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Emerging vector-borne pathogens and their vectors affecting dogs and other animals in Central Europe

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submitted by
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Contents

1. Declarations

I hereby declare that the work included in this thesis with the title “Emerging vector-borne pathogens and their vectors affecting dogs and other animals with a focus on Austria” was performed during my PhD study at the University of Veterinary Medicine Vienna, Austria, following the rules of Good Scientific Practice in all aspects. In addition, I certify that no parts of this work have been previously submitted to another academic institution for the fulfilment of any sort of degree or awards.

Vienna, December 2023

Dr. med. vet. Maria Sophia Unterköfler

2. Abbreviations

COI: cytochrome *c* oxidase subunit I

DNA: deoxyribonucleic acid

L1: first-stage larvae

L2: second-stage larvae

L3: third-stage larvae

PCR: polymerase chain reaction

VBP: vector-borne pathogen

WNV: West Nile Virus

3. Summary

The distribution of vectors and vector-borne pathogens (VBPs) is changing due to globalisation, climate change and habitat reduction, leading to the emergence of VBPs in new areas. A nematode affecting the eye is *Thelazia callipaeda*, which can now be considered endemic in Austria. It is transmitted by the fruit fly *Phortica variegata*, which we detected in Austria together with an unidentified *Phortica* sp. Dogs and numerous other mammals, including humans, can act as definitive hosts for *T. callipaeda*. *Onchocerca lupi*, a nematode forming nodules around the eye is known to be present in Greece and Portugal. It is usually found in dogs but can also be zoonotic. It is described here as the first autochthonous case in a dog from Austria. The vector is not known, but likely candidates are biting midges (Ceratopogonidae) or black flies (Simuliidae). Several species of both families are present in Austria. Nematodes affecting the cardiopulmonary system are *Angiostrongylus vasorum* and *Dirofilaria immitis*, and while the former seems to be established in Austria, the first case report in a cat is reported here for the latter. *Dirofilaria repens* is closely related, but forms skin nodules, and several autochthonous infections have been documented for this nematode. Both can be zoonotic and are transmitted by mosquitoes. The introduction of the Asian tiger mosquito (*Aedes albopictus*) might accelerate the spread in Austria in the future, as this mosquito feeds during daytime and on different hosts including dogs. In cattle, flies of the genus *Musca* transmit *Parafilaria bovicola*, the causative agent of summer bleeding. With a survey and subsequent molecular and phylogenetic analysis, we could confirm that this parasite is endemic to Austria, and we have detected four different haplotypes. *Babesia canis* are piroplasms of dogs and were previously only known in Austria as imported infections. The vector *Dermacentor reticulatus* is widespread in eastern Austria and by analysing clinical cases, we could demonstrate that *B. canis* can be considered endemic to Austria. Wildlife often plays a key role in the distribution and transmission of VBPs. In wildcats from Germany, we detected the protozoal parasites *Hepatozoon silvestris*, *H. felis*, and *Cytauxzoon europaeus*, as well as the bacteria *Candidatus* Mycoplasma haematominutum, and *Bartonella* spp. in spleen and blood. These pathogens can also induce clinical disease in domestic cats. With the emergence of VBPs of medical and veterinary importance in Austria and other Central European countries, future research in this area is mandatory and should focus on the VBPs, their vector(s), and mammalian host(s), including possible wildlife reservoirs.

4. Introduction

4.1. General introduction

Vector-borne pathogens (VBPs) are transmitted by invertebrate, mostly arthropod vectors. These usually not only transmit the pathogen but are also necessary for the life cycle of the pathogen and act as intermediate or definite hosts. The definite host, also called final host, is the species in which the VBPs reproduce sexually. The intermediate host is necessary for the development of the asexual or juvenile stages of the VBP. In Filarioidea, this usually includes the development of first (L1) to third-stage larvae (L3) and takes place in the arthropod vector. By contrast, in haematozoan protozoa, asexual multiplication (merogony) takes place in the intermediate host, while sexual reproduction (gamogony), takes place in the definite host, which is usually the arthropod vector (Deplazes et al. 2021, Hemphill and Gottstein 2006).

Some VBPs have a narrow host range and can only infect single host species, while others can infect a wide range of hosts. If different host species can be infected, they often have a different suitability as host. Reservoir hosts are necessary for VBPs to be maintained, i.e. endemic in a certain geographic area. They often only develop mild or no disease. On the other hand, aberrant hosts have a poor suitability for the development of VBP, but often develop severe disease. Side hosts are hosts where the VBP can in principle develop, but they are not of importance for the transmission and spread of the pathogen (Deplazes et al. 2021, Hiepe 2006).

4.2. Study region

As an exemplary country in the centre of Europe, most of the studies within this work were conducted in Austria, apart from one study that was undertaken in Germany. Austria is a country with diverse habitats. High mountainous areas are present in the Alps in central and western Austria, while lowland plains are found in the east. With an area of 83 900 km², Austria has over 2 000 km² of nature reserves. Currently it has around 9 100 000 inhabitants mostly in urban areas. It is separated into 9 federal states: Vorarlberg and Tyrol in the west, Carinthia and Styria in the south, Lower Austria, Vienna (capital city) and Burgenland in the east, and Upper Austria and Salzburg in the northwest. As a landlocked country in Central

Europe, it is confronted with the introduction of pathogens from surrounding countries. Neighbouring countries are Germany, Switzerland, and Liechtenstein in the west, Italy and Slovenia in the south, Hungary and Slovakia in the east, and Czechia in the north (Baier et al. 2011, https://www.statistik.at/, 27.09.2023, https://de.statista.com/, 27.09.2023).

Austria has three climate zones: The east has a Pannonian (Central European) climate with low precipitation and hot summers, and moderately cold winters. In the Alpine regions high precipitation, short summers and long winters prevail. In the rest of the country, a transient climate dominates (https://www.austria.info/en/service-and-facts/about-austria/nature-climate, 02.12.2023).

4.3. Changes in distribution

In recent years, interest in VBPs has increased due to observed shifts in vector and pathogen distribution. Several factors are thought to be important (Harrus and Baneth 2005): Firstly, global warming is changing climatic conditions. Many invertebrates including arthropods benefit from warmer temperatures and can inhabit new areas. In addition, higher temperature can favour more rapid development of VBP within their vectors, thus producing more generations per year (Cuthbert et al. 2023). On the other hand, areas might become too dry for some arthropods and might no longer offer suitable habitats. Secondly, globalisation and travel of humans and pets introduces pathogens to new regions (Beugnet and Chalvet-Monfray 2013). Vectors can be carried via land (e.g. with trucks) or air (e.g. by planes), especially in cargo which is transported around the world (Medlock et al. 2012). Thirdly, the adaption of arthropod vectors to human settlements facilitates their dispersal to new inhabited areas. Habitat change due to urbanization and deforestation leads to selection pressure on both vectors and vertebrate hosts, and populations that are better adapted to man-made habitats can have a higher chance of survival (Gottdenker et al. 2014).

4.4. Zoonotic potential

Some of the most important pathogens of humans are transmitted by arthropod vectors, such as parasites causing malaria, whose life cycle is sustained by humans and mosquitoes (Alemayehu 2023). Additionally, many VBPs of animals are considered zoonotic and can have a high impact on human health. Infection of humans as side or aberrant hosts usually occurs only in high endemic regions and in infants or immunosuppressed humans (Molina et

al. 2003, Quinnell and Courtenay 2009). The adaption of the main vector of a VBP to the reservoir host determines the likelihood of transmission to another species. An example depicting the often complex epidemiological situation is the transmission of West Nile Virus (WNV). The vector *Culex pipiens* form *pipiens* mainly feeds on birds, which act as reservoir hosts for the virus, whereas *Cx. pipiens* form *molestus* mainly feeds on mammals, especially humans. These two *Culex* species can form hybrids, which can then act as so-called bridging vectors (Osório et al. 2014, Zittra et al. 2016).

4.5. Mammalian hosts

As mammalian hosts, humans, their pets, livestock, as well as wildlife, can be affected by vector-borne diseases. Pets are an important part of the lives of the Austrian population, with 35 out of 100 households having a pet (https://www.statistik.at/, 27.09.2023). VBPs affecting farm animals, especially grazing stock, represent a significant economic problem worldwide (Aubry and Geale 2011).

4.5.1. Dogs

Dogs (*Canis lupus familiaris*) act as reservoir hosts for various zoonotic VBPs such as *Leishmania* spp. or *Dirofilaria* spp. Besides the potential to transmit disease to humans, dogs' health as pets is also important (Dantas-Torres 2007, Genchi et al. 2011, Quinnell and Courtenay 2009). In Austria, 13 out of 100 households have a dog (https://www.statistik.at/, 27.09.2023). Dogs are often adopted from other regions, especially eastern and southern Europe, with a risk of introducing non-endemic VBPs to Austria (Fuehrer et al. 2016, Leschnik et al. 2008, Sonnberger et al. 2021).

4.5.2. Cats

Cats (*Felis catus*) are even more popular than dogs in Austria, with 22 out of 100 households having a cat (https://www.statistik.at/, 27.09.2023). In urban areas cats are usually kept indoors. In periurban and rural areas, it is common for cats to have unrestricted outdoor access. They can act as reservoir hosts of zoonotic VBPs, albeit to a lesser degree than dogs (Asfaram et al. 2019, Genchi et al. 2011).

4.5.1. Cattle and small ruminants

Cattle (*Bos taurus*) farming is an important economic sector in Austria, with approximately 1 850 000 cattle registered. They are used for milk and meat production and are kept at low altitudes as well as in high alpine regions (https://www.statistik.at/, 27.09.2023). Husbandry of cattle must include pasturing as regulated by law, putting them in closer contact with vectors (Bundesrepublik Österreich 2022). Besides cattle, sheep (*Ovis gmelini aries*) and goats (*Capra aegagrus hircus*) are also farmed in Austria, although only in small numbers compared to semi-arid and arid regions of the world (https://www.statistik.at/, 27.09.2023).

4.5.2. Wildlife

Wildlife can play in important role in the epidemiology of VBPs. For VBPs of domestic dogs, wild canids such as red foxes (*Vulpes vulpes*) often act as reservoir hosts (Battisti et al. 2020, Duscher et al. 2015, Millán et al. 2016). Other wild canids native to Europe are grey wolves (*Canis lupus*) and golden jackals (*Canis aureus*). Due to their lower abundance, their role as reservoir host is probably less important. However, they move larger distances than red foxes do and might therefore contribute to the spread of certain VBPs (Duscher et al. 2013, Hodžić et al. 2020, Kuručki et al. 2022, Mitková et al. 2017, Széll et al. 2020, Wymazał et al. 2023).

The common raccoon dog (*Nyctereutes procyonoides*) is a canid recently introduced to Europe from Asia (Kauhala and Kowalczyk 2011, Süld et al. 2014). The raccoon (*Procyon lotor*) is also new to Europe and originally comes from North America (Stope 2023). These two species are considered invasive species in Europe and might contribute further to the spread of VBPs (Daněk et al. 2023, Duscher et al. 2017, Hildebrand et al. 2018).

The European wildcat (*Felis silvestris silvestris*) and Eurasian lynx (*Lynx lynx*) are wild felids, that could act as reservoir hosts of VBPs of domestic cats. Wildcats have a very secretive lifestyle but often live close to domestic cats, so that hybrids regularly occur (Velli et al. 2023). This favours the transmission of VBPs between domestic and wildcats. In Austria, the population of wildcats is small with only a few individuals (Slotta-Bachmayr et al. 2016). In Germany, however, there are larger populations of estimated 5 000 to 10 000 individuals in different areas (https://www.iucnredlist.org/, 10.10.2023).

4.6. Vectors and intermediate hosts

Most arthropod vectors are found in the classes of Arachnida or Insecta, important vectors among the latter belonging to the order Diptera (Service 2008). Arthropods often live in different habitats during their development. The distribution therefore depends on dispersion and the availability of suitable habitats for their different developmental stages (Petrić et al. 2014).

For an arthropod to be suitable vector for a certain pathogen two requirements must be fulfilled: it needs to be able to support the development, survival, and transmission of the pathogen (i.e. vector competence) and environmental and ecological factors must support an interaction of host and vector (i.e. vector capacity) (Becker 2020, Capinera 2008). Vector competence can be assessed in laboratory infection studies (Villavaso and Steelman 1970). However, they are elaborate and therefore missing for many suspected vectors. Additionally, cryptic species and species complexes (e.g. *Cx. pipiens* sensu lato) further complicate the correct vector identification (Haba and McBride 2022). Molecular screening can provide information whether a species is a likely candidate as vector, especially if the abdomen is separated from the thorax and head. Evidence of the pathogen in other body parts than the abdomen indicates development and longer survival inside the arthropod (Cancrini et al. 2007).

4.6.1. Mosquitoes

Mosquitoes (Culicidae) can be found in very different habitats and some species are adapted to humans (Facchinelli et al. 2023). They use plants as their main food source, but females need a blood meal for the development of eggs. This makes mosquitoes important vectors for VBPs in both humans and animals. Some species have a strong host preference and only feed on e.g. birds (ornithophilic), mammals (mammalophilic) or prefer humans (anthropophilic). Other species do not have a host preference, and by feeding on different species are important bridge vectors of zoonotic VBPs (Becker 2020, Weaver 2005).

After the blood meal, the female produces eggs and deposits them near or on the water. The larvae hatch in the water and grow into fourth instar larvae. These stay in the water as motile pupae and subsequently hatch as adult mosquitoes (Becker 2020).

In Austria, 52 different species of mosquitoes are known to occur (Bakran-Lebl et al. 2021, Bakran-Lebl et al. 2022, Fuehrer et al. 2020b, Zittra et al. 2017a), with the house mosquitoes (*Culex*) usually being the most prevalent in human settlements. In recent years, alien species from the genus *Aedes*, such as the Asian tiger mosquito (*Aedes albopictus*), have been transported around the world (e.g. in used tires and lucky bamboo plants), and are now also present in Austria. *Culex* spp. place their eggs in rafts on the water surface, whereas *Aedes* spp. place the eggs on dry ground near water. The eggs can survive for long dry periods and only hatch when they get in contact with water (Becker 2020). This feature has enabled them to be successfully distributed globally (Medlock et al. 2012).

4.6.1. Black flies

Like mosquitos, black flies (Simuliidae) need water for development. However, while mosquitos use mainly stagnant water bodies, black flies need flowing water. After six to eleven larval instars, the mature larvae pupate by spinning a cocoon, which is attached to objects such as submerged vegetation or rocks. Many species emerge almost simultaneously. As adults, females take blood meals for their egg development (Service 2008). In Austria, a total of 45 species are known to be present. Of these, four are from the subgenus *Wilhelmia*, which are considered to most relevant to human and veterinary medicine (Car and Lechthaler 2002, Ebmer et al. 2023).

4.6.2. Biting midges

Biting midges (Ceratopogonidae) are very small, only 1-2.5 mm long. The eggs are laid in humid soil, where the larvae develop. Females take blood-meals and often occur in high numbers, making them a considerable nuisance and more importantly a vector for bluetongue virus (Meiswinkel et al. 2008, Service 2008). In Austria, 35 species have been documented (Zittra et al. 2020).

4.6.3. Sand flies

Like biting midges, sand flies (Phlebotominae) are very small. Their body and wings are covered with hair. Larval development takes place in humid substrates. Again, only the females take a blood meal (Service 2008). In Austria, sand flies have only recently been documented,

and appear to be spreading from their former endemic region northwards due to climate change. Two species have so far been found in Austria (Kniha et al. 2021, Naucke et al. 2011, Poeppl et al. 2013).

4.6.4. Flies

Flies of the genus *Musca* are ubiquitous in stables of livestock around the world. Their larvae develop in decomposing material, such as rotting feed or manure. For pupation they seek drier ground. As adults they like to feed on bodily fluids such as wound exudate or lachrymal fluid (Service 2008).

4.6.5. Fruit flies

Fruit flies of the genus *Phortica* like to feed on lachrymal fluid as well as fruit juices (Otranto et al. 2006). Males especially prefer lachrymal fluid, which is unusual, considering that in other species, females seek a high protein diet (e.g. blood) for egg production. Most likely, males form a spermatophore to provide females with the proteins for egg production during copulation (Máca and Otranto 2014). Females deposit their eggs on a suitable substrate, such as fruits, where the larvae hatch. These larvae moult twice and then pupate to subsequently hatch as adults (Otranto et al. 2012).

In Europe, the closely related species *Phortica variegata* and *Phortica semivirgo* are widespread. A more distantly related species formerly known from Africa, *Phortica oldenbergi* is now also present, while *Phortica erinacea* has only been reported from Bulgaria (Bächli et al. 2004).

4.6.6. Ticks

Within the class of Arachnida, the most important vectors are found in the order Ixodida. Ticks are separated into soft ticks, which often feed on birds, and hard ticks, which prefer mammals. Before every moult and for egg production the ticks need a blood meal. After oviposition the female dies. Some VBPs are transmitted only between stages (transstadially), while others can also be transmitted transovarially from the female tick to the offspring (Deplazes et al. 2021). In Austria the most abundant tick is the castor bean tick, *Ixodes*

ricinus. Another common tick is the ornate dog tick, *Dermacentor reticulatus*, which is mainly found in eastern Austria (Rubel and Brugger 2022, Vogelgesang et al. 2020).

4.6.7. Gastropods

Beside arthropods, also gastropods are important intermediate host. In trematodes, the miracidium hatches from the egg in water. They are ciliated and actively search for a suitable intermediate host, which are often snails. After development and multiplication, they leave their intermediate host and – depending on the species – are ingested by a second intermediate host (e.g. *Dicrocoelium denditriticum*) or the final host (e.g. *Fasciola hepatica*) (Deplazes et al. 2021). Also, metastrongyloids, which affect the lungs of various mammals, are dependent on slugs and snails as intermediate host. With the faeces of the mammal host, the L1 are excreted and ingested by snails and slugs. After the development to the L3, the final host is infected through the ingestion on snails and slugs, paratenic hosts, or by transmission through water (Deplazes et al. 2021, Giannelli et al. 2015).

Tab. 1: Overview of the vector-borne parasites included in this work.

Pathogen	Family	Vector/Intermediate host	Main host (Z=zoonosis)	Localisation
Dirofilaria immitis	Onchocercidae	Culicidae	Canidae (Z)	cardiovascular system
Dirofilaria repens	Onchocercidae	Culicidae	Canidae (Z)	subcutis
Angiostrongylus vasorum	Metastrongylidae	Gastropoda	Canidae	cardiovascular system
Onchocerca lupi	Onchocercidae	unknown	Canidae (Z)	periorbital tissue
Thelazia callipaeda	Thelaziidae	*Phortica* spp.	Canidae (Z)	conjunctiva
Parafilaria bovicola	Filariidae	Muscidae	Bovidae	subcutis
Babesia canis	Babesiidae	*Dermacentor reticulatus*	Canidae	erythrocytes
“Cytauxzoon europaeus”	Theileriidae	unknown	Felidae	leukocytes, erythrocytes

Hepatozoon felis	Hepatozoidae	unknown	Felidae	heart, spleen, muscle, lung
Hepatozoon silvestris	Hepatozoidae	unknown	Felidae	heart, spleen, muscle, lung

4.7. Vector-borne pathogens

Parasites, bacteria, and viruses can be transmitted by arthropod vectors and intermediate hosts. Vector-borne multicellular helminths can affect different organ systems, such as the circulatory system in *Dirofilaria immitis* and *Angiostrongylus vasorum*, the skin in *Dirofilaria repens* and *Parafilaria bovicola*, or the eye in *Thelazia callipaeda* and *Onchocerca lupi*. Unicellular vector transmitted parasites include, among others, *Babesia* spp., *Cytauxzoon* spp., or *Hepatozoon* spp. The obligatory switch of hosts in vector-borne parasites often make their life cycle and epidemiology very complex (Deplazes et al. 2021).

4.7.1. *Dirofilaria* spp.

Dogs and wild canids are reservoir hosts for *D. immitis* and *D. repens* (Genchi et al. 2011). *Dirofilaria immitis* adults are found in the heart and pulmonary arteries, while *D. repens* forms subcutaneous nodules. The females release microfilariae (L1) into the blood of the definitive host, which are ingested by mosquitoes during the blood meal. Inside the mosquito the larvae develop to infective third stage larvae to be transmitted to a new definitive host during the next blood meal (Fig. 1). Canine dirofilariosis is common in southern Europe and can also affect cats as side hosts and humans as aberrant hosts. Due to the subcutaneous location of the adults, *D. repens* usually does not cause severe clinical disease in the carnivorous hosts but can cause zoonotic infections with significant clinical alterations (Miterpáková et al. 2017, Simón et al. 2022). In contrast, *D. immitis* can lead to severe cardiopulmonary disease (Capelli et al. 2018, McCall et al. 2008). Distribution and number of cases of dirofilariosis are expected to increase in the future due to climate change (Gutiérrez-Jara et al. 2022, Riebenbauer et al. 2021).

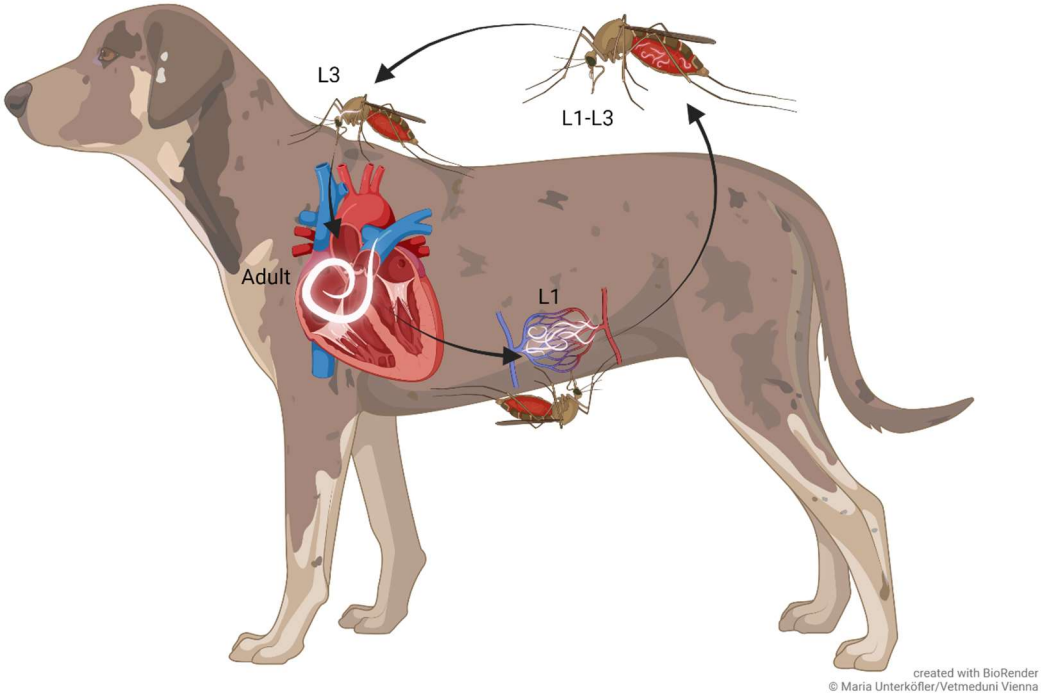

Fig. 1: Lifecycle of *Dirofilaria immitis*.

4.7.2. *Angiostrongylus vasorum*

Angiostrongylus vasorum is a nematode of the heart and pulmonary arteries of dogs and other canids, especially red foxes (Gillis-Germitsch et al. 2020). Beside cardiopulmonary signs, infected dogs can develop life-threatening coagulopathies. The adult females release eggs into the blood stream, which are trapped in the capillaries of the lung. The larvae hatch there and penetrate the endothelium to reach the bronchioles. They are ejected by coughing, subsequently swallowed, and released to the environment with the faeces. This parasite is not transmitted by arthropod vectors but uses snails and slugs as intermediate hosts, which take up the L1 by coprophagy. The L3 can infect a new host e.g. when the snail or slug is taken up by a definitive host (Elsheikha et al. 2014). In Austria, this parasite is rare (Globokar et al. 2021).

4.7.3. Onchocerca lupi

The vector for *O. lupi*, which infects canids and felines, is not known. In dogs, *O. lupi* infects the periorbital tissue and leads to nodule formation, while the microfilariae can be found in

the skin (Fig. 2). Humans can be infected as aberrant hosts, possibly leading neurological complications when the nematode erroneously enters the spinal cord, but this is rare (Grácio et al. 2015, Rojas et al. 2021). In Europe, cases are mainly reported from Greece, Portugal, and Hungary (Sréter and Széll 2008). *Onchocerca volvulus*, the causative agent of tropical river blindness, and *Onchocerca cervicalis* of equids are transmitted by black flies and biting midges, respectively (Basáñez et al. 2009, Mellor 1975).

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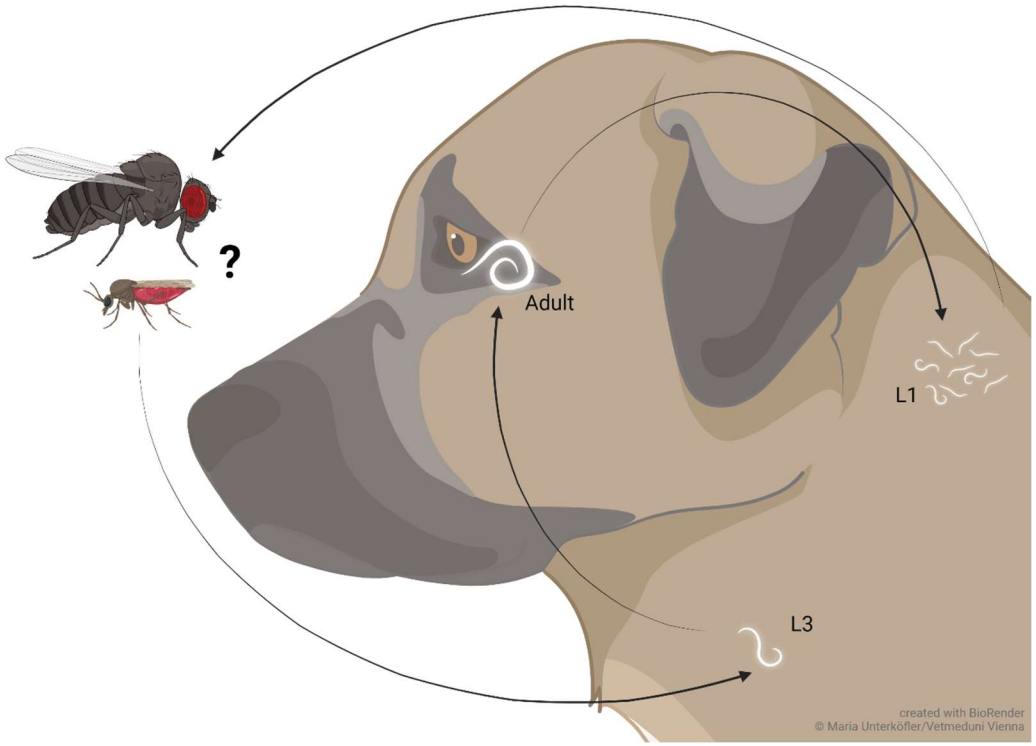

Fig. 2: Lifecycle of *Onchocerca lupi*.

4.7.4. *Thelazia callipaeda*

Another nematode affecting the eye of dogs and various other mammals, including humans, is *T. callipaeda* (Otranto et al. 2021). This parasite is also called “oriental eyeworm” because it originated in Asia. The adults float on the conjunctiva and are often found under the nictitating membrane of dogs, cats, several wild animals, and even humans. First stage larvae are released into the lachrymal fluid and taken up by *Phortica* fruit flies (Fig. 3) which act as vectors and intermediate hosts (Otranto et al. 2005b). *Thelazia callipaeda* in Europe

was first reported from Italy (Rossi and Bertaglia 1989). Other *Thelazia* spp. of cattle and horses (*Equus caballus*) are transmitted by muscid flies (Otranto and Traversa 2005).

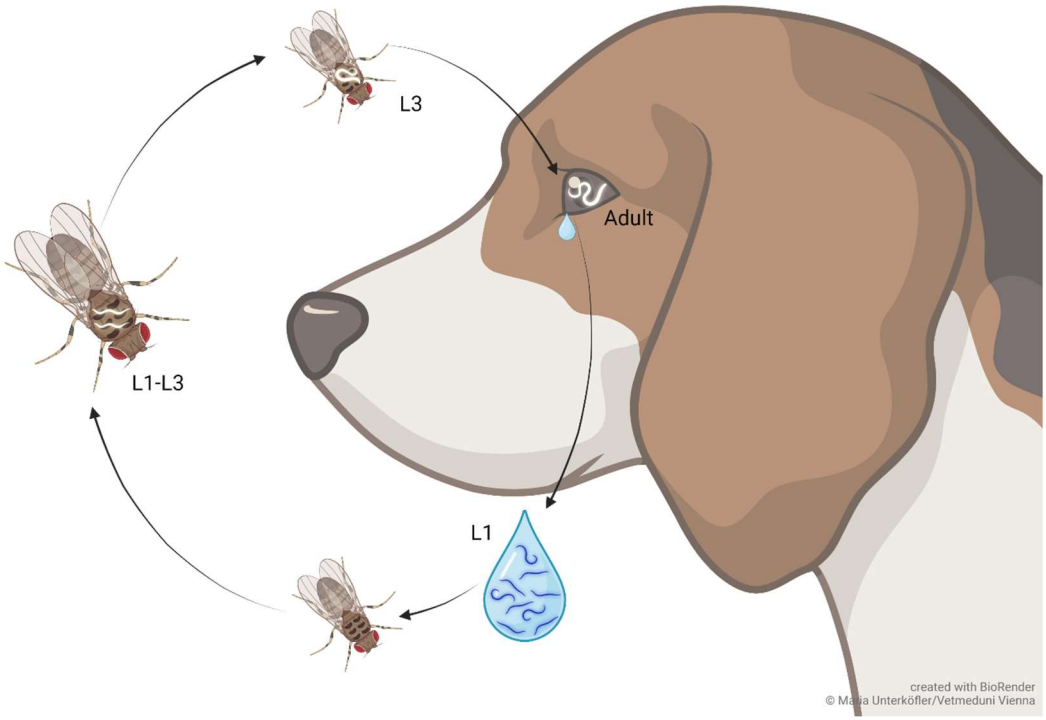

Fig. 3: Lifecycle of *Thelazia callipaeda*.

4.7.5. Parafilaria bovicola

Female *P. bovicola* living in subcutaneous tissue of cattle penetrate the skin, and eggs and larvae are released together with serosanguinous fluid through this opening (Fig. 4). This leads to the clinical presentation of so-called “summer bleeding”. Muscid flies are attracted by the serosanguinous fluid and take up the microfilaria (L1) together with the fluid. After development of infectious L3 and infection of a new host the parasite establishes in the tissue of the subcutis (Gibbons et al. 2000). *Parafilaria multipapillosa* is a closely related nematode which affects equids (Deplazes et al. 2021).

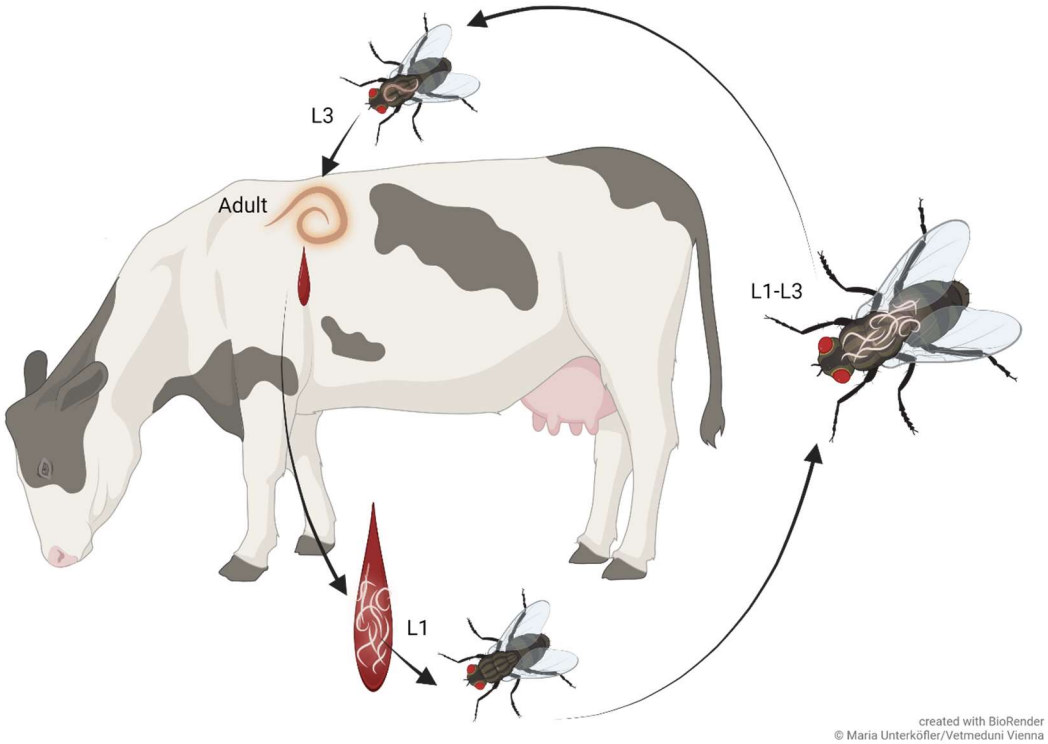

Fig. 4: Lifecycle of *Parafilaria bovicola*.

4.7.6. *Babesia* spp.

The genus *Babesia*, a member of the haematozoan family Piroplasmida, is transmitted by hard ticks and comprises a large number of species with different host spectra. Ticks are definitive hosts and can maintain the parasite in the tick population via transovarial transmission, whereas the mammalian host harbours the asexually reproducing stages that induce clinical symptoms. Different tick species prefer different intermediate hosts which is decisive for the transmission of *Babesia* spp. to different mammalian species (Deplazes et al. 2021). *Babesia canis*, for example, is transmitted by *D. reticulatus*, and multiplies in the erythrocytes in dogs. This causes haemolytic anaemia and systemic inflammatory response syndrome, leading to multiorgan-dysfunction (Solano-Gallego and Baneth 2011). Babesiosis is less common in European domestic cats, even though *Babesia* sp. can be found in the blood of healthy cats (Panait et al. 2023). Transmission of the protozoan occurs during a blood meal.

4.7.7. *Cytauxzoon* spp.

Whereas *Cytauxzoon felis* is transmitted by the tick *Amblyomma americanum* in North America (Reichard et al. 2010), the lifecycle of European *Cytauxzoon* spp. has not yet been investigated. Three species have been described in Europe based on phylogenetic characteristics and named “Cytauxzoon europaeus”, “Cytauxzoon banethi”, and “Cytauxzoon otrantorum” (Panait et al. 2021). However, the international code of zoological nomenclature was not entirely adhered to and therefore the names must be considered nomina nuda (https://www.iczn.org/the-code/the-code-online/, 13.12.2023). Infection with European *Cytauxzoon* spp. in domestic cats is mostly subclinical (Carli et al. 2022). This is not the case for infections with *C. felis* in cats that are naïve to this pathogen (Cohn et al. 2011, Conner et al. 2015, Rizzi et al. 2015).

4.7.8. *Hepatozoon* spp.

Another protozoan genus related to *Babesia* and *Cytauxzoon* is *Hepatozoon*. Various species with different definitive and intermediate hosts have been described. Most of them are parasites of amphibians and reptiles, but some species also occur in mammals (Smith 1996). *Hepatozoon canis* is transmitted by ticks, although, unlike the other two genera, transmission to the intermediate host occurs via ingestion of the vector during grooming. The sporozoites released in the gastrointestinal tract infect different organ systems, leading to variable clinical signs, ranging from subclinical to severe disease (Baneth and Allen 2022). The transmission routes of *Hepatozoon felis* and *Hepatozoon silvestris* in cats are not known.

4.7.9. Non-parasite VBPs

Numerous bacteria and viruses are transmitted by arthropod vectors, such as members of the intracellular Rickettsiales, including *Anaplasma phagocytophilum*, which are transmitted by ticks, while vector-borne viruses such as WNV are often transmitted by mosquitoes (El Hamiani Khatat et al. 2021, Rizzoli et al. 2015).

Mycoplasma spp. in cats can cause haemolytic anaemia. Fleas, as well as other arthropods, are suspected vectors. Fleas are also the vector for *Bartonella henselae*, the causative agent for cat scratch disease in humans (Lappin 2018).

4.8. Methods

Different approaches can be used to investigate the distribution and prevalence of VBPs. Not only the prevalence of domestic animals and vectors is important in this aspect, but also that of related wild animals, as they might serve as reservoir hosts (Duscher et al. 2015, Millán et al. 2016). In domestic animals, the possibility of a prior stay in a country in which VBPs are endemic is crucial to consider for correct diagnosis (Fuehrer et al. 2016).

4.8.1. Diagnostic methods

Pathogens can be indirectly detected using serological tests. Since antibodies are formed after contact with the pathogen, the test can be negative in an early phase of the infection or positive without pathogens still being present. However, these tests are very useful in determining the prevalence of VBPs. Direct pathogen detection includes classical methods like blood smears for the detection of e.g. *B. canis*, coproscopical examination for the detection of e.g. *A. vasorum*, or clinical observation for the detection of e.g. *T. callipaeda*. Furthermore, molecular tools can be used for diagnosis. Pathogen detection by PCR can be used to screen for VBPs in the tissue of definitive or the intermediate hosts (Sonnberger et al. 2021). However, vector competence cannot be assessed with this approach, as it only shows that the arthropod has ingested the VBP from an infected animal. Taking this limitation into account, however, PCR is a very sensitive detection method that has been widely used to assess the occurrence of VBPs in vectors and mammalian hosts (Clark et al. 2019, Otranto et al. 2005b).

4.8.2. Phylogenetic analysis

Analysing pathogens and their genetic relationships can shed light on the geographic introduction and distribution of different genotypes. A genetic marker frequently used in multicellular animals is the 5’ end of the cytochrome *c* oxidase subunit I (*COI*). This DNA fragment has the advantage that it has almost the same lengths in all eukaryotic animals, leaving no gaps when sequences are aligned. In most cases it is also very specific at the species level and can be used for species identification (Waugh 2007). This genetic region has therefore also been called the “barcoding” region, and a database has been established focusing on this region (http://boldsystems.org/, 26.09.2023). All of this makes it an ideal genetic fragment for phylogenetic analysis.

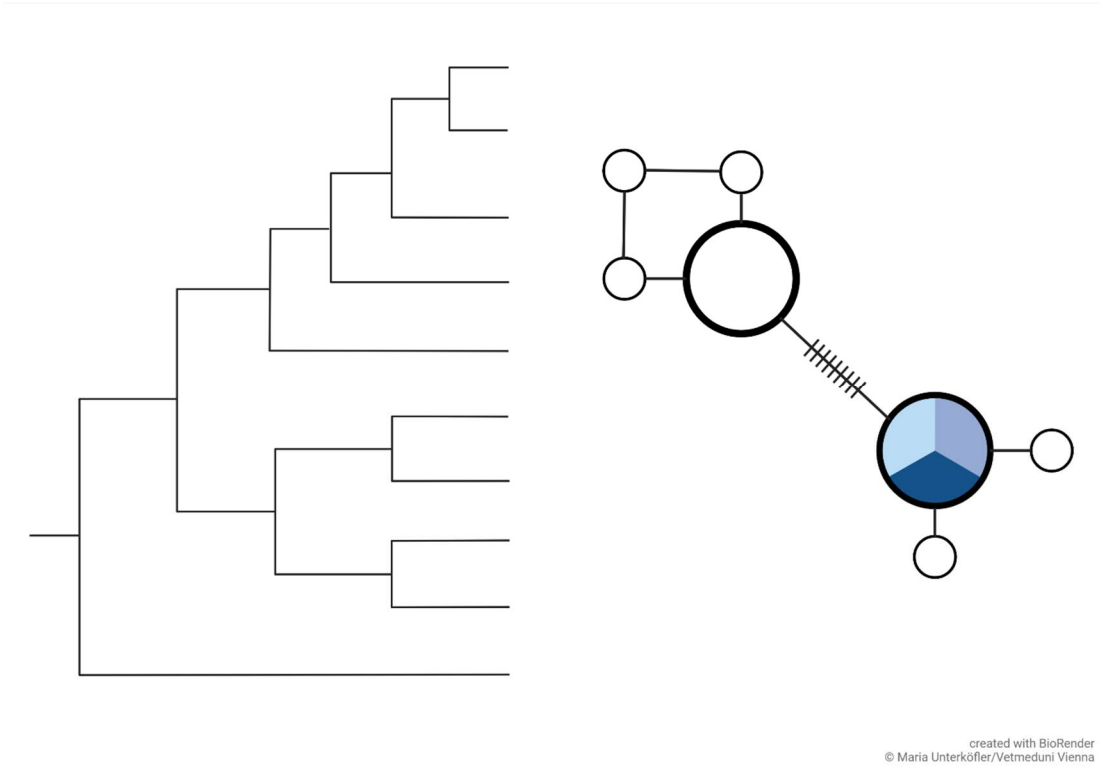

Fig. 5: Schematic depiction of a phylogenetic tree (left) and a haplotype network analysis (right). In the phylogenetic tree every haplotype is represented by a branch. In the haplotype network analysis, circles represent haplotypes, the size of the circles represent the number of individuals, and bars on branches connecting haplotypes represent the number of substitutions. Additional information can be represented by pie-charts in the circles.

The comparison with already published sequences enables the generation of a phylogenetic tree (Fig. 5), and subsequent species or genotype identification. A genotype consists of a distinct pattern of nucleotide sequence, with several nucleotides being different to other genotypes in the investigated gene fragment. Within a genotype, different haplotypes are possible. In a haplotype at least one nucleotide position is different to another haplotype. In haplotype network analysis, the different haplotypes can be compared by frequency of occurrence and other information, such as geographic origin or host (Bandelt et al. 1999, Hall 2018, Mardulyn 2012). This can help in getting an estimate on how likely transmission between hosts can occur, and if this is linked to certain haplotypes. In addition, the occurrence of several haplotypes, in contrast to only a few, indicates that the VBP is not new, but is already endemic to the region. The comparison with geographic locations can give information regarding the origin of a VBP.

4.8.3. Mapping

Distribution maps are helpful for estimating the current exposure risk in a certain area, and they permit a comparison with prior or later time points (Self et al. 2019). It is important to map both the locations where the vector or VBP has been detected, as well as the locations that have been investigated, but the vector or VBP was absent. Mapping can be done using an estimated area or using points to map the investigated locations more precisely.

5. Hypotheses and aims

The aim of this study was to evaluate which VBPs (if any) are emerging in Austria, bearing in mind the necessity to differentiate between true emergence of a parasite and an increased detection due to increased research efforts. Thus, the current distribution of VBPs which have not previously been documented in Austria, or where insufficient data was available for a baseline to compare with future studies, was evaluated. Emergence of VBPs is dependent on the vector as well as on suitable hosts, which often are wild animals. Possible reservoir hosts have also been investigated in this study.

1. Hypothesis:

Canine VBPs known to be formerly only endemic to southern or eastern Europe are emerging in, or are now endemic to, Austria.

2. Hypothesis:

Parafilaria bovicola in cattle is endemic to Austria.

3. Hypothesis:

Wildcats harbour emerging VBPs relevant for the health of domestic cats.

6. Publications

6.1. Occurrence of *Thelazia callipaeda* and its vector *Phortica variegata* in Austria and South Tyrol, Italy, and a global comparison by phylogenetic network analysis

Unterköfler MS, Dengg P, Niederbacher M, Lindorfer S, Eberle A, Huck A, Staufer K, Zittra C, Wortha LN, Hodžić A, Duscher GG, Harl J, Schlüsslmayr G, Bezerra-Santos MA, Otranto D, Silbermayr K, Fuehrer H-P. 2023. Parasites & Vectors, 16 (1): 294.

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5-year Impact Factor: 3.6 (https://jcr.clarivate.com/jcr/home, 13.12.2023)

Own contributions:

- concept and design
- morphological analysis
- PCR analysis
- sequence analysis
- phylogenetic analysis
- mapping
- drafting the manuscript

Other authors' contributions:

PD, MN, SL, AE, GS: acquisition and analysis of samples

AHU, KS: acquisition of samples, medical care and report

CZ: concept and design, acquisition of samples, analysis of samples, revising the manuscript

LW: analysis of samples

AHO: concept and design, revising the manuscript

JH: analysis of data, revising the manuscript

MBS, DO: analysis of samples, revising the manuscript

KS: concept and design, revising the manuscript

HPF: concept and design, supervision, analysis of data, revising the manuscript

Parasites & Vectors

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Occurrence of *Thelazia callipaeda* and its vector *Phortica variegata* in Austria and South Tyrol, Italy, and a global comparison by phylogenetic network analysis

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Abstract

The zoonotic nematode *Thelazia callipaeda* infects the eyes of domestic and wild animals and uses canids as primary hosts. It was originally described in Asia, but in the last 20 years it has been reported in many European countries, where it is mainly transmitted by the drosophilid fruit fly *Phortica variegata*. We report the autochthonous occurrence of *T. callipaeda* and its vector *P. variegata* in Austria. Nematodes were collected from clinical cases and fruit flies were caught using traps, netting, and from the conjunctival sac of one dog. Fruit flies and nematodes were morphologically identified and a section of the mitochondrial cytochrome *c* oxidase subunit I gene (*COI*) was analysed. A DNA haplotype network was calculated to visualize the relation of the obtained *COI* sequences to published sequences. Additionally, *Phortica* spp. were screened for the presence of DNA of *T. callipaeda* by polymerase chain reaction. *Thelazia callipaeda* and *P. variegata* were identified in Burgenland, Lower Austria, and Styria. *Thelazia callipaeda* was also documented in Vienna and *P. variegata* in Upper Austria and South Tyrol, Italy. All *T. callipaeda* corresponded to haplotype 1. Twenty-two different haplotypes of *P. variegata* were identified in the fruit flies. One sequence was distinctly different from those of *Phortica variegata* and was more closely related to those of *Phortica chi* and *Phortica okadai*. *Thelazia callipaeda* could not be detected in any of the *Phortica* specimens.

Keywords Oriental eye worm, Canine thelaziosis, Zoophilic fruit fly, Vector-borne disease, Emerging zoonotic disease, *COI*, Cytochrome *c* oxidase subunit I

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Background

Thelazia callipaeda is a parasitic nematode of the order Spirurida that affects the eyes of various mammals and is transmitted by the fruit fly *Phortica variegata*. Among domestic animals, dogs (*Canis lupus familiaris*) are frequently affected, but infections in cats (*Felis silvestris catus*) and rabbits (*Oryctolagus cuniculus*) have also been reported [1–4]. As humans can also be infected, though less frequent, *T. callipaeda* is also a zoonotic nematode and therefore of importance to public health [5, 6]. Among wildlife, *T. callipaeda* has been found in species of the families Canidae, Felidae, Ursidae, Mustelidae, Procyonidae, Suidae, and Leporidae, which represent many possible potential reservoir hosts [7–12].

Clinical signs of infection with *Thelazia callipaeda* can vary widely and have been divided into four stages, ranging from the absence of clinical signs to corneal ulcers [3]. *Thelazia callipaeda* is usually found under the eyelid and the nictitating membrane, and is easily distinguishable from other nematodes that can affect the eye, such as *Onchocerca lupi*, which is embedded in the tissue around the eye and often associated with nodule formation [13]. In combination with the removal of the nematodes from the eye, macrocyclic lactones, such as moxidectin and milbemycin oxime, are useful for both the prevention of infection and its treatment [14–17].

Thelazia callipaeda is also known as the 'oriental eye worm' due to its original distribution in Asia. In Europe, *T. callipaeda* has been documented in most southern and central countries and is predicted to spread further across the continent [18–20]. The commonly used DNA barcode region, a section of the cytochrome *c* oxidase subunit I gene (*COI*), is useful for the molecular identification of *T. callipaeda* and to distinguish different haplotypes. In the European population of *T. callipaeda*, only one haplotype has been detected, whereas the Asian population is highly diverse, with over 20 different known haplotypes [21, 22].

The sexual reproduction of *T. callipaeda* takes place in mammals, which act as the definitive hosts, while zoophilic fruit flies of the genus *Phortica* are the intermediate hosts. Male *Phortica* spp. feed on lacrimal fluid and take up first-stage larvae of *T. callipaeda* during feeding [22]. In the intermediate host, the larvae can survive for up to 147 days and develop into third-stage larvae, which can be transmitted to a new definitive host when the fruit fly next feeds on lacrimal fluid [23].

In Europe, the main vector of *T. callipaeda* is *P. variegata*, whereas in Asia it is *P. okadai* [22]. *Phortica oldenbergi* is also a competent vector under laboratory conditions, but its vector capacity under field conditions needs to be assessed [24]. Dissection and polymerase chain reaction (PCR) can be used to detect larvae of *T. callipaeda* in *Phortica* spp.; however, live fruit flies are necessary for the nematode's detection through dissection [25, 26].

For the collection of *P. variegata*, fruit fly traps can be hand-made cost efficiently from easily available components. Alternatively, netting placed around the eye of a human or dog can be used for this purpose. Although this is a time-consuming and less efficient method than using fruit fly traps, more male specimens can be collected when using this approach [27]. Identification can be done by using morphological features or by analysing the *COI* barcode region [28–31].

Suitable habitats for *P. variegata*, which are mountainous areas at 600–1200 m above sea level, can be found in large parts of Europe, and in particular central Europe. *Phortica variegata* fruit flies are mainly active at 20–25 °C and their lachryphagous activity increases with air temperature [31, 32].

Few records of *P. variegata* exist for Austria, but recent autochthonous infections of *T. callipaeda* have been reported [33–36]. The few records of *P. variegata* that were available at the start of this study dated from before 1988, and it is not clear if this drosophilid fruit fly is still endemic in Austria and, if it is, how widespread it is [35, 36].

A first report of *P. variegata* in Burgenland, Austria, was published only recently [37], and the genetic diversity of *T. callipaeda* and that of its vector *P. variegata* have not yet been investigated in this region. The aims of this study were to examine whether *P. variegata* and *T. callipaeda* occur in different parts of Austria, and to assess their genetic diversity. The occurrence of *P. variegata* and *T. callipaeda* was also investigated in South Tyrol, which is located in Italy and borders Austria, as this should provide further information on the distribution of these species, which are endemic in some other Italian regions.

Methods

Sample collection

A questionnaire designed to identify cases of *T. callipaeda* infections recorded in private veterinary practices was sent to all of the veterinarians in the database of Boehringer Ingelheim (Vienna, Austria) who had given their consent to receive customer mailings. The questionnaire could be completed online between October and November 2020. The veterinarians had the possibility to send the *T. callipaeda* specimens that they had collected previously or from a subsequent clinical case to the Institute of Parasitology, University of Veterinary Medicine Vienna. Material of clinical cases was included for 2015 to 2022, and included that from a published case report [34]. Specimens collected from

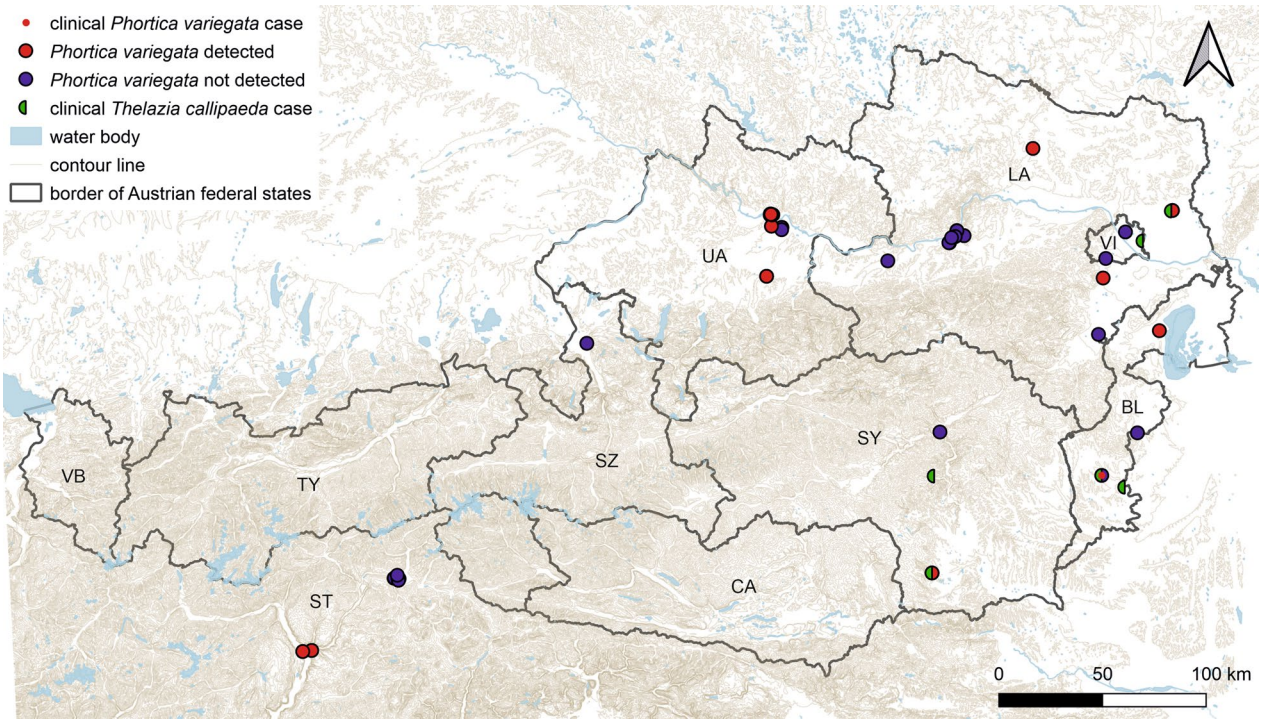

Fig. 1 Geographical distribution of sampling sites for *Phortica variegata* and location of the residence of the infected animal, or if not available, that of the clinic of the treating veterinarian of clinical cases of *Thelazia callipaeda* included in this study. *BL* Burgenland, *CA* Carinthia, *LA* Lower Austria, *SZ* Salzburg, *ST* South Tyrol, *SY* Styria, *TY* Tyrol, *UA* Upper Austria, *VI* Vienna, *VB* Vorarlberg. [Map created using QGIS v.3.22.3 (Free Software Foundation, Boston, MA)]

pets that had previously been abroad were excluded from the study.

To collect *P. variegata*, fruit fly traps built out of disposable plastic bottles were set up near forests, fruit trees, and dog-walking areas, as described in detail by Roggero et al. [27]. Every 2 weeks, the fruit flies were collected from the nets and frozen at − 20 °C until further analysis, and the chopped fruit which was used as the bait was changed. In July and August 2020, two traps in each of four sites were sampled. Two of the sites were in areas where infection with *T. callipaeda* had been reported (the town of Deutschlandsberg in Styria and the town of Gänserndorf in Lower Austria), and the two other sites were selected independently of known cases (Floridsdorf district, Vienna and Rohr im Kremstal municipality, Upper Austria). In July, August, and September 2021, eighteen traps were set up in Lower Austria, 17 in Upper Austria, 16 in South Tyrol, and 11 at participating veterinary practices that had reported cases of *T. callipaeda* infections, as well as sites provided by other volunteers (Fig. 1).

Additionally, eight fruit flies were collected from the eyes of a dog, and three by netting. Two *P. variegata* found during another study, which took place during the same period of time as the present study [37], were also included.

All of the samples were morphologically identified at the Institute of Parasitology, University of Veterinary Medicine Vienna or at the Department of Veterinary Medicine, University of Bari.

DNA extraction, PCR amplification, and sequencing

DNA was extracted from whole specimens of *P. variegata* and *T. callipaeda* using the QIAGEN DNeasy Blood and Tissue kit (QIAGEN, Hilden, Germany). Samples were incubated at 56 °C overnight and processed according to the manufacturer's protocol. To screen *P. variegata* samples for the presence of DNA of *T. callipaeda* and for genetic identification of *T. callipaeda* samples, PCRs targeting 649-base pair (bp) and 674-bp sections of mitochondrial *COI* were performed using the primers COIintF/COIintR [38] and H14FilaCOIFw/H14FilaCOIRv [39], respectively. DNA barcoding of *P. variegata* was done with the primers Lep-F1/LepR1 [40] and LCO1490/HCO2198 [41], which respectively target 665-bp and 658-bp sections of the *COI* gene. One *Phortica* sp. sample, which was genetically different from *P. variegata*, was further analysed by targeting a different region of the *COI* gene using the primers UEA7/UEA10 [42]. PCR products

were analysed by electrophoresis in 1.8% agarose gels stained with Midori Green Advance DNA stain (Nippon Genetics Europe, Germany). PCR-positive samples were sent to a commercial company (LGC Genomics, Germany) for sequencing using the PCR primers.

Phylogenetic analysis

For phylogenetic analysis, nucleotide sequences available from GenBank (National Center for Biotechnology Information) and Barcode of Life Data System (BoldSystems) databases were searched with the Basic Local Alignment Search Tool (BLAST) function, using one of the sequences obtained for each organism. In GenBank, the organism group was specified as *Thelazia* (taxid 103826) for the *T. callipaeda* sequences and *Phortica* (taxid 462262) for the *P. variegata* sequences, with the number of maximum target sequences set to 5000. For *P. variegata*, only the species belonging to *Phortica* sensu stricto were included. The sequences were aligned and sorted using the default option (FFT-NS-2) in multiple alignment using fast Fourier transform (MAFFT) v.7.311 [43] and sequences not covering the fragment of the sequences obtained in this study were excluded. All sequences featuring obvious sequencing errors and ambiguity characters were removed from the alignment and were excluded from the analysis.

To provide an overview of the diversity of haplotypes, maximum likelihood and Bayesian inference trees were calculated for each organism based on alignments, and included 110 sequences (617 nucleotide positions) for *T. callipaeda* (Additional file 1) and 280 sequences (647 nucleotide positions) for *P. variegata* (Additional file 2). The sequences were collapsed to haplotypes using data analysis in molecular biology and evolution (DAMBE) v.7.0.5.1 [44], leaving 42 haplotypes for *T. callipaeda* and 188 haplotypes for *P. variegata*. A sequence of *Mastophorus muris* (GenBank accession number MK867476) was used as an outgroup for *T. callipaeda*, and a sequence of *Anopheles gambiae* (GenBank accession number MG753768) was used as an outgroup for *P. variegata*. Maximum likelihood bootstrap consensus trees (1000 replicates) were calculated using the W-IQ-TREE web server (http://iqtree.cibiv.univie.ac.at/; [45]) applying the models TIM2+F+I+G4 for *T. callipaeda* and TIM+F+I+G4 for *P. variegata*, which were suggested as the best fit for the data sets in the model test according to the corrected Akaike information criterion. The Bayesian inference trees were calculated using MrBayes v.3.2.7 [46], applying the next complex model GTR+G+I because the same models were not available in this program. The analyses were run for 10^6 generations (number of chains, 4), sampling every thousandth tree. The first 25% of the trees were discarded as burn-in and 50% majority-rule consensus trees were calculated based on the remaining 7500 trees.

Based on the results of the consensus tree, clades were selected for the calculation of median-joining haplotype networks using Network 10.2.0.0 (Fluxus Technology, Suffolk, UK), applying the default settings. Networks were graphically prepared and provided with information on the countries and hosts in Network Publisher v.2.1.2.3 (Fluxus Technology) and finalized with CorelDRAW 2021 (Corel, Ottawa, ON).

Results

In total, the questionnaire was filled out by 183 participating veterinarians. Of these, 16 practitioners stated that they had detected *T. callipaeda* and specified the hosts as dogs ($n=11$), cats ($n=2$), and a horse ($n=1$), from Burgenland ($n=5$), Lower Austria ($n=1$), Salzburg ($n=1$), Styria ($n=5$), and Vienna ($n=1$). The report of *Thelazia callipaeda* in a horse came from Carinthia and was assumed to be a misidentification since horses have never been reported as hosts of *T. callipaeda* but rather as hosts of *Thelazia lacrymalis* [47, 48]. In total, 12 *T. callipaeda* specimens from six dogs were collected during the period 2015–2022. The dogs had not travelled abroad prior to diagnosis and originated from Styria ($n=2$), Lower Austria ($n=1$), Vienna ($n=1$), and Burgenland ($n=2$) (Fig. 1).

Phortica variegata ($n=45$) was detected in five of the seven investigated provinces (Table 1; Fig. 1). Thirty-two specimens were caught in the fruit fly traps; of these, 17 were females, eight were males and seven were unidentified (Table 1). Eight fruit flies were found in the eye of a 2-year-old male Doberman Pinscher from Burgenland (Fig. 1; Table 1). Both eyes of this dog showed ocular discharge, which had started 2 weeks previously. It was treated with a combination compound containing moxidectin (2.5 mg/ kg BW) and imidacloprid (10 mg/kg BW) Spot-On (Advocate; Bayer, Leverkusen,

Table 1 *Phortica variegata* analysed in this study

Province	Female	Male	Sex not determined	Total	Collection site
Burgenland	1	5	2	8	Dog eye
Burgenland	0	1	0	1	[37]
Lower Austria	0	1	0	1	[37]
Lower Austria	0	1	2	3	Netting
Lower Austria	0	0	1	1	Traps
Styria	2	0	0	2	Traps
South Tyrol	10	6	5	21	Traps
Upper Austria	5	2	1	8	Traps
Total	18	16	11	45	

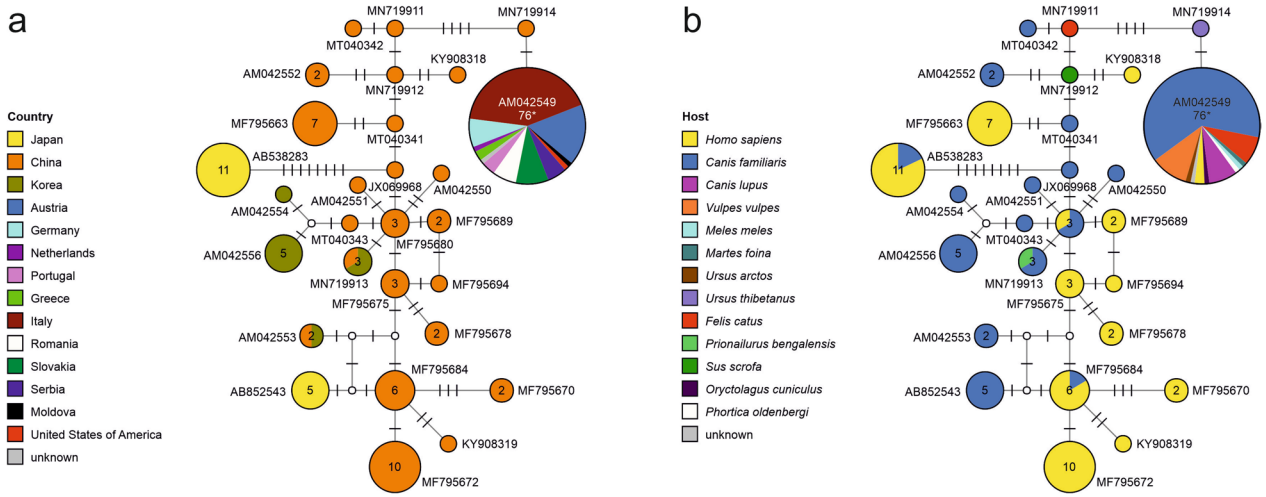

Fig. 2 Median-joining haplotype network of the cytochrome *c* oxidase subunit I gene (*COI*) sequences (617 nucleotide positions) of *Thelazia callipaeda* showing the geographical distribution (**a**) and the reported hosts (**b**). Circles represent haplotypes; numbers within the circles represent the number of individuals; if no number is shown, then only one individual is represented. Representative GenBank accession numbers of the haplotypes are shown next to the circles; white circles represent intermediate nodes; bars on branches connecting haplotypes represent the number of substitutions; asterisks indicate haplotypes of the individuals obtained in the present study

Germany) as well as with a topical ointment containing tobramycin and dexamethasone (Tobradex; Novartis, Basel, Switzerland). The dog continued to show ocular discharge and was presented at the surgery after another week. Further examination revealed the presence of dead fruit flies in the conjunctival sac of both eyes. The flies were removed using cotton swabs, after which the ocular discharge resolved.

The sequences obtained in this study were uploaded to BoldSystems (process identifiers PAVEA165-22–PAVEA176-22, PAVEA183-22, PAVEA184-23–PAVEA227-23) and GenBank (accession numbers OP620892–OP620903, OQ507612, OQ359791–OQ359834, and OQ689078).

All *T. callipaeda* corresponded to haplotype 1, which is the only haplotype that has been found in Europe so far (Fig. 2). In the fruit flies, 43 sequences could be assigned to *P. variegata*, with a total of 22 different haplotypes. In the case of one fruit fly from a dog's eye, it was not possible to obtain a sequence of sufficient quality and it was therefore excluded from the phylogenetic analysis. One sequence was different from those of *P. variegata* and more similar to those of species previously found in Asia, such as *Phortica chi* and *Phortica okadai* (Fig. 3). Analysis of this sample using a different region of the

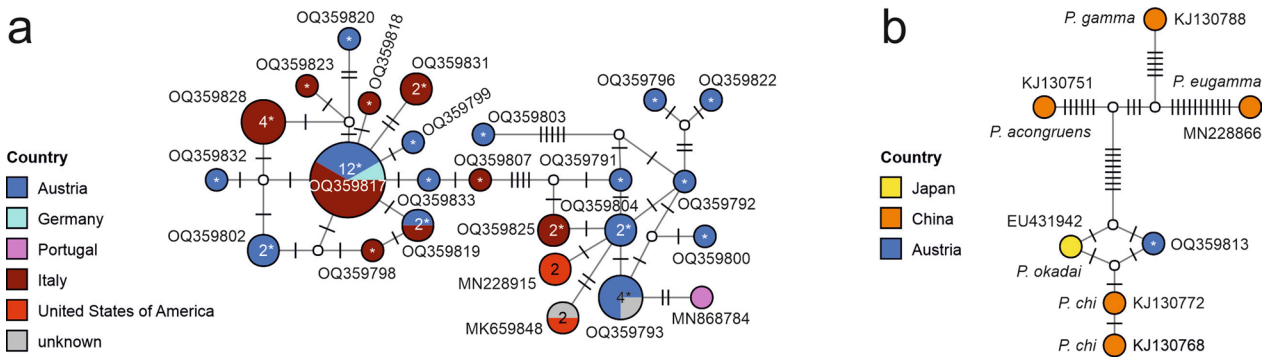

Fig. 3 Median-joining haplotype network of the *COI* sequences (647 nucleotide positions) of *Phortica variegata* sensu stricto (**a**) and *Phortica* spp. closely related to the unknown specimen from the present study (**b**) showing the geographical distribution. Circles represent haplotypes; numbers within the circles represent the number of individuals; if no number is shown, then only one individual is represented. Representative GenBank accession numbers of the haplotypes are shown next to the circles; white circles represent intermediate nodes; bars on branches connecting haplotypes represent the number of substitutions; asterisks indicate haplotypes of the individuals obtained in the present study

COI gene showed 99.67% similarity to one *P. okadai* (GenBank accession number EU431942), 92.16% similarity to another *P. okadai* (GenBank accession number: EF576924), and only 93.46% and 92.32% similarity to *Phortica variegata* and *Phortica semivirgo*, respectively (GenBank accession numbers MK659848 and EF576935, respectively). *Thelazia callipaeda* could not be detected in any of the *Phortica* specimens.

Discussion

Thelazia callipaeda and its vector *P. variegata* were found in different parts of Austria in the present study. Although *P. variegata* had been previously reported from Burgenland, Lower Austria, Styria, and Vienna [35–37], it was detected for the first time here in Upper Austria. New areas in the distributions of *P. variegata* and *T. callipaeda* were identified in the present study, but the presence and absence of the parasite in Austria could not be seamlessly mapped, as not all Austrian provinces were sampled and the locations of the traps were based on the presumed suitability of the habitats for the host species and not according to a systematic grid.

Female *P. variegata* were mainly caught in the fruit fly traps, and males predominantly around the eyes of the dogs. The preference of male *P. variegata* for the eye was not unexpected as this has also been observed in other studies, and only male *P. variegata* are considered to act as vectors of *T. callipaeda* [25, 27, 49]. To the best of our knowledge, clinical signs caused by the presence of *P. variegata* fruit flies in the conjunctival sac of a dog have not been reported up until now. It is likely that the obtained fruit flies were caught in the eyes while feeding on lacrimal fluid.

As expected, only *T. callipaeda* haplotype 1, which is the only haplotype detected in Europe to date, was found in Austria. In contrast, there is a high haplotype diversity in Asia [21, 50]. It is presumed that *T. callipaeda* is not native to Europe and that its introduction into Europe occurred as a single event. This hypothesis is also supported by the fact that *T. callipaeda* was first reported in Italy in 1989, after which it spread to other European countries due to the presence of *P. variegata*, which acts as an intermediate host [25, 51–53].

In both Europe and Asia, *T. callipaeda* is commonly found in dogs, although several wild animals have also been reported as hosts in Europe. Interest in this parasite has increased since its presence in Europe was first reported, and reports of it presence in new hosts are probably partly due to increased research efforts. Recent reports indicate that it is likely that wild animals in Asia are also frequently infected [10, 11]. Although there are case reports of human infections with *T. callipaeda* in Europe, these are more common in Asia. The infection rate in animals in Europe is probably not yet high enough to lead to many human cases. However, this may change in the future if this parasite becomes more prevalent in Europe [3, 18]. That the current prevalence of *T. callipaeda* in Austria is probably low was indicated by the low number of reported clinical cases of thelaziosis in the present study and the fact that none of the investigated *Phortica* fruit flies were positive for this parasite.

Many species within the *P. variegata* complex are not monophyletic at the *COI* barcoding region. However, *P. variegata* sensu stricto was shown to be monophyletic in both a previous study [54] and in the present one. Two haplotypes of *P. variegata* have been reported in the USA and 23 different haplotypes in Europe, including the 20 new ones reported in this study. The diversity of haplotypes found in Austria can be attributed to the fact that this fruit fly has long been native to Europe [35].

The *COI* barcoding sequence that differed from the sequence of *P. variegata* was more closely related to those of *P. chi* and *P. okadai*, which have not yet been reported from Europe. These latter two species are not monophyletic or clearly separated from their closely related morphospecies or cryptic species, and therefore delineating them through use of the *COI* gene is limited [54, 55]. *Phortica chi* and *P. okadai* have only been reported from Asia, but *P. semivirgo*, another species of the *P. variegata* complex, has been found in Europe [29, 56, 57]. Since no reference sequence of the *COI* barcode region was available at the time of analysis, another region of the *COI* gene was additionally analysed to determine whether the sample might be from a *P. semivirgo* specimen. While the sequence was 99.67% similar to one reported *P. okadai* sequence (GenBank accession number EU431942) it was not closely related to one reported for *P. semivirgo* (GenBank accession number EF576935), and was only 92.16% similar to the *P. okadai* sequence (GenBank accession number EF576924) used in a phylogenetic study comparing European *Phortica* spp. [29].

Conclusions

Further analysis of *Phortica* spp. with the use of additional genetic markers is needed to clarify the significance of the new sequence found in the present study and to assess its occurrence in other parts of Europe. *Thelazia callipaeda*, as well as its vector *P. variegata*, can be considered endemic in Austria.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-023-05913-y.

Additional file 1: Bayesian interference (BI) tree featuring mitochondrial cytochrome *c* oxidase subunit I gene (*COI*; 617 nucleotide positions) sequences of *Thelazia* spp. Nodes are marked with BI posterior

probabilities and maximum likelihood bootstrap values. Clades which are marked in red were used for calculation of the median-joining haplotype (hpt) network containing the sequences obtained in this study. Scale bar indicates the expected mean number of substitutions per site according to the model of sequence evolution applied.

Additional file 2: BI tree featuring *COI* (647 nucleotide positions) sequences of *Phortica* sensu stricto. Nodes are marked with BI posterior probabilities and maximum likelihood bootstrap values. Clades which are marked in red were used for calculation of the median-joining hpt network containing the sequences obtained in this study. Scale bar indicates the expected mean number of substitutions per site according to the model of sequence evolution applied.

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MSU: concept and design, analysis of samples and data, drafting the manuscript. PD, MN, SL, AE, GS: acquisition and analysis of samples. AHU, KS: acquisition of samples, medical care and reporting. CZ: concept and design, acquisition of samples, analysis of samples, revising the manuscript. LNW: analysis of samples. AHO, GGD: concept and design, revising the manuscript. JH: analysis of data, revising the manuscript. MBS, DO: analysis of samples, revising the manuscript. KS: concept and design, revising the manuscript. HPF: concept and design, supervision, analysis of data, revising the manuscript. All of the authors have read and agreed to the final version of the manuscript.

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Declarations

Ethics approval and consent to participate
The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as the obtained samples were gathered during procedures that were deemed of medical necessity. The pet owners were informed and gave their consent for the publication of the data obtained.

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The authors declare that they have no competing interests.

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6.2. Autochthonous *Onchocerca lupi* infection of a domestic dog in Austria

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- concept and design
- morphological analysis
- PCR analysis
- sequence analysis
- phylogenetic analysis
- drafting the manuscript

Other authors' contributions:

AH: concept and design, acquisition of samples, medical care and report, revising the manuscript

KS: concept and design, revising the manuscript

HPF: concept and design, supervision, analysis of data, revising the manuscript

Parasites & Vectors

BRIEF REPORT

Open Access

Autochthonous *Onchocerca lupi* infection of a domestic dog in Austria

Maria Sophia Unterköfler[1], Alexandra Huck[2], Katja Silbermayr[3] and Hans-Peter Fuehrer[1*]

Abstract

Onchocerca lupi is an emerging canine ocular pathogen with zoonotic potential. In Europe, known endemic areas are the Iberian Peninsula and Greece, but the parasite has also been found in Romania, Hungary, and Germany. A 5-year-old Irish Wolfhound was presented in August 2021 with ocular discharge. A subconjunctival granulomatous nodule containing several nematode fragments was removed. Molecular analysis of the mitochondrial *cytochrome c oxidase subunit I* gene confirmed the presence of *O. lupi* genotype 1. This is the first report of autochthonous *O. lupi* infection in a dog from Austria.

Keywords Canine onchocercosis, *Cytochrome c oxidase subunit I* gene (*COI*), Ocular helminthosis, PCR, Zoonotic

Background

Species of the family Onchocercidae parasitize many different vertebrate hosts and include pathogens relevant to human health such as *Onchocerca volvulus*, the causative agent of river blindness [1]. *Onchocerca lupi* was first described in a wolf (*Canis lupus*) from Russia in 1967 and affects dogs (*Canis lupus familiaris*) and, to a lesser degree, cats (*Felis silvestris catus*). Moreover, humans can be infected as well [2–5]. The adult worm is most frequently found in the subconjunctival or subcutaneous tissue, but in humans spinal cord infections have been also reported [5–7]. Clinical signs may vary, and animals that present no obvious clinical signs may not be diagnosed for several years [8]. Based on the vector capacity of other *Onchocerca* spp. and the findings of *O. lupi* DNA in Simuliidae, these have been suggested as potential vectors [9]. Other arthropods have also been considered, but evidence of competent transmission is still missing [10–12]. This parasite has been documented in Europe, America, Africa, and Asia [1, 13–15]. In Europe, the Iberian Peninsula and Greece are known to be endemic areas, but cases have also been reported from Romania, Hungary, and Germany [8, 16–19]. Diagnosis can be based on adult specimen identification in clinical cases or by skin snips and detection of microfilariae [17, 20, 21]. Morphological identification can be confirmed by PCR of, for example, the mitochondrial *cytochrome c oxidase subunit I* (*COI*) gene [22]. Treatment recommendations include surgical removal of the parasite and the use of drugs such as macrocyclic lactones [1, 23]. The present report describes the first autochthonous *O. lupi* infection in Austria.

Methods

In August 2021 a 5-year-old Irish Wolfhound living in Güssing district (Burgenland), which was born in Austria and had never left the country, was presented with ocular discharge. No other clinical signs were noted at physical examination. Subconjunctival granulomatous nodules containing nematodes were detected in both eyes and removed with forceps. The nodules were placed in saline solution and sent to the University of Veterinary Medicine, Vienna, where it was stored at −20 °C until

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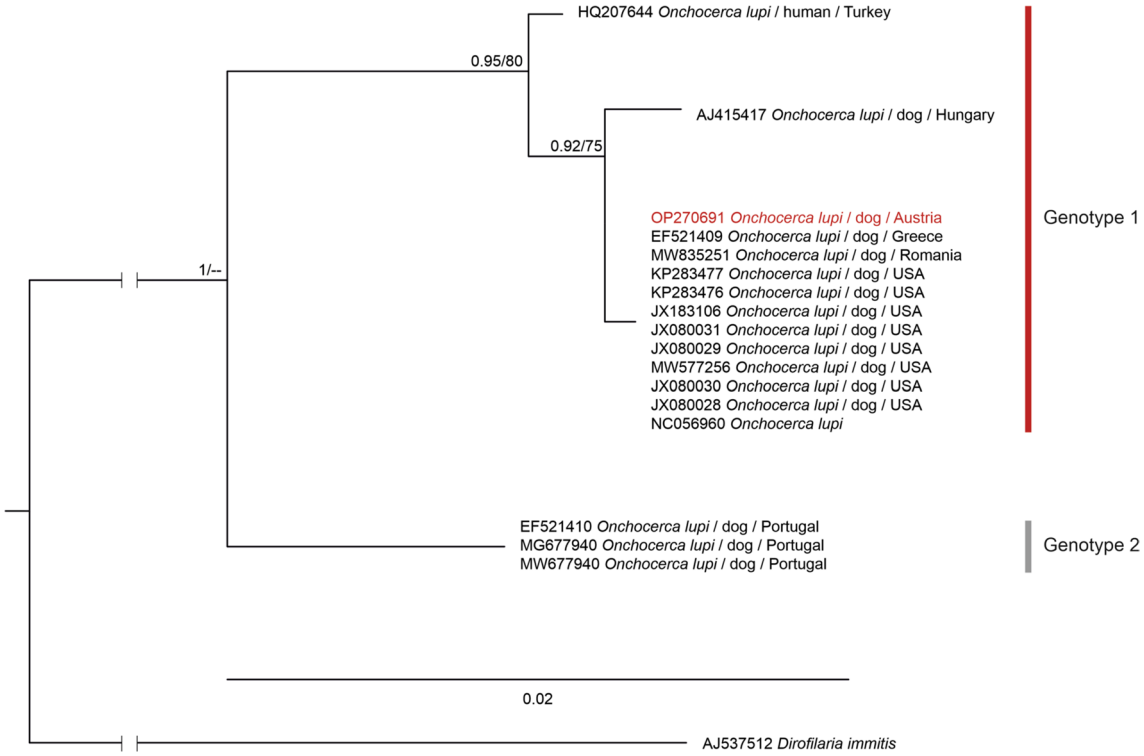

Fig. 1 Bayesian interference (BI) tree featuring mitochondrial *cytochrome c oxidase subunit I* (*COI*) sequences (649 nucleotide positions) of *Onchocerca lupi*. Nodes are marked with BI posterior probabilities and ML bootstrap values. The sequence marked in red was obtained in the present study. Scale bar indicates the expected mean number of substitutions per site according to the model of sequence evolution applied

further analysis. Nematodes were examined morphologically under a stereomicroscope, and DNA was extracted from fragments using a commercial DNA extraction kit (DNeasy® Blood & Tissue Kit; QIAGEN, Hilden, Germany) according to the manufacturer's instructions. To obtain a fragment of the *COI* gene with 649 nucleotide positions, PCR was done on a fragment of one nematode using primers COIintF/COIintR [24] with the following amplifying temperature profile: initial denaturation at 95 °C for 2 min, followed by 35 cycles of 95 °C, 50 °C, and 72 °C each for 1 min, and final extension at 72 °C for 7 min. PCR products were run on 2% agarose gels stained with Midori Green. The PCR product was further analysed by Sanger sequencing (LGC Genomics, Berlin, Germany). The sequence was compared to available sequences using the BOLD and GenBank nucleotide basic local alignment search tool.

For phylogenetic analysis, nucleotide sequences of *O. lupi* available on the NCBI GenBank database were searched by using the BLAST function, using the sequence obtained in this study. The sequences were aligned and sorted using the default option (FFT–NS–2) in MAFFT v.7.311 [25], and sequences not covering the fragment of the sequences obtained in this study were excluded. Maximum likelihood (ML) and Bayesian inference (BI) trees were calculated based on the alignment, including 17 sequences (649 nucleotide positions). Sequences were collapsed to haplotypes using DAMBE v.7.0.5.1 [26], leaving four haplotypes. As outgroup, a sequence of *Dirofilaria immitis* (GenBank accession number: AJ537512) was used. ML bootstrap consensus trees (1000 replicates) were calculated using the W-IQ-TREE web server (http://iqtree.cibiv.univie.ac.at/; [27]) applying the model TIM3 + F + G4, which were suggested as best fit for the data set in the model test according to the corrected Akaike information criterion. The BI trees were calculated using MrBayes v.3.2.7 [28], applying the next complex model GTR + G, because the same model was not available in this program. The analysis was run for 10^6 generations (number of chains: 4), sampling every 1000th tree. The first 25% of trees were discarded as burn-in, and a 50% majority-rule consensus tree was calculated based on the remaining 7500 trees.

Results and discussion

The dog was treated twice at an interval of 2 weeks with a combination compound containing moxidectin (2.5 mg/kg BW) and imidacloprid (10 mg/kg BW) Spot-On

(Advocate®; Bayer AG, Leverkusen, Germany) for the control of *O. lupi* and with a topical ointment containing tobramycin and dexamethasone (Tobradex®, Novartis AG, Basel, Switzerland) to promote the healing of the eye inflammation. The clinical signs disappeared and did not recur within the follow-up period of 1 year. Treatment with moxidectin has been reported to be successful [23]. However, whether medical treatment or the surgical removal alone resolved clinical signs cannot be concluded with certainty. In addition, it is not clear whether the treatment eliminated all nematodes as no skin biopsies could be obtained before and after treatment because of the lack of owner consent. Morphological examination of the nodule revealed several worm fragments. The DNA sequence obtained was 100% identical to an *O. lupi* sequence documented in a dog from Greece (GenBank accession number: EF521409) and has been uploaded to BoldSystems® (Process ID: PAVEA164-22) and GenBank (accession number: OP270691). This haplotype has been referred to as genotype 1 (Fig. 1), which occurs in northern America, southwestern Asia, and Europe, with the exception of the Iberian Peninsula, where genotype 2 is present [15].

In total, 45 species of Simuliidae, which could potentially act as vectors, are known to exist in Austria [29, 30]. In the Lafnitz River near Heiligenkreuz town (Burgenland), located near Güssing (Burgenland), *Simulium erythrocephalum*, *Simulium ibariense*, and *Simulium ornatum* have been found [29].

Coyotes (*Canis latrans*) have been considered as reservoir hosts in America [31]. In Europe, coyotes are not present, but other wild canids could probably fulfil this role. Another more likely mode of introduction is through pets travelling from endemic regions and subsequent establishment of the parasite in areas where it has not been present before [32]. To determine the current prevalence of *O. lupi* in Austria, a prevalence study should be performed in dogs and/or wild canids using skin snips and/or serology [8, 17, 33].

Conclusion

Information on the treatment but also on transmission and distribution of this parasite is still scarce. This case report highlights that *O. lupi* can also be present in countries not yet classified as endemic and underlines the need to raise awareness of this zoonotic parasite.

Abbreviations

BI	Bayesian inference
COI	Mitochondrial *cytochrome c oxidase subunit I*
ML	Maximum likelihood

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Author contributions

MSU: concept and design, analysis of samples and data, drafting the manuscript. AH: concept and design, acquisition of samples, medical care and report, revising the manuscript. KS: concept and design, revising the manuscript. HPF: concept and design, supervision, analysis of data, revising the manuscript. All authors have read and approved the final manuscript.

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Availability of data and materials

Additional data can be provided on request.

Declarations

Ethics approval and consent to participate

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as the obtained sample was gathered through a procedure necessary for medical reasons.

Consent for publication

Not applicable.

Competing interests

None declared.

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Publisher's Note

6.3. First autochthonous infection of a cat with *Dirofilaria immitis* in Austria

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- morphological analysis
- attempt of PCR analysis
- writing of minor parts of the manuscript
- revising the manuscript

Other authors' contributions:

LMK: involved in the patient's diagnosis process and follow-up, wrote the manuscript

MSU: wrote the manuscript, identified the *Dirofilaria* specimens, revised the final version of the manuscript

HPF: identified the *Dirofilaria* specimens, revised the final version of the manuscript

VJ, MP, and MS: involved in the patient's diagnosis process and follow-up, revised the final version of the manuscript

LV: identified the *Dirofilaria* specimens, revised the final version of the manuscript

ML: involved in the patient's diagnosis process and follow-up, revised the final version of the manuscript

pathogens

MDPI

Case Report

First Autochthonous Infection of a Cat with *Dirofilaria immitis* in Austria

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Abstract: This case report is about a seven-year-old male neutered European Shorthair cat infected by *Dirofilaria immitis* as the first reported autochthonous *Dirofilaria immitis* infection in Austria. There was no history of periods abroad. Echocardiography showed suspected *D. immitis* in the right cardiac chamber with increased pulmonary pressure and ascites. Surgical removal of the heartworms was performed. Twenty adult heartworms were removed by transvenous jugular approach under general anesthesia and stored in 4% formalin. Five out of 20 specimens were examined via light and stereomicroscopy and feline heartworm infection was confirmed. Amplification of a 203 bp or 724 bp fragment of the cytochrome c oxidase subunit I gene was unsuccessful. After surgery the cat developed acute renal failure but recovered quickly. One year later, the cat underwent a control examination including echocardiography and blood work. There were no more *D. immitis* detectable at echocardiography. Lung pressure was mildly increased. Complete blood count and creatinine were unremarkable. The Knott's test and *Dirofilaria*-Antigen-test produced negative results. The cat did not show any clinical signs during the follow-up period. The aim of this case report is to highlight the growing risk of acquiring infection with *D. immitis* not only for Austrian dogs, but also for cats. This case report represents the first report of autochthonous *D. immitis* infection in Austria. Moreover, even if the prognosis in cats with caval syndrome due to feline heartworm disease is guarded to poor, surgical removal of the filariae can be a successful treatment option.

Keywords: dirofilariosis; feline heartworm disease; vector borne; caval syndrome; heartworm associated respiratory disease

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1. Introduction

Feline heartworm disease can be quite a challenging diagnosis for veterinarians due to its unique nature and physiopathology [1]. *Dirofilaria immitis* belongs to filarioid nematodes and represents the underlying agent for feline heartworm disease (FHWD) and heartworm associated respiratory disease (HARD). *D. immitis* infects mainly dogs but also cats, ferrets, wild carnivores and humans, and more than 70 different species of culicid mosquitos can act as vectors [2,3]. *D. immitis* infection has been reported mainly in temperate, tropical and subtropical areas of the world. The largest endemic area in Europe can be found in the Po River Valley in northern Italy, where the prevalence in non-preventive-treated dogs ranges up to 80% [2]. *D. immitis* emerges in new countries due to globalization and increased travel as well as the import of infected dogs. In addition, climate change and the adaptability of vectors play major roles in the spreading of *D. immitis* [4]. Between 2014 and 2018, the number of imported *D. immitis* cases in dogs more than doubled, and it is

suspected that Austria is facing pre-endemic status [5]. In Austria two mosquito species, *Aedes vexans* and the *Culex pipiens* complex are already known to act as potential vectors for *Dirofilaria* parasites. One main reason for the delayed introduction and establishment of *D. immitis* is the lack of microfilaraemic dogs as a consequence of less common kennelling or outdoor keeping of dogs in Austria [6]. Prevalence of feline heartworm infections is generally considered to be five to 20% of the canine counterpart population in the affected area [7].

Cats represent imperfect hosts for *D. immitis*. Compared to dogs, only a low number of L3 larvae develop to the adult stage, which also takes about seven to nine months. What is more, microfilariae (L1 larvae) are able to develop in only 20% of cats with mature female and male worms. Unlike in cats, significant microfilaraemia that can last for years develop in dogs. The lifetime of adult heartworms in cats up to four years old is shorter than in dogs, and adult *D. immitis* in cats are also smaller. Moreover, about 25% of cats are naturally resistant to infestation with *D. immitis* [2,7]. Cats with outdoor access seem to have a three-fold higher risk of being antigen-positive and male cats have been found to be more likely to develop mature infections [8]. On the other hand, heartworm disease as a differential diagnosis in indoor cats cannot be ruled out, but it is less likely [7]. Further proof that cats are imperfect hosts is the aberrant migration in body cavities, systemic arteries and the central nervous system, which occurs more frequently in cats than in dogs [2,7].

Severe pathological and life-threatening changes despite low parasite load of one to six adult worms per cat can be found early in cats [9]. After inoculation with L3 larvae they develop to Stage L4 and migrate to the pulmonary arteries 70–90 days post infection. The first phase is characterized by an intense eosinophilic pulmonary reaction. Most of the L4 die in this stage of disease and this stage is often misdiagnosed as feline asthma or chronic bronchitis, although this intense reaction is part of heartworm associated respiratory disease [10]. Sudden death in 20% of infected cats can be due to excessive inflammatory and thromboembolic response and is accompanied by haemothorax resulting from pulmonary artery dissection [10–13]. Caval syndrome is quite rare in cats, but it usually arises when one or two worms are located in the right heart causing tricuspid regurgitation. Most cats show moderate to mild symptoms, but owners also report chronic vomiting, anorexia and/or cachexia and respiratory signs [14].

To diagnose FHWD, a multimodal approach is necessary. A combination of diagnostic tools like thoracic radiographs, serum antibody tests, echocardiography and serum antigen tests are recommended. Necropsy is the gold standard for detecting adult worms [1,10,11]. Microscopic detection of microfilariae and ELISA to detect circulating antigens have low sensitivity in cats [1]. The Knott's test for detecting circulating microfilariae is less successful, but when present, the FHWD diagnosis is confirmed [13].

In contrast to dogs, adulticidal therapy is not recommended because FHWD self-cures in most cases within 18–48 months. Surgical removal of the adult filariae can be performed in symptomatic cats. It is important to remove intact worms to avoid anaphylaxis. Monthly administration of macrocyclic lactones is strongly recommended in endemic areas [12,15].

To our knowledge, there are no reports of FHWD in Austria. In this case report, we describe a clinical case of autochthonous heartworm infection in a cat in Austria.

2. Case Report

A male neutered European Shorthair cat, seven years of age, 6 kg weight, was referred to the Veterinary Hospital Parndorf in the province of Burgenland, Austria for echocardiography in March 2020 (Figure 1). Prior to admittance, the cat had a short history of dyspnoea and increased abdominal circumference. At this time, the cat was the only animal in the household and had unrestricted outdoor access. The cat was in possession since kitten age, spending its lifetime in the same area, which is 47°34′57.443′′ N, 16°32′33.673′′ E. The cat was neither regularly vaccinated nor dewormed and had no history of periods abroad.

Figure 1. Male neutered European Shorthair cat referred for echocardiography.

On clinical examination the cat showed calm and attentive behaviour, slightly pale mucous membranes, preserved skin elasticity, increased breathing, a systolic heart murmur Grade IV/VI best heard on the right hemithorax, moderate vesicular breath sounds, weak femoral pulse and lymph nodes within normal limits.

The pretreatment by the referral vet included Benazepril 0.4 mg/kg once daily and Furosemide 1.6 mg/kg twice daily with no improvement of clinical signs. The referral vet also took thoracic and abdominal radiographs. The thoracic radiographs showed a diffuse bronchointerstitial pattern of the pulmonary parenchyma with enlarged pulmonary arteries. The abdominal radiographs revealed decreased delimitation of the abdominal organs—most likely due to ascites.

2.1. Echocardiography

Echocardiographic exams were performed by a single experienced operator with GE vivid™ S6, equipped with a microconvex GE 7s probe. The right atrium showed severe dilatation, and several double lined hyperechoic echoes close the tricuspid valve were seen (Figure 2). The suspected diagnosis based on the echocardiographic findings and the clinical signs was an infection with *D. immitis*.

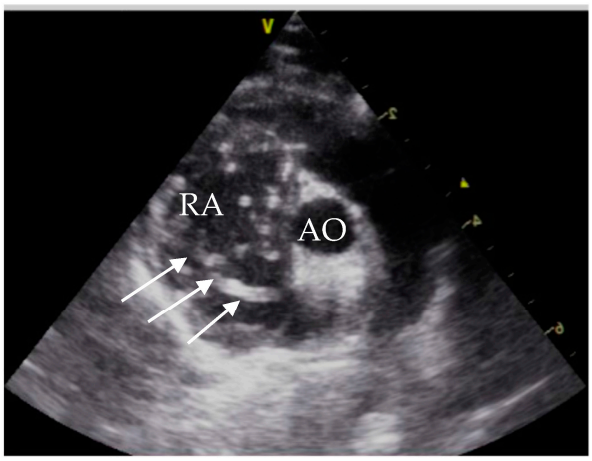

Figure 2. Echocardiography showing heartworms, right parasternal short axis view. RA = right atrium; AO = aorta; white arrows = heartworms.

After a period of reflection, the owner opted for a surgical attempt. Surgical removal of the suspected heartworms was scheduled two days after first presentation.

2.2. Complete Blood Count and Blood Chemistry

Before anaesthesia, blood analysis (CBC, biochemistry shown in Table 1) was performed via Idexx Procyte DX® (Idexx Laboratories, Westbrook, ME, USA) and Idexx Catalyst DX® (Idexx Laboratories, Westbrook, ME, USA). The cat showed a severe hyperchromic, macrocytic regenerative anaemia. What is more, the cat showed a mildly increased count of leucocytes with mildly increased neutrophils, lymphocytes and monocytes. Creatinine was 2.4 µmol/L which was in the upper reference interval. The Combo+ Snaptest® (Idexx Laboratories, Westbrook, ME, USA) for infection with feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) showed a positive result for FeLV. No antigen testing for *D. immitis* was done before surgery due to the owner's cost restrictions.

Table 1. CBC and blood chemistry—March 2020 and July 2021.

Parameter	Value		From	To	Measuring Unit
	March 2020	**July 2021**			
Erythrocytes (RBC)	2.72	7.75	6.54	12.20	T/L
Haematocrit (HCT)	12.0	39.50	30.30	52.30	L/L
Haemoglobin	5.20	9.90	9.80	16.20	mmol/L
MCV	44.10	39.40	35.90	53.10	fL
MCH	19.10	12.80	11.80	17.30	fmoL
MCHC	43.30	32.50	28.10	35.80	mmol/L
Reticulocytes	168.40	10.90	3.0	50.0	G/L
Leukocytes (WBC)	19.10	11.80	2.87	17.02	G/L
Neutrophils	15.68	8.47	2.30	10.29	M/L
Lymphocytes	1.91	1.93	0.92	6.88	M/L
Monocytes	0.94	0.42	0.05	0.67	M/L
Eosinophil	0.54	0.95	0.17	1.57	M/L
Thrombocytes (PLT)	175	594	151	600	GL
Creatinine	2.4	1.9	0.8	2.4	mg/dL

2.3. Surgical Removal of D. immitis

The cat was placed in left lateral recumbency for surgery. The right jugular vein was incised for a transvenous approach to the vena cava. A pair of flexible alligator forceps with 40 cm length—which usually finds use in endoscopy—was used to remove the suspected heartworms guided by echocardiography and C-arm. By use of this technique, which was described by Glaus et al., 1995 [16] the successful removal of 20 adult worms was possible (Figure 3, Video S1). Echocardiography revealed two to three dirofilariae left in the right atrium. After several subsequent attempts the right jugular vein was blocked, and removal of the remaining worms failed. The right jugular vein was ligated afterwards with a monofilament, absorbable suture material (Monosyn® 3/0, Braun®) and the skin incision was also closed with the same suture material in a routine manner.

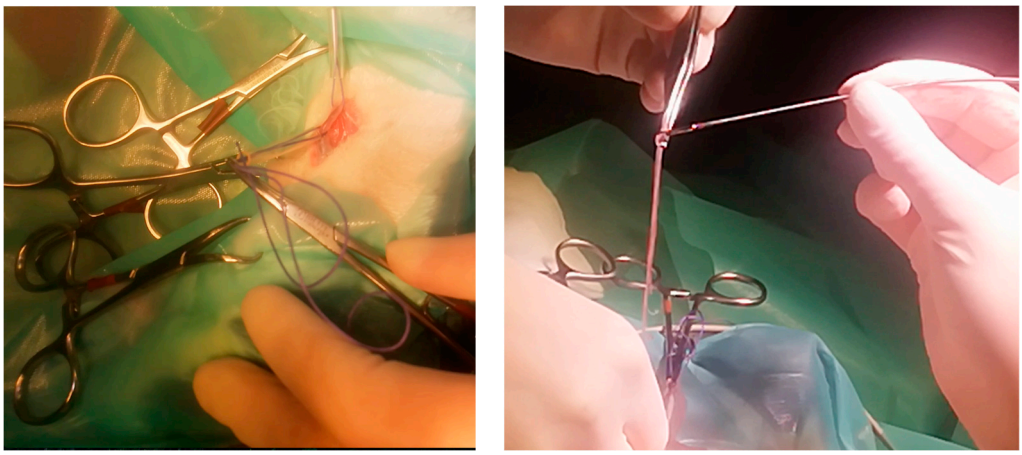

Figure 3. Surgical removal of the *Dirofilaria* specimens.

2.4. Identification of Dirofilaria Specimens

Dirofilaria specimens were stored in formalin for a year. DNA extraction of the middle piece of three specimens was performed with the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, tissue was homogenized in 180 µL buffer and three 2.8 mm ceramic beads (Precellys Ceramic Beads, Peqlab Biotechnologie GmbH, Erlangen, Germany) with a TissueLyser II (Qiagen, Hilden, Germany) for three minutes. Overnight digestion with 20 µL of Proteinase K was done at 56 °C. Buffer and ethanol were added and centrifuged in a Minispin column. After two washing steps, the DNA was collected with an elution buffer. Additionally, DNA was extracted with the QIAamp DNA FFPE Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's guidelines. Tissue was rehydrated with 200 µL of 99.9% alcohol and subsequent centrifugation. The supernatant was discarded and 200 µL of 70% alcohol was added following centrifugation and discarding of the supernatant. Remaining alcohol was removed through evaporation and tissue was digested overnight with 180 µL buffer and 20 µL proteinase K at 56 °C. After incubation at 90 °C for one hour buffer and ethanol were added and centrifuged in a Minispin column. After two washing steps, the DNA was collected with an elution buffer. Amplification of the extracted DNA was performed with two different PCR protocols. Primers H14FilaCOIFw and H14FilaCOIRv targeted a 724 bp fragment and primers DI COI-F1 and DI COI-R1 targeted a 203 bp fragment of the mitochondrial cytochrome c oxidase subunit I gene and are published along with the PCR conditions elsewhere [17,18]. To improve amplification results, PCRs were repeated with the GoTaq® Long PCR Master Mix (Promega, Madison, WI, USA). PCR products were separated in 2% agarose gels stained with Midori Green Advanced DNA stain (Nippon Genetics Europe, Düren, Germany) by electrophoresis. Amplification of a 203 bp or 724 bp fragment of the cytochrome c oxidase subunit I gene was unsuccessful.

Using light microscopy (Nikon Eclipse Ci, Tokyo, Japan) and stereo microscopy (Nikon SMZ1270, Tokyo, Japan) five specimens were analysed of which three were female and two were male. Female specimens were 212 to 250 mm long and 0.74 to 1.08 mm wide. Male specimens were 97 to 116 mm long and 0.63 to 0.66 wide; however, in one specimen, the tail was missing. The cuticle was smooth and lacking significant longitudinal cuticular ridges (Figure 4A), showing cephalic extremity slightly thin and rounded (Figure 4B), no clear demarcation between the oesophageal muscular and glandular regions (Figure 5A), visible uterus (Figure 5B) and straight tail in females (Figure 6A) and tail wound-up in a corkscrew manner in males (Figure 6B). All morphological features were compatible with adult stages of *D. immitis*.

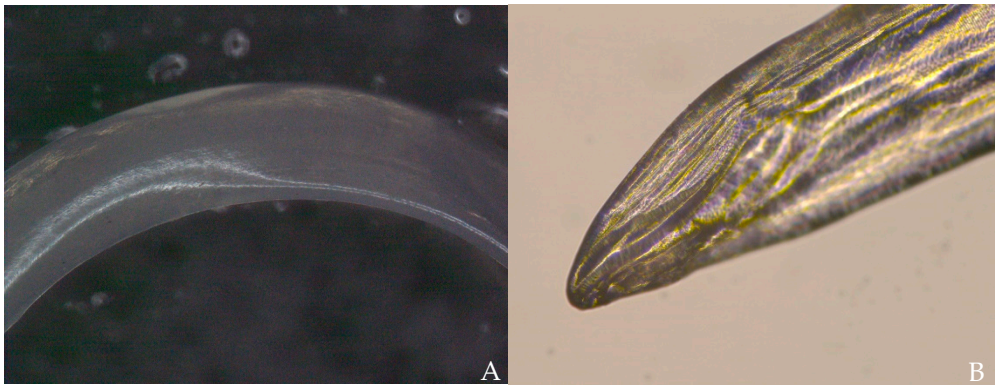

Figure 4. (**A**) Stereomicroscopic image of the body of adult D. immitis. The cuticle is smooth and does not have longitudinal cuticular ridges as could be found in adult D. repens. (**B**) Light microscopic image of the cephalic extremity of adult D. immitis.

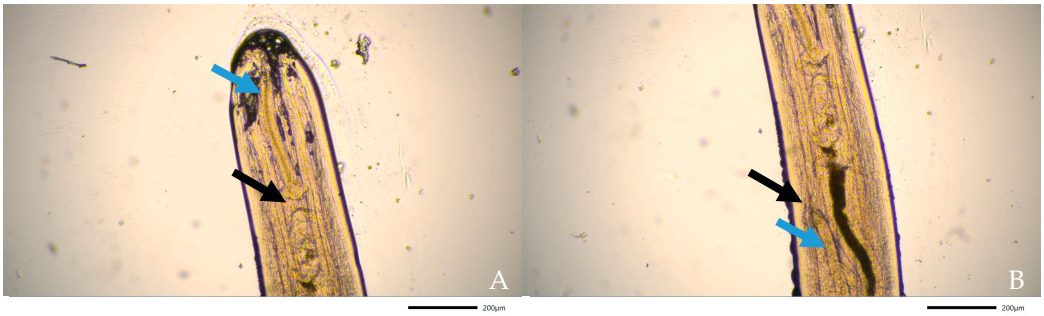

Figure 5. (**A**) Light microscopic image of cephalic extremity adult *D. immitis*. The oesophagus (blue arrow) and intestine (black arrow) are visible. (**B**) Light microscopic image of adult female *D. immitis*. Vulvar opening (black arrow) and uterus (blue arrow) are visible.

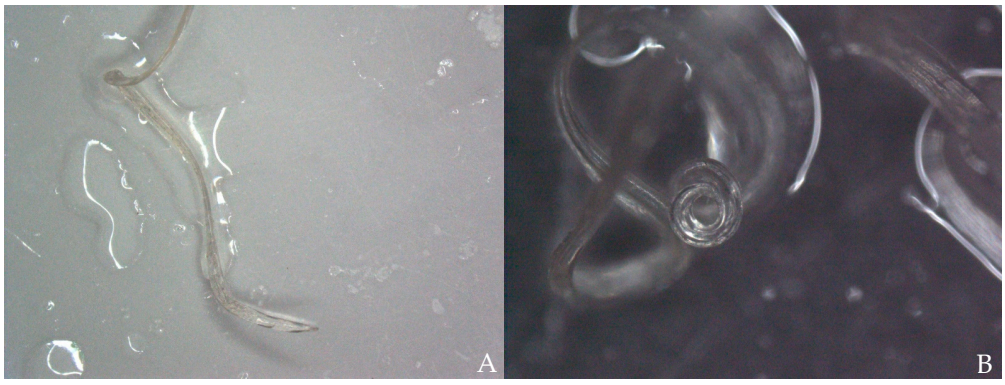

Figure 6. (**A**) Stereomicroscopic image of the straight tail of adult female *D. immitis*. (**B**) Stereomicroscopic image of the tail of adult male *D. immitis*. The tail in males is wound up in a corkscrew manner.

2.5. Postoperative Course

After surgery, the cat's creatinine increased to 5.1 mg/dL and the haematocrit decreased to 12.8%—but showed still high regeneration with a reticulocyte count of 158.7 G/L. It was suspected that the cat had an episode of acute kidney failure due to the removal of the heartworms. The therapy included buprenorphine 10 µg/kg three times a day, amoxicillin–clavulanic acid 20 mg/kg twice daily, doxycycline 5 mg/kg twice daily, spironolactone 2 mg/kg twice daily, pimobendan 0.25 mg/kg twice daily, and calcium carbonate to lower the phosphorus level. Fortunately, the cat's medical condition improved, and it was sent

home three days after surgery with a creatinine of 3.2 mg/dL and a stable haematocrit of 12%.

The prescribed treatment included pimobendan 0.25 mg/kg twice daily, clopidogrel 1.5 mg/kg once daily and milbemycin 4 mg/kg monthly. Further control examinations were performed at the referral vet.

2.6. Follow Up

The cat was presented 16 months after surgery for a control examination including thoracic radiographs and echocardiography at the Veterinary Hospital in Parndorf, Burgenland. The owner reported that the cat was not showing any clinical signs despite of infrequent, spontaneous coughing and receiving the prescribed medication daily. Clinical examination remained unremarkable. The thoracic radiographs showed a moderate bronchointerstitial lung pattern without enlarged pulmonary arteries (Figure 7). This bronchointerstitial lung pattern could be due to heartworm associated respiratory disease (HARD).

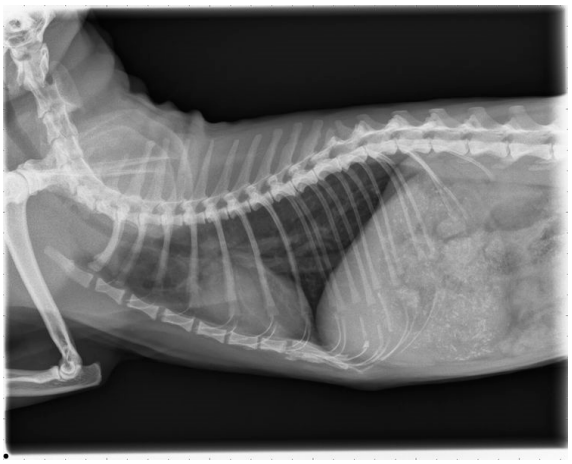

Figure 7. Thoracic radiograph, right laterolateral view.

Additionally, an echocardiography was performed. The cat showed a tricuspid insufficiency (2.8 m/s), a mild mitral insufficiency and a pulmonary artery flow of V_{max} 1.1 m/s (probably suggesting mild pulmonary hypertension). Comparing the two echocardiograms the cat's hemodynamic status significantly improved (Table 2) with increased left ventricle preload. No heartworm was detectable (Figure 8). LA/Ao could not be measured in the initial echocardiography due to the enlarged right atrium.

Complete blood count and creatinine were repeated, and CBC showed no abnormalities with a haematocrit of 30.5% and creatinine of 1.9 mg/dL (Table 1).

What is more, the Knott's test to detect microfilaria was negative. In addition, the Heartworm-Snap-Test® (Idexx Laboratories, Westbrook, ME, USA) showed a negative result. Felichek-3® (Bionote), a chromatographic immunoassay showed negative results for feline heartworm antibodies as well as feline immunodeficiency virus antibody and feline leukaemia virus antigen.

At this time, in July 2021, there was no sign of reinfection with *D. immitis* in this cat.

Table 2. Echocardiograms compared (March 20 to July 21).

Date	IVSd	LVIDd	LVPWd	LA/Ao
4 March 2020	0.58 cm	1.20 cm	0.60 cm	-
14 July 2021	0.55 cm	1.75 cm	0.42 cm	1.35

IVSd = interventricular septal end diastole, LVIDd = left ventricular internal diameter end diastole, LVPWd = left ventricular.

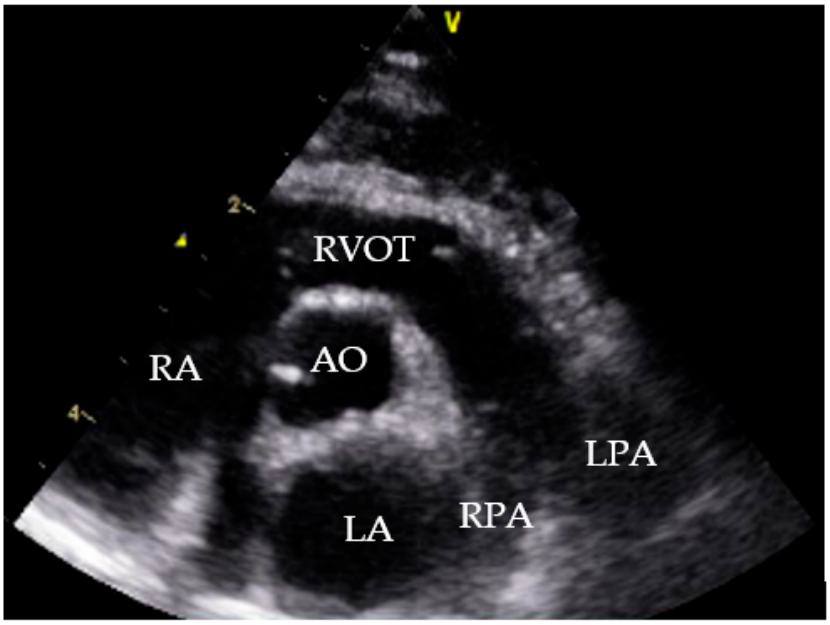

Figure 8. Echocardiogram July 2021, right parasternal short axis view. RA = right atrium; RVOT = right ventricle outflow tract; AO = aorta; LA = left atrium; RPA = right pulmonary artery, LPA = left pulmonic artery.

3. Discussion

Dirofilaria immitis has shown a rapidly increasing prevalence over the past few years, especially in Central Europe. FHWD can be detected in the same areas as canine heartworm disease at up to 20% of the rate in unprotected dogs [7,19]. The cat in this case report lives in Horitschon, which is approximately 4.5 km linear distance from the Hungarian border. Due to climate change and abundance of mosquito vectors, the development and transmission of *D. immitis* and *D. repens* has increased over the past few years and Hungary now also belongs to one of the endemic areas in Europe [20]. Male cats are reported to have a larger home range than female cats. It is reported that desexing male cats should decrease their home range, because their behaviour will be focused more on foraging than mating [21]. In a study with fourteen house cats, the home range for wandering cats was 5.1 ha. The longest linear distance travelled by a house cat in this study was 1.17 km. In another study from 2015, the home range was between 2.66 and 5.52 ha for free-ranging farm cats. One study of 2020 including 925 cats, only three cats exceeded the usual home range of less than 1 km^2 [22–24]. In this case report we cannot completely rule out that the cat crossed the Hungarian border and got infected there or that mosquitos crossed the border and infected the cat at its home farm, even if it seems improbable. As part of a study showing the incidence for *D. immitis* in shelter dogs and mosquitoes, 205 mosquito species were trapped in Austria and 115 dogs were tested for *D. immitis* infection. Forty-six of these mosquitoes were found in Burgenland. In none of these 205 mosquitoes has DNA of *D. immitis* been found to date but several dogs in a local shelter, all originating from Hungary, tested positive for *D. immitis* [25].

At initial presentation, the cat showed dyspnoea, ascites and double hyperechoic parallel lines in the echocardiography, which is the typical presentation for *D. immitis* in echocardiography [26]. The sensitivity of echocardiography for detection of *D. immitis* is operator-dependent and is reported between 88% and 100%. False positive results can be caused by right ventricular chordae tendineae [27].

As a differential diagnosis, *Angiostrongylus chabaudi* was assumed. At necropsies, immature nematodes of *A. chabaudi* can be found in the pulmonary arteries without evidence of L1 in faeces [28].

A. chabaudi was first described in 1957 in a wild cat in Central Italy. More case reports of cats with *A. chabaudi* in Greece, Romania, Italy, Bulgaria, Bosnia-Herzegovina and Germany

do exist but it is not known yet if angiostrongylosis caused by *A. chabaudi* is clinically relevant or not. Pathological lesions like granulomatous pneumonia, hyperplasia of pulmonal arteries and thrombosis are reported [29]. In our case report we assume that FHWD is the most likely diagnosis due to clinical presentation and findings in echocardiography. Currently no publications exist regarding *A. chabaudi* being detectable in echocardiography. In dogs, *Angiostrongylus vasorum* cannot be seen in echocardiography, so it also seems unlikely that *A. chabaudi* can be seen in echocardiography [30].

Another differential diagnosis with emphasis on the radiographic changes are infections with *Aelurostrongylus abstrusus* and *Troglostrongylus brevior*. The most common signs of aelurostrongylosis are dry or productive cough, dyspnoea, tachypnoea as well as weight loss, anorexia and fever. Pleural effusion or pneumothorax caused by *A. abstrusus* can lead to death. Secondary pulmonary hypertension is caused by the local inflammation triggered by parasite stages. Clinical presentation of cats with *T. brevior* is similar, although the nematode seems to be more pathogenic in kittens and young animals. Due to the paucity of clinical studies on this disease, knowledge on the radiographic features of troglostrongylosis is still poor [31,32]. To our knowledge, no publications exist regarding *A. abstrusus* and *T. brevior* being detectable in echocardiography, what makes these differentials unlikely.

The cat also showed right atrial as well as right ventricular enlargement, which underlined our suspected diagnosis of FHWD. These findings seem to appear quite rarely in cats and are commonly observed in dogs [1]. What is more, the cat showed diffuse bronchointerstitial lung pattern and enlarged pulmonary arteries in the thoracic radiographs, which were taken by the referral vet. These findings in the radiographs are also described as common changes in cats with FHWD although the diffuse parenchymal pattern can also occur in cats with asthma or aelurostrongylosis [33]. In this case report, no test for microfilaraemia was performed during initial diagnosis. Even using special techniques like the Knott's test microfilaraemia is only detected in less than 20% of cats with adult heartworms. When microfilariae are present, it is considered a definitive diagnosis for FHWD [34]. Antigen testing is still considered the gold standard. One disadvantage of serological testing is that antibodies and antigen circulate for an indeterminate length of time after the parasite has been cleared [35]. Antibodies against *D. immitis* are found two months post infection. False positive test results as consequence of clearing the infection can be found as well as false negative results in asymptomatic cats. Running antigen and antibody tests can improve the sensitivity compared to running one test alone [15,34]. In our case report, neither an antigen nor antibody test was performed due to the indication of FHWD in echocardiography and thoracic radiographs.

Usually, the worm burden is low and infections with only male or female adults reduce the sensitivity of the antigenic reaction. Detection of antigen cannot confirm the presence of immature stages of the parasite. A negative antigenic test cannot be the basis for ruling out an infection with *D. immitis*. Therefore, the result should be recorded as "no antigen detected" [1,10]. In this case, the cat's worm burden with 20 removed adult heartworms was unusually high. As part of a study, cats were experimentally infected with 100 L3 larvae, three to ten adult heartworms developed in 75% of the cats in this study population [1]. This high worm burden could either be due to multiple bites by infected mosquitoes or decreased immune response, as the cat was seropositive for FeLV. One study showed no association between heartworm infection and co-infection with FIV or FeLV. Male uncastrated cats had a higher risk of infection with heartworm, FeLV and FIV than females. Another study from 2017 found that cats with retroviral infections—especially FIV—had a marked increase of seropositivity for *D. immitis*. They postulate that this is not necessarily related to a relationship with heartworm infection but might be due to common predisposing factors, such as outdoor roaming. Contrary to this study, no apparent correlation with FeLV and FIV infection was noted in a study of 2011 [8,20,34]. In addition, the cat showed negative results for FeLV 16 months after surgery. This could be due to an abortive or regressive infection [36]. No further testing for FeLV provirus was

done. To our knowledge, a worm burden as high as was found in our subject has not been reported before.

The cat showed also severe hyperchromic, macrocytic regenerative anaemia. Anemia is described in cats and dogs with caval syndrome due to haemolysation [37].

In this case, we decided to remove the adult nematodes surgically. Acute death of cats can occur when even only one worm is present [1,10]. In most cases, the prognosis for caval syndrome is poor, so surgical removal seemed the only realistic chance for this cat. Different authors have described techniques for the removal of heartworms. We decided to use a transvenous approach through the right jugular vein. A limiting factor of this technique can be the body size of small cats. Major complications range from iatrogenic damage that results in thrombus formation, damage to the endo- or myo-cardium, tricuspid valve or chordae tendinae as well as iatrogenic damage that cause pneumothorax [37,38]. If a transvenous approach is not possible, right atriotomy using total venous inflow occlusion is prescribed. One advantage of this technique is the in-situ removal of heartworms, whereas removal with alligator forceps can break the heartworms and cause a shock-like reaction induced by the worm's body fluid. In addition, main pulmonary arteriotomy as well as right auriculotomy are described as therapy for cats with caval syndrome. The cat developed acute kidney failure postoperatively. Hepatorenal dysfunction is also reported in cats with *D. immitis*. It is associated with poor tissue perfusion and hyporexia of these organs. In necropsy of dogs with caval syndrome tubular necrosis and haemosiderosis was found [14,35,38].

DNA extraction of formalin fixed tissue is challenging, and usually only short fragments can be amplified. A study comparing two DNA extraction kits showed better results with the QIAamp DNA FFPE Tissue kit (Qiagen, Hilden, Germany), which is designed for DNA extraction from formalin fixed paraffin embedded tissue but is recommended for the use of tissue that has been fixed in formalin no longer than 24 h. In this study, up to 171 bp sequences could be amplified from historic formalin fixed tissue, albeit with low purity [39]. Considering this outcome, we did not expect successful amplification of the 202 bp sequence and did not attempt to extract DNA from the formalin fixed tissue any further. Recently published, more advanced protocols might overcome these limitations, but do require special equipment [40]. A definitive morphological identification was possible and comparable to other publications [41].

After surgery, the cat was treated with doxycline 5 mg/kg twice daily orally to target *Wolbachia*. *Wolbachia* spp. plays an important role in the survival of filarioid nematodes and gets amino acids for bacterial growth in turn. In dogs, pre-treatment with doxycycline before adulticidal therapy helps to reduce pulmonary pathology. In cats, this benefit has not yet been evaluated. That is the reason why doxycycline is not recommended as an adjunctive therapy in cats at the moment [1,34]. Clopidogrel was prescribed to prevent thromboembolism although there is lack of evidence in literature. In asymptomatic cats adulticidal therapy is not recommended due to the self-limiting infection within 18–48 months. Melarsomine, which is used as adulticidal therapy in dogs is not safe in cats and can trigger pulmonary thromboembolism and anaphylactic reactions as result of parasite death. Melarsomine is toxic to cats at doses as low as 3.5 mg/kg [1,10,15].

To prevent reinfection, the owner was advised to give milbemycin 4 mg/kg monthly following surgery. Monthly chemoprophylaxis is recommended from eight weeks of age year-round to kill L3 and L4 larvae. Ivermectin and milbemycin oxime, both administered orally, as well as topical moxidectin and selamectin can all be used for the prevention of FHWD [15].

Considering the lack of clinical signs and detectable abnormalities at clinical, sonographic and laboratory examination at 16 months post-surgery, the cat can be considered fully recovered. Coughing can be a long-term effect of HARD. Parasite death can be associated with severe pulmonary thromboembolism and eosinophilic inflammatory response in the lungs, causing HARD. Chronic, histologic evident myofibrocyte proliferation can be observed up to 18 months after infection [42].

In a study with asymptomatic cats with HWD, it was quite impossible to predict the outcome if the infection was diagnosed early. Three cats in this study died suddenly after 38–40 months post-diagnosis. In addition, if the duration of infection from diagnosis to death exceeds 1000 days, it is too long to implicate HWD as cause of death. Another retrospective study of symptomatic cats showed a median survival time of 1.5 years overall [43,44]. Our cat in this case report now nearly exceeds this median survival time.

The spreading of *D. immitis* worldwide due to climate change, globalization and increased travel of infected dogs is the reason Austria is facing the pre-endemic status [5,6]. This case report shows the first cat in Austria with an autochthonous *D. immitis* infection. Austrian veterinarians should be aware of the zoonotic potential of *D. immitis*. Increased and more intensive communication about prevention with owners living in close owner-pet-relationships is necessary. FHWD should be considered as a differential diagnosis if cats are living in border regions of surrounding countries. Macrocyclic lactones should not be used only for deworming (e.g., milbemycin oxime) but also for prevention of HWD.

4. Conclusions

The spreading of *D. immitis* worldwide due to climate change, globalization and increased travel of infected dogs is the reason Austria is facing pre-endemic status [5]. This case report describes what is potentially the first documented autochthonous *D. immitis* infection in Austria. In our case, a cat was infected with *D. immitis*. Furthermore, considering that the cat is not the natural reservoir of the parasite, it must be deduced that in the same area there were dogs infected by *D. immitis* and not diagnosed. Increased and more intensive communication about prevention with owners living in close owner-pet-relationships is necessary. FHWD should be considered as a differential diagnosis if cats are living in border regions of surrounding countries. Macrocyclic lactones should not be used only for deworming (e.g., milbemycin oxime) but also for prevention of HWD.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pathogens10091104/s1, Video S1: Surgical removal of *Dirofilaria immitis*.

Author Contributions: L.-M.K., V.J., M.P., M.S., M.L. were involved in the patient's diagnosis process and follow-up. L.-M.K. and M.S. wrote the manuscript. M.S.U., H.-P.F. and L.V. identified the *Dirofilaria* specimens. L.-M.K., M.S.U., M.L., H.-P.F., L.V., V.J., M.P. and M.S. revised the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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6.4. *Dirofilaria* spp. and *Angiostrongylus vasorum*: Current risk of spreading in Central and Northern Europe

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- merging the text
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HPF: conceived and planned the manuscript, organized the review, and drafted sections 1 and 4

SM: organized the review, and drafted sections 1 and 4

AB, DDS, RF, GG, MH, PJ, TK, ML, MM, DM, HHP, KS, AVR, MS, and CS: reviewed and drafted section 2 and 3, and provided information for section 4

All authors participated in preparing the final version of the article and have approved its content.

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Review

Dirofilaria spp. and *Angiostrongylus vasorum*: Current Risk of Spreading in Central and Northern Europe

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Abstract: In the past few decades, the relevance of *Dirofilaria immitis* and *Dirofilaria repens*, causing cardiopulmonary and subcutaneous dirofilariosis in dogs and cats, and of *Angiostrongylus vasorum*, causing canine angiostrongylosis, has steadily increased in Central and Northern Europe. In this review, a summary of published articles and additional reports dealing with imported or autochthonous cases of these parasites is provided for Central (Austria, Czechia, Germany, Hungary, Luxemburg, Poland, Slovakia, Slovenia, and Switzerland) and Northern (Denmark, Finland, Iceland, Norway, and Sweden) Europe. Research efforts focusing on *Dirofilaria* spp. and *A. vasorum* have varied by country,

and cross-border studies are few. The housing conditions of dogs, pet movements, the spread of competent vectors, and climate change are important factors in the spread of these nematodes. Dogs kept outside overnight are a major factor for the establishment of *Dirofilaria* spp. However, the establishment of invasive, diurnal, synanthropic, competent mosquito vectors such as *Aedes albopictus* may also influence the establishment of *Dirofilaria* spp. The drivers of the spread of *A. vasorum* remain not fully understood, but it seems to be influenced by habitats shared with wild canids, dog relocation, and possibly climatic changes; its pattern of spreading appears to be similar in different countries. Both *Dirofilaria* spp. and *A. vasorum* merit further monitoring and research focus in Europe.

Keywords: Central Europe; Northern Europe; *Dirofilaria immitis*; *Dirofilaria repens*; *Angiostrongylus vasorum*

1. Introduction

In the past few decades, arthropod-borne and gastropod-borne pet diseases have changed their distribution. A series of drivers, including wildlife-habitat reduction, urbanization, climatic changes, increased movements of pets traveling with their owners, and animal rehoming, have favored the geographical spread of specific arthropod-borne and gastropod-borne diseases within endemic areas, and their emergence in previously free areas [1–7]. In Europe, this has led to the modification of the epizootiological picture of diseases with key relevance in veterinary medicine, e.g., of cardiopulmonary and subcutaneous dirofilariosis caused by *Dirofilaria immitis* and *Dirofilaria repens*, respectively, and of canine angiostrongylosis due to *Angiostrongylus vasorum*, which have expanded their geographical distribution [4,8–13].

Canines are the main definitive hosts and primary reservoir of *D. immitis* and *D. repens* [9,14,15]. Nevertheless, these filarioids have moderate host specificity and are able to infect a wide range of vertebrates, also including cats and humans [9,14–19].

Adults of *D. immitis* live in the pulmonary arteries and, occasionally, the right chambers of the hearts of definitive hosts. After mating, females release first-stage larvae (L1), known as microfilariae, into the bloodstream. During the blood meal, microfilariae are picked up by mosquitoes, within which they develop to the third infective larval stages (L3). When feeding on vertebrate hosts, infected mosquitoes transmit L3, which undergo two further larval stages and then reach the adult stage with patency of six months [14,15]. Canine heartworm disease (HWD), caused by *D. immitis*, is usually a chronic cardiorespiratory disease that can be fatal if not treated [20]. Clinical signs include cough, exercise intolerance, dyspnea, and ascites; in severe cases, pulmonary hypertension, heartworm thromboembolism, and heart failure may occur [15,20]. Cats are less suitable hosts than dogs; they usually harbor a low number of adult *D. immitis*, and patent infections are rare [17]. In cats, the arrival and early death of immature adults in the pulmonary arteries causes a marked inflammatory response known as heartworm-associated respiratory disease (HARD), characterized by dyspnea, cough, anorexia, and vomiting [17,21]. Cats that survive the HARD phase can become subclinically infected until the death of adult heartworms, which may result in a sudden fatal outcome [17]. Human infections with *D. immitis* usually result in pulmonary granulomas known as "coin lesions", and can be asymptomatic or present with cough and nonspecific signs [14,22]. The available literature has no reports of human infections with microfilaremia.

The lifecycle of *D. repens* is very similar to the one of *D. immitis*, with the main difference represented by the final localization of the adult stages in the vertebrate hosts, i.e., the subcutaneous and intramuscular connective tissues [9]. Most canine infections are subclinical; in the case of the appearance of clinical signs, non-painful subcutaneous nodules, pruritus, erythema, alopecia, and papulae can be observed [23–25]. The disease in cats is similar, with pruritus, alopecia, erythema, and papulae as the most frequent clinical manifestations [24]. *Dirofilaria repens* has higher zoonotic potential if compared

to *D. immitis*, and thereby has public-health relevance in Europe [9]. Human infection with *D. repens* can present as subcutaneous nodules, mostly in the facial region, perioral and periorbital tissue, scrotum and testicles in men, and breasts in women; it is rarely microfilaremic [9,26].

Dirofilaria immitis and *D. repens* are traditionally endemic in Southern and Eastern Europe [9,10,27]. Nevertheless, a recent increase in the number of cases was reported in Central and Northern Europe, and adjacent regions for both nematodes [9–13,18]. Global warming is regarded as a key factor involved in the spreading of *Dirofilaria* spp., as it enhances the development of these filarioids inside mosquitoes [9,28].

Canine angiostrongylosis has been a predominant disease in canine veterinary medicine in the last 10 years. Similar to *D. immitis*, adults of *A. vasorum* inhabit the pulmonary arteries of definitive hosts [8]. After mating, females lay eggs that hatch and release L1, penetrate the alveoli, reach the pharynx, and then are swallowed and excreted with feces into the environment [8,29,30]. Thereafter, L1 penetrate or are ingested by a terrestrial gastropod (e.g., snails or slugs), within which they develop to L3. Dogs become infected when ingesting infected molluscs [29,31]. The diagnosis of canine angiostrongylosis is challenging for veterinary practitioners. Clinical pictures may be extremely variable, as (i) they range from subclinical to hyperacute, and (ii) infected dogs can display a plethora of different clinical signs that are both cardiorespiratory and nonspecific, gastrointestinal, neurological, and related to coagulation disorders [32–36]. The definitive host spectrum of *A. vasorum* is narrower than that of *Dirofilaria* spp., as it infects almost exclusively canids, with only few descriptions in other animals [8,37–40]. A single case of a natural non-patent infection was documented in a domestic cat [41], though the importance of feline angiostrongylosis due to *A. vasorum* or *Angiostrongylus chabaudi* is considered to be minimal at present, with patent infections reported only in wildcats [42,43]. At present, *A. vasorum* does not have any relevance to human health.

Several new records in both definitive and intermediate hosts documented a geographical expansion of *A. vasorum* in previously free areas, including the Iberian Peninsula [44,45] and Eastern [46–50], Central, and Northern Europe [51–54].

The geographical expansion of *Dirofilaria* spp. and *A. vasorum* in areas that were previously considered to be non-enzootic requires constant epizootiological surveillance (Figure 1). These changes could partly be attributed to the increased interest of scientists and the pharma industry (funding studies), also strongly contributing to the development of new treatment options. An increase in the awareness of local veterinarians and owners is warranted, as there is a lack of updated data in many countries, and dogs may be at risk of infection with both *Dirofilaria* spp. and *A. vasorum*, even in areas where their presence is currently unexpected. The situation is a challenge for the veterinary profession, and regarding the zoonotic *Dirofilaria* spp., also for medical profession, particularly in areas where the parasites have recently emerged or are not yet established [55,56].

Although the number of studies has increased in the last few years, epizootiological knowledge on *Dirofilaria* spp. and *A. vasorum* in Central and Northern Europe is still fragmentary. Therefore, the aim of the present study is to comprehensively review epizootiological data from countries of Central and Northern Europe in order to provide an updated and accurate picture on canine dirofilariosis and angiostrongylosis.

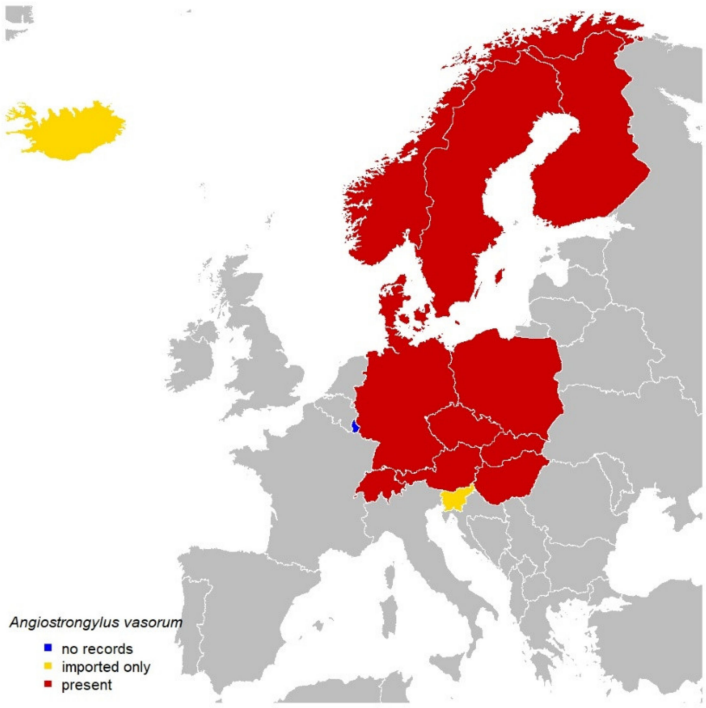

Figure 1. Distribution of *Angiostrongylus vasorum* in Central and Northern Europe.

2. *Dirofilaria* spp.

2.1. Central Europe

2.1.1. Austria

The first diagnosed cases of imported canine *D. immitis* infection in Austria were published in 1987 and 1988 [57,58]. The first documented canine *D. repens* infection and mixed dirofilarial infection in Austria, all of them imported cases, were published in 2001 [59]. Between 2000 and 2007, another six *D. immitis* and four *D. repens* infections in imported dogs were documented [60]. In a local survey in Eastern Austria in 2008, seven out of 98 canine blood samples tested positive for *D. repens* by PCR—two dogs were not reported to have had any stay abroad; this may have documented the first autochthonous infections [61]. Seven years later, a review on canine and human dirofilarial cases in Austria reported 37 dogs with *D. repens* infection (including the seven possibly autochthonous infections) and 25 dogs with *D. immitis* infection till 2014—a total of 62 cases within 18 years [62]. In the four following years, 84 more cases were documented till 2018, mostly *D. immitis* infections from imported dogs, and 10 additional dogs with coinfections of *D. repens* and *D. immitis* [63]. The most recent surveys in dogs and mosquitos in Austria focused on the possible risk for the development of new local endemic foci in and near Austrian dog shelters, and possible infections in kenneled military dogs. In total, 115 shelter dogs from 14 animal shelters located in five different Austrian states were examined in 2018 and 2019. Blood samples were screened for *D. immitis*, using rapid diagnostic devices (SNAP 4Dx Plus, IDEXX Laboratories, Inc., Westbrook, ME, USA), PCR, and microscopical examination for microfilariae. In total, 91.0% of the dogs originated from countries endemic for dirofilariosis. Eleven dogs (9.6%), all originating from Hungary, tested positive for *D. immitis*. All mosquitos (n = 205) trapped in animal-shelter proximity tested negative for *Dirofilaria* spp. by PCR. Of these mosquitos, 98.5% belonged to a species proven or even suspected to transmit *Dirofilaria* spp. [64]. In the Military Working Dog Training Centre in Eastern Austria, two of 96 dogs tested positive for *D. repens* – one from Hungary and one originating from Austria [65]. Moreover, the first autochthonous case of *D. immitis* was recently documented in a cat from Burgenland [66]. Neither *D. repens* nor *D. immitis* have yet been reported from wild canids in Austria (e.g., red foxes (*Vulpes vulpes*)) [67].

The first reported human case of subcutaneous dirofilariasis in an Austrian woman was published in 1981. It was assumed that this infection had been imported from the Mediterranean area [68]. In 2006, the first autochthonous infection with *D. repens* in an Austrian woman near the Hungarian border was diagnosed [69]. From 1978 to 2014, 33 cases of human dirofilariasis caused by *D. repens* were reported in Austria (30 cases), and three cases caused by *D. immitis*, rising to a total of 39 cases in 2020. Over the past four decades, incidence has markedly increased, particularly after 1998 [62,70,71]. In 2018, Austria was classified as endemic for *D. repens* (but not for *D. immitis*) with sporadic human cases [9].

D. repens in mosquitos in Eastern Austria was first detected by PCR in 2012. A low local prevalence was supposed, as two of 437 pools of collected mosquitoes close to the Hungarian border gave a positive result. All 18 individuals of one positive pool belonged to the *Anopheles maculipennis* (Meigen, 1880) group, and 14 individuals in the other positive pool were the *Anopheles algeriensis* (Theobald, 1903) species [72]. In 2013–2015, 45,848 mosquitos were sampled and analyzed for filarioid DNA by PCR. The DNA of *D. repens* was found in an *Anopheles plumbeus* mosquito close to the Slovakian border, confirming that *D. repens* is still endemic in low prevalence in Eastern Austria [73]. Potential invasive mosquitoes are competent vectors of *D. repens* and *D. immitis*. *Aedes japonicus* eggs were identified in Lower Austria, Styria, and Burgenland. *Ae. japonicus* was first found in Vienna in July 2017 during a routine sampling of adult mosquitoes [74]. A survey in Western Austria in 2018 found *Ae. albopictus* and *Ae. japonicus* eggs at highways and urban areas in both East and North Tyrol [75]. *Ae. albopictus* was first recorded in Vienna, Austria in August 2020. The species occurred in three sites within the capital city of Austria [76].

2.1.2. Czechia

Based on older data from the literature, the Czech Republic is regarded to be an endemic country of both *D. immitis* and *D. repens* species. However, while cases of *D. repens* are commonly reported in dogs in South Moravia, no recent reports of *D. immitis* are available. In the Czech Republic, *D. immitis* was only reported by Svobodová et al. [77,78]. This steeply contrasts the virtual absence of heartworm disease in the Czech Republic, and the autochthonous infection by the parasite has not been detected since then. Two recent studies [79] failed proving *D. immitis* in large sets of dogs examined by rapid diagnostic devices and PCR. Imported cases are sporadic and usually associated with imports of dogs from endemic regions in South Europe. Cases are not systematically reported by private vets.

Dirofilaria repens is well-known among veterinary clinicians in South Moravia, and its presence was also confirmed in mosquitoes [80]. Two studies reported human cases in the same region [81,82]. In the most recently published study, Miterpáková et al. [79] provided comparative data on distribution of *D. repens* in Slovakia and the Czech Republic, reporting 1.9% prevalence of *D. repens* in dogs from the Czech Republic. However, distribution shows a strong geographic pattern and is not homogenous; prevalence in endemic regions is higher. *Dirofilaria repens* is well-established in the domestic dog population in lowland areas along the Dyje and Morava rivers, extending northwards to the Kroměříž region. This distribution corresponds well with previously diagnosed cases of human subcutaneous dirofilariosis [81,82].

2.1.3. Germany

In Germany, reports on imported *Dirofilaria* spp. infections started at the end of the last century, although no epidemiological framework was usually given. For the period of 1991–1993, Leuterer and Gothe [83] identified *D. repens* and *D. immitis* infections in three and 12 dogs, respectively, which had been imported from or traveled to endemic areas. In 1993–1996, a total of 155 imported or traveled dogs were diagnosed with filarioid infections [84–87], 10 of them with *D. repens*, 115 with *D. immitis*, and one dog with a coinfection of *D. immitis* and *Dipetalonema reconditum*. Two other dogs were mono-infected

with *D. reconditum*, whereas for the remaining 27 Knott´s test-positive dogs, native blood smears were not available for histochemical filariae identification.

In the following years, reports on the occurrence of dirofilariae in Germany stopped until the first autochthonous case of *D. repens* infection was diagnosed in a Southwestern German dog in 2004 [88]. Three years later, further autochthonous *D. repens* infections were reported from a sledge-dog kennel in Northeastern Germany [89], and the parasite was also identified in three of 44 Southwestern German hunting dogs with no history of traveling [90]. With these reports, prevalence data on *Dirofilaria* spp. in Germany gained importance, and retrospective studies including several thousand samples detected 1.1–3.1% *D. immitis* antigen-positive imported or traveling dogs during 2004–2008 [91–94]. In a similar period (2004–2009), Knott´s test revealed microfilariae in 6.4–7.7% of dogs; however, no species differentiation was carried out [93–95]. In a study only including traveling dogs, none of the individuals tested positive for *D. immitis* antigen (380 dogs) or microfilariae (223 dogs) [96].

Additionally, autochthonous *D. repens* infections stimulated research on mosquito vectors. While no *Dirofilaria* DNA was found in more than 80,000 mosquitos collected throughout Germany between 2009 and 2010 [97], *D. repens* DNA was observed in 2011 in a pool consisting of *Culiseta annulata*; in 2012, in two pools of *An. maculipennis* s.l. and each one of *Anopheles daciae* and *Aedes vexans*; and in 2016, in one *Anopheles messeae* pool [98–100]. Furthermore, *D. immitis* DNA was amplified in two *Culex pipiens/torrentium* pools in 2012 [100]. Both *D. repens* and *D. immitis* were found in mosquitoes originating from Southwestern and Northeastern Germany, more precisely from the federal states of Baden-Wurttemberg, and Berlin and Brandenburg, from which the autochthonous *D. repens* infections in dogs were also reported. Moreover, both federal states were considered to be climatically suitable for dirofilarial development in the mosquito vector and classified as risk regions for stable endemicity [101–103]. However, DNA detection in the mosquitoes just proves that they had a blood meal on a (local) microfilaremic animal, but cannot be equated with established transmission cycles. Nevertheless, Sassnau et al. [104] reported that the number of *D. repens*–infected individuals in the sledge-dog kennel increased from five in 2007 to 11 in 2012. Likewise, in the German federal state of Saxony-Anhalt, which borders Brandenburg to the west, an autochthonous infection was diagnosed in a dog in 2010 [105]; in 2014, the first German autochthonous human case was reported [106]. However, the screening of 122 red-fox and 13 raccoon-dog (*Nyctereutes procyonoides*) lung samples from 2009 [107], and of 1023 dog-blood, 179 red-fox-blood, and 195 red-fox-spleen samples from 2013 and 2014 [108] did not provide evidence of endemic occurrence of *Dirofilaria* in Brandenburg.

The most recent data on filarial infections in Germany refer again to imported or traveling pets. In dogs imported between 2007 and 2015, a prevalence of 7.3% was found in the 178 tested individuals. Of the 13 positive dogs, eight were diagnosed with *D. immitis*, three with *D. repens*, one with *D. reconditum*, and no differentiation was performed in one dog [109]. In 133 German dogs traveling to endemic areas in 2007–2018, one dog (0.8%) became infected with *D. immitis* [110]. Fortunately, the first data on cats living in Germany have recently become available. Of the 618 cats subjected to *Dirofilaria* spp. PCR included in the feline travel profile, one (0.2%) tested positive, but no further species differentiation was conducted [111].

Overall, as the German climate allows for dirofilarial development in the mosquito vector [101–103], imported and traveling pets should be thoroughly monitored and, if positive, treated against dirofilariae to prevent the autochthonization of *D. immitis* and endemization of *D. repens* in Germany.

2.1.4. Hungary

On the basis of human cases reported since 1879 without confirming the identification of worms, Kotlán [112], Szénási [113] and others suspected that *D. repens* had been present in Hungary since the end of the 19th century. Although human dirofilariosis is not a

notifiable disease in the country, a few dozen ocular and subcutaneous dirofilarioses were reported during the last two decades [113–115].

The first autochthonous *D. repens* infections of dogs were described only at the end of the 1990s [116,117]. In nationwide epidemiological surveys [118–120], 11.1–19.6% of dogs and two cats were positive for *D. repens* microfilariae. Many infected dogs probably remain undetected due to the subclinical nature of the disease, and to the lack of rapid and reliable diagnostic tools. A significant cluster of microfilaremic dogs were found in the southern part of the country [120], where *D. repens* was the most frequent filarioid parasite in mosquito samples [121]. These veterinary reports confirmed that this nematode species is present in local dogs, representing a continuous risk of human infection in many regions of Hungary.

Heartworm infection was pathologically diagnosed only in dogs imported from the USA until 2000 [122,123]. The first autochthonous *D. immitis* infection was detected in a Hungarian Vizsla dog that lived in the eastern part of the country [124]. Since that time, the examinations of dogs [120,125–128], red foxes, golden jackals (*Canis aureus*) [129], and one ferret (*Mustela furo*) [130] revealed that *D. immitis* is endemic in the country, and the Great Hungarian Plain is hyperendemic. Mixed infections of dogs by both *Dirofilaria* spp. were also detected in some counties [120]. No human heartworm infection was diagnosed in Hungary.

It cannot be definitively excluded that *D. immitis* had been present in the country before the 21st century because no epidemiological surveys were carried out, and no reliable diagnostic methods were available earlier. However, it is more plausible that this filarioid species has only recently been introduced to Hungary, because neither its microfilariae nor adult worms were found in local dogs [118], and red foxes before [131]. Hunting dogs from Italy with patent heartworm infection may have acted as microfilarial reservoirs for the local mosquito population during their stay in the area, resulting in the development and transmission of infective L3 to native dogs. The role of infected wild canids arriving from neighboring countries might not necessarily be considered regarding the geographical distribution of heartworm infections in Hungary because only a few red foxes and two golden jackals were infected with a low number of worms without microfilaremia [129].

The occurrence and spread of both filarioid species in Hungary are not surprising, because the local climate and the abundance of mosquito vectors around their breeding sites offer suitable conditions for the development and transmission of these parasites. Stray dogs and dogs adopted from shelters pose an especially high risk in the epidemiology of both dirofilarioses because these animals are unlikely to receive proper examination and prevention.

2.1.5. Luxembourg

So far, there are no data in the literature on infections with *D. immitis* and *D. repens* in Luxembourg. To the authors' knowledge, no specific studies have been conducted on *D. repens* in Luxembourg. Between 2014 and 2020, the first serological tests were carried out, using the rapid diagnostic device to detect antibodies against *D. immitis*, among others. Serum from road-kill red foxes (n = 50) and raccoons (*Procyon lotor*) (n = 81) was analyzed across Luxembourg. All the tested red foxes and raccoons were negative. Given the presence of mosquitoes that can serve as vectors in Luxembourg (*Cx. pipiens* s.l. and *Ae. vexans* [132]), further studies should be conducted.

2.1.6. Poland

The first autochthonous *D. repens* infections of dogs were reported in Central Poland, Mazovia between 2009 and 2011 [133–136]. Since then, the number of reported cases has been growing [137,138]. Epidemiological studies carried out in 2014 in the canine population in Mazovia revealed a surprisingly high prevalence of *D. repens*, especially in dogs from suburban and rural areas [139,140]. In a study of Demiaszkiewicz [139], 462 dogs aged 1.5–14 years were examined, using the Knott method. Microfilariae of *D. repens* were found in the blood of dogs

originating from the city of Warsaw and from 18 districts of Mazovia (Mazowieckie). Overall prevalence was 25.8%. The highest prevalence (53.0%) and the highest intensity of infection were found in the Radom district (Southern Mazovia). In a study [140] among sled dogs living in Mazovia sampled between 2010 and 2013, *D. repens* DNA was detected in 15 of 34 dogs (44.0%). Prevalence was especially high (50.0–57.0%) in two sled-dog kennels situated near Grodzisk Mazowiecki (Southern Mazovia).

In a nationwide epidemiological study [141], 1588 dogs were examined for dirofilariosis. *Dirofilaria repens* microfilariae were found in 11.7% of the blood samples of dogs originating from all 16 provinces of Poland. The highest prevalence (25.8%) was found in Mazovia, Central Poland. About 12.0–16.0% of dogs were positive in Eastern Poland, while much lower prevalence was noted in western and northern areas of the country.

In another study [18], 147 blood samples from cats from Central Poland, and 257 blood samples from dogs from Central, Northern, Southern, and Western Poland were collected in the period of 2013–2015. No positive dogs were noted from Kraków (Southern Poland), Wrocław (Western Poland), and Gdańsk (Northern Poland). The DNA of *D. repens* and/or *Wolbachia* was identified in two cats (1.4%) from Central Poland. The DNA of *D. repens* was detected only in dogs in Mazovia (38.0%).

In the most recent studies, the prevalence of *D. repens* in dogs from the area of Poland was about 12.0% (2017, 2019, and 2020) [11,142]. This decrease in prevalence was accompanied by increased awareness of this parasitosis, both among dog owners and veterinary practitioners, and may reflect the increased application of preventive measures during the season of mosquitoes activity [11]. The DNA of *D. repens* was detected in samples containing a mixture of *Cx pipiens* and *Ae. Vexans* mosquitoes, collected in Mazovia during the summer months of 2010–2012 [143].

The awareness and endemic status of dirofilariasis due to *D. repens* have risen and been confirmed only in the last decade, following the recognition of autochthonous cases in dogs and humans. The first cases of human dirofilariasis (*D. repens*) preceded the reports on *D. repens* in dogs, and were between 2007 and 2009 [144–146]. In 2012, a paper reviewing the cases of dirofilariasis was published [147]. Between 2007 and 2011, a total of 18 *D. repens* infections were detected in humans in Poland. Parasitic lesions were located in various parts of the body in the form of subcutaneous nodules containing single nematodes surrounded by granulation tissue (15 cases). In three cases, subconjunctival localization was found. Of the 18 described cases, 17 were in Central Poland. In this area, autochthonous infections were identified in three women who had never left Poland. The first was found in 2010 in Grójec, and the next two in 2011 in Białobrzegi and Warsaw [147]. Since that time, the number of published human cases has increased, with reports on the unusual localization or manifestation of *D. repens* [148–151].

In 2012, the first, likely autochthonous, case of *D. immitis* infection was recognized in a dog in Gdynia, Northern Poland [152]. No additional cases have been reported to date, imported or autochthonous [142,148]. Results of three epidemiological studies revealed very low or zero prevalence: in the largest study in 2014, 3094 healthy dogs from the area of Poland were tested by rapid diagnostic devices. Only 0.2% of the samples tested positive (n = 5), with no information on clinical signs or origin (imported vs. autochthonous) of dogs [153].

In 2019, a rapid diagnostic device test was carried out on 167 healthy sled dogs from Lithuania (n = 46), Latvia (n = 24), Estonia (n = 20), and Poland (n = 35), and on 42 healthy pet dogs from Poland, including 20 dogs positive for *D. repens* [11]. No positive results were obtained, and no cross-reaction with *D. repens*–infected dogs was detected.

In 2020, 160 dogs from Eastern Poland were tested for *Dirofilaria* spp. (PCR, rapid diagnostic device). These dogs were selected on the basis of demographic features (kept outdoors, no ectoparasite prophylaxis) and the presence of clinical signs compatible with *D. immitis* infection (exercise intolerance, cough). Microfilariae of *D. repens* were identified by PCR in 20 dogs (prevalence, 12.0%), but no samples tested positive for *D. immitis* [142].

2.1.7. Slovakia

In Slovakia, autochthonous canine dirofilariosis was recorded for the first time in 2005, when *D. repens* was confirmed in 13 dogs, and *D. immitis* in two other dogs from Bratislava and Komárno districts situated in the southwestern part of the country bordering Austria and Hungary. All infected dogs were asymptomatic [154].

In February 2007, the first monitoring covering two areas of Southwest and Southeast Slovakia was carried out. The study, encompassing 287 dogs of different age, gender, and breed, revealed microfilariae in 99 dogs, representing an overall prevalence of 34.5%. In all positive dogs, *D. repens* was detected, and in six of them, coinfection with *D. immitis* was confirmed. Only in seven dogs could an autochthonous source of the infection not be unambiguously evidenced, and only in four infected dogs was a health state alternation, including dermal changes, observed. Within this research, the utilization of dogs was revealed as an important risk factor for the infection. Police, guard, and hunting dogs, with prevalence rates of 51.1%, 50.0%, and 40.0%, respectively, were more often found to be infected when compared with companion dogs (an average prevalence rate of 7.8%). On the basis of this study, in the territory of Slovakia, highly endemic areas of *D. repens* were identified [155].

Between September 2007 and February 2010, a monitoring program of canine dirofilariosis aimed at working (police and military) dogs was performed in Slovakia. All 710 (591 police and 119 military) dogs from all Slovak regions were examined for *Dirofilaria* spp. presence. Microfilariae were detected in blood of 128 (18.0%) dogs (118 police and 10 military). DNA analyses revealed *D. repens* mono-infection in 125 dogs and mixed *D. repens*/*D. immitis* infection in three dogs. This survey confirmed the highest prevalence rates in southwestern parts of Slovakia identified as endemic for *D. repens* in previous study. In all infected dogs, the autochthonous origin of the infection was acknowledged. Evaluating the questionnaire data, it was highly presumable that the majority of the examined police dogs had become infected during their stay in training and breeding centers situated in the endemic area of Western Slovakia [156].

A comprehensive study summarizing research of canine dirofilariosis in Slovakia between 2005 and 2015 was published in 2016 [157]. During the 10-year study, a total of 4043 dogs from all Slovak regions were examined for *Dirofilaria* spp. Microfilariae were found in the peripheral-blood system of 450 dogs, representing an average prevalence of 11.1%. DNA analysis confirmed *D. repens* mono-infection in 440 animals, mixed *D. repens* and *D. immitis* infection in nine dogs, and one dog was infected only with *D. immitis*. The spatial distribution of *Dirofilaria* spp. showed significant regional differences. The highest above-average prevalence rates were steadily recorded in the southern regions of Nitra (over 25.0%), Trnava (18.4%), and Košice (12.7%). In the northern regions of Slovakia (Žilina and Prešov) bordering Poland, prevalence ranged between 2.0% and 4.0% [157].

An independent serological study tested newly developed commercial rapid diagnostic devices in 2015. During this study, blood and sera from 180 dogs originating from the southwestern and southeastern regions of Slovakia were investigated for the presence of microfilariae and circulating *D. immitis* antigen. Microfilariae were observed in 12 of 180 examined dogs, and subsequent DNA analyses confirmed *D. repens* in all the positive samples. In parallel, using the rapid diagnostic device, circulating *D. immitis* antigens were detected in the serum samples of five dogs (2.8%). In two *D. immitis*-seropositive dogs, microfilariae of *D. repens* were also found. Regarding DNA analyses not revealing *D. immitis* presence, all five cases can be considered to be an occult form of the infection. One of the *D. immitis*–positive dogs came from Southeast Slovakia, and the remaining four from Komárno district, in the southwest, where *D. immitis* was confirmed in previous studies [158].

However, after 2015, an evident increasing trend of *D. immitis* cases in Slovakia has been observed. The first outbreak of heartworm infection was recorded in a dog-breeding establishment in the district of Dunajská Streda, Trnava region, near the border with Hungary. Out of 25 examined dogs (22 Newfoundlands, two Central Asia shepherd dogs,

and one Sarplaninac), dirofilariosis was diagnosed in 18 animals (72.0%), using several different diagnostic approaches (Knott test, DNA analysis, histochemical staining, rapid diagnostic device). Ten of the infected dogs were positive only for *D. immitis*, two for *D. repens*, and mixed infection was confirmed in six dogs. Occult *D. immitis* infections without circulating microfilariae were recorded in six dogs. No dogs showed clinical signs of heartworm disease. Regarding travel history, the autochthonous origin of the infection could unambiguously be confirmed in seven dogs [159].

The first registered fatal case of canine heartworm disease was recorded in 2019. In two seven-year-old Tibetan Mastiff siblings from the Košice region, Southeast Slovakia, raised in the same household, *D. immitis* was confirmed. The course of the infection in the two dogs markedly differed. Although the female dog manifested no health-status alternation, the male dog exhibited severe clinical signs, including elevated creatinine and urea levels, increased liver hyperechogenicity, and hepatomegaly. The dog died five days after hospitalization. Subsequent postmortem examination revealed adult *D. immitis* worms in the right heart ventricle [160].

The most recent epidemiological study on canine dirofilariosis in Slovakia was carried out in late 2019. Within the study, 644 randomly selected dogs were examined for the presence of *Dirofilaria* spp. Microfilariae were present in 68 blood samples with an overall prevalence of 10.6%. Subsequent DNA analysis confirmed *D. repens* mono-infection in 38 (5.9%) dogs, a single *D. immitis* infection in 21 (3.3%) animals, and both *Dirofilaria* species were detected in nine (1.4%) samples. These data indicate an increasing number of *D. immitis* cases in Slovakia, previously considered to be endemic only for *D. repens* [79].

In Slovakia, besides dogs, the presence of *D. repens* DNA was confirmed in the spleen samples from one individual of beech marten (*Martes foina*) and red foxes [161,162]. The results of the study showed 105 of the 183 examined red foxes being infected, representing an overall prevalence above 57.0%.

Regarding *Dirofilaria* vectors, research focused on mosquitoes is still in its infancy and mostly regionally oriented in Slovakia. The first screening for dirofilariosis in mosquitoes was performed in 2013 in Eastern Slovakia, and showed that the *Ae. vexans* species was incorporated into the life cycles of both *D. repens* and *D. immitis* [163,164]. During the next xenomonitoring carried out in Bratislava, Western Slovakia, *D. repens* was detected in *An. Messeae*, *An. maculipennis*, and *Cx pipiens* complexes, and *D. immitis* in *Coquillettidia richiardii* and *Cx. pipiens pipiens*. Both dirofilarial species were also found in *Ochlerotatus sticticus* [165].

The first case of human dirofilariosis in Slovakia (at that time Czechoslovakia) was reported in 1992, when the presence of a wormlike formation in the vitreous body of a patient was discovered at the ophthalmological examination. Nevertheless, retrospective view of this case reveals some doubts about the diagnosis [166]. The first autochthonous and unambiguously confirmed case of human dirofilariosis was reported in 2007, two years after the first finding of dirofilarial parasites in dog population. Since then, between 2007 and 2020, 23 cases (subcutaneous, ocular, and pulmonary) were confirmed in Slovakia. In all cases, *D. repens* was validated as the causative agent [167,168].

2.1.8. Slovenia

The first case of *D. immitis* in Slovenia was recorded in 1986. A clump of nematodes was found in the right ventricle and pulmonary artery of only one dog. The researchers hypothesized that a factor in the spread of dirofilariosis in Slovenia might be dogs imported from Italy and tourism flows with pets, which led them to expect an increase in the number of infected dogs. They cautioned that, with these epidemiological data, human cases should also be expected. They assumed that, since *D. repens* was present in neighboring Italy, its occurrence in Slovenia should also be expected [169]. The following year, the first case of the subcutaneous form of dirofilariosis in a red fox, caused by *D. repens*, was described in Slovenia by Brglez and Verbančič [170]. They found a high number of mature and juvenile parasites in a red fox killed on the road. In 1998, a human case of subcutaneous

dirofilariasis was described in the occipital region of a 61-year-old Slovenian woman, caused by *D. repens*. The authors identified a trip to Canary Islands, Spain as the probable site of infection, because the subcutaneous tumor was diagnosed seven months later. The authors suggested that human cases of dirofilariasis are most likely under-reported, as many cases are undiagnosed or unpublished [171]. Currently, there is no officially reported number of human cases of dirofilariasis in Slovenia. A study of imported canine filarioid infections in Germany from 2008 to 2010 reported that *D. repens* was found in a dog imported from Slovenia. Although no prevalence studies of dirofilariosis in dogs were available, the authors considered Slovenia to be endemic for *D. repens* [92]. Currently available data from the Institute of Microbiology and Parasitology at the Veterinary Faculty of the University of Ljubljana show that, out of 400 blood samples from dogs acquired and tested for *Dirofilaria* spp. between April and October 2018, only two were positive for this parasite (Vergles Rataj, personal communication).

2.1.9. Switzerland

Due to the perceived spread of *D. immitis* in the USA in the 1960s [172], the presence of the parasite in countries neighboring Switzerland, such as Italy [173,174] and France [175,176], and the report of an imported case in Germany [177], in a review article, Thun (1975) alerted Swiss veterinarians about the relevant aspects of *D. immitis* infections in dogs [178]. At the end of the 1980s, the first imported cases of canine dirofilariosis were diagnosed at the Institute of Parasitology, University of Zurich by the detection of circulating antigens and characterising microfilariae by acid phosphatase activity. The first clinical case of *D. immitis* dirofilariosis was diagnosed at the Animal Hospital of the Veterinary Faculty in Zurich, in a Siberian husky living in Milan (Italy) [179]. While clarifying the situation of another husky living in the same kennel, this dog was negative for *D. immitis* but positive for microfilariae of *D. repens*, thereby representing the first diagnosed and imported case of cutaneous dirofilariosis in a dog in Switzerland. Moreover, two stray dogs of unknown origin were diagnosed positive for *D. immitis* in Ticino, Southern Switzerland, and several dogs originating from the Mediterranean basin were diagnosed positive for *D. immitis* or *D. repens* at the Institute of Parasitology in Zurich [179]. In a follow-up study in which 217 stray dogs and 154 unwanted dogs from Ticino had been investigated, microfilaria were isolated from the blood of four dogs; these were confirmed as *D. immitis* by morphology and antigen detection. In all these cases, the import of dogs from Italy could not be excluded, thus not confirming the autochthonous presence of *Dirofilaria* spp. in the country. However, on the basis of further mentioned cases diagnosed close to the border with Italy and in suitable temperatures for the development of *D. immitis* in mosquitoes in the same area, the establishment of the parasite was anticipated [180]. In fact, when testing 479 blood samples from that region, three (0.6%) and eight (1.6%) dogs were positive for *D. repens* and *D. immitis*, respectively. For a single dog, local transmission was confirmed by excluding traveling abroad by the owner [181]. The investigation of dog samples from both sides of the borders, Switzerland and Italy, confirmed higher prevalence in Italy, while contemporaneously identifying four dogs positive for *D. repens* (n = 2) and/or *D. immitis* (n = 3) from Ticino. Due to the limited number of cases in Southern Switzerland despite the widespread presence of suitable vectors [182,183], Southern Switzerland is considered as the border of the endemic area of both *Dirofilaria* spp.; therefore, prevention measures were recommended and are currently regularly implemented. These include the treatment of all infected dogs with microfilariae in order to decrease the risk of transmission [184]. To our knowledge, no autochthonous case of dirofilariosis north of the Alps was determined (Deplazes, personal communication). In fact, most of the cases have a clear history of import [185] or traveling.

Similarly, three human patients of dirofilariasis diagnosed in Swiss hospitals originated from abroad (India) [186], had a travel history to the Mediterranean area [187] or to Southern Switzerland and Northern Italy [188]. Different organs were affected: the epididymis in the first case, pulmonary nodules in the second, and the subconjuctival tissue

in the third patient. Two were confirmed as caused by *D. repens*, and one was attributed to *D. immitis* (pulmonary nodules), but not confirmed by laboratory techniques.

2.2. Northern Europe

Results of a questionnaire study among veterinarians showed that 11.0% of the participating veterinarians who were practicing in Nordic countries reported having seen dogs with *D. immitis* infection, 3.0% reported having seen dogs with *D. repens* infection in 2016, and a majority of the cases were reported to be in dogs with a history of travel or import [56]. The situation was very different to that in the nearby Baltic countries, in particular regarding *D. repens*: almost a fourth of veterinarians practicing in Baltic countries reported having seen dog(s) with *D. repens* infection, and none of these had a history of travel or import [56]. *Dirofilaria repens* emerged in the Baltic countries in 2008–2012 and became endemic [11,13,189,190].

2.2.1. Denmark

There are no published reports of *Dirofilaria* spp. in animals in Denmark, while a human case of *D. repens* in a 39-year-old woman was reported in 2014. However, the woman was likely infected on Crete [191]. The vectors of both *D. repens* and *D. immitis* are present in Denmark (Huus Petersen, personal communication) and, during a period of 15 years, two locations in Denmark reached in July at least once the 130 heartworm development units (HDU, the total environment heat required for the development of *Dirofilaria* from microfilaria to infectious L3 within the mosquito), but none of them reached the 130 HDU based on average temperature [192]. There are no published records of surveillance studies on dirofilariosis in wild-living canines from Denmark, and to the authors' knowledge, no surveillance studies have been performed.

2.2.2. Finland

There is one published case report of autochthonous human *D. repens* infection from Southeast Finland from 2015 [193]. There are no published reports of autochthonous *D. repens* nor *D. immitis* infections in dogs from Finland. The first *D. repens* finding in an imported dog was in 2014 [193]. *Dirofilaria ursi* is present in brown bears (*Ursus arctos*) in the eastern part of Finland, but no human cases have been reported [193,194]. During a period of 15 years several places in Finland reached in July at least once the 130 HDU, but none of them reached the 130 HDU based on average temperature [192].

2.2.3. Iceland

There are no reported cases of *D. immitis* or *D. repens* from Iceland. Moreover, competent mosquito vectors are not present on Iceland.

2.2.4. Norway

The first published case report, published in 1991, of apparently imported human *D. repens* infection in the Nordic countries was from Norway [195]. *Dirofilaria repens* infection was reported in dogs imported to Norway from South Africa [196] and Hungary [197]. *Dirofilaria immitis* was also reported in imported dogs [198]. During a period of 15 years, some places in Southern Norway reached in July at least once the 130 HDU, but none of them reached the 130 HDU based on average temperature [192].

2.2.5. Sweden

Endemic cases of *Dirofilaria* spp. have not been found in Sweden to date, but in addition to *A. vasorum*, these infections are notifiable, and the following cases were reported during the last five years: three cases in 2015, two cases in 2016, no cases in 2017, two cases in 2018, and seven cases in 2019 [199,200]. Unfortunately, *Dirofilaria* spp. infection is reported at the genus level, i.e., the record of notified cases is not discriminating between *D. immitis* and *D. repens*, so it is not possible to describe which of the two parasites has been

diagnosed more often. According to the most recent studies of the northward expansion of *Dirofilaria* infection in Europe, during a period of 15 years some places in Sweden reached in July at least once the 130 HDU, but none of them reached the 130 HDU based on average temperature [192].

3. *Angiostrongylus vasorum*

3.1. Central Europe

3.1.1. Austria

A. vasorum was detected in gastropods in Austria in two studies [51,201]. One study investigated their occurrence in 1320 gastropods collected in the Austrian provinces of Styria, Burgenland, Lower Austria, and in metropolitan Vienna. Metastrongyloid larvae were microscopically detected in 25 samples, and sequence analysis confirmed the presence of *A. vasorum* in one slug (*Arion vulgaris*; 0.1%) [51]. The first cases of canine angiostrongylosis reported in Austria were imported from endemic areas of France [202,203]. In a retrospective study, 1040 fecal samples of Austrian dogs were analyzed by using the Baerman method [204]. L1 of *A. vasorum* were documented in 1.3% of the dogs originating from Vorarlberg (Western Austria), Styria (Southeastern Austria), Lower Austria, and Vienna (Northeastern Austria). Moreover, 1.2% of 1279 dogs were positive for specific antigens, and 1.5% for specific antibodies at serological tests. These dogs originated from all Austrian provinces (with the exception of Burgenland), namely Lower Austria, Upper Austria, Vienna, Styria, Carinthia, Salzburg, and Tyrol [204]. However, although helminth L1 antigens and antibodies were reported at many locations, these data indicate a very low prevalence of *A. vasorum* in dogs in Austria [204].

3.1.2. Czechia

The occurrence of *A. vasorum* was not surveyed on larger sample set. A relatively recent study by Hajnalová et al. [205] found 4.7% of dogs (nine of 193) to be positive for circulating antigen by ELISA. L1 were detected in one of the 253 examined dogs. Infection is sporadically detected in necropsied red foxes; however, systematic research is not conducted. On the basis of feedback from small-animal practitioners, *A. vasorum* is not yet considered an issue, though conditions for the transmission are ubiquitous.

3.1.3. Germany

The first reference on angiostrongylosis in Germany describes phagocytosis of the parasite by giant cells in a histological section of a five-year-old royal poodle suffering from verminous pneumonia caused by *A. vasorum*, necropsied in 1964 [206]. Shortly thereafter, angiostrongylosis was reported in a four-year-old dachshund that was euthanized in 1965, due to incurable heart damage [207]. In both cases, nothing is mentioned about a possible travel history, so it remains unclear whether the dogs had traveled or not. A few decades later, in 2003, another case was diagnosed in a Southern German dog living on the border to Switzerland. On the basis of anamnestic data, the authors considered an autochthonous infection [208], whereas subsequently published cases reported an import from or travel history to France, Italy, or Portugal [209,210].

The first German prevalence data are available from 1999 to 2002, where *A. vasorum* L1 were found in 0.3% of diagnostic dog fecal samples [211]. In the following years, prevalence increased to 0.9% in 2010 and 1.6% in 2016 [212,213]. *A. vasorum* occurrence spatially clustered in Southwest Germany. This pattern was also observed in a seroprevalence study resulting in 0.5% antigen-positive and 2.3% antibody-positive dogs; however, the vast majority of samples originated from West German federal states [214]. In a study conducted in Central Germany, 1.2% clinically healthy sheep-herding dogs tested coproscopically positive [215].

In dogs with clinical signs indicative for lungworm infection, *A. vasorum*-positive fecal samples ranged between 1.1% and 7.4% [216–219]. The most recent study on lungworm-suspected dogs evaluating more than 12,000 fecal samples reported both a significant

increase in *A. vasorum* prevalence, and an accumulation of positive dogs in Northeast and Southwest Germany, indicating a potential spread or awareness of the parasite (from these parts of Germany, autochthonous *D. repens* infections were also reported; see above section). A study in red foxes confirmed the endemicity of *A. vasorum* in Northeast Germany by DNA detection in 9.0% of the lungs [107]. Nevertheless, the prevalence of 27.0% in red foxes in Rhineland-Palatinate [220] still reveals Southwest Germany to be a highly endemic region [221].

Lastly, the increasing *A. vasorum* prevalence and the accumulation cluster in Southwest and Northeast Germany raise the question of what happens in the intermediate parts of the country. New data are desirable, especially as samples from these intermediate regions were underrepresented in all studies evaluating geographical distribution.

3.1.4. Hungary

In 1960, Kotlán [222] first mentioned the sporadic occurrence of *A. vasorum* in a dog and red foxes in Hungary. A few decades later, angiostrongylosis was found in red foxes [129,131] and golden jackals [49], indicating that wild canids play an important role in the distribution and establishment of this parasitic species, since the mollusc intermediate hosts are broadly distributed in Hungary [223]. The first cases of dogs infected by *A. vasorum* were two asymptomatic animals kept in gardens close to the Croatian border, where five slugs were found carrying larvae of this parasitic species [224]. These infections were considered autochthonous because both dogs were born where they lived and never left their villages. In a large-scale combined serological survey of 1247 pet dogs, 1.4% of them were positive by two ELISA [225]. A considerable number of cases were observed in Budapest, and in the southern part bordering Croatia. The results of this serological survey confirmed the endemic occurrence of *A. vasorum* in dogs in different parts of Hungary.

3.1.5. Luxembourg

There are no reports in the literature on the presence of *A. vasorum* in Luxembourg. In a current study (Heddergott, personal communication), 27 fresh road-kill red foxes, mainly from the eastern part of the country (administrative districts Diekirch, Echternach, and Grevenmacher), were examined for infection with *A. vasorum*. At necropsy, the heart, lungs, and adjacent vessels and from the rectum of the cadavers were taken. The genetic diagnosis of fecal samples was performed by SAF technique, *Giardia* and *Cryptosporidium* coproantigen ELISAs, and by duplex copro-PCR. All examined red foxes were negative.

3.1.6. Poland

The first finding of *A. vasorum* in Poland (northeast, Augustowska Primeval Forest) was in red foxes in 2013 [226]. Adult nematodes were found in 4/76 red foxes (5.0%). In 2014, the first clinical case was described in a dog in Lublin (Eastern Poland) [227].

In a large epidemiological study conducted in 2013, the sera of 3345 healthy dogs from veterinary clinics all over the country were tested; specific antibodies against *A. vasorum* were found in 60 animals (1.8%), and parasitic antigens in 43 dogs (1.3%) [228].

In another study, *A. vasorum* L1 were detected by using coproscopic methods in 7.0% of the 58 fecal samples of grey wolves from the Bieszczady Mountains (Southeast Poland) [229].

3.1.7. Slovakia

The first autochthonous case of canine angiostrongylosis in Slovakia was officially reported in 2013 in a seven-month-old Maltese pinch dog in Košice, southeastern part of the country [230]. The physical examination revealed no remarkable clinical signs in the patient [230].

In the same year and location, *A. vasorum* was diagnosed in an 18-month-old Bernese mountain dog. The infection was accompanied by serious clinical signs and almost fatal course; inter alia, irritating cough, dyspnea, vomiting, bilateral scleral bleeding, and acute

physical collapse were observed in the patient. The infected dog excreted L1 in high numbers (more than 800 L1 were counted in 10 g of feces) [231].

On the basis of these first cases, a serological survey was conducted to assess the current distribution of *A. vasorum* in the dog population of Slovakia. Serum samples from 225 dogs originated from 22 districts were tested by ELISA for the presence of circulating *A. vasorum* antigens and for the detection of specific antibodies. Fourteen (6.2%) dogs were positive in at least one ELISA; seven dogs (3.1%) were only antibody-positive, four animals (1.8%) were positive only for circulating *A. vasorum* antigen, and three individuals (1.3%) were positive in both ELISAs. Seropositive dogs came from different regions with the highest accumulation of the cases in Southwest Slovakia. Three dogs positive for circulating antigen and specific antibodies originated from Bratislava region on the border with Austria [232].

Another survey based on the Baermann technique and modified-flotation method revealed that 14 of 339 (4.1%) examined dogs had been infected by *A. vasorum* [233].

A rare case of canine angiostrongylosis was described in 2019, when *A. vasorum* was detected in the anterior eye chamber of an 18-month-old beagle from the northeastern part of Slovakia. The dog's feces were examined for the presence of L1 with negative results, but the final diagnosis was confirmed by DNA analyses and sequencing [234].

The circulation of *A. vasorum* in populations of free-living carnivores was confirmed in two independent surveys. Between 2014 and 2016, 571 fecal samples from red foxes, originating from all Slovak regions, were investigated for L1 of *A. vasorum*. The parasitic presence was confirmed in 31 animals, representing an average prevalence of 4.4%. In five positive red foxes, infection with *Crenosoma vulpis* was also diagnosed. Within this study, the potential influence of selected environmental variables on the occurrence of *A. vasorum* was quantified, using logistic regression. The distribution of *A. vasorum* showed typical spatial clustering and occurred in endemic foci mainly in the eastern part of Slovakia. A cluster of *A. vasorum* infection foci was found in both humid and the driest areas of Slovakia. A multivariable model for *A. vasorum* also revealed tendency of the parasite to prefer areas with higher shares of arable land and lower proportions of forests [48].

Besides the red fox, the grey wolf (*Canis lupus*) is considered to be another suitable reservoir host for *A. vasorum*. Between 2015 and 2016, the first systematic parasitological examination of the wolf population living in two national parks and in one protected landscape area of Slovakia was carried out. Overall, 256 wolf fecal samples were gathered and examined for *A. vasorum* presence, using the modified-flotation method with zinc sulfate solution. *Angiostrongylus vasorum* L1, subsequently confirmed by DNA analysis, were detected in two samples, in one wolf originated from Tatra National Park and in one individual from Pol'ana Protected Landscape Area [235].

3.1.8. Slovenia

In several dozen samples per year sent to the Institute of Microbiology and Parasitology at the Veterinary Faculty of the University of Ljubljana for diagnosis of lungworms, *A. vasorum* was diagnosed only once in a hunting dog imported from Hungary, whose cause of death was angiostrongylosis (unpublished data).

3.1.9. Switzerland

In Switzerland, the first cases of *A. vasorum* were reported from a dog-breeding station in Zurich in 1968 [236], but only decades later did Staebler et al. [208] report five dogs infected with *A. vasorum* in the northern part of Switzerland, and three dogs coming from Southern Ticino, all diagnosed between 1999 and 2004. In addition, in 2001, two infected red foxes originating from the region of Basel were reported [237].

By means of serological tests that were developed for circulating antigen and specific antibody detection [238], in a first epidemiological study with more than 4000 sera an overall seroprevalence of 1.0%, 2.8%, and 3.1% for dogs positive in both ELISAs, in antigen ELISA and antibody ELISA, respectively, was detected. Spatial analysis showed that

positive dogs were distributed over large areas of the country, and a cluster of antibody-positive dogs in the northern area of Switzerland bordering Germany was identified [239]. Approximately in the same period, a grid-cell-based noninvasive fecal-sampling scheme for red fox samples indicated an overall prevalence of 8.8%, and revealed that land use and altitude affected prevalence rate [240]. Both dog and red-fox studies showed that prevalence rates increased with decreasing altitudes (and corresponding temperature variations), and that trend prevalence was higher in and around the first known endemic foci where *A. vasorum* was initially present. Investigating the Swiss red-fox population further, it was hypothesized that the transmission of *A. vasorum* among red foxes started to increase at the end of the 20st century due to the higher density of red foxes [241], increasing contamination of the environment, thereby infecting intermediate hosts and dogs. In fact, working up blood samples back from the past three decades from throughout the country, a drastic *A. vasorum* emergence from 2.4% to 62.0% was identified, reaching currently regional prevalence of more than 80.0% [4]. In particular, around 2000, a marked increase in seropositive red foxes correlated with the first accumulations of cases of canine angiostrongylosis. Locally, prevalence based on red-fox necropsy increased fourfold in only six years [4]. A group of captive meerkats (*Suricata suricatta*), housed in such a locally known highly endemic area was *A. vasorum*-positive, with L1 excretion in seven of 17 animals. Their natural infection was supported by the identification of positive mollusc intermediate hosts in their immediate surroundings [37]. The very first global identification of a naturally occurring infection with *A. vasorum* in a cat may trace back to a highly endemic area; however, because cats do not become patent, such infections are highly challenging to diagnose intra vitam and are possibly underestimated [41]. Overall, these data evidenced the important role of red foxes as reservoir hosts, and also helped to understand the increasing number of dog cases along with significant prevalence in the red fox populations in other European countries in the last decade.

3.2. Northern Europe

3.2.1. Denmark

In Denmark, the first case of *A. vasorum* was described in 1983 in a five-year-old Cairn terrier from North Zealand [242]. The dog was euthanized due to bronchitis; at necropsy, *A. vasorum* were observed in the arteria pulmonalis, and the smaller arteria and arterioles. L1 were also demonstrated in a fecal sample [242]. The dog had visited Southern France several times and probably acquired the infection there. The next case was observed in 1989, also in a dog from North Zealand that had also been visiting France (Huus Petersen, personal communication). In 1990/1991, clinical cases were diagnosed in a considerable number of Danish dogs, none of which had ever been outside Denmark, but all from North Zealand [243,244]. In the same period, 12 of 15 adult red foxes from North Zealand were found positive for L1 in the feces, and/or adult *A. vasorum* in the right atrium of the heart and the pulmonary arteries [245]. The parasite had not previously been detected in a1973 parasitological survey of 100 wild red foxes from Denmark [246]. Since then, North Zealand has been a hyperendemic focus of *A. vasorum* in red foxes and domestic dogs for decades [245,247], while the parasite was either absent or with low prevalence in red foxes in the remainder of Denmark (0.0–1.1%) [247–249]. The latest study of *A. vasorum* in Denmark was conducted in 2017/2018 on 1041 wild animals, including 367 red foxes [40]. The study showed that *A. vasorum* prevalence in red foxes originating from the remainder of Zealand (37.0%) was now similar to the prevalence in the hyperendemic North Zealand (37.5%). This indicates that the hyperendemic area expanded to include all of Zealand [40]. In Jutland, the prevalence of *A. vasorum* in red foxes was much lower (1.7–2.3%), but higher than what had previously been reported. This indicates that *A. vasorum* is spreading in the red-fox population both in New Zealand and in Jutland. In addition, raccoon dogs (15 of 476) and polecats (*Mustela putorius*) (7 of 14) constitute a reservoir for *A. vasorum* in Jutland with prevalence ranging from 2.1% to 3.6% and from 50.0% to 100.0%, respectively.

3.2.2. Finland

Angiostrongylus vasorum appears to be multifocally present in Finland. Two autochthonous *A. vasorum* findings in dogs, from 2014 and 2017, have been described in detail [53]. There is also a single case report of imported *A. vasorum* infection in a domestic dog [250]. A questionnaire survey among veterinarians indicated that a limited number of more domestic dogs with the infection would have been seen in the country, including a third autochthonous case [53]. The parasite was described in red foxes in the 1960s [251], and in a single red fox in the southern part of the country more recently [252].

3.2.3. Iceland

A dog, a Siberian husky, imported in December 2017 to Iceland from Switzerland, was reported to be positive for *A. vasorum* [253]. In this study, more than 5000 imported dogs had been examined since 1989, and only this single *A. vasorum* case was found.

3.2.4. Norway

No autochthonous *A. vasorum* findings have been reported from domestic dogs from Norway. The parasite was detected in red foxes in the country for the first time in 2016, and further findings were reported from active surveillance [254,255]. In 2019, *A. vasorum* was detected also in Northern Norway [256].

3.2.5. Sweden

The first endemic case of *A. vasorum* was described in Sweden in 2003, when a dog from the island of Sydkoster, province of Bohuslän, was euthanized, and the diagnosis was confirmed at necropsy [257]. The demonstration of the endemic presence of the parasite came from the finding of parasitic L1 in two fecal samples from red foxes from Sydkoster; later, *A. vasorum* adults and L1 were found at necropsy in a dead red fox coming from the same island [257]. During 2011–2015, the parasite was detected in 0.7% of fecal samples (n = 20 of 2882 samples) analyzed with the Baermann test at the National Veterinary Institute (SVA, Uppsala, Sweden); these findings came from different parts of the country [54]. During the same period, *A. vasorum* was found at necropsy in red foxes, representing an occurrence ranging between 0.3% and 1.4% of necropsied red foxes, but the necroscopic investigations were not aimed at detecting specifically *A. vasorum* [54]. Regarding other potential final hosts, *A. vasorum* was not found in grey wolves (n = 20) hunted in Sweden [258]. A large national seroprevalence study was performed on serum samples collected between 2013 and 2014, and it showed that 0.1% of dogs were positive in both parasite antigen tests and antibody tests [54]. Since the disease is notifiable in Sweden, the following cases were reported during the last five years: 11 cases in 2015, eight cases in 2016, no cases in 2017, two cases in 2018, and two cases in 2019 [199,200].

4. Factors Influencing the Prevalence and Establishment of *D. immitis* and *D. repens*

According to Simón et al. [15], the transmission of *D. immitis* and *D. repens* is limited by two main preconditions: (i) the presence of a mosquito species capable of transmitting the parasite and (ii) the presence of a minimal number of dogs infected with adult nematodes shedding microfilariae. The distribution of *D. immitis* and *D. repens* is further influenced by human behavior (e.g., the housing conditions and travel activity of dogs, and imports), and climatic conditions allowing the presence of competent mosquito vectors and larval development [15]. Although infections with *D. immitis* and *D. repens* were documented in various wild canids, wildlife seem to play a limited role in the spread of these pathogens.

Three factors majorly impacted the prevalence, distribution, and establishment of populations of *D. repens* and *D. immitis* in Central and Northern Europe:

4.1. Dogs Staying Outside Overnight

Currently, human behavior is the major factor for the spread or import of *Dirofilaria* spp. in Central and Northern Europe. Both *D. immitis* and *D. repens* are frequently

imported to non-endemic countries from endemic countries (e.g., stray dogs from Spain and Greece) [64]. The introduction of microfilaremic dogs to non-endemic areas may lead to local autochthonous outbreaks such as in military-dog facilities with kennel keeping [65]. The way of dog keeping majorly impacts the establishment of populations of these parasites. Stray dogs and cats, and private kennel keeping are not (very) common in Central and Northern Europe. Kennel or outdoor keeping of military, hunting, and sled dogs and keeping dogs in animal shelters are common practices in certain regions in Central and Northern Europe. In several Central European countries, more than 30.0% of the dogs are estimated to stay outside overnight (Figure 2), and so they are at a higher risk for mosquito bites compared to dogs staying inside. In addition, nocturnal house mosquitoes of the *Cx. pipiens* complex are competent vectors for *D. immitis* and *D. repens*, and those are the mosquitoes with the highest abundance in the vicinity of humans.

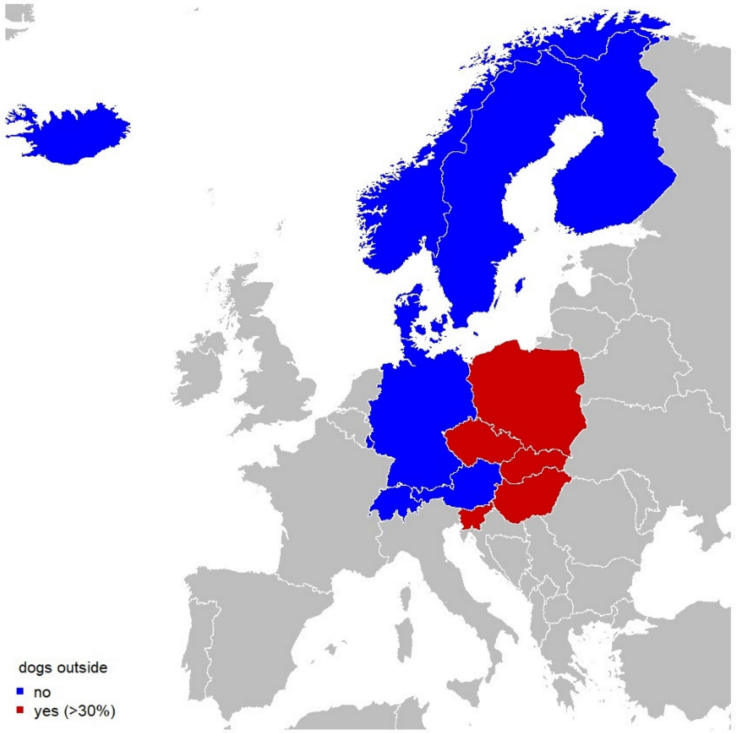

Figure 2. Dogs staying outside overnight (no = uncommon that dogs are kept outside overnight; yes $\geq$ 30% of dogs stay outside overnight).

4.2. Diurnal Vector Activity

Diurnal vector activity: More than 60 mosquito species are vectors of *D. immitis* or *D. repens*. During blood meals, microfilaria are ingested, move to the Malpighian tubes, and L3 move to mouthparts and enter the labium (after passing the cibarium). Vector competence can be proven in laboratory studies [259]. At xenomonitoring studies where pooled mosquitoes are screened by PCR, vector competence can only be estimated [73]. Findings of L3 in caught wild mosquitoes (microscopically and not DNA in an entire mosquito only) can indicate vector competence. Furthermore, the molecular analysis of the head or thorax vs. abdomen can indicate vector competence [260,261]. However, laboratory suitability does not automatically mean that infections in the field occur frequently. Mosquito ecology and preferences differ from species to species (such as different habitats or blood-meal preferences) [262,263].

Several species of the *Aedes*, *Culex*, and *Anopheles* genera were demonstrated as competent vectors [15,262,264]. Synanthropic mosquito species with high abundance at human settlements might be the most important vectors for the establishment of parasite populations. In Central and Northern Europe, house mosquitoes of the *Cx. pipiens* complex fill

this gap. These mosquitoes have a nocturnal activity pattern [265], so dogs staying outside overnight are more prone to these mosquitoes than those staying inside are.

In recent decades, several potential invasive mosquito species such as Asian tiger mosquito *Ae. albopictus* were introduced to Europe, primarily through the transport of goods (such as used tyres) [266]. In Southern Europe, the tiger mosquito rapidly established, and this invasive species is spreading northwards [267]. The tiger mosquito has already established in certain regions in Central Europe, and is regularly reintroduced in others (Figure 3) [75].

The Asian tiger mosquito is both a competent vector for *D. immitis* and *D. repens*, and an annoying day-active biter that outcompetes *Cx. pipiens* s.l. [268]. The establishment of populations of tiger mosquitoes would allow for the transmission of microfilariae to dogs during daytime and increase the risk of dogs to acquire an infection. The increased probability of *Dirofilaria* infections in areas where tiger mosquitoes established was reported from Italy [269,270].

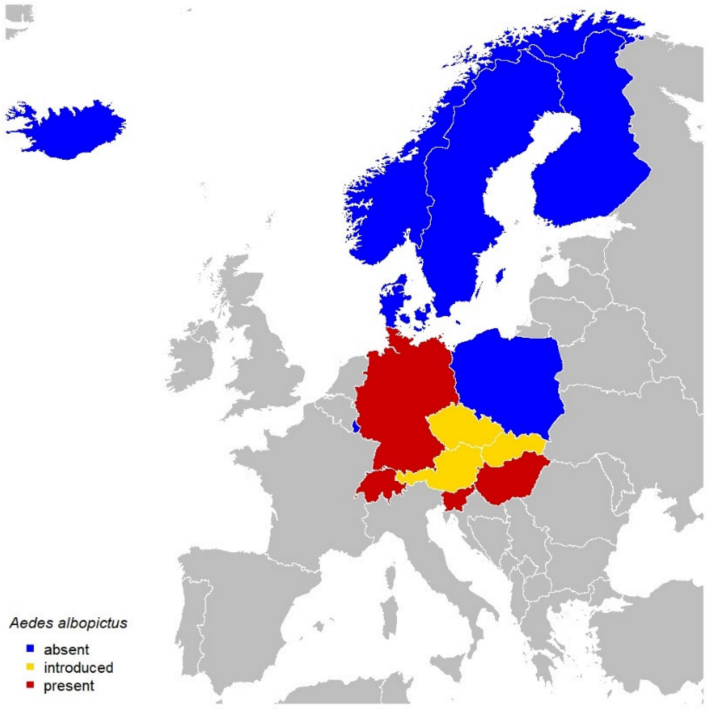

Figure 3. Distribution of Asian tiger mosquito (*Aedes albopictus*) in Central and Northern Europe according to the ECDC in March 2021 (introduced, no stable populations known yet; present, established populations at certain areas in the country). For detailed and updated information, please visit https://www.ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/mosquito-maps (accessed on 14 August 2021).

4.3. Climate Change

Temperature has an important influence on the development of mosquito vectors and parasites. On the one hand, longer warm periods per year allow for more generations of parasites and vectors per year. On the other hand, increasing temperatures allow for a faster development from eggs to adult mosquitoes. Environmental temperature is also the key factor for microfilariae development in mosquito vectors (e.g., L3 require 16–20 days at 22 °C) [15]. Microfilariae do not develop to L3 at temperatures below 14 °C [27]. Several models showed that the expansion from Southern to Central and Northern Europe (but also in North America) is probable [27,192,271]. Both the heartworm predictive model (based on growing degree days) and the *Dirofilaria* development units show parts of Central Europe suitable for the establishment of these parasites [28]. In Central Europe, the possible transmission period for *D. immitis* is estimated to be three to four months, while 20 days

to 2 months are estimated for some Northern European regions [15]. However, climatic changes might prolong these periods and allow for spreading to areas that are currently climatically unsuitable for these parasites and certain vectors.

5. Factors Influencing the Prevalence and Distribution Dynamics of *Angiostrongylus vasorum*

Angiostrongylus vasorum generally has patchy distribution with hyperendemic foci surrounded by low prevalence areas [272–274]. Its recent spread in various European territories calls for higher awareness by local veterinarians, as the absence of records in a given area may often be due to lack of information [8].

The emergence of *A. vasorum* in Central and Northern Europe is likely driven by a combination of factors, including wildlife movement to urban areas, increased dog movements, and possibly climatic changes.

Compared to *D. immitis* and *D. repens*, wildlife reservoirs are highly relevant for the distribution of *A. vasorum*. The prevalence of *A. vasorum* in wild canids in Europe, mainly red foxes and golden jackals, which act as natural reservoir of this parasite [4], is regionally high. Red foxes are ubiquitous, share recreational areas with dogs around urban contexts, and are usually subjected to reinfections that can lead to high worm burdens, as they do not reach effective immunity following *A. vasorum* infection [4,241,275]. As a consequence, they can act as a continuous source of environmental contamination, favoring the infection of gastropod intermediate hosts [276], and thereby the local establishment of the parasite [277].

Climatic changes may also play a role in the increase in the distribution of angiostrongylids, including *A. vasorum*, as their development from L1 to L3 inside snails may be positively influenced by the increase in environmental temperature, contrarily to crenosomatids [278–281]. Thus, it cannot be excluded that the current global warming may drive the further spread of *A. vasorum*. Water availability strongly affects the biology of intermediate *A. vasorum* hosts; thus, increased precipitations can act synergistically with increased temperatures in the spread of this metastrongyloid [274].

Lastly, dog relocation from endemic to non-endemic areas may cause the spread of parasites, including *A. vasorum*, and introduce the parasite into new areas [203,277,282–287].

Overall, the *A. vasorum* spread pattern seems to be multifocal and later coalescing, highlighting the need for larger and multicentral studies in order to support targeted interventions such as prophylactic anthelmintic treatments or testing [274].

6. Conclusions

Dirofilaria immitis, *D. repens*, and *A. vasorum* are spreading in Europe, and the relevance of these parasites is steadily increasing for dogs and veterinary practitioners in Central and Northern Europe. Housing conditions of dogs, increased animal movements, and climate change are important factors in the spread of these nematodes. Keeping dogs outside overnight seems to be a major factor for the establishment of *D. immitis* and *D. repens.* However, the establishment of invasive, diurnal, synanthropic, competent mosquito vectors such as *Ae. albopictus* may also influence the spread of these filarioid helminths. Although the reasons for the spread of *A. vasorum* are not definitely clarified, habitat sharing and increased chances of contact with red foxes seem to play a major role in the epidemiology of this parasite, which may also be influenced by increased temperature and precipitation, and dog relocations.

Research efforts focusing on these parasites vary by country, and cross-border studies are few. The available data are not easily comparable. Both *Dirofilaria* spp. and *A. vasorum* merit monitoring and further studies in Europe.

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data for the figures. All authors participated in preparing the final version of the article and have approved its content. All authors have read and agreed to the published version of the manuscript.

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6.5. Canine babesiosis in Austria in the 21st century - A review of cases

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KBL: software, formal analysis, visualization

HPF: conceptualization, validation, supervision

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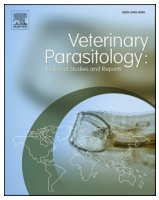

Original Article

Canine babesiosis in Austria in the 21st century – A review of cases

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Retrospective analysis

ABSTRACT

A retrospective study on 699 cases of canine babesiosis presented to veterinary clinics in eastern Austria were evaluated for the location where infection had presumably taken place. Of these, 542 (77.54%) had acquired the infection in Austria, while the majority of non-autochthonous cases came from neighboring countries, most notable Hungary. Both groups were recorded primarily in Vienna, eastern Lower Austria and Burgenland, but cases from the southern (Styria, Carinthia) and western (Upper Austria, Tyrol, Salzburg) provinces of the country were also recorded. Records were made all year round, with most cases in spring (46.6%) and fall (48.4%). The annual cases ranged from four to 58 (mean: 31.8) with large fluctuations and no visible trend for an in- or decrease. The tick vector of *Babesia canis*, *Dermacentor reticulatus,* is present in Austria but displays a very patchy distribution, and its occurrence and activity are not readily foretold, which might be a reason why its presumably increasing density in Europe is not reflected by increased incidences of canine babesiosis. Another factor that may influence the numbers of cases per year could be the application (or non-application) of acaricidal or repellent compounds. A limitation of this study is that bias is exerted by the location of the participating clinics, and by the unknown rate of infections that does not induce clinical symptoms and is likely not presented in veterinary practices and clinics. The data, however, clearly show that at least the lowlands of Austria are endemic for *B. canis*, and appropriate tick control must be advised all year round.

1. Introduction

Canine babesiosis can be caused by a variety of *Babesia* species. In Europe, *Babesia vogeli*, transmitted by the Brown Dog tick *Rhipicephalus sanguineus*, prevails in the Mediterranean and southeastern regions where the vector is abundant, while *Babesia canis*, transmitted by the ornate dog tick or meadow tick *Dermacentor recticulatus*, is prevalent in central and western Europe. In addition, rather focal occurrence of "small" canine *Babesia* species, i.e. *Babesia gibsoni* and *Babesia vulpes* (syn. *Theileria annae, Babesia microti*-like), has been reported (Solano Gallego and Baneth, 2011; Baneth et al., 2019; Bajer et al., 2022a, 2022b). Recently published works strongly indicate a spread of *D. reticulatus* in central and northern Europe (Zygner et al., 2009; Radzijevskaja et al., 2018; Drehmann et al., 2020; Grochowska et al., 2022; Daněk et al., 2022), and, with it, an increased reporting of *B. canis* infections in some countries (Dwużnik-Szarek et al., 2022; Helm et al., 2022). By contrast, in Switzerland temporal epidemic foci were previously described that did not seem to have persisted in more recent times (as reviewed by Bajer et al., 2022a, 2022b).

Babesia canis infects erythrocytes and can cause subclinical or mild, but also life-threatening infections. Thrombocytopenia, anemia, and other hematological changes can develop from low to baseline to severe levels and lead to lethargy, inappetence, coagulopathies, renal failure, and occasionally neurological signs with coma and even fatal outcomes (Strobl et al., 2020; Beletić et al., 2021). The presence of *B. canis* in Austria has previously been described (Leschnik et al., 2008, 2012; Duscher et al., 2013; Strobl et al., 2020; Sonnberger et al., 2021; Bajer et al., 2022a, 2022b), and we hypothesized that annual cases were increasing. Moreover, it was assumed that the majority of these cases were autochthonous and are reported not only from eastern Austria (where a high abundance of *D. reticulatus* ticks is recorded (Rubel et al., 2020; Dirks et al., 2021; Sonnberger et al., 2022) but throughout the

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country. For this we retrieved data on 699 cases referred to veterinary clinics in eastern Austria from 2001 to 2022 and evaluated their geographical distribution, whether they were autochthonous or imported, and changes in in their rates over seasons and years.

2. Materials and methods

Cases of canine babesiosis reported from two large veterinary clinics and two associated diagnostic laboratories in eastern Austria from September 2001 until March 2022 were included. The clinics and laboratories were located in northern Burgenland, and Vienna. The diagnosis had been made by detection of parasite stages in stained blood smears or by molecular detection (PCR) of Babesia DNA in blood conducted in the veterinary diagnostic laboratories. In addition, all dogs showed clinical signs of acute babesiosis such as pallor (confirmed in hematology as anemia), hemoglobinuria, jaundice, petechiae or ecchymosis in skin or mucous membranes, lethargy, fever and/or gastrointestinal disturbances.

Of all dogs, age, gender, breed, and the place of residence for the previous three weeks or longer were recorded.

Since no central register of dogs in Austria is available, we estimated a "virtual" dog population for the provinces and districts by relating the Austrian dog population with the human population making the following calculations:

$$\frac{\text{Percentage of households with one or more dogs (per province)}}{100} \times \text{number of households (per district)}$$

($n = 629.120$ dogs; Statistik Austria Konsumerhebung 2019/20; www.statistik.at; accessed 20.08.2022).

For a more detailed evaluation of this proxy calculation, data only for Vienna (available at Statistiken zu Hunden in Wien - Offizielle Statistik der Stadt Wien) were calculated separately and returned a mean deviation of 38.6% (min 10.2%, max 63.0%) from our calculation based on the total populations of citizens and dogs (for details see Suppl. file 2).

Autochthonous infections were defined as infections of animals that had not spent time abroad within three weeks prior diagnosis of canine babesiosis (according to the owner's information). Animals with a stay in endemic countries outside Austria within three weeks (including dogs originating from abroad) prior to the occurrence of clinical signs were grouped as "imported infections".

Maps for the distribution of cases (grouped as "autochthonous" or "imported"), divided into two decades (2001–2011 and 2012–2022) and grouped according to the season were created using R version 4.2.0 (R Foundation for Statistical Computing, Vienna, Austria) based on the zip code by district. Open access shapefiles were retrieved from the Statistik Austria homepage (www.statistik.at; accessed 01.03.2022).

Data regarding age of the included dogs and cases per year were tested for normality (Shapiro-Wilk test) and logistic regression (glm) in R.

3. Results

A total of 699 cases of canine babesiosis reported from veterinary clinics in eastern Austria from September 2001 until March 2022 were included.

The age of the animals ranged from 1 month to 17 years (median: 5 years; interquartile range: 6 years) and was not normally distributed; around half of the positive dogs were between two and six years old (see Suppl. File 1). They were of mixed gender (144 intact females, 161 neutered females, 300 intact males, 93 neutered males, 1: no information) and of different (pure or mixed) breeds.

3.1. Cases by year and season

Over the evaluated years, the annual cases reported ranged from four to 58 (Fig. 1) with an annual mean of 31.8 ± 16.9 cases. Cases were normally distributed over time and logistic regression calculation did not reveal an increase or decrease over time.

The majority of cases were reported in fall, September to November (48.40%) and spring, March to May (46.60%), while in summer (4.00%) and winter (7.00%) only few cases were noticed (Fig. 2).

In the two decades that were evaluated, 367 cases (52.5%) were reported 2001–2011, and 332 cases (47.5%) were reported in 2012–2021 with similar annual caseloads (33.4 ± 14.4 respectively 30.2 ± 19.7 cases/year).

3.2. Autochthonous vs. imported cases

Based on the distinction between autochthonous and imported cases, the majority of reported dogs (77.5%) originated from Austria at the time of submission and had not been outside the country three weeks before submission to veterinary hospital according to the owner's information, while the rest originated mostly from (or had spent time in) Hungary (15.9%), Serbia (1.6%) Slovenia (1.1%), Slovenia, Croatia, Poland, Romania, Ukraine (0.6% each), Bosnia (0.3%) and in single cases from other countries (Table 1).

Both the cases considered as autochthonous and those that were considered as imported were mostly recorded from the eastern part of Austria, the provinces of Burgenland (most notably in Neusiedl/See with 23 non-imported and three imported cases over the whole period), Lower Austria and Vienna and, to a lesser extent, Styria where single cases occurred in a number of districts from 2002 to 2012.

The most westerly autochthonous infections were noted in in Tyrol with single infections in the districts of Imst, Innsbruck-Land, and Kufstein 2007–2016 (Fig. 3).

3.3. Origin of dogs in autochthonous cases

When all non-imported cases were considered by district of residence, primarily dogs from Vienna and neighboring districts were recorded (Fig. 4**, left panel**).

Calculating the proportion of cases by population density of the districts, however, more districts from Lower Austria and Burgenland stood out (Fig. 4**, right panel**).

3.4. Geography and climate of districts with reported cases

Considering the autochthonous cases by mean elevation of the district they were recorded in 167 districts (excluding Vienna) with average 272 (±146.9) m above sea level, with the majority below 200 m (61; 36.5%; including most of the districts around lake Neusiedl) or between 200 and 400 m (145, 50.3%, including districts in the Vienna Basin in Lower Austria). The highest districts with positive cases are listed in Table 2. For comparison, the city (and province) of Vienna (with a total number of 117 cases) is elevated from 151 m (Lobau, 22nd district, Donaustadt) to 542 m (Hermannskogel, 19th district Döbling) and is on

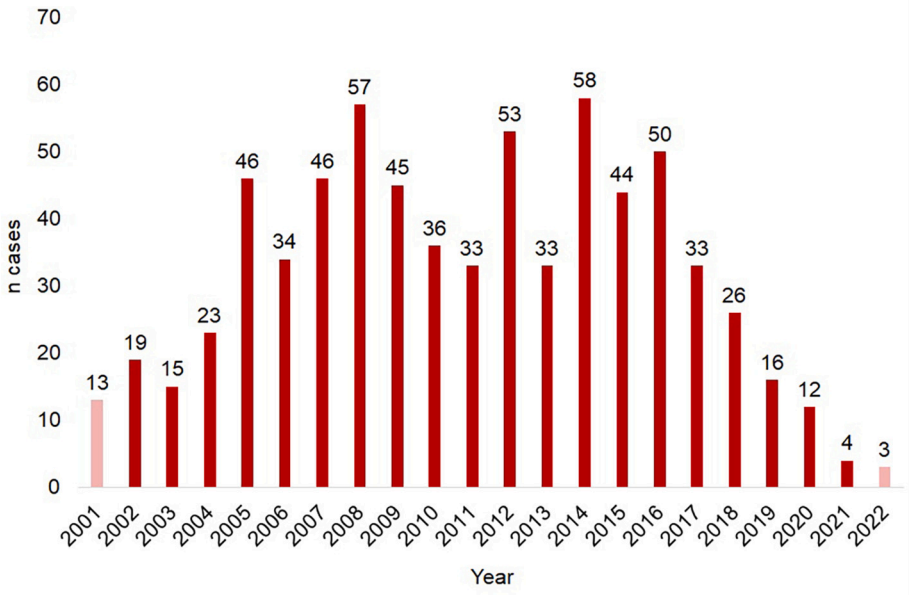

Fig. 1. Annual cases of canine babesiosis in Austria recorded from Sept 2001 to March 2022.

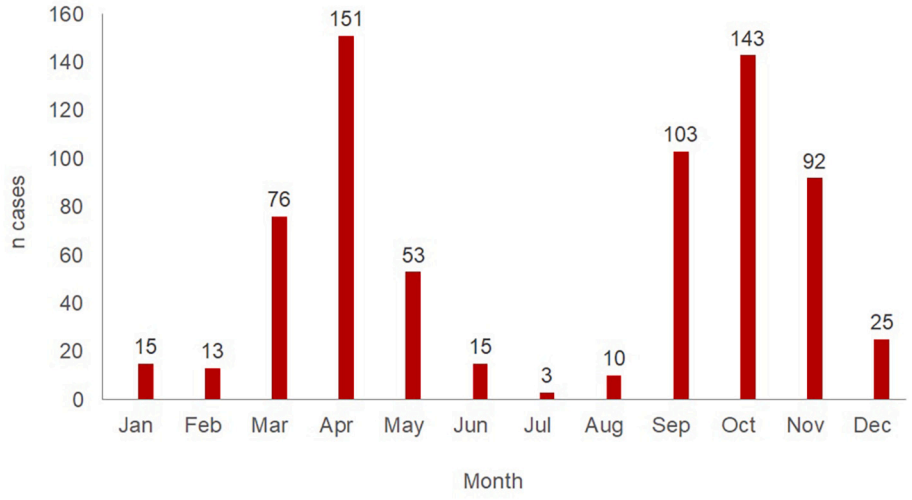

Fig. 2. Monthly cases of canine babesiosis recorded in Austria from Sept 2001 to March 2022.

Table 1
Country where *B. canis* transmission/infection had occurred; $n = 699$.

Country	Year(s)	N dogs [Percent]
Austria	2001–2022	542 [77.54]
Bosnia	2012,2015	2 [0.29]
Croatia	2009,2010,2013	4 [0.57]
Germany	2015	1 [0.14]
Greece	2015	1 [0.14]
Hungary	2001–2020	111 [15.88]
Italy	2005	1 [0.14]
Poland	2004	4 [0.57]
Portugal	2004	1 [0.14]
Romania	2016,2017	4 [0.57]
Serbia	2009–2011, 2013, 2015,2017,2019,2020	11 [1.57]
Slovakia	2005,2007,2009,2010,2012,2015,2016	8 [1.14]
Slovenia	2005,2008,2013,2018	4 [0.57]
Thailand	2014	1 [0.14]
Ukraine	2011,2014,2018,2022	4 [0.57]

average about 200 m above sea level (www.wikipedia.org; accessed 06.07.2022; www.topographic-map.com/maps/64z2/%C3%96sterreich/; accessed 06.07.2022.

4. Discussion

In this study we evaluated 699 cases of canine babesiosis presented at veterinary clinics in Eastern Austria in 2001 to 2022. The majority of cases was located in Vienna, Lower Austria and Burgenland, which was expected as these areas represented the most probable origin of clients of the participating clinics in eastern Austria. In addition, Vienna and lower Austria are the provinces with the largest populations; www.statistik.at; accessed 20.08.2022). Consequently, the "top ten" of the districts with cases of canine babesiosis also located nearby two large clinics that provided most case records. When we related the numbers of cases to the presumed size of the dog population in the districts, differences were noted, and one district stood out, Rust, a small community

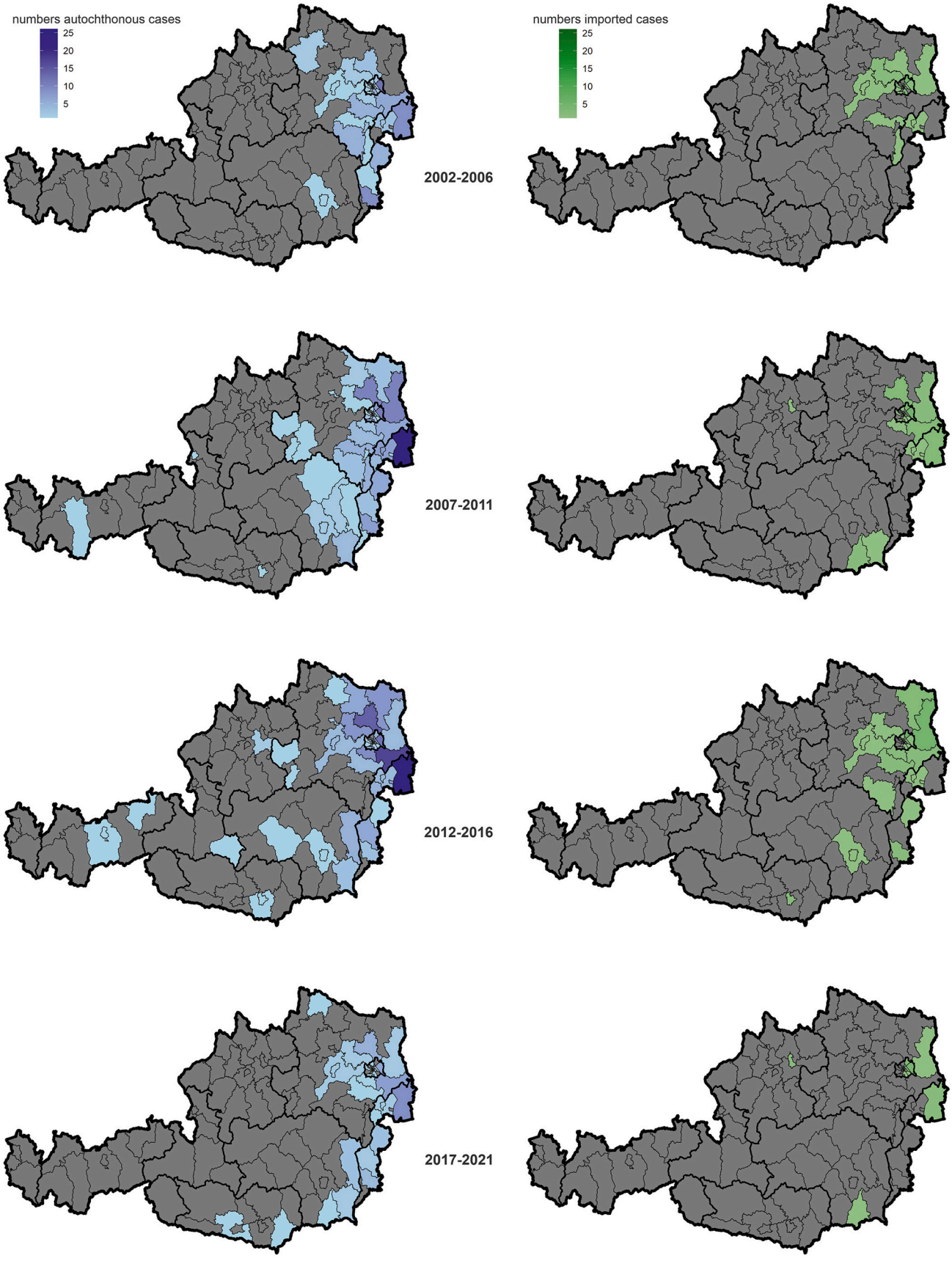

Fig. 3. Autochthonous (left, in blue) and imported (right, in green) cases 2002–2021 by geographic area (based on districts) in five year-intervals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

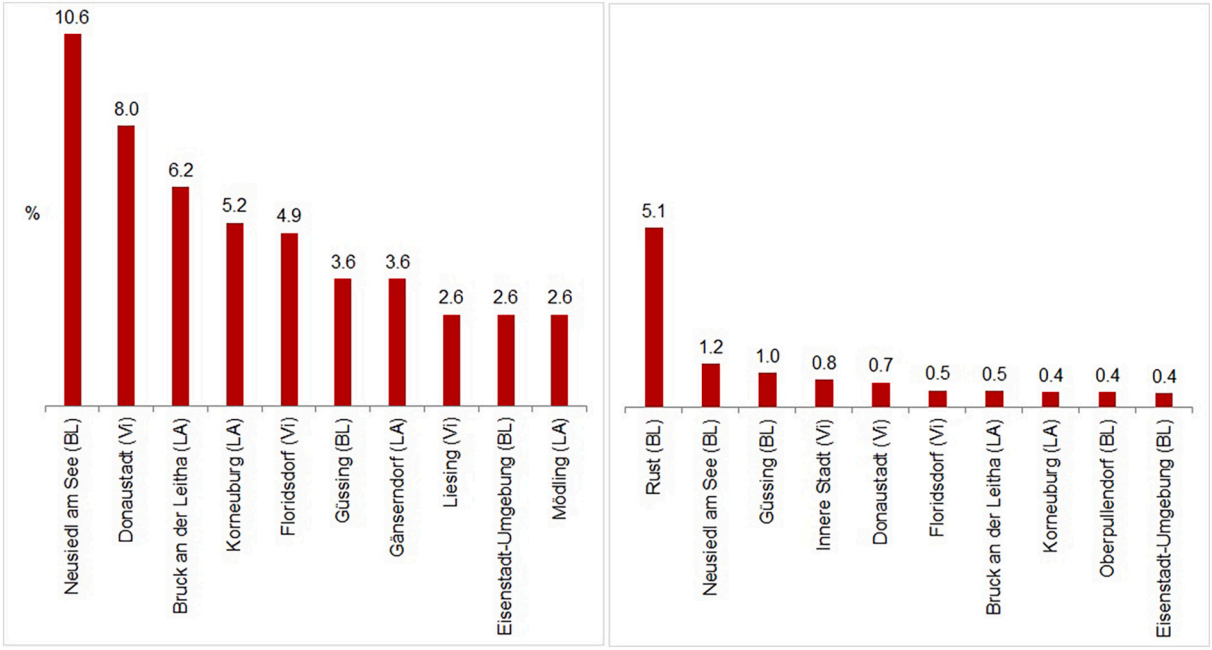

Fig. 4. Cases by district (in brackets: province - BL: Burgenland, VI: Vienna, LA: Lower Austria) (a) Top ten districts as proportions of all cases; (b) Top ten districts as proportions of all cases in relation to the population in the districts (Statistik Austria: Statistik des Bevölkerungsstandes, Statistik der natürlichen Bevölkerungsbewegung, Wanderungsstatistik. Provided: 29.06.2021. https://www.statistik.at/blickgem/pr1/g30604.pdf; Statista Research Department, https://de.statista.com/statistik/daten/studie/1098254/umfrage/hunde-in-oesterreich/; accessed: 21.01.2022) over the period of 2001 to 2022. For details on the locations see Suppl. File 3.

Table 2
"Top ten" districts from which positive cases were reported by mean elevation above sea level (www.wikipedia.org; accessed 06.07.2022; www.topographic-map.com/maps/64z2/%C3%96sterreich/; accessed 06.07.2022). For details on the locations see Suppl. File 2.

District	Mean elevation above sea level [m]	N positive dogs
Ferndorf (Villach-Land, Carinthia)	560	5
Kindberg (Bruck-Mürzzuschlag, Styria)	565	11
Hall in Tirol (Innsbruck Land, Tyrol)	574	9
Friedberg (Hartberg-Feistritz, Styria)	601	1
Schweiggers (Zwettl, Lower Austria)	633	4
Sankt Marein-Feistritz (Murtal, Styria)	698	4
Lichtenegg (Wiener Neustadt/Bucklige Welt, Lower Austria)	770	34
Grafenschlag (Zwettl, Lower Austria)	780	4
Arzl im Pitztal (Imst, Tyrol)	880	7
Sankt Margarethen im Lungau (Tamsweg, Salzburg)	1065	6

on lake Neusiedl, which is known for its large community of visitors especially during summer. However, it was not clear whether all the positive dogs located there were originally from Rust or had just visited from other areas of Austria, as the lake is a prominent holiday location for the population of Vienna and surroundings. As dogs travel frequently with their owners, we could not fully exclude "travel infections" in the sense of locally acquired infections, although the dogs' travel history was part of the anamnestic routine in the enrolled veterinary clinics. As regards the most frequently listed district for cases in total as well as cases in relation to the calculated dog population, 7/10 of the districts with the highest rates in each of the two categories overlapped, indicating the possibility of increased infection risks in these areas.

In the large majority of cases the infection appeared to be acquired in Austria, however, the case numbers or rates most likely do not reflect Austria as a whole due to the bias of the location of the clinics. A questionnaire survey conducted in 2010 among small animal practitioners in Western Europe revealed frequent cases of *B. canis* infections (163 cases reported by 151 veterinarians (ca. 15% of all registered practices in Austria), mostly from Burgenland (annual incidence 2.0–5.5) but also from Lower Austria, Styria, and Tyrol with low annual incidences of <0.2% in 2010 (Halos et al., 2014). A previous study that evaluated 240 cases of canine babesiosis in the Clinic of the University of Veterinary Medicine, Vienna, Austria, determined 59.6% of the cases to be autochthonous (Strobl et al., 2020). In the present study the rate of infections acquired in Austria was higher (77.5%), and the difference can be attributed to the larger data set that also included cases recorded in a second clinic and a veterinary laboratory.

On the basis of the obtained data, a number of cases was suspected to be acquired outside Austria, especially in Hungary, which borders

Table 3
Records for canine *B. canis* infections in European countries that were documented for suspected non-autochthonous *Babesia* infections (ref. Table 1).

Country	Reference(s)
Bosnia	Ćoralić et al. (2018)
Croatia	Mrljak et al. (2017)
Germany	Zahler and Gothe (1997); Barutzki et al. (2007); Silaghi et al. (2020); Helm et al. (2022)
Greece	Diakou et al. (2019)
Hungary	Földvári et al. (2005); Máthé et al. (2006); Hamel et al. (2012)
Italy*	Morganti et al. (2022)
Poland	Welc-Faleciak et al. (2009), Dwużnik-Szarek et al. (2021)
Portugal	Dordio et al., (2021)
Romania	Hamel et al. (2012)
Serbia*	Davitkov et al. (2015); Kovačević Filipović et al. (2018); Strobl et al. (2021)
Slovakia*	Majláthová et al. (2011); Víchová et al. (2016)
Slovenia**	Duh et al. (2004)
Ukraine	Hamel et al. (2013); Bajer et al. (2022a, 2022b)

* Also endemic for *B. gibsoni* (Davitkov et al., 2015; Kovačević Filipović et al., 2018; Strobl et al., 2021; Víchová et al., 2014; Carli et al., 2021; Víchová et al., 2014.

** Also endemic for *B. vogeli* (Duh et al., 2004).

Austria to the East and is a popular holiday destination for Austrians and their dogs. Hungary also offers specific hunting trips (e.g. https://www.jagdreisen.at/laender/jagen-in-ungarn; accessed 15.08.2022) – a travel opportunity also for hunting dogs and possibly a number of parasite as well, since neither Hungary nor Austria request antiparasitic treatment prior to entering the country. For the majority of the countries in question, the presence of *B. canis* has been recorded in the literature (Table 3). In the single case of a dog imported from Thailand the infectious agent was diagnosed as *B. vogeli* which is endemic in South-East Asia (Piratae et al., 2015; Buddhachat et al., 2020; Colella et al., 2020).

The reported cases consisted of slightly more male than female dogs (56.3 vs. 43.7%). As gender data are not available for the Austrian dog population as a whole, a correlation between sex and parasite infection could not be established. Previous works suggested such testosterone as an influential factor (Hughes and Randolph, 2001), and an influence of sex was noted in infections with *Babesia rossi* (Mellanby et al., 2011); however, Strobl et al. (2020) could not confirm this for *B. canis* infections. This issue clearly warrants further investigations.

Canine babesiosis can take a variety of clinical courses in affected individuals (Solano Gallego and Baneth, 2011), so the exact time point of infection could not be determined in the evaluated cases. However, evaluation of the monthly cases showed year-round reports but two distinct peaks in spring and fall which is in line with the activity of *D. reticulatus*, the vector of *B. canis* (Drehmann et al., 2020; Duscher et al., 2013). Along with this, the majority of cases was reported from eastern Austria where this tick species is considered most prevalent (Rubel et al., 2020). While the clustering of the imported and probably the majority of the autochthonous infections in Vienna, eastern Lower Austria and Burgenland are attributable to the location of the clinics providing the data, cases from Styria, Upper Austria, Carinthia Salzburg and Tyrol and the most northerly tip of Lower Austria indicate that *B. canis* is probably spread throughout Austria. Here it was reported most frequently from the dry and warm lowlands of eastern Austria, but also from more humid areas of central and western Austria with elevations of up to 1000 m above sea level (for data on climate zones of Austria ref. Hiebl and Frei, 2016, 2018). We previously also documented *B. canis* in a red fox in Western Austria (Hodžić et al., 2018). The assumption that these are indeed endemic areas is also supported by the finding that through the five-year periods, cases were repeatedly reported from some of these regions. As *D. reticulatus* prefers, but is not limited to, areas with high humidity, it most likely prefers the vicinity of rivers and natural lakes, as well as the preference of deer as a suitable host (Silaghi et al., 2020), and it is widespread tick species that is considered to be expanding (Földvári et al., 2016); however, it has a very patchy distribution and its populations are difficult to assess (Enigk, 1958), and the infection rates of *D. reticulatus* specimen with *B. canis* are highly variable and not always correlated with infections in dogs reported from an area (as reviewed in Silaghi et al., 2020). It can also be speculated that the rate and yearly frequency of antiparasitic treatment for tick control may have influenced the annual rate of presented cases over time. Over the observation period, a number of acaricidal and/or tick repellent compounds have reached the veterinary pharmaceutical market. However, whether (and to what extent) this has influenced treatment applications and in turn transmission rates for tick-borne pathogens (separately or in relation to any changes of tick densities that may have occurred seasonally or over time) could not be uncovered here. Previous investigations on tick control and pathogen transmission in dogs in eastern Austria showed a poor owner compliance with the recommendations for tick control and consequently poor control of transmission of pathogens (Leschnik et al., 2013); this is somewhat mirrored in the present study where cases of *B. canis* infections did not seem to decrease during the observation period.

Since the data shown here were based on veterinary records, it can also be assumed that the numbers of *B. canis*-infections in the different areas were probably higher, firstly because affected animals were referred to other clinics, secondly because subclinical infections (Sonnberger et al., 2021), including reinfections of immune animals, were likely not presented. Therefore, molecular diagnostic tools are recommended to diagnose patients with submicroscopic parasitemia. Various PCR protocols can be used for the diagnosis of canine babesiosis causing parasites ranging from pan-apicomplexan protocols and RFLP to species-specific protocols (Zahler et al., 1998; Jefferies et al., 2007; Zintl et al., 2011).

Over the evaluated period, the number of annual cases fluctuated distinctly, but an increase of cases over time was not visible. In addition, an increase in records in eastern areas of the country or a spread of *B. canis* within Austria over the last 20 years was not detected, indicating that fluctuations in annual incidences are most likely not driven by spread of the parasite (or the vector) or at least not alone, but possibly by weather, focal presence of infected ticks or individual risk behavior, such as waiving the use of an acaricide or repellent on dogs or regular dog walks in preferred vector habitats (Leschnik et al., 2013), and have to be monitored over a longer time period, not compared on an annual basis.

Regarding the risk of increased transmission in the presence of infected canine hosts, the partial overlap of autochthonous with imported cases does not unequivocally explain this, due to the aforementioned bias of the locations. This phenomenon needs more in-depth analysis and larger data collection.

5. Conclusion

Retrospective evaluation of 699 confirmed cases of canine babesiosis diagnosed in dogs from Austria over the past 20 years shows that the majority of cases were autochthonous and occurred throughout the year, primarily in spring and autumn. Fluctuations but no steady in- or decrease in annual cases was recorded over the observed period. Most cases were reported from eastern parts of the country, which is presumably at least in part due to the location of the participating practices, however cases were also repeatedly reported from central and western districts, and also from areas with an average elevation of 750 m or more. Thus, it must be assumed that, except for the high-altitude alpine areas, Austria is generally endemic for *B. canis*. Prospective surveillance studies, including the vector tick *D. reticulatus*, should be conducted to monitor occurrence and possible spread of *B. canis* and its vector, and genetic typing of *Babesia* isolates should be carried out to determine possible connections between foci of infection. In addition, measures related to tick control on dogs should be monitored, evaluated and further promoted to keep tick-borne pathogens including *B. canis* at check.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vprsr.2022.100820.

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6.6. Emergence of *Parafilaria bovicola* in Austria

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JR: conceptualization, investigation, writing – review and editing

BSB: investigation, writing – review and editing

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JH: methodology, formal analysis, writing – review and editing, visualization

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animals

MDPI

Article

Emergence of *Parafilaria bovicola* in Austria

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Simple Summary: Bovine parafilariosis is a disease caused by the helminth *Parafilaria bovicola* (Filariidae, Nematoda). Flies transmit the parasite, which grows to adulthood in an unknown location in the affected animals. The adult female worms are located in nodules under the skin, which they penetrate and lay their eggs in the fluid leaking from the site. There is virtually no information about *Parafilaria bovicola* in Austria. In this study, these parasites were documented in the provinces of Lower Austria, Upper Austria, Styria, Salzburg, Carinthia, and Tyrol. With a high number of cases during the 2020 study period, it can be assumed that the number of reports will increase in the near future.

Abstract: Veterinarians reported cases of cutaneous bleeding in cattle in Austria in the spring and summer of 2020. It was our goal to confirm the tentative diagnosis of parafilariosis by identifying *Parafilaria bovicola* in exudate samples using molecular methods for the first time in Austria. We asked veterinarians in the field to collect exudate from typical lesions on cattle. We performed polymerase chain reactions (PCRs) and sequenced a 674-bp section of the mitochondrial cytochrome oxidase subunit I in all positive samples. Overall, in 57 of 86 samples, *P. bovicola* was confirmed by PCR in cattle from Lower Austria, Upper Austria, Styria, Salzburg, Carinthia, and Tyrol. Sequencing detected four different haplotypes or genotypes, respectively, indicating multiple routes of introduction. We conclude that parafilariosis has spread in Austria and we expect that the number of reports of clinical signs and losses due to carcass damage will increase in the future.

Keywords: *Parafilaria bovicola*; cattle; parafilariosis

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1. Introduction

Bovine parafilariosis is a parasitic disease caused by the nematode *Parafilaria bovicola* that was first described by Tubangui [1] in the Philippines. Parafilariosis is characterized by the appearance of raised nodules on the neck and body of cattle, which may bleed profusely [2]. These nodules contain adult ovoviviparous females of *P. bovicola*, which penetrate the skin and release eggs and microfilariae (L1 larvae) into the serosanguinous fluid leaking from the site. The L1 larvae are ingested by *Musca* spp. (such as *M. autumnalis*, a species known to be endemic in Austria) and develop into infective L3 larvae, which are transmitted to cattle and cause cutaneous bleeding after a long period of prepatency of seven to ten months [3,4].

In Europe, Daslakow [5] identified a parasite he thought was identical to *P. bovicola* as described by Tubangui [1] at an abattoir in Sofia, Bulgaria in 1944. Tubangi had only

described two female adult worms morphologically, whereas Daslakow found parafilariosis in 60 of 410 examined cattle. In these, he isolated up to 124 male and female *P. bovicola* specimens per animal. In 1948, cases of parafilariosis in Transylvania, an area that is in the center of present-day Romania, were reported. The author found the parasite in many locations and concluded that it must be widely spread in Romania already [6].

The disease was then described in Sweden in 1978 [7] and again in 2000 [8], where it is now regarded as endemic, but it was not found in Finland, the neighboring country [9]. Parafilariosis was first diagnosed in Belgium in 2009 [10] and was later found to be spreading in several Belgian provinces [11]. Single cases were described in Ireland in 1997 and in The Netherlands in 2007, both in bulls imported from France [12,13]. In both cases, the disease did not seem to spread any further. There are other reports of parafilariosis in Charolais cattle imported from France, for example in Canada [14,15]. In France, the disease seems to be present in the regions of Charolais and the southwest including Piemont Pyrénéen and Piemont du Massif Central, but has rarely been described [16,17]. Bech-Nielsen et al. [18] assumed that the parasite was of little economic concern and thus ignored in France.

Parafilariosis was first confirmed recently by microscopy of filariid eggs and parts of an adult worm retrieved by biopsy in two locations of Bosnia and Herzegovina [19]. The authors conducted a telephone survey with veterinarians in the possible endemic area but only three of 28 veterinarians had observed the symptoms in the past.

In Austria, the clinical symptom "spontaneous cutaneous hemorrhage" became of interest as a differential diagnosis to bovine neonatal pancytopenia [20]. Symptomatic cattle were first observed in the provinces of Carinthia, Styria, and Salzburg in 2009 and attributed to *P. bovicola* based on clinical signs and the epidemic situation [21]. *Parafilaria bovicola* has since been considered endemic in parts of Carinthia.

The route of introduction to Austria is unknown, but lesions typical of *P. bovicola* were described in the neighboring countries, in southwestern and southern Germany and later in Italy. In both countries, species identification of *P. bovicola* was based on morphological characteristics using microscopy only [22,23]. Molecular methods have since been established to identify nematodes on a species level using the mitochondrial gene cytochrome c oxidase 1 (*COI*); *COI* haplotypes can be used to studying population structure and genetic diversity [24,25].

It was the goal of our study to identify the cause of cases of cutaneous bleeding in cattle in new areas in Austria and to confirm our clinically tentative diagnosis of parafilariosis by identifying *P. bovicola* in exudate samples using molecular genetic methods. In all samples that were polymerase chain reaction (PCR) positive, we sequenced a 674 bp section of the mitochondrial cytochrome oxidase subunit I.

2. Materials and Methods

2.1. Sample Collection

Several cases of punctual bleeding from the skin of cattle were reported to the University Clinic for Ruminants at the University of Veterinary Medicine Vienna. We suspected parafilariosis, drafted an information letter together with a sample submission sheet and a questionnaire (Table 1), and distributed these to veterinarians in Austria via the Animal Health Services (Tiergesundheitsdienste) of the federal states. We asked veterinarians in the field to collect exudate from typical lesions on cattle using sample collection tubes and to freeze the samples at −20 °C. The samples were then collected by the medical logistics company medlog© and transported to the Institute of Parasitology at the University of Veterinary Medicine Vienna for further analysis. Some veterinarians were not able to collect samples themselves so J.R. collected samples on farms in Styria and Lower Austria.

Table 1. History questions for farmers as distributed through veterinarians.

History Question
What is the type of farm: dairy, suckler cow, or fattening?
How many cattle are housed at the affected farm?
How many cattle show the symptoms typical of parafilariosis (skin bleeding)?
Did you observe any changes in behavior or a decrease in production in the affected cattle? If yes, please describe.
Do the affected animals have access to pasture or an outdoor pen?
Have you observed the symptoms in the past? If yes, since when?
Are you using fly control on the farm? If yes, what do you use?
Do you deworm the animals on your farm? If yes, what do you use?
Have you submitted samples of the bleeding lesions? If yes, what was the result?

2.2. Laboratory Analysis

DNA was extracted from the exudates using a DNeasy® Blood and Tissue DNA extraction kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Conventional PCRs, targeting a 674 bp section of the mitochondrial cytochrome c oxidase subunit I (*COI*) gene using the primers H14FilaCOIFw and H14FilaCOIRv, were performed as reported previously [26]. PCR products were separated by electrophoresis in 2% agarose gels stained with Midori Green Advance DNA stain (Nippon Genetics Europe, Düren, Germany). All positive PCR products were sequenced in both directions using Sanger sequencing at LGC Genomics GmbH, Berlin, Germany. The sequences were analyzed using Bioedit 7.5.0.3 [27]. The resulting sequences were compared for similarity to sequences available in GenBank (http://www.ncbi.nlm.nih.gov/BLAST) (accessed on 7 July 2021) and BOLD Systems (https://www.boldsystems.org/) (accessed on 7 July 2021). Moreover, sequences were uploaded to GenBank and BOLD Systems (accession numbers: MZ563376-MZ563429).

2.3. Data Analysis

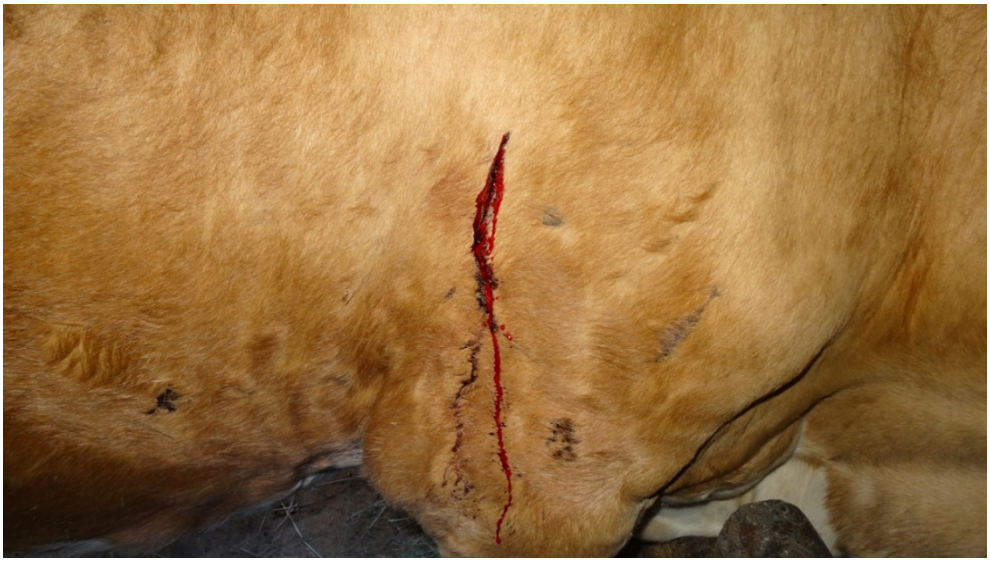
A maximum likelihood tree was calculated for the *Parafilaria* sequences using the W-IQ-TREE web server (http://iqtree.cibiv.univie.ac.at/ (accessed on 7 July 2021) [28]) by applying the best-fit model TIM3+G4+F and performing 1000 bootstrap replicates. A sequence of *Dirofilaria repens* (MW590257) was used as the outgroup.

A median-joining haplotype network was calculated with Network 10.2.0.0 (Fluxus Technology Ltd., Suffolk, UK), applying the default settings. The network was graphically prepared and provided with information on the counties in Network Publisher v.2.1.2.3 (Fluxus Technology Ltd.) and finalized with Adobe Illustrator CC v.2015 (Adobe Inc., San José, CA, USA).

To illustrate the phylogenetic relationships of the genus *Parafilaria*, a maximum-likelihood tree was calculated with the *COI* sequences of other members of the order Spirurida. The sequences were obtained by blasting a *COI* sequence of *P. bovicola* against the Spirurida in the NCBI GenBank (accessed on 25 September 2021). The sequences were then aligned and sorted using the default option (FFT-NS-2) in MAFFT v.7.311 [29]. Since most sequences did not cover the 674 bp section analyzed in the present study, the alignment was trimmed to 576 bp. All sequences featuring obvious sequencing errors and ambiguity characters were removed from the alignment and the sequences were collapsed to haplotypes using DAMBE v. 7.0.5.1 [30]. To reduce the size of the alignment, only two sequences were kept per species, resulting in 239 haplotypes. A sequence of *Ascaris suum* (KY045800) was used as the outgroup. The tree was calculated using the W-IQ-TREE web server (http://iqtree.cibiv.univie.ac.at/) (accessed on 25 September 2021) by applying the best-fit model TIM3+G4+F and performing 1000 bootstrap replicates. The sequence alignment is provided in Supplementary File S1.

3. Results

Photographs of affected cattle provided by veterinarians and farmers showed punctual bleeding in cattle with dried and fresh bloody streaks of exudate in the typical areas of the dorsal aspect of the body including the head, neck, shoulders, withers, dorsal part of the ribs, and the gluteal region. Examples can be seen in Figure 1.



(**a**)

(**b**)

Figure 1. Cattle affected by *P. bovicola* with bleeding spots on (**a**) the thorax in the shoulder region and (**b**) the neck. Photo credit: Susanne Möser (**a**), Johannes Reithofer (**b**).

Overall, 86 samples from 62 cattle from Lower Austria, Upper Austria, Styria, Salzburg, Carinthia, and Tyrol were submitted to the Institute of Parasitology of the University of

Veterinary Medicine Vienna (Table 2). In 57 of these 86 samples (n = 41 animals), *P. bovicola* was confirmed by PCR and sequencing. One sample did not contain enough exudate for the test to be performed. If multiple samples from the same animal were submitted, the results of the PCRs were consistent in all animals, except for one case, meaning that *P. bovicola* DNA was detected either in all samples or none. In one animal, two of three samples were positive. All but three samples from three different animals featured sequences of high quality and could be assigned to one of four haplotypes (haplotype 1: GenBank accession number MZ563421, haplotype 2: MZ563418, haplotype 3: MZ563406, haplotype 4: MZ563380). Interestingly, in four animals, two different haplotypes were identified in different samples (haplotype 1 and 2 (2x), haplotype 2 and 4, and haplotype 2 and 3). A map showing the geographic distribution of sampling locations and haplotypes is provided in Figure 2. The haplotypes showed a close resemblance, differing by 1–4 bp from each other. A Maximum likelihood tree, DNA haplotype network and an alignment showing the nucleotide differences in the *COI* between *P. bovicola* haplotypes is provided in Figure 3. A maximum-likelihood tree was calculated with the *COI* sequences of *P. bovicola* and other members of the order Spirurida (Supplementary File S2). The genus clades were mostly well-supported, but the deeper nodes in the tree obtained only low bootstrap values. Based on the 576 bp *COI* section, *Parafilaria* is closest related to *Thelazia* and the two genera cluster in a clade with maximum support.

Table 2. Overview of the number of samples, affected cattle, herds, and haplotypes detected.

Federal State	Samples Subm.	Animals Subm.	Herds Subm.	Samples Positive	Animals Positive	Herds Positive	Haplotypes
Lower Austria	34	23	9	32	22	8	1, 2, 4
Styria	23	15	13	18	12	11	1, 4
Upper Austria	11	8	7	2	2	2	1
Salzburg	13	11	7	4	3	3	1, 2, 3
Carinthia	3	3	3	0	0	0	n.d.
Tyrol	2	2	2	1	1	1	1
Total	86	62	41	57	40	25	

n.d., not detected; subm., submitted.

Figure 2. Location of haplotypes and number of positive samples submitted by town. Please visit https://www.google.com/maps/d/edit?mid=1zjFBQwKYfcNieR0q0vtQLkYcYkbKObSy&usp=sharing (accessed on 20 August 2021) for an interactive map.

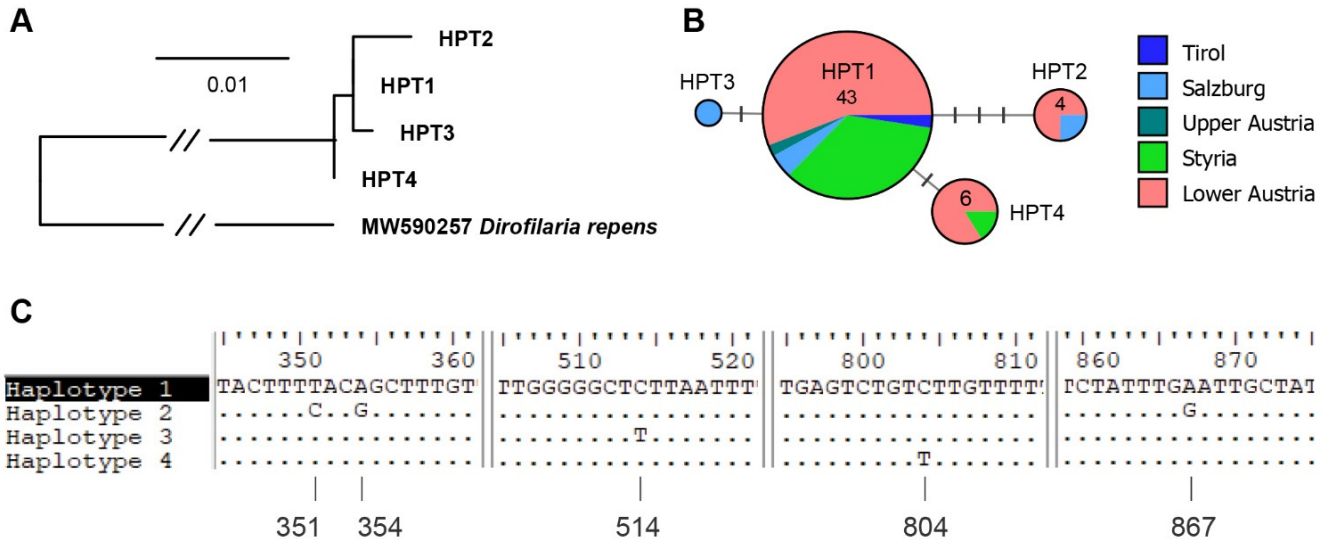


Figure 3. (**A**) Maximum likelihood tree of the four cytochrome c oxidase subunit I (*COI*) haplotypes (HPT1–4) with a *Dirofilaria repens* sequence from GenBank (accession number: MW590257) as the outgroup; (**B**) median-joining haplotype network showing the distribution of different haplotypes per federal state and the number of individuals; (**C**) sequence alignment showing the nucleotide positions (counting from the 5′-end of the *COI*) differing between the four *COI* haplotypes.

Most samples were collected in June and July, with only one and two negative samples collected in August and September, respectively. Of the 37 samples collected in June, 19 were positive, and of the 28 samples collected in July, 27 samples were positive for *P. bovicola*.

Most samples originated from animals kept on dairy farms (53 individuals from 34 farms) and only samples of seven animals came from suckler cow herds on five different farms. No further information or history was submitted with the samples from two animals. The farms kept between 2 and 95 cattle (mean: 43.5, median: 35 cattle) and the farmers reported that between 1 and 8 cattle (mean 3.3, median 2.5) were or had been affected by bleeding from the skin typical of *P. bovicola*. The animals were between 7 months and 10 years old, with a mean age of 52 months and a median age of 47 months. Only two animals were male.

Of those studied, 54 animals had access to pasture or an outdoor pen. Of the eight animals where access to the outdoors was unknown or not given, only one animal in each category was positive for *P. bovicola.* Eight farmers reported that they had observed the symptoms from as early as six years ago up until early 2020, the year of sample collection. However, *P. bovicola* was only detected in cattle from three of those farms. At the farms where 32 of the animals were kept, fly control was conducted using adhesive or insect electrocutor traps or using pour-on formulations containing pyrethroids. On farms where 34 of the animals were kept, the cattle received a regular anti-parasitic treatment using macrocyclic lactones. However, even though preventive measures were taken, in 26 of 32 animals from farms where fly control was applied and in 28 of 34 animals from farms where the cattle were dewormed, *P. bovicola* was detected. In 21 of 24 animals from farms where both fly control and deworming were performed, *P. bovicola* DNA was detected.

Loss of production or abnormal behavior in relation to the occurrence of parafilariosis were only reported at one farm where the somatic cell count of the cattle had increased. Two farms reported no information, and when samples were sent from the others, nothing was noted. One farmer reported that the affected animal had aborted a calf six weeks before the calculated calving date but did not attribute this to the skin bleeding. No *P. bovicola* could be detected in the sample of this particular animal.

4. Discussion

Parafilaria bovicola was present in 25 cattle herds in five states of Austria. Based on the questionnaire sent out, participants collected samples from cattle showing the typical symptoms of parafilariosis, namely, localized bleeding from the skin. We assume that most cattle showing these symptoms were affected by *P. bovicola*. Though trauma or insect bites could cause the same symptoms [10], we suspect that, instead, a lack of genetic material in some samples was likely caused due to a suboptimal sampling technique or timing, or high temperatures during storage or shipping. The fact that one veterinary practice submitted nine negative samples from five farms collected in June supports our proposal. Only negative samples were submitted in August and September, indicating that there may be differences in the presence of eggs and/or larvae through the season. This may lead to a lower number of positive samples by the end of the season but should not have had an effect on samples collected in June.

We did not obtain information on the breed of the affected animals. However, in Austria, about 75% of the cattle population consists of *Fleckvieh*. Breeds like Charolais or Blonde d'Acquitane that introduced parafilariosis from France to countries like Canada or Belgium [12–15] only make up about 1% of cattle in Austria [31]. However, it is not impossible that breeders introduced the parasitosis by purchasing subclinically affected breeding stock from endemic regions like in the case of *Besnoitiosis* in Switzerland and Germany [32,33].

Sequencing resulted in the detection of four haplotypes. Only one entry of *P. bovicola* was available on GenBank (accession number: MG983751) for comparison [34], which showed 100% identity to haplotype 1 with a query coverage of only 96%. Therefore, the sample was not included in the analysis.

Three different haplotypes were detected in both Lower Austria and Salzburg. Together with reports of parafilariosis in several neighboring countries [22,23,34], this leads to the conclusion that it is unlikely that the infections originated from a point source, but rather from different routes of introduction. The first suspected cases of parafilariosis—which were not confirmed using molecular methods—were reported in Austria over a decade ago and the disease is considered endemic in parts of Carinthia [21]. Likely, cattle in other parts of Austria have displayed symptoms before, but we suspect a surge of clinical cases in 2020, which might have exceeded the threshold required to be noted as unusual.

The true extent of the problem, the epidemiological situation in Austria, is unknown because our study is based on a convenience sample and we relied on the voluntary participation of veterinarians and farmers. However, we are convinced that most Austrian veterinarians received our information letter distributed by the Animal Health Services in all federal states, meaning that everyone who was interested had a chance to participate.

Lesions were usually observed between December and July in the northern hemisphere [2,3]. Even though the call to submit samples was sent out in June, which is late in the typical "bleeding season", we received a substantial number of samples from five federal states. Therefore, we conclude that *P. bovicola* has spread in Austria and is most likely endemic in most parts of the country. Many farms in Austria are not closed operations, meaning that farmers buy animals at cattle markets or directly from other farms. This livestock movement allows for the distribution of asymptomatic animals that carry *P. bovicola* larvae. Once these animals start showing symptoms, the reproductive cycle of the parasite can be completed because the vector flies are ubiquitous [35]. Thus, the parasitic disease can spread in the new herd. Bech-Nielsen et al. [18] observed an expansion of the endemic *Parafilaria* area in Sweden of about 50 km/year through airborne transport by vector flies and the movement of these flies and cattle via transport vehicles.

Responses from the questionnaire indicate that many cattle were affected by parafilariosis on farms where fly control was performed and cattle were regularly treated with macrocyclic lactones. Unfortunately, antiparasitic treatment is ineffective against early larval stages. Hence, the metaphylactic treatment of animals from affected herds is useless. Symptomatic animals that have been treated show a rapid resolution of lesions [36,37] but may start bleeding again after only a few weeks [13].

The finding that farmers noted little to no effect of *P. bovicola* on the condition of affected animals are in accordance with previous reports [3,19]. The main cause for economic losses associated with *P. bovicola* is the carcass quality. The parasite causes edematous changes that may turn the form yellow to greenish, covering an area of 490.7 cm^2 on average, leading to the condemnation of 1.23 up to 6 kg of trimmings, especially in young bulls [4,38]. Most lesions are superficial but extensive involvement of the muscles are found in more severe cases [39,40]. Superficial lesions may be mistaken for contusions that occurred during handling or transport [4] and were, therefore, not reported in our study.

5. Conclusions

With a substantial number of positive samples from all over Austria, we conclude that *P. bovicola* has spread and will become endemic in the country in the near future if this is not the case already. We expect that reports of symptoms and lesions will occur more frequently as veterinarians and farmers become increasingly aware of the disease. It would be beneficial to implement a voluntary surveillance program where farmers and veterinarians submit samples to gain a better understanding of the true situation in Austria and other European countries. As the voluntary participation in our study was taken up well, we would expect such a program to yield valuable results and help us to understand the mode and pace of the spread of *P. bovicola* in different climatic zones and landscapes.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ani11102966/s1, File S1: COI sequence alignment, species names and accession numbers for the maximum likelihood tree, File S2: Maximum likelihood tree (1000 replicates) featuring COI sequences (576 bp) of *Parafilaria bovicola* and other members of the suborder Spirurida.

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Institutional Review Board Statement: This study was discussed and approved by the institutional ethics and animal welfare committee in accordance with GSP guidelines and national legislation (ETK-090/05/2020).

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6.7. Molecular analysis of blood-associated pathogens in European wildcats (*Felis silvestris silvestris*) from Germany

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- PCR analysis
- sequence analysis
- phylogenetic analysis
- statistical analysis
- drafting the manuscript

Other authors' contributions:

JH: supervision, data analysis, visualization, revising the manuscript

BSB: investigation, revising the manuscript

JS: investigation, resources, revising the manuscript

KH: investigation, resources, revising the manuscript

FM, DJ, OA, HA: sample acquisition

HPF: conceptualization, supervision, resources, methodology, revising the manuscript

MH: conceptualization, sample acquisition, supervision, methodology, revising the manuscript

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Molecular analysis of blood-associated pathogens in European wildcats (*Felis silvestris silvestris*) from Germany

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ABSTRACT

European wildcats (*Felis silvestris silvestris*) have not been investigated in large numbers for blood-associated pathogens in Germany, because wildcats, being a protected species, may not be hunted, and the collection of samples is therefore difficult. Thus, spleen tissue and whole blood from 96 wildcats from Germany found as roadkill or dead from other causes in the years 1998–2020 were examined for the prevalence of blood associated pathogens using molecular genetic tools. PCR was used to screen for haemotrophic *Mycoplasma* spp., *Hepatozoon* spp., *Cytauxzoon* spp., *Bartonella* spp., Filarioidea, Anaplasmataceae, and Rickettsiales, and positive samples were subsequently sequenced. Phylogenetic analyses were performed for *Mycoplasma* spp. and *Hepatozoon* spp. by calculating phylogenetic trees and DNA haplotype networks. The following pathogens were found: *Candidatus* Mycoplasma haematominutum (7/96), *Mycoplasma ovis* (1/96), *Hepatozoon silvestris* (34/96), *Hepatozoon felis* (6/96), *Cytauxzoon europaeus* (45/96), and *Bartonella* spp. (3/96). This study elucidates the prevalence of blood-associated pathogens in wildcats from Germany.

1. Introduction

Arthropod vectors are responsible for the transmission of several blood-associated parasites and bacteria, which can cause disease in wild and domestic animals, as well as in humans. Their relevance is increasing due to climate change driven migration into regions that are more temperate. Other factors promoting the introduction and spread of vector-borne pathogens (VBP) include, for instance, globalization, habitat change, loss of biodiversity, and pollution (Harrus and Baneth, 2005; Aguirre, 2009). Different wildlife species have different effects on the density of vectors (Takumi et al., 2019), and the role of wildlife in the transmission of VBP to humans and pets is not yet fully elucidated (Mackenstedt et al., 2015). Wild carnivores are important for the maintenance of the sylvatic cycle, therefore understanding the epidemiology of their VBP is crucial (Battisti et al., 2020). Wild canids and felids can spread disease-causing pathogens to their domestic counterparts and vice versa. The close relationship of pet dogs and cats to humans increases the risk of the emergence of zoonotic disease (Otranto

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et al., 2015).

The European wildcat (*Felis silvestris silvestris*) is closely related to the domestic cat (*Felis silvestris catus*) and inhabits pristine forests in different parts of Europe, with one main population group in Germany (Mattucci et al., 2016). Since the 17th their population has been significantly reduced as a result of human activities century, and the species is now listed as endangered (Heddergott et al., 2018; Meinig et al., 2020). However, species protection and habitat restoration has enabled the wildcat to thrive again, and there is now an estimated population size of 5000–10000 individuals (Balzer et al., 2018; European Topic Centre on Biological Diversity, 2019). Nonetheless, anthropogenic habitat fragmentation and possible genetic introgression from domestic cats still threaten their genetic integrity, and conservation measures are still essential (Hertwig et al., 2009; Eckert et al., 2010; Witzenberger and Hochkirch, 2014; Mattucci et al., 2016). Understanding the prevalence of potential pathogens in wildcat populations can be important in this context (Poirson and Dutilleul, 2014).

Only few studies on blood-associated parasites and bacteria in these animals are available due to their secretive lifestyle and, compared to the ubiquitous red fox (V*ulpes vulpes*), small population size, making it difficult to obtain samples from these animals (Hodžić et al., 2018a). Apicomplexan parasites such as *Hepatozoon* spp., *Cytauxzoon* spp., and *Babesia* spp. are frequently found in wildcats (Zaeemi et al., 2015; Gallusová et al., 2016; Veronesi et al., 2016; Hodžić et al., 2018a; Hornok et al., 2022). *Hepatozoon* spp. use a wide range of vertebrates as intermediate hosts and hematophagous invertebrates as definitive hosts and vectors. The transmission to their vertebrate host is mainly through ingestion of the vector but can also happen through predation via tissue cysts or transplacental transmission (Nordgren and Craig, 1984; Johnson et al., 2009; Baneth et al., 2013). Among the Hepatozoidae, *Hepatozoon felis* is the predominant species found in cats and wildcats, but *H. silvestris* and have also been described (Giannelli et al., 2017a; Hodžić et al., 2017). The vectors of feline hepatozoonosis are unknown (Hodžić et al., 2017). Among the family Theileriidae, *Cytauxzoon felis*, *C. manul,* and three newly described species, namely *C. europaeus*, *C. otrantorum,* and *C. banethi*, have been reported from felids. The tick species *Amblyomma americanum* and *Dermacentor variabilis* were identified as vectors for *C. felis* in America (Blouin et al., 1984; Reichard et al., 2010). The vector is not known for European *Cytauxzoon* spp., but *Ixodes ricinus* has been suggested due to its high abundance (Panait et al., 2021b). Bacterial pathogens transmitted by vectors to domestic cats include *Bartonella* spp., *Anaplasma* spp., *Rickettsia* spp., and *Mycoplasma* spp. (Lappin, 2018). Of these, *Mycoplasma* spp. has also been reported in wildcats (Willi et al., 2007; Hodžić et al., 2018a). *Candidatus* Mycoplasma haemominutum was first described by Foley and Pedersen (2001); it affects domestic cats as well as wild felids (Foley and Pedersen, 2001; Willi et al., 2007; Cerreta et al., 2022). Oren (2017) suggested correcting the name to *Candidatus* Mycoplasma haematominutum for the sake of linguistic accuracy.

Reports of Filarioidea in wildcats are only sporadic and the role of wildcats in the maintenance of the sylvatic cycle of Filarioidea, such as *Dirofilaria immitis,* is considered to be low (Penezić et al., 2014; Ionică et al., 2017).

In the present study, we screened blood and spleen tissue of wildcats from Germany for blood-associated pathogens using molecular genetic methods, to estimate the possibility of pathogen transmission between wildcats and domestic cats by vectors or other transmission routes.

2. Materials and methods

2.1. Sample collection

Between 1998 and 2020, 96 wildcats found as roadkill or dead from other causes were collected in Germany (Fig. 1) and stored at −20 °C until necropsy. The individuals originated from six federal states: Bavaria (n = 2), Hesse (n = 30), Lower Saxony (n = 37), Rhineland-Palatinate (n = 1), Saxony-Anhalt (n = 4), and Thuringia (n = 22) (Fig. 2). During dissection, 1–5 ml of blood were collected from 55 individuals and spleen samples from 41 individuals. The samples were stored at −20 °C until final processing at the University of Veterinary Medicine, Vienna. The cats were classified as wildcats according to the intestinal index (Braunschweig, 1963) and cranial index (Schauenberg, 1969). Individuals, where classification was not clear or not possible due to severe destruction, were genetically tested (Steyer et al., 2016). The age determination of the wildcats was based on the growth lines in the enamel of a mandibular canine (Ansorge, 1995; Heddergott et al., 2016). According to Piechocki and Stiefel (1988), the cats were assigned to two age classes: juvenile/subadult (≤24 months; none or one growth line) and adult (≥25 months; two or more growth lines).

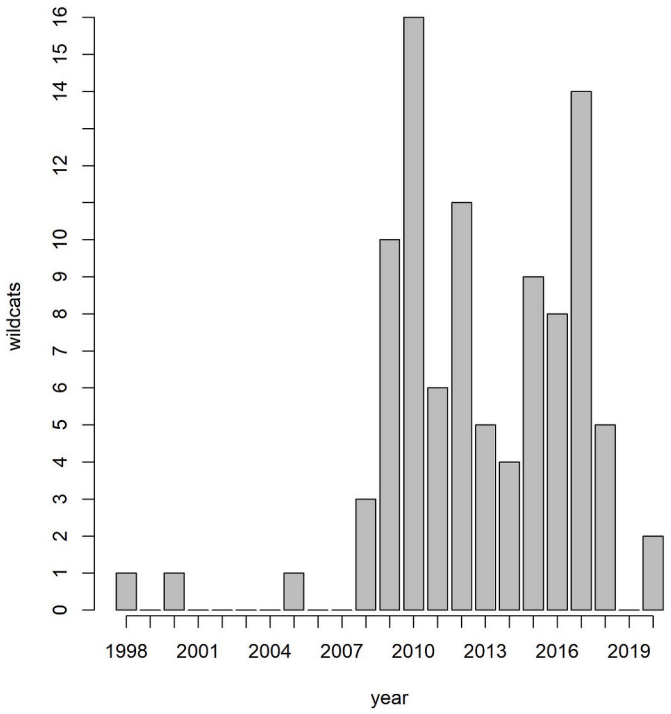

Fig. 1. Distribution of wildcat samples in total number of wildcats (y-axis) collected per year (x-axis).

2.2. DNA extraction, PCR amplification, and sequencing

DNA was isolated from spleen samples and whole blood using the QIAGEN DNeasy Blood and Tissue kit (QIAGEN, Hilden, Germany). Samples were incubated at 56 °C overnight and processed according to the manufacturer's protocol. Samples were screened for the presence of various blood-associated pathogens using specific broad-range PCR assays (Table 1) targeting the following fragments: *Mycoplasma* spp. Within the 16 S rRNA gene, and if positive, a larger fragment of the 16 S rRNA gene; Piroplasmida and other Apicomplexa within the 18 S rRNA gene, and if positive, for *Hepatozoon* spp. or *Cytauxzoon* spp. a larger fragment of the 18 S rRNA gene; and for *Cytauxzoon* spp. additionally the cytochrome *b* gene (*CytB*); *Bartonella* spp. Within the 16 S–23 S rRNA gene, and if positive, the citrate synthase gene (*gltA*); *Rickettsia* spp. the 23 S–5S rRNA gene; Anaplasmataceae within the 16 S rRNA gene; and Filarioidea targeting a fragment of the mitochondrial cytochrome *c* oxidase subunit I gene (*COI*). Positive and negative controls were used to validate results. PCR products were analyzed by electrophoresis in 2% agarose gels stained with Midori Green Advance DNA stain (Nippon Genetics Europe, Germany). Positive samples were sent to a commercial company (LGC Genomics GmbH, Germany) for sequencing using amplification primers.

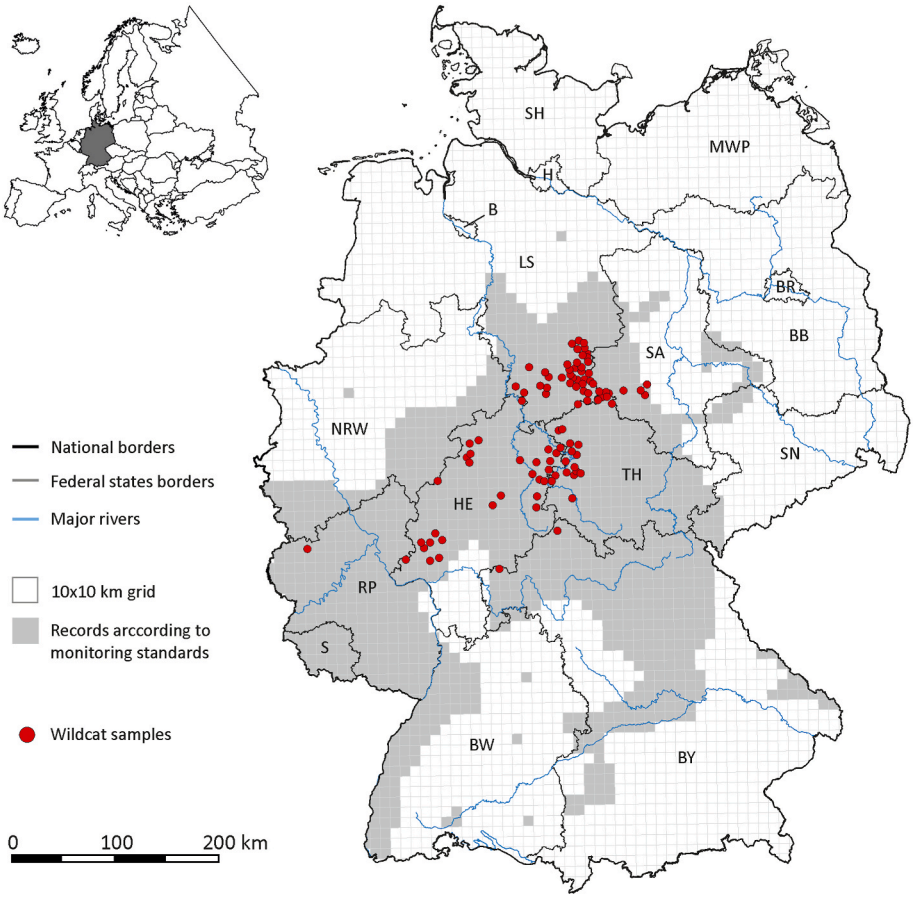

Fig. 2. Geographic origin of the 96 European wildcats (*Felis silvestris*) from Germany included in this study. The gray area represents the geographic distribution of wildcats in Germany according to the National FFH Report 2019, plotted on the 10 × 10 km reference grid ETRS89-LAEA5210 EEA according to a compilation of the German Federal Agency for Nature Conservation (BfN) and monitoring data of the federal states (Bundesamt für Naturschutz, 2020). Abbreviations: Brandenburg (BB), Bremen (B), Berlin (BR), Baden-Württemberg (BW), Bavaria (BY), Hamburg (H), Hesse (HE), Mecklenburg-West Pomerania (MWP), Lower Saxony (LS), North Rhine-Westphalia (NRW), Rhineland-Palatinate (RP), Schleswig-Holstein (SH), Saarland (S), Saxony (SN), Saxony-Anhalt (SA) and Thuringia (TH).

2.3. Phylogenetic analysis

The 18 S rRNA sequences of *C. europaeus* and the 16 S–23 S rRNA sequences of *Bartonella* spp. Were analyzed using the BLAST function on NCBI GenBank. For *C. europaeus* the *CytB* sequences were compared to those of *Cytauxzoon* spp. published by Panait et al. (2021b) to determine the species. For phylogenetic analysis, nucleotide sequences available on the NCBI GenBank database were searched by using the BLAST function, using one of the sequences obtained for each organism. The organism group was specified as Mycoplasma (taxid:2093) for the *Mycoplasma* spp. sequences and Adeleorina (taxid:75,740) for the *Hepatozoon* spp. sequences, with the number of maximum target sequences set to 5000. The sequences were aligned and sorted using the default option (FFT–NS–2) in MAFFT v.7.311 (Katoh and Standley, 2013) and sequences not covering the fragment of the sequences obtained in this study were excluded. All sequences featuring obvious sequencing errors and ambiguity characters were removed from the alignment and were excluded from the analysis. The chosen sequences included selected *Mycoplasma* spp. (based on their similarity in the alignment) and *Hepatozoon* spp. as well as other members of the suborder Adeleorina. Sequences used for analysis were uploaded to GenBank (GenBank accession numbers: ON202709-ON202711, ON180678-ON180682, OL415842-OL415874, ON380442-ON380486, ON855993-ON856037, and OL697395-OL697397).

To provide an overview of the diversity of haplotypes, Maximum Likelihood (ML) and Bayesian Inference (BI) trees were calculated for each organism based on alignments, including 158 sequences (975 nucleotide positions) for *Mycoplasma* spp. and 537 sequences (585 nucleotide positions) for *Hepatozoon* spp. Alignment gaps were removed using TrimAl v.1.3 (http://phylemon2.bioinfo.cipf.es/; Sánchez et al., 2011) and sequences were collapsed to haplotypes using DAMBE v.7.0.5.1 (Xia and Xie, 2001), leaving 84 haplotypes (969 nucleotide positions) for *Mycoplasma* spp. and 183 haplotypes (539 nucleotide positions) for *Hepatozoon* spp. As outgroup for *Mycoplasma* spp. one sequence of *Mycoplasma pneumonia* (GenBank accession number: NR041751) and for *Hepatozoon* spp. two sequences of *Adelina bambarooniae* (GenBank accession numbers: AF494058, AF494059) were used. ML bootstrap consensus trees (1000 replicates) were calculated using the W-IQ-TREE web server (http://iqtree.cibiv.univie.ac.at/; Trifinopoulos et al., 2016) applying the models TIM3+F + I + G4 for *Mycoplasma* spp. and K81u (K3P)+F + I + G4 for *Hepatozoon* spp., which were suggested as best fit for the data set in the model test according to the Bayesian inference criterion (BIC). The BI trees were calculated using MrBayes v.3.2.7 (Ronquist et al., 2012), applying the next complex model GTR + G + I, because the same models were not available in this program. The analysis was run for 10^6 generations (Number of chains: 4), sampling every thousandth tree. The first 25% of trees were discarded as burn-in and a 50% majority-rule consensus tree was calculated based on the remaining 7500 trees.

Median-joining haplotype networks were calculated with Network 10.2.0.0 (Fluxus Technology Ltd., Suffolk, UK), applying the default settings. If only one haplotype was present, pie charts were created in Excel (2016) (Microsoft Corporation, Redmond, USA). Networks and pie charts were graphically prepared and provided with information on the countries and hosts in Network Publisher v.2.1.2.3 (Fluxus Technology Ltd., Suffolk, UK) and finalized with CorelDRAW 2021 (Corel, Ottawa, Canada). Calculation of *p*-distances was performed with MEGA version 11 (Tamura et al., 2021).

2.4. Statistical analysis

Binary logistic regression was conducted to test the association

Table 1
Oligonucleotide sequences of primers used in the present study.

Target organism (genetic marker)	Primer sequences (5′→3′)	Product size	Reference
Mycoplasma spp. (16 S rRNA)	HBT-F: ATA CGG CCC ATA TTC CTA CG HBT-R: TGC TCC ACC ACT TGT TCA	600 bp	Criado-Fornelio et al. (2003)
Mycoplasma spp. (16 S rRNA)	UNI_16 S_mycF: GGC CCA TAT TCC TAC GGG AAG CAG CAG T UNI_16 S_mycR: TAG TTT GAC GGG CGG TGT ACA AGA CCT G	1000 bp	Volokhov et al. (2011)
Hepatozoon spp. (18 S rRNA)	H14Hepa18SFw: GAA ATA ACA ATA CAA GGC AGT TAA AAT GCT H14Hepa18SRv: GTG CTG AAG GAG TCG TTT ATA AAG A	620 bp	Hodžić et al. (2015)
Piroplasmida (18 S rRNA)	BTH-1F: CCT GAG AAA CGG CTA CCA CAT CT BTH-1R: TTG CGA CCA TAC TCC CCC CA	700 bp	Zintl et al. (2011)
	GF2: GTC TTG TAA TTG GAA TGA TGG GR2: CCA AAG ACT TTG ATT TCT CTC	561 bp	
Cytauxzoon spp. (18 S rRNA)	7549 F: GTC AGG ATC CTG GGT TGA TCC TGC CAG 7548 R: GAC TGA ATT CGA CTT CTC CTT CCT TTA AG	1726 bp	Millán et al. (2007)
	Cyt-SSU-F2: CAT GGA TAA CCG TGC TAA TTG Cyt-SSU-R4: AGG ATG AAC TCG ATG AAT GCA	1335 bp	Panait et al. (2021b)
Cytauxzoon spp. (*CytB*)	Cytaux_cytb_F1: CTT AAC CCA ACT CAC GTA CC Cytaux_cytb_R3: GGT TAA TCT TTC CTA TTC CTT ACG	1434 bp	Schreeg et al. (2013)
	Cytaux_cytb_Finn: ACC TAC TAA ACC TTA TTC AAG CRT T Cytaux_cytb_Rinn: AGA CTC TTA GAT GYA AAC TTC CC	1333 bp	Panait et al. (2021b)
Bartonella spp. (16 S–23 S rRNA)	bartgd_for: GAT GAT GAT CCC AAG CCT TC B1623_rev: AAC CAA CTG AGC TAC AAG CC	179 bp	Jensen et al. (2000)
Bartonella spp. (*gltA*)	BhCS.781p: GGG GAC CAG CTC ATG GTG G BhCS.1137n: AT GCA AAA AGA ACA GTA AAC A	379 bp	Norman et al. (1995)
Rickettsia spp. (23 S–5S rRNA)	ITS-F: GAT AGG TCG GGT GTG GAA G ITS-R: TCG GGA TGG GAT CGT GTG	350-550 bp	Vitorino et al. (2003)
Anaplasmataceae (16 S rRNA)	EHR16SD_for: GGT ACC YAC AGA AGA AGT CC EHR16SR_rev: TAG CAC TCA TCG TTT ACA GC	345 bp	Parola et al. (2000)
Filarioidea (*COI*)	H14FilaCOIFw: GCC TAT TTT GAT TGG TGG TTT TGG H14FilaCOIRv: AGC AAT AAT CAT AGT AGC AGC ACT AA	724 bp	Hodžić et al. (2015)

Note: Supplementary data associated with this article.

between detection of pathogens (summarized per genus) and tissue investigated, age and sex of the animals (each fitted as fixed categorical effects with two levels), and over time. Effects were considered statistically significant if $P < 0.05$. No multiple testing was necessary. Statistical analysis was performed using R version 4.2.0 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

All individuals included in this study were European wildcats (*Felis silvestris*). The data set evaluated in this study consisted of 61 males and 35 females (comprising 25 juveniles/subadults, 70 adults, and one of unknown age). Pathogens detected were *Candidatus* Mycoplasma haematominutum (n = 7; 7.29%), *Mycoplasma ovis* (n = 1; 1.04%), *Hepatozoon silvestris* (n = 34; 35.42%), *H. felis* (n = 6; 6.25%), *Cytauxzoon europaeus* (n = 45, 46.88%) and *Bartonella* spp. (n = 3; 3.13%). All PCRs for Filarioidea, Anaplasmataceae, and Rickettsiales were negative (Fig. 3). In total, pathogens were found in 67/96 (69.79%) wildcats. One pathogen only was documented in 40/96 (41.97%) cats, two different pathogens were found in 25/96 (26.04%), and three different pathogens were detected in 2/96 (2.08%) animals (Fig. 4). Logistic regression did not detect any association between pathogen occurrence and tissue investigated, and age and sex of the animals. There was a statistically significant increase in detecting *C. europaeus* ($P = 0.038$, McFadden $R^2 = 0.05$) over time, but no other association over time was detected.

The genetic analysis of *Candidatus* Mycoplasma haematominutum revealed one haplotype identical to a haplotype found in domestic cats in Switzerland, Italy, Hungary, the United Kingdom, and Brazil (Fig. 5). This haplotype was placed within the clade (BI posterior probability (BI pp): 1; ML bootstrap value (ML bs): 100) of other *Candidatus* Mycoplasma haematominutum sequences in the consensus tree (Suppl. 1). The sequence of *M. ovis* obtained in this study showed 100% identity to a *M. ovis* sequence found in a goat (*Capra hircus*) from China (GenBank accession number: KU983745).

The sequences of *H. silvestris* were 100% identical to the haplotype detected in wildcats in Bosnia and Herzegovina and one domestic cat in Switzerland. The sequences of *H. felis* were 100% identical to the haplotype found in a wildcat from Hungary (Fig. 6). In the consensus tree, *H. felis* and *H. silvestris* were placed in a clade (BI pp: 0.65; ML bs: 75) together with *Hepatozoon* spp. mainly found in Canidae, Suidae, and Mustelidae (Suppl. 2). Within that clade, the two species were not closely related, and most sequences of *H. felis* were placed in a separate clade in both the BI tree (BI pp: 0.65) and the consensus tree (BI pp: 0.93; ML bs: 99), except for two sequences that fell outside this clade. Two *H. felis* sequences were placed in another distinct clade (BI pp: 0.92; ML bs: 99) together with *H. luiperdjie*.

The *18S* rRNA sequences of *C. europaeus* showed high similarity to other European *Cytauxzoon* spp. (99.77–100% identity to *C. europaeus* with the GenBank accession number MT904044). The alignment of *CytB* sequences with *C. europaeus* (GenBank accession number: MT916191), *C. otrantorum* (GenBank accession number: MT916204), *C. banethi* (GenBank accession number: MT916193), and *C. felis* (GenBank accession number: MT916203) showed the highest similarity to *C. europaeus* with a *p*-distance < 0.011.

The three *Bartonella* spp. sequences obtained in this study were distinct from each other and showed 100% identity to *Bartonella* sp. found in a yellow-necked mouse (*Apodemus flavicollis*) from Slovakia (GenBank accession number: KX267683), 100% identity to *Bartonella* sp. found in a common mole (*Microtus arvalis*) from Poland (GenBank accession number: GU338968) and 100% identity to *Bartonella* sp. found in a bank vole (*Clethrionomys glareolus*) from Slovakia (GenBank accession number: KX267679), respectively.

4. Discussion

The large sample size of the present study allowed us to obtain an overview of blood-borne pathogens harboured by wildcats in Germany. The pathogens detected and their prevalence are comparable to those obtained by studies on wildcats from other parts of Europe using molecular genetic tools (Willi et al., 2007, 2022; Gallusová et al., 2016; Veronesi et al., 2016; Hodžić et al., 2018a; Panait et al., 2021b).

Our results of *Hepatozoon* spp. are comparable with the results in

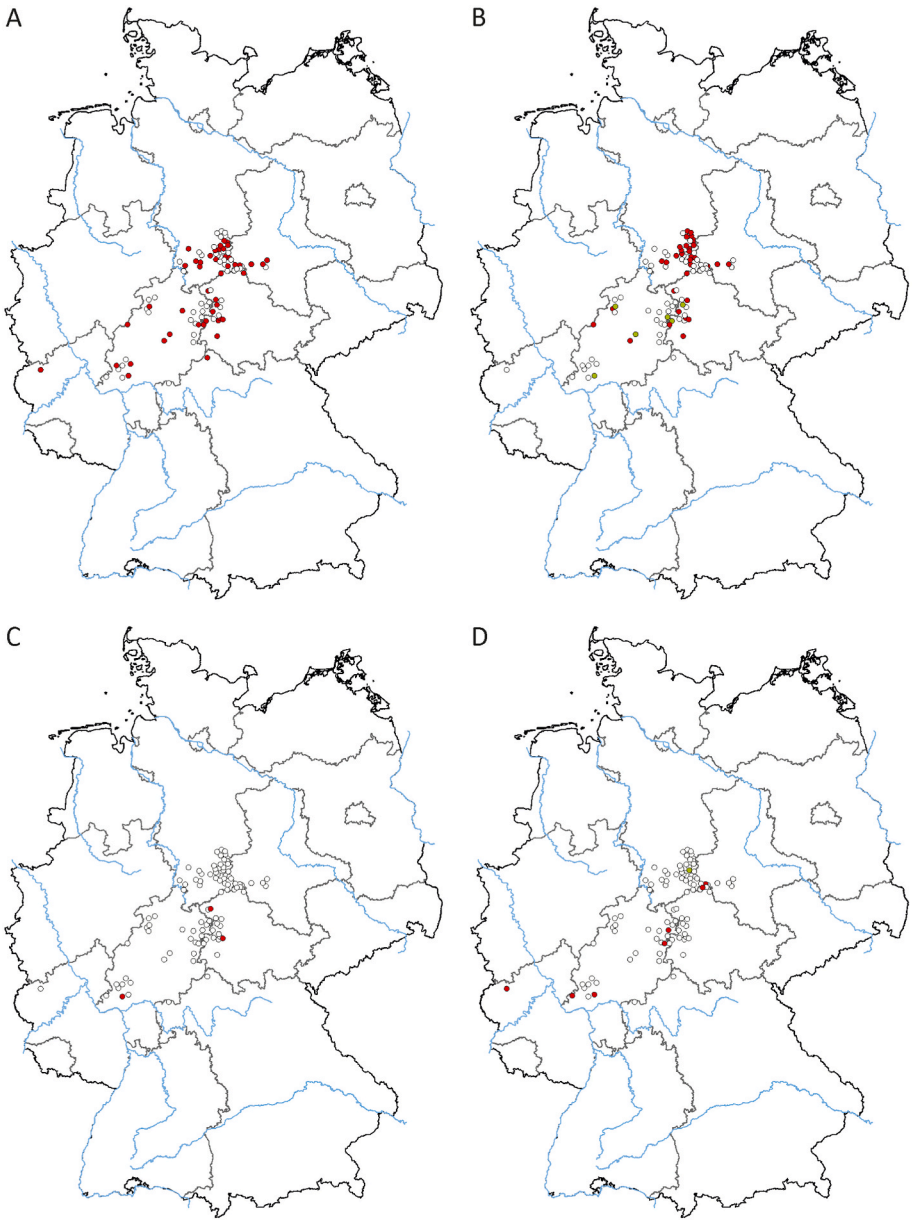

Fig. 3. Geographical distribution of uninfected (white dots) and infected European wildcats (*Felis silvestris*) from Germany according to detected pathogens. A: red dots represent detection of *Cytauxzoon europaeus*; B: red dots represent detection of *Hepatozoon silvestris*, green dots represent detection of *Hepatozoon felis*; C: red dots represent detection of *Bartonella* spp.; D: red dots represent detection of *Candidatus* Mycoplasma haematominutum; green dots represent detection of *Mycoplasma ovis*; blue lines represent major rivers; and black lines represent borders of federal states. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

wildcats from Bosnia and Herzegovina, where *H. felis* (3/9), *H. silvestris* (2/9), and unidentified *Hepatozoon* species (2/9) were detected (Hodžić et al., 2018a). In the same study, *Cytauxzoon* sp. (10/18) was found, which is consistent with results in wildcats from Romania (9/12) and with our study (Gallusová et al., 2016; Hodžić et al., 2018a). *Cytauxzoon* sp. Was also shown to be present in wildcats in Italy (4/21), albeit with a lower prevalence (Veronesi et al., 2016). Panait et al. (2021b) analyzed European *Cytauxzoon* spp. in more detail and described three new species. They found *C. europaeus* in wildcats from Germany (30/46), Romania (9/31), the Czech Republic (5/11), and Luxembourg (9/13). This is also comparable with the findings of *C. europaeus* in wildcats from France (10/34) and with our results (Willi et al., 2022).

Rickettsiales such as *Anaplasma phagocytophilum* are widespread tick-borne pathogens in many mammals in Europe, and have also been reported in domestic cats from Germany. However, we did not detect these pathogens in our study (Stuen, 2007; Morgenthal et al., 2012; Bergmann et al., 2015; Bergmann and Hartmann, 2017; Schäfer et al., 2022). Likewise, García-Pérez et al. (2016) did not detect Anaplasmataceae in wildcats from Spain (0/8), although the sample size may have been too small for detection. This is not the case with the sample size in our study. Considering that serological detection revealed a higher prevalence than molecular detection in studies conducted in domestic cats, our results may suggest few active infections with this pathogen, rather than an absence of infection in wildcats (Morgenthal et al., 2012; Schäfer et al., 2022).

Candidatus Mycoplasma haematominutum was detected in our study as well as in wildcats from France (6/13), where *Candidatus* Mycoplasma turicensis (11/31) was also found (Willi et al., 2007). In the study by Hodžić et al. (2018a) mentioned above *Mycoplasma* spp., which were genetically distinct from *Candidatus* Mycoplasma haematominutum, were found in wildcats from Bosnia and Herzegovina (4/18). Surprisingly, we detected *M. ovis* in a wildcat in our study. Since *M. haemofelis* was used as a positive control, contamination of the sample is unlikely. This pathogen is usually found in sheep and other small ruminants, but it was also recently reported in horses from Iran (Kalantari et al., 2020). *M. ovis* was likely only transiently present in the blood of the one wildcat from our study, as there are no other reports of *M. ovis* in carnivores to the authors' knowledge.

Bartonella spp. sequences obtained in our study were distinct from each other but were all 100% identical to sequences found in rodents. It

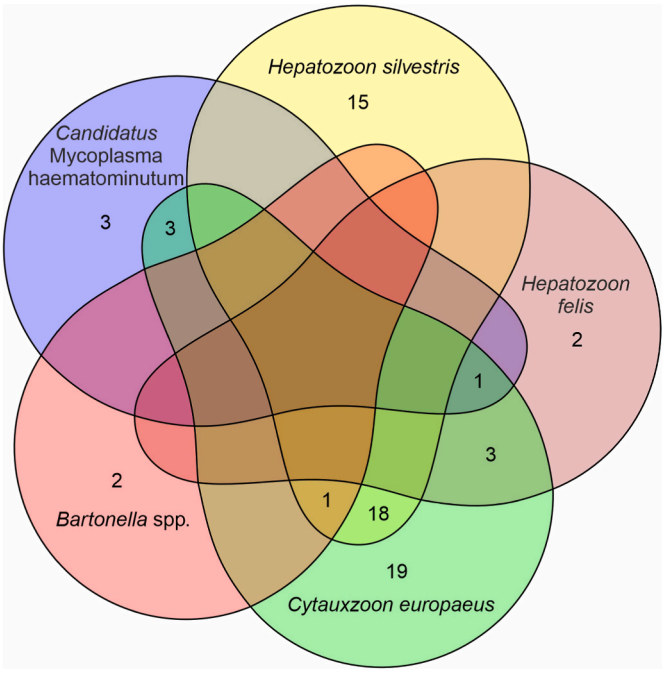

Fig. 4. Co-infection scheme of detected pathogens, excluding *M. ovis*. Numbers represent counts of European wildcats (*Felis silvestris*) with respective pathogen (s) detected.

is also possible that these pathogens were temporarily present in the blood, or that transmission to wildcats occurred through predation. This hypothesis, however, would need further investigation. Generally, fleas are known to play an important role in the spread of *Bartonella* spp. and are also discussed as vectors for haemotrophic *Mycoplasma* spp. (Millán et al., 2021). In fact, *Candidatus* Mycoplasma haematominutum was detected in cat fleas (*Ctenocephalides felis*) in Hungary (Hornok et al., 2010). Findings of Millán et al. (2021) support this theory, as co-infection of *Bartonella* spp. and haemotrophic *Mycoplasma* spp. often occur, although ingestion of the cat flea does not seem to play a role in transmission (Woods et al., 2006). This co-infection was not detected in our study, possibly due to the low prevalence of *Bartonella* spp. In the same review, Millán et al. (2021) state that detection of haemotrophic *Mycoplasma* spp. is also dependent on the tissue investigated, being more prevalent in blood compared to spleen tissue, probably because the pathogen is eliminated more quickly from the spleen compared to the blood. Likewise, in our study, *Mycoplasma* spp. Was detected more often in the blood compared to spleen tissue, although this difference was not statistically significant. Modelling of transmission pathways for *Candidatus* Mycoplasma haematominutum suggests a concurrent role of vectors as well as direct transmission (Kellner et al., 2018).

Similarly, there are known routes of transmission for Piroplasmida, for example by hard ticks, but they are not fully elucidated for all species (Giannelli et al., 2017b; Thomas et al., 2018). Based on the overlapping distribution of the pathogens detected in our study, *Ixodes ricinus*, *Dermacentor reticulatus*, *Ixodes hexagonus*, and *Ixodes inopinatus* could act as possible vectors (Rubel et al., 2021), but other routes of transmission are also suggested to play a role, such as direct transmission through bites or diaplacentar transmission (Hornok et al., 2013; Hodžić et al., 2018a, 2018b). The high number co-infections with *C. europaus* and *H. silvestris* might indicate a common transmission route for these pathogens, but since these pathogens were the most prevalent, a high rate of co-infection is expected.

Although climate change is considered to promote the distribution of vectors (Harrus and Baneth, 2005; Aguirre, 2009; Cunze et al., 2022), for most pathogens we found no evidence of a trend over time for possible or definite vector transmission. However, it could be that a trend in this and other transmission routes, was not yet detectable, but will become apparent as climate change progresses. In that case, this study will provide essential baseline data. Nevertheless, an increase over time was observed in *C. europaeus*, although unknown confounding factors may not have been accounted for in the model, as indicated by the low McFadden R^2. Although Willi et al. (2022) demonstrated that *C. europaeus* could be detected in wildcat samples between 1995 and 1996 and for this reason do not consider this pathogen as emerging, an increase in prevalence in wildcats over time may have led to spill over into domestic cats and therefore explain the recent more frequent detection in domestic cats Legroux et al. (2017); Panait et al. (2021b); Willi et al. (2022).

Interestingly, *H. silvestris* was more widespread in central Germany than *H. felis*, which was detected more in the western part of Germany. This distribution might reflect the separation of the German wildcat population into a western and central population (Mattucci et al., 2016).

The phylogenetic analysis of *Mycoplasma* spp. and *Hepatozoon* spp. sequences focused on generating a network to illustrate the distribution of haplotypes according to hosts and countries with closely related sequences. For this reason, *M. haemofelis* and *Candidatus* Mycoplasma turicense were not included in our phylogenetic tree, due to their dissimilarity, although these pathogens have been found in wild felids and their phylogenetic relation was described by Willi et al. (2007). For *Hepatozoon* spp., the BI tree only supported the clade of *H. felis* with a posterior probability of 0.65, and the clade was not supported in the ML tree. However due to the high similarity of the sequences this clade was chosen for the network analysis.

The *H. felis* haplotype found in the present study and in a wildcat from Hungary was not closely related to the only other haplotype found in Europe in domestic cats from Spain (Criado-Fornelio et al., 2006; Hornok et al., 2022). Furthermore, Hornok et al. (2022) described two distinct genotypes of *H. felis* in wildcats from Hungary. Comparison with the consensus tree calculated in the present study shows that these genotypes refer to the clade containing the *H. felis* sequences obtained here, and to the clade containing sequences of *H. luiperdjie* described by van As et al. (2020) in a leopard (*Panthera pardus*). These findings support the hypothesis that *H. felis* is not a phylogenetically well-defined species, but rather a species complex that requires further investigation (Hodžić et al., 2017; van As et al., 2020; Hornok et al., 2022).

Apart from one report in wildcats from France (Willi et al., 2007), which had a different haplotype, this is the first report of this *Candidatus* Mycoplasma haematominutum lineage in wildcats. All sequences from the present study belong to the same haplotype, which is also the major haplotype reported, but has only been detected in domestic cats until now (Tasker et al., 2001; Willi et al., 2006; Hornok et al., 2008; Aquino et al., 2014). The haplotype of *H. silvestris* reported in the present study is identical to the only haplotype reported so far in wildcats from Bosnia and Herzegovina, and also in a domestic cat from Switzerland (Hodžić et al., 2017; Kegler et al., 2018). This indicates that wildcats and domestic cats do share blood-associated pathogens.

The clinical impact of the pathogens detected in wildcats is unknown. Although there are case reports in domestic cats with severe disease (Hornok et al., 2008; Legroux et al., 2017; Kegler et al., 2018; Basso et al., 2019), the high prevalence of *Candidatus* Mycoplasma haematominutum, *Hepatozoon* spp., and *C. europaeus* more likely indicate asymptomatic infection in wildcats. This theory is supported by other studies reporting high prevalence without clinical disease (Willi et al., 2006; Grillini et al., 2021).

Hodžić et al. (2018a), as well as a study performed in Romania (Panait et al., 2021a), described the presence of *Babesia* spp. in wildcats, yet we failed to detect this parasite in the present study. In our study, Piroplasmida and other Apicomplexa were detected by a nested PCR, and detection of *Hepatozoon* spp. or *Cytauxzoon* spp. might have interfered with the detection of *Babesia* spp. This interpretation is contradicted by the fact that in the study by Hodžić et al. (2018a) the same method was used, and *Babesia* sp. Was detected in a sample that was also

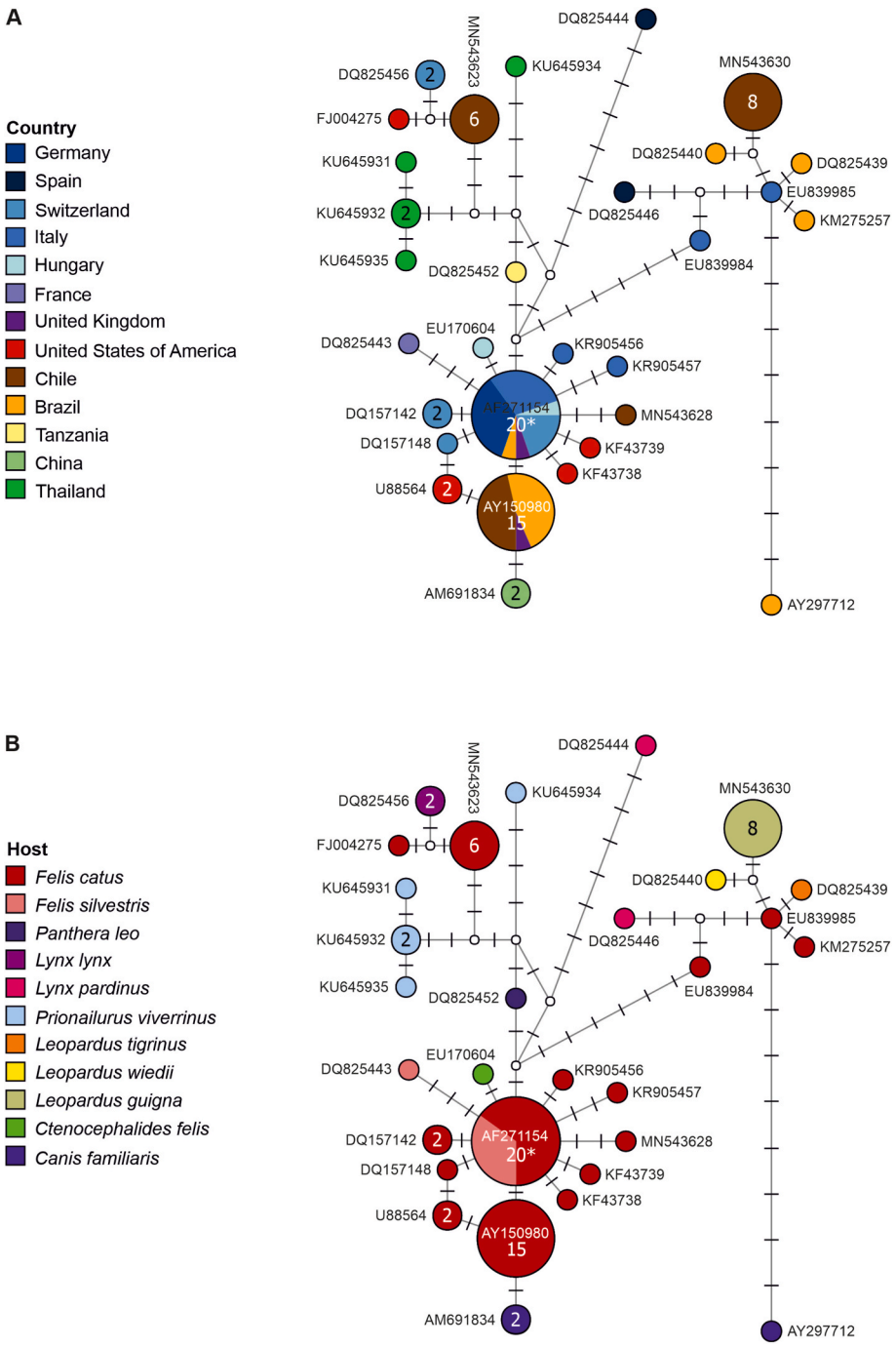

Fig. 5. Median Joining haplotype network of the 16 S rRNA sequences (983 nucleotide positions) of *Candidatus* Mycoplasma haematominutum showing the geographical distribution (A) and the reported hosts (B). Circles represent haplotypes; numbers within the circles represent the number of individuals, if no number is shown, then only one individual is represented; labels next to circles specify representative GenBank accession numbers of the haplotypes, white circles represent intermediate nodes; bars on branches interconnecting haplotypes represent the number of substitutions; and asterisks mark haplotypes containing the individuals obtained in the present study.

positive for *Cytauxzoon* sp. Another explanation might be that *Babesia* spp. associated with felids are not yet widespread in Europe (Penzhorn and Oosthuizen, 2020). Similarly, Filarioidea, such as *Dirofilaria* spp. Were not detected in our study, which is most likely due to the fact that though *Dirofilaria* spp. has been described in mosquitoes and vertebrate hosts in Germany, the prevalence is not considered to be high (Fuehrer et al., 2021). Romania is a highly endemic country for *Dirofilaria* spp., and *D. immitis* has been detected in a wildcat there (Ionică et al., 2017).

In conclusion, this study provides information on the prevalence of blood-associated pathogens in wildcats from Germany. Considering that the wildcat is an endangered species, the data can be of importance for wildlife conservation. The results are also valuable for veterinarians, as free-ranging domestic cats roam in the same area as wildcats and are therefore at risk of infection. However, additional studies are needed to elucidate the route of transmission and the clinical impact of these pathogens.

Data availability statement

The data presented in this study are contained within the article and supplementary material (Supplementary file).

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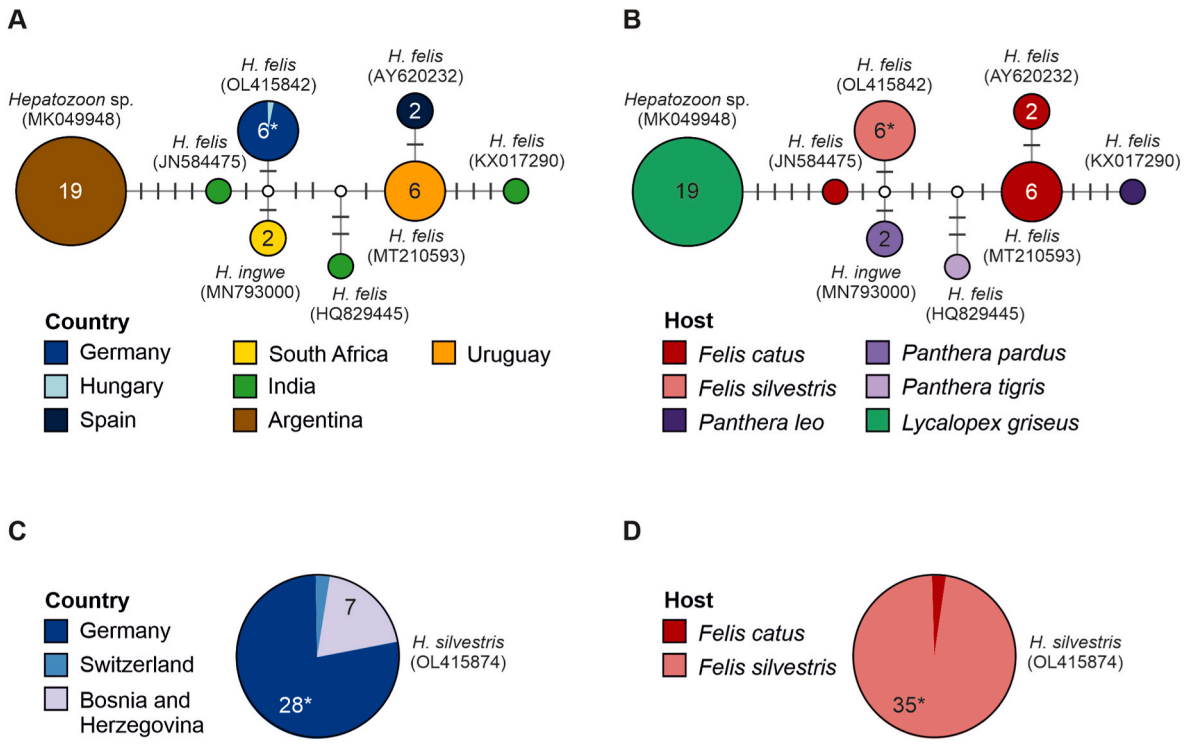

Fig. 6. Median Joining haplotype network of the 18 S rRNA sequences (561 nucleotide positions) of *Hepatozoon felis* (A, B) and pie chart of the 18 S rRNA gene (572 nucleotide positions) of *Hepatozoon silvestris* (C, D) showing the geographical distribution (A, C) and the reported hosts (B, D). Circles represent haplotypes; numbers within the circles represent the number of individuals, if no number is shown, then only one individual is represented; labels next to circles specify organism name and representative GenBank accession numbers of the haplotypes, white circles represent intermediate nodes; bars on branches interconnecting haplotypes represent the number of substitutions; and asterisks mark haplotypes containing the individuals obtained in the present study.

commercial, or not-for-profit sectors.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2022.08.012.

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7. Discussion

In view of the hypotheses put forward, the following conclusions can be drawn:

1. Hypothesis:

Canine VBPs known to be formerly only endemic to southern or eastern Europe are emerging in, or are now endemic to, Austria.

The hypothesis could be verified in several different studies. Firstly, we report here for the first time the presence of autochthonous infections of *D. immitis* and *O. lupi* in Austria. Secondly, a literature review on the current risk of spreading in central and northern Europe of *Dirofilaria* spp. and *A. vasorum* was conducted and revealed that they are spreading, and their relevance is increasing. Furthermore, the housing conditions of dogs seem to play an important role for the occurrence of *Dirofilaria* spp. Thirdly, the analysis of clinical cases of canine babesiosis showed that they are now predominantly autochthonous cases in Austria and that the prevalence of canine babesiosis fluctuated but did not increase since the beginning of the 21st century. Finally, autochthonous clinical cases of thelaziosis were described and the vector *P. variegata* was identified. All these parasites were not present in the past, but only known as import diseases in Austria. These parasites are now obviously extending their range, hypothetically due to multiple factors, such as climate change, globalisation and habitat change. They should be closely monitored so that medical and veterinary care can be prepared.

2. Hypothesis:

Parafilaria bovicola in cattle is endemic to Austria.

The hypothesis could be verified by the molecular detection of *P. bovicola* in 40 animals and the evidence of four different haplotypes. Based on this data, future studies will be able to evaluate if the parasite was underreported or is now emerging.

3. Hypothesis:

Wildcats harbour emerging VBPs relevant for the health of domestic cats.

The hypothesis could be verified by molecular detection of *H. silvestris*, *H. felis*, *Cytauxzoon europaeus*, *Candidatus* Mycoplasma haematominutum, and *Bartonella* spp. in blood and

spleen of wildcats. Although these pathogens can theoretically infect domestic cats, their impact on their health and that of wildcats needs further investigation. Currently only a small population of wildcats is present in Austria, but if the population increases their role as reservoir hosts for pathogens of domestic cats will most likely increase as well. Additionally, the route of infection is not clear for many of those pathogens and warrant further research.

Many VBPs are geographically defined. Nevertheless, if VBPs and vectors are dispersed through travel and trade or if their environment changes due to factors like climate change and changes in land use, their distribution can change as well (Harrus and Baneth 2005). In Austria, all those factors are present. Firstly, Austria as a central European country is a transit land. Additionally, global trade, tourism, and travel supports the import of new VBPs and vectors (https://www.statistik.at/, 27.09.2023). Secondly, climate change did already alter the Austrian climate, as seen by increase of the annual average temperature of the year 2023 by +2.4 °C above the temperature before 1990 (https://www.zamg.ac.at/, 15.12.2023). This supports the development of arthropod vectors as well as VBPs and can accelerate the epidemiology in endemic areas or enable VBPs to be introduced into new areas (Cuthbert et al. 2023). Thirdly, human land use is steadily increasing. In the year 2022 land claim in Austria was 5 648 km², leading to a change of the habitat for many vectors (https://www.oerok.gv.at/, 15.12.2023). All these factors promote the introduction and spread of VBPs. This process is currently evident in Austria, as shown by an increase of first reports on autochthonous cases of VBPs, as, for instance, reported for *D. immitis* and *O. lupi* in the present study (Kulmer et al. 2021, Unterköfler et al. 2023b). However, different VBPs and vectors are influenced differently by these factors, making prognosis of these complex situation difficult.

Introduction of new pathogens can be promoted by the mammal host (human, domestic or wild animal) or by the vector. Arthropod vectors have different routes of introduction into new geographic areas. New species of mosquitoes are expanding their range from the Mediterranean region northwards due to climate change, as can be seen by the detection of *Culiseta longiareolata* and *Anopheles hyrcanus* in Austria (Zittra et al. 2017b). Additionally, globalisation led to the introduction of mosquitoes from other continents, like the Asian tiger mosquito (Medlock et al. 2012). Dispersion by wind is another possibility for mosquitoes and even more for biting midges. This was most likely a major route of introduction for bluetongue-virus into Europe (Wilson and Mellor 2009). Ticks on the other hand are rather dispersed

through mammal or avian hosts. The tick *Hyalomma marginatum* for example is sporadically introduced to Austria through bird migration. However, the climate in Austria does currently not support their establishment (Duscher et al. 2022, Gray et al. 2009).

The effects of climate change on mosquitoes and ticks are similar in some respects, as milder winters will lead to a longer period of activity for both. The development of pathogens in mosquitoes will also accelerate with temperature, changing the epidemiology of mosquito-borne pathogens. However, the development of tick-borne pathogens occurs during the much longer time that they feed on a host that provides a relatively constant temperature and is therefore not altered by warmer outdoor temperatures. Changes in the distribution and prevalence of VBPs in mosquitoes are likely to be more immediate, whereas changes in tick-borne pathogens are likely to occur over decades (Ogden and Lindsay 2016).

The spread of *Dirofilaria* spp. is accelerated by climate change, as their development in the mosquito is temperature dependent (Ledesma and Harrington 2015). Formerly confined to southern Europe, *Dirofilaria* spp. are now spreading northwards (Fuehrer et al. 2021). In addition to the more suitable climate, the movement of dogs supports their distribution (Drake and Parrish 2019). Also, wild canids might play a role as reservoir hosts (Alsarraf et al. 2023, Moroni et al. 2020). Considering *D. repens* could not be detected in red foxes and racoon dogs in new endemic areas, wildlife probably plays a minor role in the dispersion in central Europe (Härtwig et al. 2016). In Austria, autochthonous *D. repens* infections are still very rare (Fuehrer et al. 2016, Sonnberger et al. 2020). Occurrence of autochthonous *D. immitis* infection in Austria has been published for the first time in this study. Considering that this case involved a cat, which is considered a side host, and that it was infected with more than 20 adult *D. immitis*, the question arises, if this region might have infected dogs as well (Kulmer et al. 2021). Nevertheless, *D. immitis* is not yet endemic to Austria, despite the fact that suitable vectors are present, and dogs with dirofilariosis are regularly imported (Sonnberger et al. 2020, 2021). The native house mosquito *Cx. pipiens* s.l. is considered a capable vector, as the development has been demonstrated in its sibling species the southern house mosquito *Cx. quinquefasciatus* and DNA of *D. immitis* has been detected in thorax and head of *Cx. pipiens* s.l. (Cancrini et al. 2007, Villavaso and Steelman 1970). The alien Asian tiger mosquito has also been demonstrated to be a competent vector (Lai et al. 2000). In contrast to house mosquitoes, they do feed during the day (Becker 2020). As this mosquito becomes more widespread, it might accelerate the distribution of *Dirofilaria* spp., as has been described in other regions (Giangaspero et al. 2013). Neighbouring countries such as Slovakia – with

similar climate and a similar dog and mosquito population – already have a higher prevalence of *D. repens* and are endemic for *D. immitis*. This is possibly due to the fact that it is more common for dogs to be kept outside there than in Austria, putting them in closer contact with the native nocturnal mosquitoes (Fuehrer et al. 2021). Prevalence studies should be carried out at regular intervals to monitor the process of introduction of *Dirofilaria* spp.

For *B. canis* the tick *D. reticulatus* is considered the reservoir host, which is expanding its range as well as its activity period, most likely due to climate change (Bajer et al. 2022, Drehmann et al. 2020, Rubel et al. 2020). This tick seems to be present in Austria at least since the Little Ice Age as genetic analysis suggests. Eastern, western, and northern populations can be differentiated genetically, of which alle three are present in Austria (Bilbija et al. 2023). In our study we could demonstrate, that canine babesiosis is endemic in Austria. Furthermore, we could not detect an increase of cases or a spread westwards during the study period of 20 years (Joachim et al. 2023). This contrasts with other studies, where the spread of *D. reticulatus* was accompanied by an increase of canine babesiosis (Bajer et al. 2022, Drehmann et al. 2020). However, it cannot be ruled out that a change in distribution of *B. canis* already took place prior to the study period or will take place in the future. Further studies should monitor the distribution of *D. reticulatus* in Austria as well as the prevalence of *B. canis* in domestic dogs to evaluate the risk of spread westwards.

Often the reservoir host of a VBP is not the arthropod vector (Genchi et al. 2011). Therefore, if only the vector is distributed northwards, the VBP is not necessarily present in a new area. However, combined with the travel to endemic countries or the import of pets from these regions, the VBP can establish itself (Harrus and Baneth 2005). This was most likely the case for the spread of *T. callipaeda* in Austria. Alternatively, the red fox is a suitable wild reservoir and was important for the spread in other areas (Hodžić et al. 2014). However, reports of thelaziosis in red foxes from Austria are lacking.

After the first record of *T. callipaeda* in Italy, the nematode has subsequently been found in other countries, including Austria (do Vale et al. 2019). In the present work we could demonstrate that the nematode and its vector are endemic to Austria, albeit rather rare. As in other European countries, only haplotype 1 was detected (Unterköfler et al. 2023a), most likely due to a single introduction event. However, it might also be possible that only this haplotype can develop in the European *P. variegata*. It is expected that *T. callipaeda* will further spread through Europe, as there are already suitable conditions for the native vector *P. variegata*

(Palfreyman et al. 2018). Climate change might promote this spread, considering that vector activity and larval development in the arthropod vector are temperature dependent (Otranto et al. 2005a, Pombi et al. 2020).

Surprisingly, a *Phortica* fruit fly infestation which had induced clinical signs was detected in the eye of a dog in Austria. This had not been reported so far. Even more surprising, one of these fruit flies could not be designated to the *Phortica* spp. native to Europe, as it was more closely related to Asian *Phortica* spp. (Unterköfler et al. 2023a). Several different *Phortica* spp. are present in Asia, and their morphological, as well as molecular identification is very complex. In many, but not all Asian *Phortica* spp., molecular identification is possible with the 5' fragment of the *COI*. For instance, species of the *P. variegata* complex cannot be reliably differentiated using this genetic region because they are either polyphyletic or cannot be delineated to other species. Investigation of further genetic markers is therefore necessary in order to elucidate their phylogenetic relationships (Huang et al. 2019). Probably more *Phortica* spp. than are currently known also exist in Europe. Alternatively, the unidentifiable *Phortica* specimen could have been imported from Asia e.g., with travellers or imported fruits. Thus, further studies to clarify this question are also necessary in Europe.

The unexpected finding of *O. lupi*, for which the vector is not known in Austria also raises many questions. Considering this was an autochthonous infection (the affected animal had never left the country), the vector should be present in Austria (Unterköfler et al. 2023b). Biting midges and black flies have been proposed as vectors, and several species are present in Austria (Car and Lechthaler 2002, Hassan et al. 2015, Roe et al. 2023, Zittra et al. 2020). However, further studies on infected vectors and vector competence are needed to clarify the life cycle of *O. lupi*. The reservoir host for this nematode in Europe is also unknown (Roe et al. 2020), although red foxes are ubiquitous and would be a likely candidate (Mackenstedt et al. 2015). Domestic dogs themselves could also be an unnoticed reservoir, as infected dogs are often asymptomatic (Otranto et al. 2013). Prevalence studies on dogs and wild canids using skin snips could clarify this question.

Another important factor for the emergence of VBPs is human expansion into natural areas, leading to closer contact with vectors and possible wildlife reservoir hosts. Often the loss of natural habitat leads to the adaption of wild animals to human settlement. This is observed in red foxes, which act as reservoir hosts for parasites such as *Echinococcus multilocularis* and *A. vasorum* (Gillis-Germitsch et al. 2020, Mackenstedt et al. 2015). Because of their close

relationship, domestic and wildcats can harbour the same pathogens (Traversa et al. 2021). If their number increases their role as reservoir of pathogens, including feline lung worms, might increase (Bisterfeld et al. 2022). However, their need for natural structure-rich habitats restricts the population increase in the near future (Hötzel et al. 2007).

Interestingly, VBPs such as *Hepatozoon* spp. and European *Cytauxzoon* spp. can be found with a high prevalence in wildcats but are not a common cause for disease in domestic cats. This is probably due to the low pathogenicity of these VBPs (Baneth and Allen 2022, Carli et al. 2022, Tuska-Szalay et al. 2023, Unterköfler et al. 2022). The situation for *C. felis* is different as naïve cats can develop severe disease (Cohn et al. 2011, Conner et al. 2015, Rizzi et al. 2015). This might be due to the more distantly related wild reservoir host, the bobcat (*Lynx rufus*) (Shock et al. 2011). In Europe, feline *Hepatozoon* spp. and *Cytauxzoon* spp. have only recently been investigated in more detail. It is not clear whether they have been endemic to Europe for a long time, or if they are currently emerging after recent establishment. Based on recent studies, future work should clarify this question.

As indicated by our findings and previous reports, the nematode *P. bovicola* is endemic to Austria (Hofer 2011, Hund et al. 2021). The fact that four different haplotypes could be detected in our study either points to multiple introduction events to this country, or that this parasite had been endemic for a long time but has not been previously described and characterised. Both scenarios are possible, as import of cattle from endemic countries is common but, on the other hand, this parasite is not considered to lead to high economic losses (Bech-Nielsen et al. 1982) and has therefore not attracted much attention from the scientific community. The present study supplies information on its distribution and prevalence in Austria, so that, based on the data now available, future studies will be able to evaluate whether this parasite is spreading further.

The transition from sporadic to endemic cases of a pathogen in an area is fluid. Detection in the vector is often interpreted as a sign of endemicity (Fuehrer et al. 2020a, Übleis et al. 2018). Screening of vectors is therefore an important tool when a change of VBPs distribution is expected and should be implemented for early detection of VBPs, especially if they have a zoonotic potential, such as *Dirofilaria* spp. Furthermore, close monitoring of new vectors needs to be carried out to assess future risks of disease spreading, as is done in some regions of Austria for mosquitoes and to a lesser degree ticks (Bakran-Lebl et al. 2021, Vogelgesang et al. 2020). These efforts should also be expanded to other regions and vectors, such as

sandflies or biting midges. Investigation into less obvious vectors, e.g. keds (Hippoboscidae) is also recommended (Peña-Espinoza et al. 2023). Citizen science can contribute to these efforts (Južnič-Zonta et al. 2022). Additionally, it offers the educational benefit for citizens, as measures for vector control are often dependent on the cooperation of local communities, such as mosquito brood site avoidance in private gardens (Lüthy et al. 2013).

Beside vectors, the mammal host should also be closely monitored. The unexpected role of lagomorphs in an outbreak of human leishmaniosis in Spain has highlighted the importance of considering alternative wildlife reservoirs (Arce et al. 2013). Also, in Austria the role of wildlife in the transmission of VBPs is complex and should be monitored closely (Duscher et al. 2015). The increase in travel and import of pets requires additional education of pet owners, animal welfare organisations, and veterinarians. Furthermore, clear legislation to rigorously test all domestic animals entering could hopefully prevent the introduction of VBPs through pets. The extended activity period of arthropod vectors also warrants adaption and accurate recommendation for preventive measures (Leschnik et al. 2013).

New or imported VBPs pose a challenge for medical staff, as they are not familiar with the clinical picture (Roure et al. 2022, Sævik et al. 2014). In the report of *O. lupi* for instance, the referring veterinarian had diagnosed thelaziosis and the nematode was only later identified as *O. lupi* in the laboratory. This highlights the importance of reporting early autochthonous cases and educating medical personnel with respect to emerging and imported VBPs.

8. Additional Results

8.1. Vector-borne pathogens in guard dogs in Ibadan, Nigeria

Gruenberger I, Liebich A-V, Ajibade TO, Obebe OO, Ogbonna NF, Wortha LN, **Unterköfler MS**, Fuehrer H-P, Ayinmode AB. 2023. Pathogens (Basel, Switzerland), 12 (3).

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Own contributions:

- sequence analysis
- revising the manuscript

Other authors' contributions:

IG: methodology, validation, formal analysis, writing—original draft preparation

AVL: methodology, formal analysis, writing—original draft preparation

TOA: methodology, writing—review and editing

OOO: methodology, writing—review and editing

NFO: methodology, writing—review and editing

LNW: methodology, writing—review and editing

HPF: conceptualization, methodology, validation, data curation, writing—review and editing, supervision

ABA: conceptualization, methodology, validation, writing—review and editing, supervision

The prevalence of VBPs in dogs is dependent on the region investigated and varies in different European regions and even more in other continents. Due to the increased import and travel of dogs, knowledge of the occurrence of VBPs outside Europe is important (Harrus and Baneth 2005). This study was performed in Nigeria.

8.2. Molecular pathogen screening of louse flies (Diptera: Hippoboscidae) from domestic and wild ruminants in Austria

Peña-Espinoza M, Em D, Shahi Barogh B, Berer D, Duscher GG, van der Vloedt L, Glawischnig W, Rehbein S, Harl J, **Unterköfler MS**, Fuehrer H-P. 2023. Parasites & Vectors, 16 (1): 179.

https://doi.org/10.1186/s13071-023-05810-4

Own contributions:

- phylogenetic analysis
- revising the manuscript

Other authors' contributions:

MPE: molecular analysis, writing—original draft preparation

DE, BSB, LVDV: molecular analysis

DB, GGD, WG, SR: sample acquisition

JH: supervision, writing—review and editing

HPF: conceptualization, supervision, writing—review and editing

Arthropods that feed on blood can potentially act as vectors. The relevance of louse flies (Hippoboscidae) as vectors has not yet been intensively investigated. Of the several species that can be found in Austria, the forest fly (*Hippobosca equina*) prefers horses and other large mammals, including cattle. This ked has wings during the entire adult life, whereas the sheep ked (*Melophagus ovinus*) are wingless. Deer keds (*Lipoptena cervi*) prefer deer but can also accidentally attack other hosts, including dogs and humans. They have wings, which they break off once the ked lands on a host (Bezerra-Santos and Otranto 2020, Buczek et al. 2020, Hermosilla et al. 2006, Small 2005). In all three species *Bartonella* spp. were detected as well as *Trypanosoma* spp. in the sheep ked and forest fly.

8.3. Mosquito Alert - Leveraging citizen science to create a GBIF mosquito occurrence dataset

Južnič-Zonta Ž, Sanpera-Calbet I, Eritja R, Palmer JRB, Escobar A, Garriga J, Oltra A, Richter-Boix A, Schaffner F, della Torre A, Miranda MÁ, Koopmans M, Barzon L, Bartumeus F, **Mosquito Alert Digital Entomology Network**, Mosquito Alert Community. 2022. GigaByte (Hong Kong, China), 2022: gigabyte54.

https://doi.org/10.46471/gigabyte.54

Mosquito Alert Digital Entomology Network: Alarcón-Elbal PM, Alexander González M, Angeles Puig M, Bakran-Lebl K, Balatsos G, Barceló C, Bengoa Paulis M, Bisia M, Blanco-Sierra L, Bravo-Barriga D, Caputo B, Collantes F, Costa Osório H, Curman Posavec M, Cvetkovikj A, Deblauwe I, Delacour S, Escartin Peña S, Ferraguti M, Flacio E, Fuehrer H-P, Gewehr S, Gunay F, Gutiérrez-López R, Horváth C, Ibanez-Justicia A, Kadriaj P, Kalan K, Kavran M, Kemenesi G, Klobucar A, Kurucz K, Longo E, Magallanes S, Mariani S, Martinou AF, Melero-Alcíbar R, Michaelakis A, Michelutti A, Mikov O, Montalvo T, Montarsi F, Paoli F, Parrondo Montón D, Rogozi E, Ruiz-Arrondo I, Severini F, Sokolovska N, **Unterköfler MS**, Stroo A, Teekema S, Valsecchi A, Vaux AGC, Velo E, Zittra C.

Own contributions:

- mosquito identification
- communication to citizens

Other authors' contributions:

ŽJZ: writing – original draft, writing – review and editing, data curation

ISC: writing – original draft, validation

RE: data curation, validation

JRBP: conceptualization, supervision, funding acquisition, software, data curation, writing – review and editing

AE: software, data curation

JG: data curation

AO: conceptualization, data curation, project administration

ARB: project administration

FS, MÁM: AIMSurv conceptualization, resources

ADT: AIMSurv conceptualization, funding acquisition, resources

LB: resources

MK: VEO conceptualization, funding acquisition, resources

FB: conceptualization, funding acquisition, supervision, writing – review and editing

MAC: investigation

Citizen science has found several useful applications in research (Roche et al. 2020), such as in the investigation of vector distribution, where the data can be very efficiently supplemented by amateur scientists (Poh et al. 2022). Using a tool called the Mosquito Alert App, citizens can report mosquitoes by submitting a photograph together with a location and date. The photograph is then analysed by three experts to evaluate if, for instance, it might be an Asian tiger mosquito or not (Miranda et al. 2022). These mosquitoes can transmit other VBPs than the native European mosquitoes (Medlock et al. 2012), and knowledge regarding their occurrence is therefore important for the prevention of disease outbreak.

9. Conclusion and Outlook

Vector-borne pathogens are and will be a highly relevant topic in the future, considering the numerous new VBPs found in Austria in the short period of the present work. The process of introduction into Austria and increased prevalence has most likely started before and research efforts should be increased to establish baseline data for future reference. Vectors as well as domestic and wild animals should be monitored and screened for VBPs. Education of the public, governmental authorities, and especially medical and veterinary staff is necessary. Prevention and intervention measurements should be planned, applied, and closely monitored for their efficacy. Legislation should be adapted to support the implementation of new findings and to prevent zoonosis, economic loss, and damage to endangered wildlife species.

It is clear, that different vectors and VBPs warrant different approaches in research, monitoring, prevention, and intervention. Technical and methodical advances and new scientific findings will hopefully help to cope with the high costs associated with the increased effort. Combined with other associated risks and dangers, it is likely that mitigating the causes of the factors driving the spread of VBPs (i.e., most importantly climate change, globalisation, loss of natural habitat and biodiversity) is more cost-effective. However, as a change in distribution has already begun, an adapted strategy to combat VBPs is inevitable.

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