outdoor lifestyle, respectively. Moreover, 10 Ab-positive cats were from Veneto (Province of BL-1 cat, PD-1, TV-1, VI-4, VR-3), and 1 was from Friuli Venezia Giulia (TS). All Ag-positive cats were from Veneto (VR, TV, PD). None of Ab-positive cats presented clinical signs, whereas 2/3 Ag-positive cats showed cardio-respiratory failure.
Most of positive cats had outdoor access exposing them to vectors' activity. Nonetheless, an indoor lifestyle can partially protect them, being some mosquito species attracted inside

human dwellings, and active both night and day. In Veneto, Ab positive results are not surprising except for BL province, where data on canine HW disease are scant and, to our knowledge, no autochthonous cases have been officially reported. Thus, the Ab positive cat suggests the potential exposure to the nematode circulation, that could be also exacerbated by the introduction in the NE areas of new invasive species, competent vector of *D. immitis* [5]. In conclusion, since cats have an inborne resistance to this parasite, there is a risk of underestimating its presence and diffusion. It is therefore essential to become aware of the actual presence and spread of HW in cats from NE Italy, in order to minimize the risk of HW transmission implementing appropriate prophylaxes protocols.

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General conclusion

The research aimed to investigate neglected VBDs (i.e., *Cytauxzoon* spp., *Hepatozoon* spp. and *Dirofilaria immitis*) in felids in North-eastern Italy in order to provide new information on the parasites' circulation, and to update the current epidemiological scenario in North-eastern Italy *(i)* improving the knowledge on the presence and distribution of *Cytauxzoon* spp., *Hepatozoon* spp. and *Dirofilaria immitis* in different felid species; *(ii)* investigating on the possible role of ticks in the transmission of *Hepatozoon* spp. and *Cytauxzoon* spp., and *(iii)* developing diagnostic protocols to provide fast and sensitive screening procedures.

Cytauxzoonosis was restricted around Trieste province in the Friuli Venezia Giulia region. This finding confirms almost ten years later (Carli et al 2012) that Trieste is an endemic site where *Cytauxzoon* commonly occurs in domestic cats with a high prevalence value of around 20%. All cats were infected by *Cytauxzoon europaeus*, the most distributed species in Europe.

Cytauxzoon-positive cats presented mild parasitaemia at the stained blood smear. They were all free-ranging cats with an outdoor lifestyle suggesting that outdoor habit could be the main risk factor allowing cats to come in contact with the potential vector present in the environment. In addition, the reduction of wildlife habitat, along with the confirmed presence in Friuli Venezia Giulia region and the expansion of wildcats distribution in the northern part of Veneto region and in the southern areas of Trentino Alto Adige region (Genovesi et al., 2014; Lazzeri et al., 2022) may favours the sympatry between wild and domestic felids in those areas where both species are present, permitting the sharing of parasites and arthropod vectors as consequence.

Hepatozoonosis resulted quite equally distributed in each considered region (i.e. Veneto, Friuli Venezia Giulia and Trentino Alto-Adige). In addition, the first Italian clinical case was described in North-western Italy (i.e., Piedmont region).

Two species of *Hepatozoon* have been molecularly detected, i.e., *Hepatozoon felis* and *Hepatozoon silvestris.*

Outdoor lifestyle was found the main risk factor for *H. silvestris* since most of the positive animals were stray cats. This feature undoubtedly leads cats to come in contact with the potentially infected vectors (i.e., ticks) and wildlife, as reported for *Cytauxzoon*, but it has been even suggested that other route of transmission should be considered) (e.g., predation of infected prey).

Nevertheless, although not statistically significant, a higher prevalence of *H. felis* was reported in indoor cats. Since indoor habit does not involve the risk of contact with the vector in the environment, the results suggested again that vertical transmission may have a potential role in the transmission of the protozoa from mother to offspring (Baneth et al., 2013).

Despite the arthropod vector is not been yet recognised, it is important to implement the use of prevention against the ectoparasites, most of all for cats with outdoor lifestyle.

Since vertical transmission could be possible, it would be recommended to neuter stray and owned cats with outdoor lifestyle and/or carry out molecular tests before breeding them.

In addition, pest control should be schedule to limit potential horizontal transmission by predation.

No *Hepatozoon*-positive cats in Italian study presented parasitaemia at the stained blood smear. These findings are conformed with studies where low level of parasitaemia in cats positive to *Hepatozoon* spp. is described (Baneth, 2011; Basso et al., 2019; Pereira et al., 2019). Among captive felids, two tigers located in zoological garden of North-eastern Italy resulted positive for *Hepatozoon* spp. showing that exotic felines may be similarly susceptible hosts if located in a risky area.

Wildcats reported a high rate of positivity for both haemoprotozoa suggesting a potential role of wild felids in the transmission of hepatozoonosis and cytauxzoonosis, although the roles of feline hosts need further clarifications. Increasing the sampling capacity may be a valid option in order to improve the sensitivity of data and obtain more accurate information.

The research aimed also to investigate the potential role of ticks in protozoa transmission. The results depict the molecular detection of *Cytauxzoon* in *I. ricinus* nymphs and *H. felis* and *H. silvestris* in both nymphs and adult of *I. ricinus*. Since the ectoparasites were collected by dragging/flagging method, it could be suggested that these positive results could not depend on an infected blood meal, but to the infected/infective ticks harbouring both protozoa. Unfortunately, ticks have not been observed for oocysts before the molecular analysis. However, further studies would be needed to clarify this hypothesis. It would be interesting to develop an experimental study able to observe how ticks react to an experimental infection of *Cytauxzoon* and *Hepatozoon*, and to confirm if the parasites are transmitted during the moult.

Finally, considering the purpose to develop a diagnostic protocol, the elaborated real-time PCR approach guarantees a high sensitivity able to simultaneously detect and discriminate both *Cytauxzoon* and *Hepatozoon* genera. As a result of melting curve analysis, it is possible to analyse different matrixes (i.e., blood, tissue, organ) and screen a considerable number of

samples. A conventional PCR assay can be adopted only if there is a need to characterize the parasites at the species level. This approach permits to quickly respond to a suspected infection and allows to promptly act in the case of clinical signs.

The lack of certificated and quantified strains for *Cytauxzoon* and *Hepatozoon* species on the market is definitely a complication, overcome using a strain of *Babesia microti* (ATCC®-PRA-398DQ) to set the sensibility of the assay.

Additionally, the research project aimed to investigate on *Dirofilaria immitis* in felids confirming the occurrence of the nematode in domestic cats in different provinces of North-eastern Italy (i.e., Belluno, Padova, Verona, Vicenza and Treviso in Veneto region, Udine and Trieste in Friuli Venezia Giulia region), and wildcat in Friuli Venezia Giulia region. Indeed, the Po Valley (i.e., the area including parts of the Piedmont, Lombardy, Emilia-Romagna, Veneto and Friuli-Venezia Giulia regions) is reported as an hyperendemic area for canine Heartworm disease (Genchi et al., 2005). No cat from Trentino Alto-Adige region reported positivity neither for antigens (Ag) nor antibodies (Ab) suggesting that the climatic condition of the regions does not allow appropriate microclimate for both vector and parasite.

Despite the typically unsuitable latitude for the presence of *D. immitis*, two cats from Belluno province (Veneto region) were positive for anti-*D. immitis* Ab. Belluno has the highest mean altitude (390 masl) among the provinces included in the study, nevertheless, it is an area colonized by an exotic mosquito since 2011. In particular, *Aedes koreicus* is reported as a competent vector for *D. immitis* thanks to its ability to resist at cold environmental temperatures (Capelli et al., 2011). These results suggest that *Dirofilaria*-free areas are also potentially at risk since the nematode range of distribution is expanding, especially northwards in areas that were considered nematode-free in the past.

Concerning wildcats, this survey shows positive results about *D. immitis* in wild felids in Italy for the first time. On the contrary, exotic captive felids from zoological parks were all negative for both Ag and Ab tests demonstrating that the adoption of prevention programmes is useful in preventing heartworm infection.

The decision on using two different tests (i.e., Ag anti-D. immitis and Ab tests) was planned according to the complicacy, in felids, to obtain a univocal result. Indeed, feline cardio-pulmonary dirofilariosis is a challenging issue due to its elusive nature which leads to uncertain effects in this host. No single test is able to detect the presence of *D. immitis* in all its stages (McCall et al., 2008; Nelson, 2008), and more diagnostic methods are necessarily combined to

confirm the exposure and/or the infection, however considering the limits of available serological tests (Venco et al., 2011; AHS, 2020). No cats tested positive for both tests.

In positive samples for Ag and Ab, *D. immitis* DNA was not amplified by conventional PCR. This is not surprising due to the low sensitivity of the method using blood as a matrix. Indeed, molecular techniques are not frequently applied for the amplification of the nematode in feline blood samples (Liu et al., 2005; Park et al., 2014).

The outdoor lifestyle is found to be the main risk factor, indeed, cats are more susceptible to vector activities night and day, as it happens equally to wildcats. Nevertheless, a lower rate of indoor cats was positive suggesting that this condition can protect them just relatively. Indeed, mosquito species are usually enticed by human dwellings (Atkins et al., 2000), and they bite as well during day and night. Due to *(i)* the mosquitos' anthropophilic habits, *(ii)* the complexity of diagnosis and *(iii)* the limitation of diagnostic methods in felines, the most important step is to implement regular preventive measures against the vector and the use of prophylaxis programmes against the nematode to avoid the risk of infection in endemic or hyper-endemic areas.

In conclusion, this research proved the circulation of *Cytauxzoon* spp., *Hepatozoon* spp. and *D. immitis* in the feline population of North-eastern Italy including domestic cats, wildcats and exotic captive felids, considering the risk factors and the diagnostic approaches to detect these protozoa through a new, time-saving and sensitive real-time PCR approach. Finally, the obtained results suggest a potential role of *I. ricinus* tick in their transmission.

All considered, a "piece" has been added to the complex "mosaic" regarding the investigated parasites, nevertheless the way to clearly understand them is still long. It is therefore extremely important to persist in studying to obtain data on life-cycles, on all the plausible ways of transmission including potential vectors, and on possible reservoirs in order to carry out adequate control measures.

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