Cardiopulmonary helminth parasites of the red fox (*Vulpes vulpes*) in the north-east of The Netherlands

RESEARCH PROJECT MASTER OF VETERINARY SCIENCE

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Abstract

Background: The wild red fox (*Vulpes vulpes*) is considered a reservoir for various cardiopulmonary helminth species of veterinary importance. Improved knowledge of fox cardiopulmonary parasite prevalence and geographic distribution in The Netherlands provides essential information for epidemiological studies about the complex relationship between wildlife, domestic animals and parasites.

Methods: The presence of four cardiopulmonary helminth species – *Angiostrongylus vasorum*, *Crenosoma vulpis*, *Eucoleus aerophilus* and *Dirofilaria immitis* – was examined in the heart and lungs of 95 Dutch wild red foxes originating from the north-eastern part of The Netherlands (Groningen and Drenthe) by the washing and sieving technique. The prevalence of each species was determined and a logistic regression was applied to examine the correlation between fox age, sex, geographical origin, bodyweight-length index and single and multiple cardiopulmonary helminth infections.

Results: 81/95 (85.3%) of the foxes were infected with one or more cardiopulmonary helminth species. Three species were identified: *Angiostrongylus vasorum* (31/95, 32.6%), *Crenosoma vulpis* (27/95, 28.4%) and *Eucoleus aerophilus* (76/95, 80.0%). These prevalences were all higher than those previously reported in Dutch wild red foxes. *Dirofilaria immitis* was not found in the cardiorespiratory tract of the examined foxes. The prevalence of *E. aerophilus* was significantly higher in male foxes than in female foxes. Juvenile foxes were significantly more often infected with *E. aerophilus* and *C. vulpis* and were more frequently infected with 2 or 3 cardiopulmonary helminth species than adult foxes. There were no significant correlations between province of origin or BWL-index of the foxes and infection rates.

Conclusions: Cardiopulmonary helminth parasites *A. vasorum*, *C. vulpis* and *E. aerophilus* are present in high prevalences in wild red foxes in the north-east of The Netherlands. Future studies should be focused in the prevalence of these helminths in the domesticated dog population and attempt to evaluate the factors involved with parasite transmission between foxes and domesticated dogs.

Keywords: Cardiopulmonary helminth, Red fox, Angiostrongylus, Crenosoma, Eucoleus

Background

Wild canids, like the red fox (*Vulpes vulpes*) harbor several cardiopulmonary helminth species which may be shared with domestic dogs and cats, and occasionally humans (Otranto *et al.*, 2015). These include the heartworms *Angiostrongylus vasorum* and *Dirofilara immitis*, and the lungworms *Crenosoma vulpis* and *Eucoleus aerophilus* (previously *Capillaria aerophila*).
It has been suggested that (a) free-roaming of foxes and (b) an expanding population of foxes due to rabies vaccinations, (c) climate changes in favor of vector-borne helminths, and (d) an increasing (international) movement of domesticated dogs can facilitate cross-infections between wild and domestic animals (Hodžić *et al.*, 2016; Lalosevic *et al.*, 2013). The role of the red fox as a reservoir for cardiopulmonary parasitic disease is of veterinary, and in the case of *D. immitis*, human concern, and calls for an improved knowledge of the complex wildlife - domestic animals-humans-parasites

relationship (Otranto *et al.*, 2015; Lalosevic *et al.*, 2013). *A. vasorum* is especially of concern, since this clinically important parasite in dogs has been established just recently in The Netherlands, and has increasingly been reported outside known European endemic foci. (Van Doorn *et al.*, 2009; Morgan *et al.*, 2005; Taylor *et al.*, 2015).

The aim of this study is to determine the prevalence of *A. vasorum* and to investigate the presence of *C. vulpis*, *E. aerophilus* and *D. immitis* in wild red foxes (*Vulpes vulpes*) from the north-east of The Netherlands (Drenthe and Groningen), in order to increase the knowledge on the distribution of these parasites in The Netherlands.

Angiostrongylus vasorum

Angiostrongylus vasorum ('French heartworm') is a nematode of the superfamily Metastrongyloidea.
As for most of the metastrongyloid lungworms, the definitive hosts (e.g., red foxes and dogs) become infected following the ingestion of gastropod intermediate hosts (Latrofa *et al.*, 2015). Due to their free-roaming behavior, red foxes have been suggested to be an important source of heart- and lungworm infections in domesticated dogs (Latrofa *et al.*, 2015).
The helminth parasitizes the right ventricle and pulmonary arteries of dogs (*Canis familiaris*) and wild carnivores such as the red fox (*Vulpes vulpes*) and Eurasian badger (*Meles meles*), causing verminous pneumonia and coagulopathy (Taylor *et al.*, 2007; Koch & Willesen, 2007; Van Doorn *et al.*, 2009).
The life cycle of *A. vasorum* is indirect, with a number of terrestrial mollusk species (slugs and snails) as obligatory intermediate hosts. Dogs and foxes can be infected by the ingestion of mollusks containing L3 larvae, or by the ingestion of frogs, birds or rodents, which serve as paratenic/transport hosts (Bolt *et al.*, 1993; Koch & Willesen, 2007, Traversa *et al.*, 2013) (**Box 1, Figure 1**).

An *A. vasorum* infection in dogs can be asymptomatic or can cause, among other things, circulatory and respiratory signs, such as coughing, dyspnea, exercise intolerance, anorexia, insufficient growth, weight loss, depression, edema and abdominal pain (Eleni *et al.*, 2014; Van Doorn *et al.*, 2009). Pathological findings in infected dogs consist of signs of right heart failure (right ventricular hypertrophy, hepatic congestion, ascites, and hydrothorax) and a pyogranulomatous interstitial pneumonia (Bourque *et al.*, 2008). In foxes, clinical signs are often milder and only in the presence of concurrent diseases, high worm burdens can provoke significant lesions in the respiratory and cardiovascular systems (Eleni *et al.*, 2014). These signs include right ventricle hypertrophy, congestion and discoloration of the lung tissue and the presence of encapsulated nodules in the lung tissue (Eleni *et al.*, 2014; Simpson, 1996).

In Europe, the distribution of *A. vasorum* in dogs is characterized by isolated endemic foci surrounded by regions where only sporadic cases occur, but there have been reports of expansion of the geographical range of *A. vasorum* and the spreading from known endemic foci to regions previously free of infection throughout Europe (Morgan *et al.*, 2005; Hodžić *et al.*, 2016). Since the detection of *A. vasorum* larvae in fecal samples of Dutch domestic dogs that had never been outside the country in 2007, *A. vasorum* is now considered endemic in The Netherlands (Van Doorn *et al.*, 2009).
In 2009, a prevalence of 0.8% (n = 4) was found in fecal samples of 485 Dutch domesticated dogs from The Hague and the north-western edge of the Veluwe, areas where *A. vasorum* infected dogs were

found in 2007 (Van Doorn *et al.*, 2009). For this study, single fecal samples of dogs not subjected to an effective *A. vasorum* anthelminthic therapy for the last 12 months were collected by veterinary practices, hunting associations and kennel clubs and examined for L1 *A. vasorum* larvae by the Bearmann technique. Three out of four positive dogs never travelled across the borders of the Netherlands and therefore must have contracted an *A. vasorum* infection in their own surroundings (Van Doorn *et al.*, 2009).

A recent study demonstrated an *A. vasorum* prevalence of 4.2% after washing and sieving the cardiopulmonary tract of 96 Dutch red foxes originating from the border region in the eastern part of The Netherlands (Franssen *et al.*, 2014). Due to the lack of systematic data focusing on the geographical distribution and identification of endemic *A. vasorum* foci in western Germany, it is unknown how the *A. vasorum* prevalence in Dutch foxes originating from the north-east is influenced by foxes or dogs along the German border (Taubert *et al.*, 2009). In general however, the European prevalence of *A. vasorum* is much higher in foxes (5-56%) than in dogs (up to 10 %) and therefore dense populations of both foxes and dogs living close together are of concern (Koch & Willesen, 2007).

Crenosoma vulpis

Like *A. vasorum*, *Crenosoma vulpis* ('fox lungworm') is a nematode of the superfamily Metastrongyloidea (Taylor *et al.*, 2007).
Little is known about the pathogenicity of *C. vulpis* in red foxes, but the clinical effects of this parasite have been studied extensively in dogs (Nevárez *et al.*, 2005; Jeffery *et al.*, 2004). With the trachea, bronchi and bronchioles as predilection sites, canine crenosomosis is characterized by clinical respiratory conditions, varying from asymptomatic to a mild respiratory disease with chronic coughing, sneezing and nasal discharge (Taylor *et al.*, 2007; Latrofa *et al.*, 2015). Pathological findings include areas of emphysema in the lungs, bronchitis, consolidation, interstitial pneumonia, and blockage or thickening of the bronchioles (Jefferey *et al.*, 2004). Infections of *C. vulpis* in dogs tend to be insidious and are often misdiagnosed and treated as allergic respiratory disease. Fatal *C. vulpis* infections are never reported in dogs (Traversa *et al.*, 2010).

In Europe, only few cases of *C. vulpis* infections have been reported, most of them being a single case of infection. In Germany, a prevalence 0.9 to 6% was found in 810 dogs with a clinical tentative diagnosis of lungworm examined for fecal *C. vulpis* larvae from 2007 to 2009 (Traversa *et al.*, 2010; Barutzki & Schaper, 2009). The prevalence of *C. vulpis* positive dogs in the Netherlands is unknown, but a single autochthonous case has been reported. In 2009, a case of a combined *A. vasorum* and *C. vulpis* infection was described in a 9 year old Dutch Bull-Terrier presented with clinical cardiorespiratory signs. An adult *C. vulpis* worm was found by bronchio-alveolar lavage, and Bearmann examination of the feces showed both *A. vasorum* and *C. vulpis* larvae (Nijsse *et al.*, 2009).
C. vulpis is endemic in red fox populations in Europe (Navàrez *et al.*, 2005). In 1984, Borgsteede reported a *C. vulpis* prevalence of 4.5% among 111 wild red foxes shot along the Dutch-German border, and more recently, a prevalence of 16.7% among 96 Dutch red foxes originating from the same area was found (Borgsteede, 1984; Franssen *et al.*, 2014).

Eucoleus aerophilus

*Eucoleus aerophilus (*previous*: Capillaria aerophila)* is another common pulmonary nematode (superfamily Trichuroidea) found in the trachea, bronchi and bronchioles in canids, felids and some omnivorous animals. It causes a mild tracheitis and/or bronchitis in dogs, and poor growth and fur quality in foxes (Lalosevic *et al.*, 2013; Navárez *et al.*, 2005; Taylor *et al.*, 2007). Furthermore, it has been suggested that *E. aerophilus* has zoonotic potential (Lalosevic *et al.*, 2013).
The knowledge on the biological life cycle of this nematode is scarce, and both a direct and indirect life cycle has been described (**Box 2, Figure 2**). While the occurrence of *A. vasorum*, *C. vulpis* and *D. immitis*, in different geographical areas is mainly influenced by the presence of the appropriate gastropods or culicoid species, the presence of *E. aerophilus* is guaranteed by the direct lifecycle and – in case of the indirect lifecycle – ubiquity of the earthworm vector (Lalosevic *et al.*, 2013).
E. aerophilus is an endemic pulmonary helminth in European red fox populations. Borgsteede (1984) found a prevalence of 46.8% among 111 Dutch red foxes, whilst Franssen *et al.* (2014) reported a prevalence of 67.7% among 96 Dutch red foxes. Little is known about the prevalence of *E. aerophilus* in dogs and cats in Europe (Traversa *et al.*, 2010).

Dirofilaria immitis

Dirofilaria immitis is a zoonotic nematode of het superfamily Filarioidea that causes cardiopulmonary dirofilariasis in both wild and domestic carnivores such as foxes, dogs and cats (Taylor *et al.*, 2007).
Various species of culicoid mosquitoes (*Culex* spp., *Aedes* spp. and *Anopheles* spp.) act as the intermediate vector in the life cycle of the nematode (**Box 3, Figure 3**) (Morchón *et al.*, 2012; Hoch & Strickland, 2008). After developing from L3 to the adult stage, adult *D. immitis* worms parasitize the right heart chambers, pulmonary arteries and occasionally vena cava of their definitive host, causing a severe condition known as 'heartworm disease' (Morchón *et al.*, 2012).

D. immitis is endemic in fox populations in many European countries, including Bulgaria, Hungary, Spain and Italy (Otranto *et al.*, 2015). To this date, only limited data is available on the prevalence of *D. immitis* in red foxes. Current European studies report a prevalence of infection ranging from 1% to 32.3%, depending mainly on the climatic conditions of the area under investigation. A suitable climate is necessary for larval development in the mosquito; therefore, the parasite is mainly located in temperate, tropical and subtropical areas in the world (Otranto *et al.*, 2014; Simón *et al.*, 2012; Traversa *et al.*, 2010; Morchón *et al.*, 2012). No autochthonous cases of canine or feline heartworm disease have been reported in The Netherlands. However, over the last few years there has been a debate regarding the risk of spread of *D. immitis* to non-endemic European countries as a consequence of climate changes and movement of dogs (Traversa *et al.*, 2010). Indeed, heartworm disease has been diagnosed in non-endemic European areas and recently larvae have been found in mosquitoes in the very south and east of Germany (Genchi *et al.*, 2014; Kronefeld *et al.*, 2014).
In The Netherlands, the presence of the culicoid vector, an occasionally favorable climate for parasite development and the import of infected dogs contribute to the risk of infections between dogs, foxes and humans. As most Dutch domesticated dogs are regularly treated with antiparasitic drugs that are effective against *D. immitis*, foxes are an interesting sentinel species for this helminth. Therefore, *D. immitis* will be included as an emerging helminth of interest in this study, even though the part of the

Netherlands were foxes are studied (north-east) is not expected to be the first to detect an infection due to the more favorable climate for culicoid vectors in the south of The Netherlands.

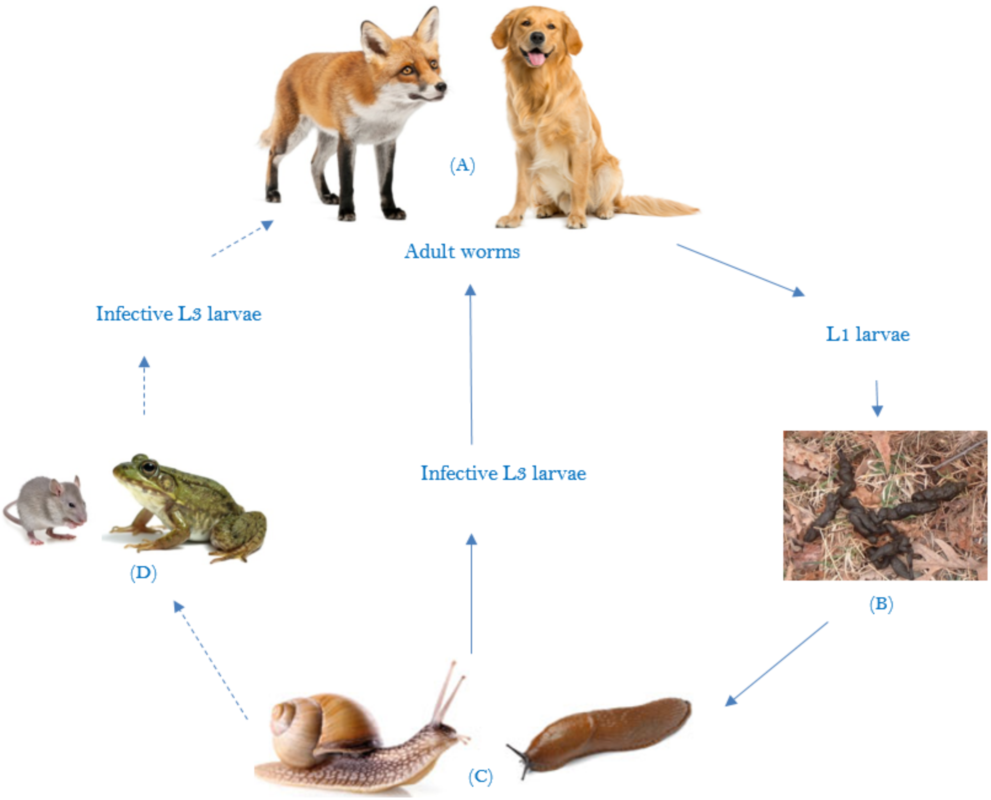

Figure 1 The life cycle of canid Metastrongyloidea (**A**) A canid species, the definite host, becomes infected by ingestion of third stage larvae (**B**) First-stage larvae are shed with the feces, (**C**) The intermediate host is a terrestrial mollusk, (**D**) Paratenic hosts like frogs may be a source of infection (adapted from Traversa *et al.*, 2013).

Box 1 The life cycle of Metastrongyloidea residing in the cardiopulmonary tract of canids

The life cycle is indirect (**Figure 1**). Adult worms live in the cardiorespiratory system of the canid definite host: right ventricle and pulmonary arteries (*A. vasorum*) or trachea, bronchi and bronchioles (*C. vulpis*) (**Figure 1A**). The female parasites lay eggs which will mature and hatch within the parenchyma. After hatching, the first stage larvae (L1) travel to the respiratory tract to the pharynx, where they are swallowed and released into the environment via the feces (**Figure 1B**). The first stage larvae develop into the third, infective larval stage (L3) in mollusk intermediate hosts (**Figure 1C**). The definite host is infected by the ingestion of an intermediate host, or a paratenic host (rodents, frogs, birds) or by larvae living freely in the mucus of the mollusk. (**Figure 1D**). After ingestion, the L3 larvae migrate through the cardiopulmonary tract by the lymphatic vessels and develop into adult worms in the different parts of the cardiopulmonary system (Traversa *et al.*, 2013).

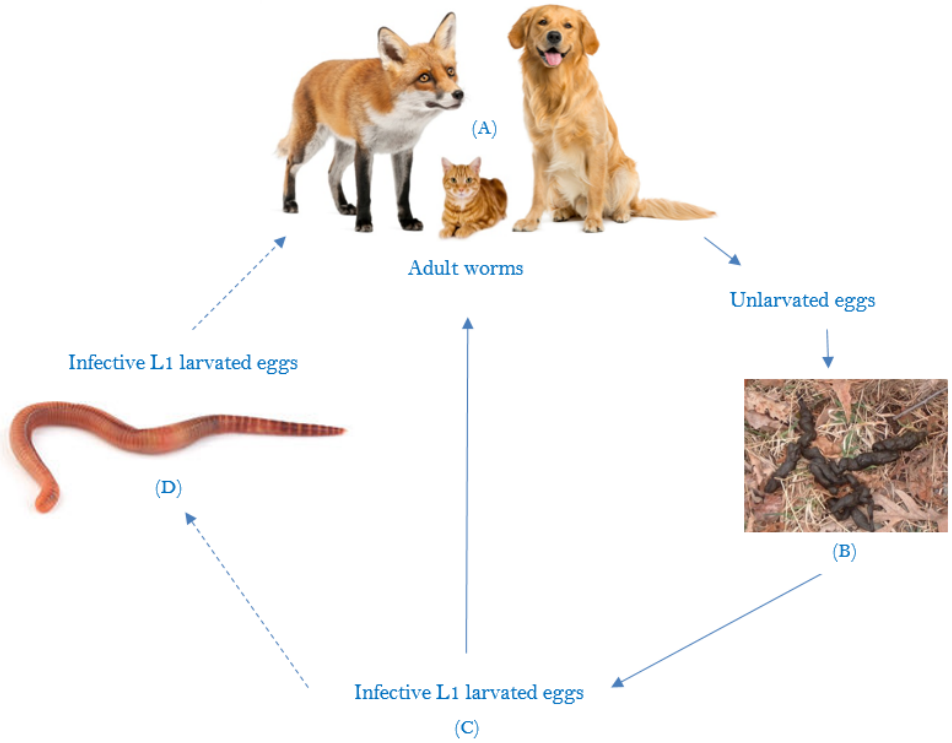

Figure 2 The life cycle of *Eucoleus aerophilus* (**A**) An omnivorous species, the definite host, (**B**) Non-larvated eggs are shed with the feces, (**C**) Eggs become infectious in the environment (**D**) Earthworms are hypothesized to act as a facultative intermediate or paratenic hosts (adapted from Traversa *et al.*, 2013).

Box 2 The life cycle of *Eucoleus aerophilus* residing in the cardiopulmonary tract of canids and felids

The life cycle is direct (**Figure 2**), although it has been suggested that earthworms could be involved as a facultative intermediate or parentenic host. The adult worms live beneath the epithelium of the bronchi and the trachea of various carnivorous hosts (foxes, dogs, cats) (**Figure 2A**). The females lay eggs that are coughed, swallowed and then released into the environment via the feces (**Figure 2B**). The eggs mature and become infectious (L1). After ingestion of environmental larvated eggs containing infectious first stage larvae (**Figure 2C**) or (possibly) parasitized earthworms (**Figure 2D**) the animal acquires an infection. When the eggs are hatched in the intestines, the larvae migrate via the bloodstream or lymphatic vessels to the lungs, where they invade the mucosa and reach the adult stage (Traversa *et al.*, 2013).

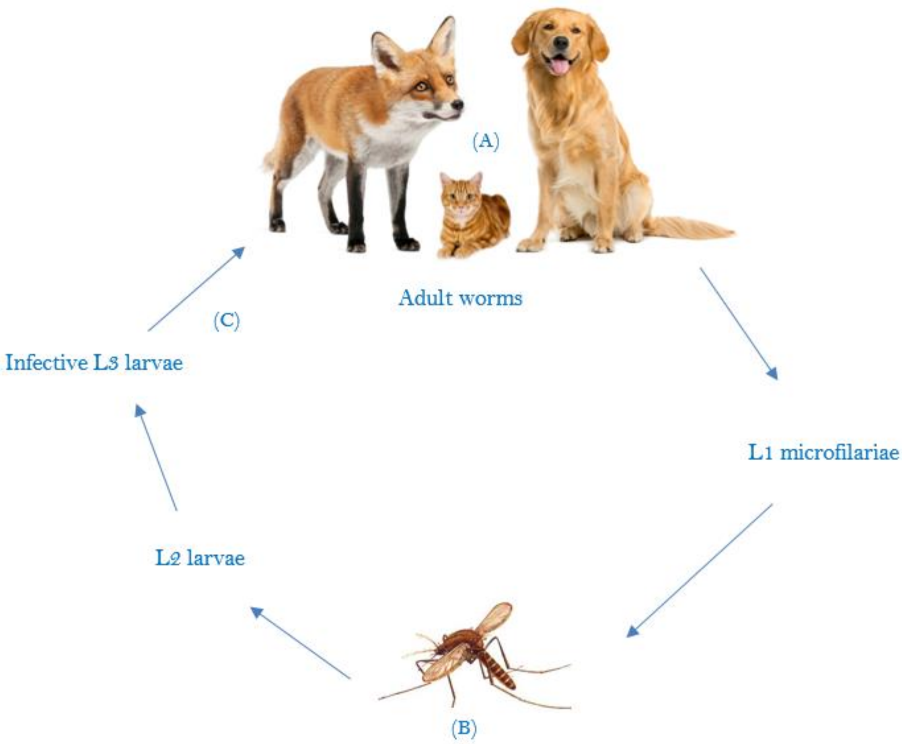

Figure 3 The life cycle of *Dirofilaria immitis*. (**A**) An omnivorous species, the definite host, (**B**) L1 larvae (microfilariae) are released into the hosts bloodstream where they can be ingested by feeding mosquitoes, (**C**) The L1 larvae molt to the infectious L3 stage within the vector (**D**) L3 can be transferred

Box 3 The life cycle of *Dirofilaria immitis* residing in the cardiovascular tract of canids and felids

The life cycle is indirect (**Figure 3**), with various culicoid mosquito species acting as the intermediate vector host. The adult heartworms live in the pulmonary arteries of the carnivorous host (foxes, dogs, cats), but can also invade the right ventricle and atrium and the caudal vena cava (**Figure 3A**). After mating, L1 larvae (microfilariae) are released into the hosts bloodstream where they can be ingested by feeding mosquitoes (**Figure 3B**). Under the right ambient temperature (at least 14°C) during a sufficient number of days, the L1 larvae molt twice within the vector (**Figure 3C**). The L3 infectious stage can then be transferred to the next host when an infected mosquito feeds (**Figure 3D**). Within the host's subcutaneous, adipose or skeletal muscular tissue, L3 larvae molt into the L4 immature worms. The final transformation from L4 to L5 occurs after the immature worms migrate to the heart and pulmonary arteries. Under the ideal conditions, the entire life cycle of *D. immitis* takes 184 to 210 days (Hoch & Strickland, 2008).

Methods

Animals

From October 2016 to January 2017, the carcasses of 95 foxes, shot as a part of a population control program, were collected by hunters in Drenthe and Groningen (**Figure 4**) and sent to the National Institute for Public Health and Environment (RIVM, Bilthoven, The Netherlands).

Upon arrival, the fox carcasses were stored at -80 °C for at least one week to inactivate the eggs of *E. multiocularis*, according to WHO guidelines (Eckert, 2001). Carcasses were then sent to the Department of Pathology of the Faculty of Veterinary Sciences (Utrecht University, The Netherlands) to be thawed and dissected. Data on geographical origin, gender, weight, body length (nose to anus) and age were collected. The age of the foxes (i.e. juvenile (<1 year) or adult (>1 year)) was estimated by examining tooth wear based on a protocol designed by the Dutch Wildlife Health Centre (DWHC, Utrecht, The Netherlands) (**Attachment A**). Body condition was estimated as the ratio of body weight (grams) over body length (millimeters) (body weight/length index, BWL-index) (Franssen *et al.*, 2014).

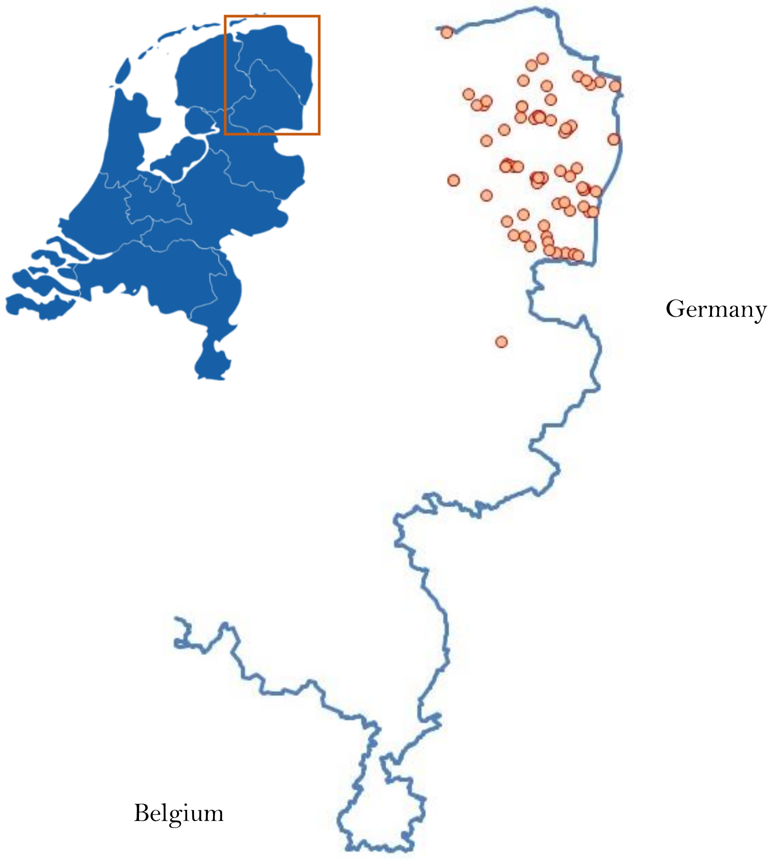

Figure 4 Geographical origin of individual foxes This figure shows the study area in the north-east of The Netherlands, with a representation of the whole country in blue. Geographical coordinates of 9 foxes were missing: 3 of these foxes originated from Groningen, and 6 from Drenthe (courtesy to F. Franssen).

Microscopical examination of cardio-pulmonary helminths

The heart and lungs of the foxes were examined by incising both ventricles transversely midway between the base and the apex of the heart. All chambers, cardiac arteries and veins and the pulmonary arterial trunk were opened. The trachea, bronchi and larger bronchioles were opened and the lung lobes were incised. Heart and lungs were washed over tap water and the washings were passed through a 150 µm mesh sieve. The residue on each sieve was collected in Petri dishes for morphological identification of adult helminths after examination under a dissection microscope (**Figure 5**) (Franssen *et al.*, 2014).

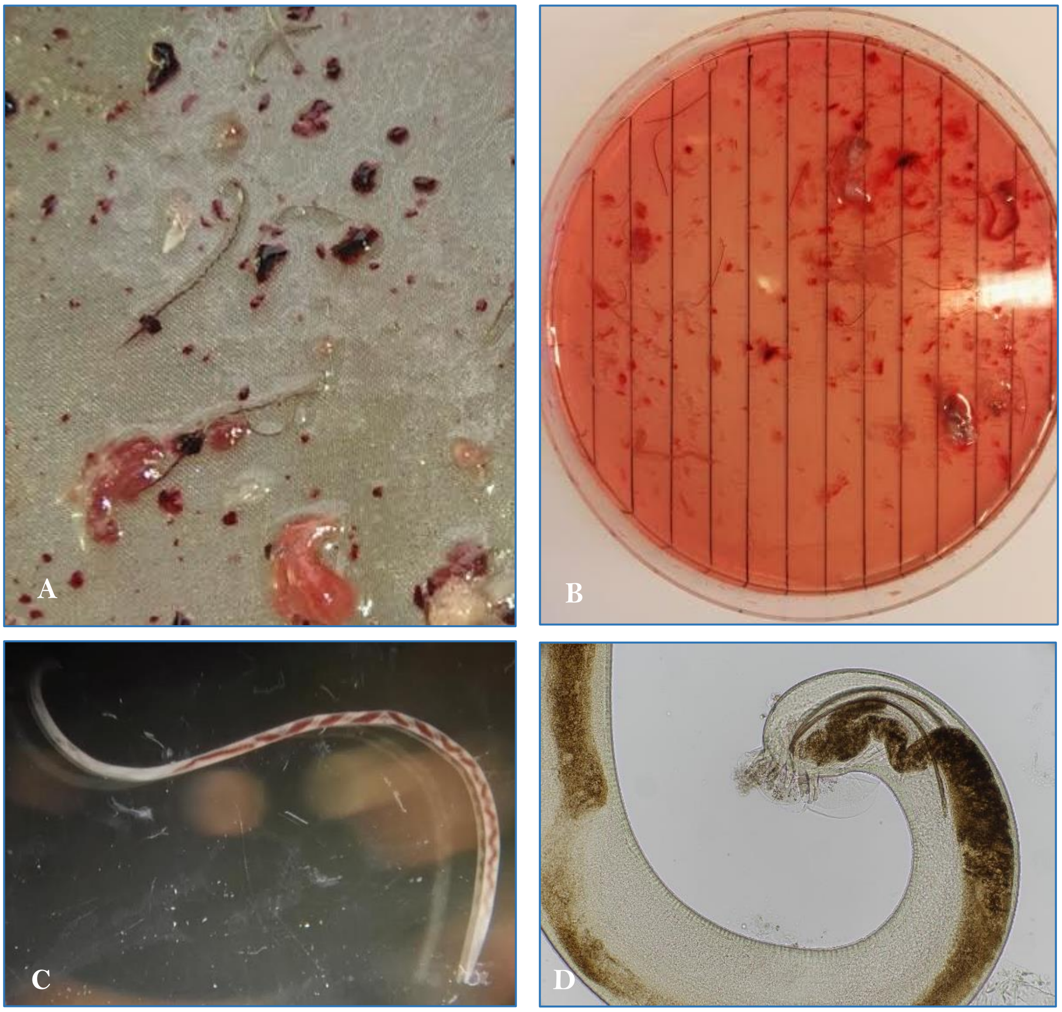

Figure 5 Macroscopic and microscopic images of *A. vasorum* adult worms retrieved from Dutch red foxes. (A) Adult worms on the 150 µm mesh sieve, (B) Multiple adult worms in the heart-lung washings, (C) Adult female with the characteristic barber's pole appearance, (D) Light microscope close-up of an adult male with the spicules and bursa copulatrix.

Statistical analysis

Statistical analysis was conducted using IBM SPSS Statistics 24.0. The Chi-square test for association was used to examine the correlation between testing positive on cardiopulmonary helminth infections and the BWL-index or fox characteristics (age, sex and geographical origin). Associations between the presence of cardiopulmonary helminths, fox characteristics and BWL-index were assessed separately for *A. vasorum*, *C. vulpis* and *E. aerophilus* using multivariate logistic regression. The association between single infections or co-infections and fox sex, age, BWL-index and geographical location was examined by Chi-square test for association. A 95% confidence interval was applied for all tests.

Results

Animal age, gender, origin and bodyweight/length index

In total, 95 foxes were collected of which 41.5% originated from Groningen and 58.5% from Drenthe. At the time of sampling, 68.4% per cent of the foxes were identified as juvenile, and 32.2% of the foxes classified as adults. The male to female ratio was 60% to 40%. The average body weight / length (BWL) index of males and females differed significantly (Chi-square, $p = 0.002$), with the males being heavier (mean BWL-index 7.9) than the females (mean BWL index 6.9). Furthermore, adult foxes had a significant higher average BWL-index (mean BWL -index 8.0) than juvenile foxes (mean BWL-index 7.2) (Chi-square, $p = 0.003$). There was no significant correlation between BWL-index and fox origin, nor between BWL-index and the presence of cardiopulmonary helminths.

Heart-lungworm infections

Table 1 provides an overview of the prevalence of cardiopulmonary helminths in this population of Dutch wild red foxes. Overall, 81/95 (85.3%) foxes were infected with one or more cardiopulmonary helminth species. The prevalence of *A. vasorum*, *C. vulpis* and *E.aerophilus* was 32.6%, 28.4% and 80.0% respectively. *D. immitis* was not found in the cardiorespiratory tract in any of the foxes.

Table 1 Overview of cardiopulmonary helminths found in Dutch red fox

	Foxes (n)	%	*A. vasorum* (n)	%	*C. vulpis* (n)	%	*E. aerophilus* (n)	%
Groningen	39	41,5	13	33,3	10	25,6	32	82,1
Drenthe	55	58,5	18	32,7	17	30,9	44	80,0
Male	57	60,0	19	33,3	19	33,3	51	89,5
Female	38	40,0	12	31,6	8	21,1	25	65,8
Juvenile	65	68,4	21	32,3	24	36,9	57	87,7
Adult	30	32,3	10	33,3	3	10,0	19	63,3
BWL 5,0 - 6,9	35	37,6	10	28,6	12	34,3	29	82,9
BWL 7,0 - 8,9	44	47,3	16	36,4	13	29,5	34	77,3
BWL 9,0 - 10,9	14	15,1	4	28,6	1	7,1	11	78,6
Overall	**95**	**100,0**	**31**	**32,6**	**27**	**28,4**	**76**	**80,0**

Cardiopulmonary helminth prevalence was higher for all three species in male foxes than in female foxes, although this was only significant for *E.aerophilus* (Logistic regression, $p = 0.008$). *C. vulpis* prevalence (Logistic regression, $p = 0.023$) and *E.aerophilus* prevalence (Logistic regression, $p = 0.032$) was significantly higher in juvenile compared to adult animals. There were no significant differences in the prevalence of the helminth species per province and correlations between BWL-index and cardiopulmonary helminth infections were absent (**Table 2**).

Table 2 Logistic regression output for fox characteristics and cardiopulmonary helminth infections

Infection	*A. vasorum*		*C. vulpis*		*E. aerophilus*	
Variable	**Sig.**	**Exp. (B)**	**Sig.**	**Exp.(B)**	**Sig.**	**Exp. (B)**
Sex	0.922	1.051	0.145	0.424	0.008*	0.159
Age	0.903	1.065	0.023*	0.160	0.032*	0.266
Province	0.961	1.022	0.512	1.408	0.883	0.916
BWL-index	0.748	1.073	0.150	0.689	0.170	0.674

**Significant at a 95% Confidence Interval*

Multiple infections per fox

Of the examined foxes, 69/81 infected animals (72.6%) were infected with more than one cardiopulmonary helminth species (**Figure 6**). All foxes infected with *C. vulpis* were also infected with at least one other cardiopulmonary helminth. The majority of *A. vasorum* infected foxes (26/31; 83.9%) and *C. vulpis* infected foxes (26/27; 96.3%) were also infected with *E. aerophilus*. Although the association between a *C.vulpis* and *E.aerophilus* infection was significant when applying the Chi-square test for association (Chi-square, $p = 0.034$), this significance was no longer present (Logistic regression, $p = 0.160$) when corrected for fox age, sex, province and BWL-index by logistic regression.

Male foxes (8.8%-47.7%) were more frequently infected with 2-3 species than female foxes (7.9-26.3%), but this difference was not statistically significant. There was however a significant difference in the prevalence of co-infections between juvenile and adult foxes: juvenile foxes (9.2-49.2%) were more often positive for 2-3 species than adult foxes (6.7-16.7%) (Chi-square, $p = 0.013$). There was no significant difference in co-infections per fox between Groningen and Drenthe or between BWL-indices.

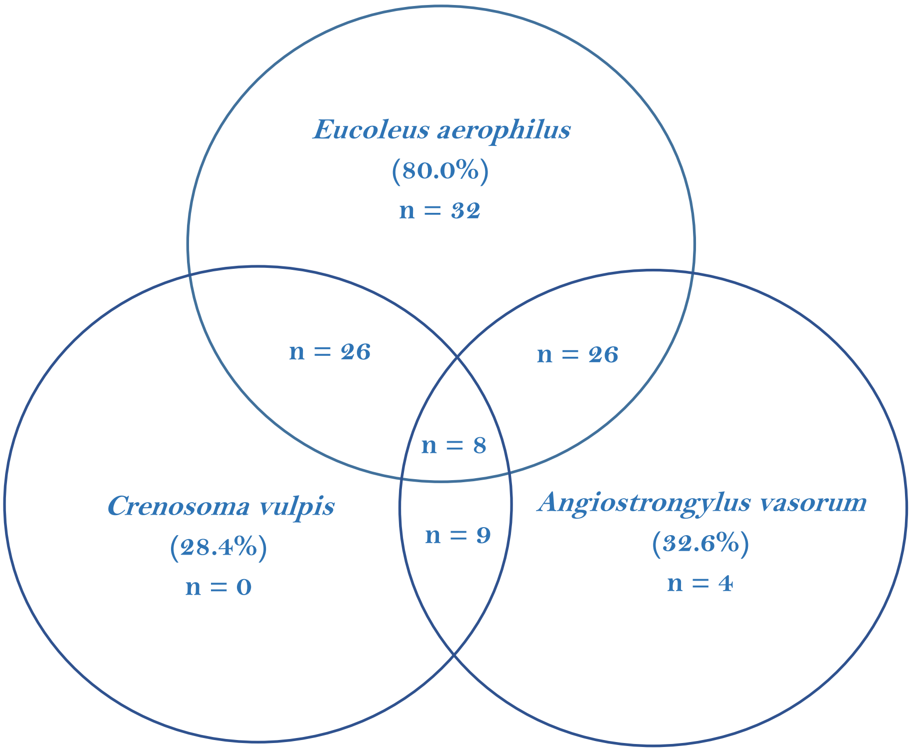

Figure 6 Venn diagram showing the overall prevalence (%) and number (n) of foxes with cardiopulmonary helminth parasites, displaying single, double and triple infections.

Discussion

The present study represents an epidemiological survey on the occurrence and distribution of four helminth species – *Angiostrongylus vasorum*, *Crenosoma vulpis*, *Eucoleus aerophilus* and *Dirofillaria immitis* – that might affect the circulatory and respiratory system of Dutch wild red foxes in the north-eastern part of The Netherlands.

The prevalences of *A. vasorum*, *C. vulpis* and *E. aerophilus* among the foxes from Groningen and Drenthe included in the present study, were all higher than those previously reported. There was no evidence for *D.immitis* in the cardiorespiratory tract of this particular population of foxes.
In the examined foxes, *E. aerophilus* was the most frequently detected parasite. *E. aerophilus* is clearly common in foxes in all parts of Europe, with a prevalence typically ranging from 28 to 88% (Davidson *et al.*, 2006). The overall prevalence observed (80.0%) was higher than that most recent reported (67.7%) in foxes from The Netherlands by Franssen *et al.* (2014). Along with the results obtained from foxes in Serbia (84%, n = 118) and Norway (88%, n = 181), this infection rate of pulmonary capillariosis is among the highest in wild canids in Europe (Davidson *et al.*, 2006; Lalosevic *et al.*, 2013) In a concurrent study, fecal samples of these foxes were also examined for eggs of *Capillaria* spp., but these were not further determined up to species level as these eggs can also be of prey origin.
The prevalence of *A. vasorum* (32.6%) was much higher than the prevalence of 4.2% reported in foxes along the Dutch-German border by Franssen *et al.* (2014), who used the same method for adult worm

screening (washing and sieving). The observation of the apparent expanding range of *A. vasorum* to area's outside the known endemic foci fits the predicted potential distribution model of Morgan *et al.* (2009) (Mccarthy *et al.*, 2016). Although in the beforementioned concurrent study fecal samples were also examined for *A. vasorum* larvae to contribute to the determination of the parasite's prevalence, identification of these larvae was compromised by freezing and thawing of the foxes.
The prevalence of infections with *C. vulpis* (28.4%) found in this study was also higher than those previously found by Borgsteede (1984) and Franssen *et al.* (2014), who reported a prevalence of 4.5% and 16.7% respectively in Dutch red foxes.

Although this particular area in the north-east of The Netherlands has not been investigated for fox cardiopulmonary helminths before, the remarkable difference in prevalences compared to those found in previous studies suggests a spread of *A. vasorum*, *C. vulpis* and *E. aerophilus* in the fox population and highlights the potential exposure of the Dutch dog population to an increased risk of cross-infection. The role of factors that might contribute to the apparently increasing prevalence of cardiopulmonary helminths in the Dutch fox population, such as a change in the presence of infected intermediate and paratenic hosts, dietary preferences, a shift towards a more vector-favorable climate and the interaction between wild and (imported) domesticated dogs are subject for future studies.

Whilst there was no evidence for *D. immitis* infections in the heart and lungs of this particular population of foxes, the prevalence of canine and feline dirofilariasis is increasing and spreading to the north-eastern and center European countries (Genchi *et al.*, 2011). It is therefore considered an emerging zoonotic parasite in both wild red foxes and dogs that should be included in future studies regarding cardiopulmonary helminth species in canids.

In the present study, *C. vulpis* had a significantly higher prevalence in young foxes than in adults. Similar findings were observed by others (Jeffrey *et al.*, 2004; Davidson *et al.*, 2006; Hodžić *et al.*, 2016; Garrido-Castane *et al.*, 2015), and according to these authors, this may be due to differences in (1) acquired immunity to reinfection, (2) behavior and (3) dietary preferences, i.e. young foxes have a greater tendency to eat intermediate gastropod hosts and experience a greater exposure to infective larvae. It is however remarkable that the same reasoning could be applied for *A.vasorum*, of which in this study no age-related difference in infection rate was found. This is in line with the findings of Morgan *et al.* (2008) and Jeffrey *et al.* (2004), who explained the lack of age-related infection by different combinations of age-dependent acquisition, acquired immunity and parasite induced host mortality (Morgan *et al.*, 2008). It should be born in mind that the applied method of age determination was crude, and not suitable to detect more subtle age dependent dynamics in parasite loads

The regression analysis revealed a positive relationship with sex and age and *E.aerophilus* inefection rates, i.e. male and juvenile foxes were significantly more frequently infected with *E. aerophilus* than female and adult foxes. Sex-related variation in parasite loads are in line with theories of immune handicap in male mammals (Wilson *et al.*, 2002). Age-infection patterns for *E. aerophilus* might be due to the same mechanisms as described for *A. vasorum* and *C. vulpis* (i.e. changes in acquired immunity and changes in exposure to parasites) (Wilson *et al.*, 2002).

Lungworm co-infections are common in foxes, but because of the lack of knowledge of the pathogenicity of cardiopulmonary helminths in foxes, it is not clear whether combined infections may lead to a worsening in health (Garrido-Castane *et al.*, 2015).
The majority (72.6%) of foxes in this study were infected with more than one type of cardiopulmonary helminth species, with the *C. vulpis* and an *E. aerophilus* co-infection being the most prominent. A high prevalence of this specific co-infection was also reported by Nevárez *et al.*, (2005), who found a *C. vulpis* – *E. aerophilus* co-infection in 75% of the examined foxes (n = 51). There was no correlation between *C. vulpis* and *A. vasorum* infections in red foxes with dual infections, although both of these helminths require a gastropod intermediate host or paratenic hosts. It has been suggested that different intermediate hosts and paratenic hosts transmit both helminths, causing the lack of infection correlation (Jeffrey *et al.*, 2004). To determine whether either *C. vulpis* or *A. vasorum* offers a mutual or cross-protective immunity against infection with the other, worm burdens should be included in the study design. In the case of a mutual or cross-protective immunity, it would be expected that animals with a single *C. vulpis* or *A. vasorum* infection would have greater worm burdens than those with dual infections (Jeffrey *et al.*, 2004).

Bodyweight-length indices were not related to *A. vasorum*, *C. vulpis* and *E. aerophilus* infections. This finding is in accordance with the findings of Franssen *et al.* (2014), who reported no correlation between BWL-index and infection classes. It differs however from studies demonstrating that fox health or body condition can decrease with parasitic infection (Jeffrey *et al.*, 2004). The non-destructive BWL-index might not have been sensitive enough to correctly evaluate the overall nutritional status of the foxes: the best multivariate predictor of total body fat in foxes is the kidney fat index combined with back fat thickness (Winstanley *et al.*, 1998).

Despite the knowledge that red foxes serve as a reservoir for *A. vasorum*, *C. vulpis* and *E. aerophilus* across Europe, the rate of transfer between foxes and domesticated dogs is unknown (Brereton, 2011). Cardiopulmonary helminth infections in foxes are more likely to give a reliable indication of the distribution of these parasites than surveys or clinical case reports in domesticated dogs, as such studies are strongly influenced by awareness of the veterinary clinician and differences is diagnostic technologies (Taylor *et al.*, 2015).
However, it is of interest to determine the prevalence of *A. vasorum* in – both symptomless and diseased - domesticated dogs in future studies, since *A. vasorum* has only recently been determined as an endemic helminth in domesticated dogs and its prevalence has increased in the fox population in the north-east of The Netherlands. Also, the risk of *A. vasorum* exposure to dogs is present, since dogs feed on the same intermediate and paratenic *A. vasorum* hosts as foxes and might occasionally share the same habitat (e.g. dog outlet areas). Anecdotal, a fecal sample of a symptomless dog of one of the researchers involved in this study, was positive for *A. vasorum* larvae.
Currently, an in vitro diagnostic antigen-test (Angio Detect™Test, IDEXX Laboratories) for the detection of an *A. vasorum* infection in domesticated dogs is available. This snap-test offers a standardized method to determine the presence of *A. vasorum* (sensitivity 98.1%, specificity 99.4%) and is, unlike current fecal testing methods, not compromised by the shedding of larvae in feces. Such standardized method could be included in future studies focusing on the prevalence of *A. vasorum* among Dutch domesticated dogs that are likely to share a habitat with the infected red foxes from the present study to increase the knowledge about cardiopulmonary helminth – fox – dog interactions.

Conclusion

The present study demonstrates that *A. vasorum*, *C. vulpis* and *E. aerophilus* are present in red foxes in the north-eastern provinces of The Netherlands, and that their prevalences are higher than those previously found.

The current data might therefore provide a baseline for future studies focusing on the prevalence and spreading of cardiopulmonary helminths shared between wild and domesticated canids. As well as surveying the parasite distribution in both dogs and foxes, future studies should attempt to evaluate the factors involved with parasite transmission between foxes and domesticated dogs. As *A. vasorum* is now an emerging parasite of clinical interest for dogs in the north-east of The Netherlands, the prevalence of *A. vasorum* in domesticated dogs, the risks and ways of transmission between foxes and dogs as well as clinician awareness in these provinces should be investigated to improve the defense against angiostrongylosis.

Disclosure statement

No competing interests exist.

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Attachment A