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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers which are referred to in the text by their Roman numerals.

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II. Laurimaa, L., Süld, K., Davison, J., Moks, E., Valdmann, H., Saarma, U. (2016). Alien species and their zoonotic parasites in native and introduced ranges: The raccoon dog example. Veterinary Parasitology 219, 24–33.

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V. Laurimaa, L., Davison, J., Süld, K., Plumer, L., Oja, R., Moks, E., Keis, M., Hindrikson, M., Kinkar, L., Laurimäe, T., Abner, J., Remm, J., Anijalg, P., Saarma, U. (2015). First report of highly pathogenic *Echinococcus granulosus* genotype G1 in dogs in a European urban environment. Parasites & Vectors 8:182.

The participation of the author in preparing the listed publications (* denotes moderate contribution, ** high contribution, *** very high contribution).

	I	II	III	IV	V
Original idea	*	*	**	**	**
Study design	**	**	**	***	***
Data collection	**	**	***	**	**
Data analysis	***	***	***	***	***
Manuscript preparation	***	***	***	***	***

1. INTRODUCTION

Zoonoses are infectious diseases that can be transmitted from animals to humans and vice versa, being an important subject of research for both veterinarians and medical doctors. It has been estimated that approximately 60% (>800 species) of infectious agents known to be pathogenic to humans are zoonotic (Taylor et al., 2001). The parasitic agents causing zoonotic diseases and thus being pathogenic to humans can vary from viruses (e.g. Ebola virus that causes Ebola virus disease), protozoa (e.g. *Toxoplasma gondii*; toxoplasmosis) and bacteria (e.g. *Salmonella* spp.; salmonellosis) to fungi (e.g. *Cryptococcus* spp.; cryptococcosis) and helminths (e.g. *Trichinella* spp.; trichinellosis). In general, humans can acquire such parasites from a direct contact with an infected animal; however, the most likely route of infection with zoonotic agents is via contaminated water and foodstuffs like fruits, vegetables and meat (Dorny et al., 2009). The consequences brought by food-borne pathogens are becoming more and more widespread due to an increase in international trade and consumer preferences for natural, minimally processed food (Dorny et al., 2009; Robertson et al., 2014). However, the characteristic of all food-borne pathogens, including zoonotic helminths, is that they cause preventable illnesses, which can be controlled among other strategies also by raising the public awareness (Zhou et al., 2008; Hegglin and Deplazes, 2013).

The term "helminth" is used for several different parasitic metazoan groups: Turbellaria, Trematoda, Cestoda, Nematoda, Nematomorpha and Acanthocephala (Auer and Aspöck, 2014a). However, helminths with high medical relevance mainly belong to trematodes, cestodes or nematodes. Most commonly, parasitic helminths occupy the gastrointestinal tract of the host. Humans may play the role of final, intermediate or aberrant hosts within the life cycle of a helminth. For example, humans are final hosts for adult stages of *Taenia saginata* and *Ascaris lumbricoides*, which are located in the small intestine and cause only mild gastrointestinal symptoms. In contrast, humans may act as accidental intermediate host for various tapeworms, including cestodes from the genus *Echinococcus*, and suffer from the larval stages of these parasites. Among the class Cestoda, the families Diphyllobothriidae, Dilepididae, Hymenolepididae and Taeniidae contain the most important clinically and epidemiologically relevant tapeworms not only in developing countries, but also in industrialised countries in Central Europe (Raether and Hänel, 2003; Auer and Aspöck, 2014b). Therefore, the health risks posed by these parasites and their abundance should be monitored globally.

Tapeworms of the family Taeniidae are important parasites of mammals. Besides genus *Taenia* the family comprises of genus *Echinococcus*, which includes parasites that cause life-threatening zoonoses called echinococcoses. There are two *Echinococcus* species present in Europe that can cause disease in humans: *Echinococcus granulosus*, the causative agent of cystic echinococcosis (hydatidosis); and *Echinococcus multilocularis*, which causes alveolar echino-

coccosis (Eckert et al., 2001). Although human infections are most commonly associated with *E. granulosus sensu stricto* (Thompson, 2008; Alvarez Rojas et al., 2014), *E. multilocularis* is regarded as the biggest threat to public health for several reasons. First, due to the rapid increase in the number of its main definitive host, the red fox (*Vulpes vulpes*), during the last decades, *E. multilocularis* has also expanded its range in Europe and has followed its host to urban environments (Deplazes et al., 2004; Davidson et al., 2012). Unlike the single cyst-forming *E. granulosus*, the larval stage of *E. multilocularis* is characterised by extensive tumour-like growth in the internal organs of human host with high mortality rate (Eckert et al., 2001; Torgerson et al., 2008). *Echinococcus multilocularis* is also considered as an emerging zoonotic helminth since infections with this species have newly appeared in many countries or have existed in unnoticed endemic areas, but are now rapidly being identified (Eckert et al., 2000; Jenkins et al., 2005; Vorou et al., 2007). For example, the historical distribution area in Western Europe covered only four central and southern European countries (France, Germany, Switzerland and Austria; Eckert and Deplazes, 1999), and it seems that the species has only recently expanded its range towards the north (the Baltic States and Sweden) (Osterman Lind et al., 2011; Marcinkute et al., 2015). In addition to direct transfer from animals to humans, the eggs of *E. multilocularis* can be transmitted with several food items: the tapeworm has recently been identified in kitchen gardens in France and Poland (Bastien et al., 2014; Lass et al., 2015) and human infections are thought to occur after consuming forest berries (Eckert et al., 2001; Lass et al., 2015).

To estimate the role of red foxes and other canids in contaminating the environment with zoonotic parasite eggs, the prevalence and infection trend of the parasites should be monitored in both urban and rural areas. For identifying helminth infection in definitive hosts, both invasive and non-invasive approaches can be used. While the necropsy is rarely applicable for privately owned dogs and hunting is prohibited in densely populated areas, a highly sensitive and specific molecular diagnostic method that can identify both parasite and host species, even from degraded faecal samples collected in urban environment, is needed.

1.1. Life cycle of *E. multilocularis*

The typical transmission cycle of *E. multilocularis* in Europe is wildlife-based, predominantly involving red foxes as definitive and arvicolid rodents (e.g. *Arvicola amphibius* and *Microtus arvalis*; Romig et al., 2006) as intermediate hosts (Figure 1). Adult tapeworms, usually less than 5 mm in length, are present in the small intestine of carnivorous mammals, where they reproduce sexually and shed eggs, which are then excreted to the environment with host faeces. In humid conditions and at low temperatures the parasite eggs are able to maintain their infectivity in the environment for up to one year (Veit et al., 1995; Eckert et al., 2001). After oral uptake of infective eggs by intermediate host, larvae

hatch from the egg and enter the blood circulation to invade different internal organs. Primary infection almost exclusively occurs in the liver; however, larval cysts of *E. multilocularis* tend to metastasise to other distant organs (Eckert et al., 2001). Eventually, the parasite life cycle is completed when fox eats infected rodent. The other known wild definitive host species for *E. multilocularis* in Europe are the arctic fox (*Vulpes lagopus*), raccoon dog (*Nyctereutes procyonoides*), grey wolf (*Canis lupus*), golden jackal (*Canis aureus*), wild cat (*Felis silvestris*) and lynx (*Lynx lynx*) (Eckert et al., 2001; Fuglei et al., 2008; Szell et al., 2013). Adult worms can additionally develop in domestic dogs and cats (Thompson et al., 2003; Kapel et al., 2006).

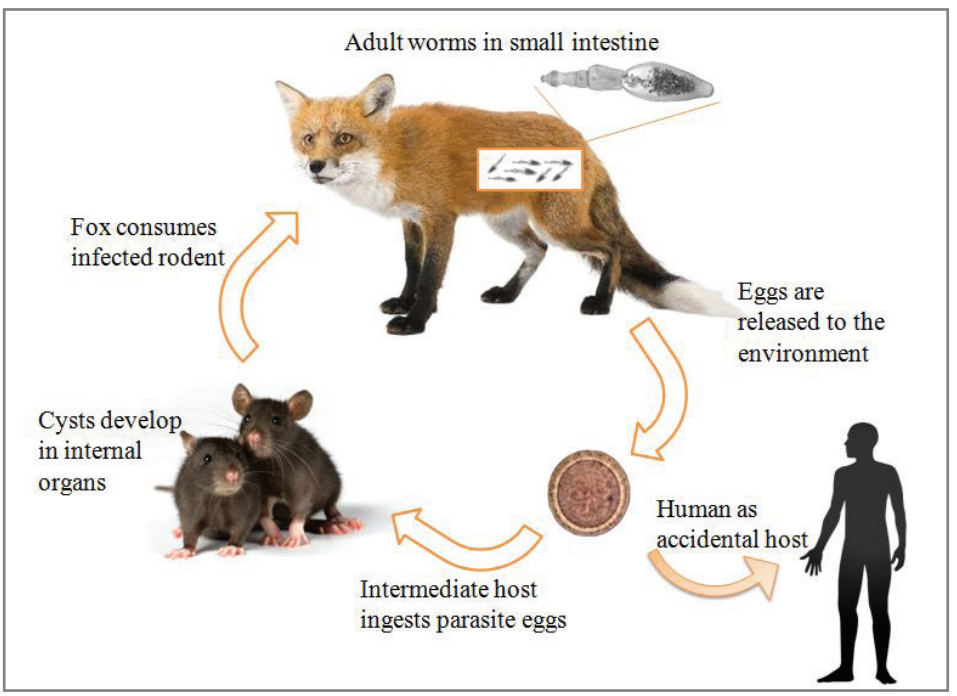

Figure 1. Life cycle of the small fox tapeworm *Echinococcus multilocularis*. Red fox serves as the main definitive host species and various rodent species as intermediate hosts for the tapeworm. Humans can also be infected by ingesting parasite eggs.

When infection with *Echinococcus* tapeworms is typically asymptomatic for the definitive host, it causes severe illness with high mortality rate in its intermediate hosts, including humans. Although humans are considered as accidental intermediate hosts, they can be infected by ingesting parasite eggs via direct contact with definitive hosts such as infected companion animals (dog, cat) or through contact with contaminated water, soil or food (Eckert et al., 2001). The predilection site for *E. multilocularis* larval form in humans is the liver, where agglomerates of rapidly proliferating small vesicles (cysts of up to 3 cm in diameter) are formed. Therefore, the signs and symptoms, including cholestatic

jaundice, epigastric pain and hepatomegaly, can be misdiagnosed as cirrhosis or liver cancer (Vuitton et al., 2015). At the later stage of infection, metastasis formation can expand to other distant organs, e.g. to brain and lungs (Eckert et al., 2001; Tappe et al., 2008). Throughout the world approximately 18,000 new human cases of alveolar echinococcosis are diagnosed annually (Torgerson et al., 2010). But since the human infection is characterised by long (5–15 years) asymptomatic incubation period (Eckert et al., 2001; Eckert and Deplazes, 2004), the number of people affected by the parasite is significantly larger. In Estonia, altogether 13 human echinococcosis cases have been reported since 1986, however the causative species (*E. multilocularis* or *E. granulosus*) have not been determined (Marcinkute et al., 2015). Alveolar echinococcosis affects people of all ages and can be life-threatening, with fatality rate of over 90% in untreated patients (Eckert et al., 2001). The treatment itself is expensive, often including radical surgery, liver transplantation and prolonged chemotherapy with administration of benzimidazoles (albendazole or mebendazole; Eckert and Deplazes, 2004; Brunetti et al., 2010). Nevertheless, modern treatment has considerably increased the life expectancy in patients with alveolar echinococcosis compared with the 1970s (Torgerson et al., 2008).

1.2. Main sources of *E. multilocularis* infection for humans

The red fox, which is the main definitive host species for *E. multilocularis*, is the most widely distributed wild terrestrial carnivore in the world with the species range of approximately 70 million km^2 (Macdonald and Reynolds, 2008). Partly as a consequence of highly successful vaccination of wildlife against rabies, red fox density appears to have increased in many countries in Europe during the last decades (Vos, 1995; Gloor et al., 2001). Therefore, informing the general public of potential risks related to foxes is becoming more and more important. Moreover, foxes are colonising urban areas in Europe and around the world with increasing pace (Harris et al., 1986; Gloor et al., 2001; Bateman and Fleming, 2012), including Estonia (Plumer et al., 2014), bringing zoonotic pathogens to the immediate neighbourhood of humans and their companion animals (Deplazes et al., 2004; Umhang et al., 2015). For example, in addition to the wide distribution of *E. multilocularis* among rural foxes (in 20 countries in Europe; Davidson et al., 2012) infected urban foxes have also been recorded in major European cities including Zürich, Geneva and Copenhagen (Hofer et al., 2000; Deplazes et al., 2004; Reperant et al., 2009). In general, due to their anthropogenic diet, urban foxes are less likely to be infected with the tapeworm (Fischer et al., 2005; Hegglin et al., 2007; Robardet et al., 2008). However, as the fox population densities can be significantly higher in urban areas and some of the foxes move between urban and rural areas, just as much infectious eggs are probably released into the environment

as in rural areas (König and Romig, 2010). This leads to increased risk to human health in urban areas, where the contact with infectious eggs occurs more likely.

As a result of global urbanisation, the number of companion animals, including domestic dogs and cats, has also increased in urban settlements. It has been estimated that approximately 81 and 99 million pet dogs and cats, respectively, live in Europe (FEDIAF, 2014). In addition, there are numerous stray dogs and cats that may act as reservoir host for *E. multilocularis* by sustaining the parasite in urban areas. Although cats can serve as definitive host for *E. multilocularis*, the infection is characterised by low worm burden and reduced development of the worm, resulting in lower egg production compared to foxes, raccoon dogs and dogs (Kapel et al., 2006). Therefore, the epidemiological role of the cat in spreading alveolar echinococcosis to humans is estimated to be low (Umhang et al., 2015). Dogs, on the other hand, are more suitable definitive hosts for the tapeworm (Thompson et al., 2003; Kapel et al., 2006), and represent a significant public health risk due to their close contact with humans. For example, many dog owners allow the pet to lick their face or allow them in bed (Overgaauw et al., 2009). The described transmission path from infected definitive host to human can be highly suitable for *Echinococcus* tapeworms. Moreover, Kern et al. (2003; 2004) showed that owning a dog is an important risk factor for acquiring alveolar echinococcosis. Besides acquiring the parasite eggs directly from the infected dog, humans can possibly also be infected after petting a healthy dog, which fur have become contaminated with infective parasite eggs after smelling or rolling in fox faeces or infectious soil. In general, *E. multilocularis* infection is reported to be low (<0.5%) in privately owned dog populations in Central Europe (Deplazes et al., 1999; Dyachenko et al., 2008). However, dogs that catch rodents and tend to roam are more susceptible to the disease and their infection rates can vary from 3% to 8% (Svobodova and Lenska, 2002; Antolova et al., 2009; Deplazes et al., 2011).

The raccoon dog is an alien canid species, introduced to Europe from the Far-East of Russia in 1929–1958 (Heptner and Naumov, 1998). Though with low prevalence, *E. multilocularis*-infected animals have been reported already in 1970s in their natural distribution area in Far-East Russia – Primorje and Amur regions (Judin, 1977). In comparison, the first infected raccoon dog in Europe was reported only in 2001 in Germany (Thiess et al., 2001). Characteristics including omnivorous diet, high reproductive potential and the ability to hibernate at high latitudes have allowed the raccoon dog to successfully colonise new areas (Kauhala and Kowalczyk, 2011). As a result, the raccoon dog is now well-established in northern, eastern and central parts of Europe and continues to expand its range towards the west and south (Kauhala and Kowalczyk, 2011). As the raccoon dog is both abundant and susceptible to *E. multilocularis*, the species should be considered as important agent in contaminating the environment with zoonotic parasite eggs.

1.3. Other zoonotic helminths transmitted by wild canids

Besides *E. multilocularis* there are two other helminth taxa frequently reported in foxes throughout Europe and causing zoonotic infections in humans: *Trichinella* spp. and *Toxocara canis* (Smith et al., 2003; Letkova et al., 2006). In the genus *Trichinella* there are 12 taxa of roundworms, however only four (*T. spiralis*, *T. nativa*, *T. britovi* and *T. pseudospiralis*) are present in Europe (Gottstein et al., 2009). While the adult *Trichinella* nematodes are located in the intestinal mucosa, the larval stage of this parasite is characterised by extensive migration to the muscle tissue of various animal species (mammals, birds and reptiles). In general, *Trichinella* larvae prefer and penetrate those muscles that have good blood supply (highly oxygenated muscles like diaphragm, forearm, masseter, tongue). In order to be transmitted, the infected tissues containing parasite larva must be eaten by another host. Human infections in Europe mostly occur after consuming raw or inadequately cooked meat of wild boar (*Sus scrofa*) or domestic pig. Despite the efforts to eradicate *Trichinella* from Europe, the parasite is still prevalent, especially in Eastern Europe where improper hunting practices are still a problem, e.g. leaving the carcasses of skinned fur animals to the forest that makes them readily available to foxes and other scavengers (Casulli et al., 2001; Pozio et al., 2001; Malakauskas et al., 2007; Kirjušina et al., 2015). *Trichinella* spp. prevalence among red fox populations in Eastern Europe can vary from 2% in Hungary and Slovakia (Letkova et al., 2006; Tolnai et al., 2014) to 29% in Bulgaria (Kirkova et al., 2011). The situation is even worse in the Baltic States, where infection rates between 29–47% have been reported (Malakauskas et al., 2007; Bružinskaite-Schmidhalter et al., 2012).

Similarly to *Trichinella* spp. the nematode *Toxocara canis* causes severe symptoms in its larval stage. Humans can acquire infective parasite eggs orally, either directly from the environment or ingest these with contaminated foodstuff. Upon infection the larvae hatch from the egg and migrate to various tissues of the host. As *T. canis* larvae fail to develop to adult nematodes in humans, they can wander around the host body for up to several years, sometimes even reaching to the eye and causing blindness (Despommier, 2003). However, if a canid host is infected, the larvae migrate to the lungs, where they are coughed up and swallowed, thus reaching to the small intestine and maturing to adult nematodes. Canids can additionally acquire *T. canis* infection in utero (transplacentally) from infected mother. In general, the parasite is widely distributed among European red foxes and raccoon dogs with the infection rate varying from 9% to 62% (Smith et al., 2003; Segovia et al., 2004; Brochier et al., 2007; Magi et al., 2009; Miterpakova et al., 2009; Siko Barabasi et al., 2010; Bružinskaite-Schmidhalter et al., 2012; Al-Sabi et al., 2013; Franssen et al., 2014). In comparison, the prevalence rate among Estonian red foxes can reach to 29% (Moks, 2008). Besides wild canids, *T. canis* is also

prevalent among domestic dogs in Europe, including Estonia, with the infection rate between 4–34% (Talvik et al., 2006; Overgaauw and van Knapen, 2013). As the eggs of *T. canis* are very adhesive and can stick to dog fur, this can represent another possible route of infection for humans. Moreover, *Toxocara* eggs have been found in dog fur (Aydenizöz-Özkayhan et al., 2008; Roddie et al., 2008) and in environmental samples (Habluetzel et al., 2003; Talvik et al., 2006; Dubna et al., 2007) throughout the Europe. The observed high prevalence rates of *T. canis* in both wild canids and domestic dogs lead to increase in environmental contamination with the parasite eggs, thus representing a significant health risk for humans.

Otranto et al. (2015) have reviewed zoonotic parasites infecting European canids and showed that there are a minimum of 15 helminth species reported in European wild canids with zoonotic importance. However, trematode *Alaria alata*, missing from the review, should also be considered as a zoonotic parasite species in Europe for its ability to occasionally infect humans (Wasiluk, 2009). *Alaria alata* is a parasitic fluke widely distributed among wild canids, its definitive hosts in Europe: infected foxes and raccoon dogs have been reported in the Baltic States (Moks, 2008; Bružinskaite-Schmidhalter et al., 2012; Esite, 2012), Ireland (Murphy et al, 2012), Iberian peninsula (Segovia et al., 2004), Germany (Manke and Stoye, 1998; Thiess et al., 2001), Denmark (Al-Sabi et al., 2013), Poland (Borecka et al, 2009), Belarus (Shimalov and Shimalov, 2002, 2003), Hungary (Szell et al., 2013), and Balcan countries (Rajkovic-Janje et al., 2002; Siko Barabasi et al., 2010; Kirkova et al., 2011). Although the prevalence in red foxes in most countries is relatively low, it can reach up to 90% in the Baltic region (Moks, 2008; Bružinskaite-Schmidhalter et al., 2012; Esite et al., 2012). Furthermore, the larvae of *A. alata* are increasingly being found in wild boar meat during official *Trichinella* inspection (Riehn et al., 2010; Portier et al., 2011). Thus, consuming inadequately cooked wild boar meat could represent a potential source of infection for humans (Möhl et al., 2009). Since the definitive hosts are responsible for environmental contamination with *A. alata* eggs, it is important to monitor the parasite distribution primarily in those host species.

Additionally, there is at least one ectoparasite of wild canids capable of infecting humans. The highly contagious itch mite *Sarcoptes scabiei* has previously been reported to cause significant reduction in the number of red foxes in different parts of Europe during epizootics (e.g. in Scandinavia, Spain and Britain; Soulsbury et al., 2007). Since the mite is usually transmitted via direct contact with infected animal, its distribution and prevalence depends on host density. Upon infection the mite causes painful itching as the parasite consumes living cells and tissue fluid of the host while burrowing into the upper layer of the skin (Pence and Ueckermann, 2002). As a result, patches of sores and thick crust emerge on the host skin, leading to loss of hair and potential hypothermia. Although the species *S. scabiei* encompass morphologically indistinguishable varieties that are highly host specific (including human specific variety), animal scabies can cause temporary itching in humans (Arlian, 1989; Heukelbach and Feldmeier, 2006).

1.3. Main diagnostic methods for identifying *Echinococcus* parasites in definitive hosts

To estimate the role of red foxes and other canids in contaminating the environment with zoonotic parasite eggs, the prevalence and infection trend of the parasites should be monitored in both urban and rural areas. As wild carnivores are constantly being hunted, it is possible to obtain the carcasses from hunters and identify parasite infection during necropsy. Sedimentation and counting technique (SCT), which is used for assessing the sensitivity and specificity of other techniques, is considered as the gold standard in identifying *Echinococcus* infection in definitive hosts (Eckert et al., 2001). SCT is an invasive analysis based on washing the contents of the intestine, including attached helminths, to a vessel and examining the sediment microscopically. However, recent comparative study with highly specific copro-PCR detection method by Isaksson et al. (2014) showed that the SCT was negative for a number of samples with positive PCR result, meaning that instead of formerly reported ~100% the actual sensitivity of the method can reach only about 80%. Second widely used necropsy method is intestinal scraping technique (IST). It is similarly based on morphological identification of parasites; however several mucosal scrapings of the intestine are taken and examined microscopically. As the SCT and IST methods are known to be very laborious, they are most suitable for morphological identification of different parasites from a relatively small number of animals, e.g. about 100 and 150 animals can be processed in one week with SCT and IST, respectively (Conraths and Deplazes, 2015). Nevertheless, such invasive methods have been used in several European countries (e.g. Shimalov and Shimalov, 2002; 2003; Smith et al., 2003; Bružinskaite-Schmidhalter et al., 2012; Al-Sabi et al., 2013), making it possible to compare the results obtained from various studies. While the necropsy is rarely applicable for privately owned dogs and hunting is prohibited in densely populated areas, immunological or molecular techniques on faecal samples of companion animals and urban foxes should be used instead.

Immunological techniques (e.g. coproantigen-ELISA), which are used to detect parasitic infection in animals, are based on detecting parasite specific antigens in host faeces, but the method can also be used for estimating soil contamination. As the eggs of *Echinococcus* parasites are highly resistant to degradation in the environment (*E. multilocularis* eggs up to one year and *E. granulosus* eggs over 3 years; Veit et al., 1995; Thevenet et al., 2003), it is a suitable method for analysing old samples. Immunological techniques can also be used for mass screening as up to 800 samples can be processed in one week (Conraths and Deplazes, 2015). However, it is possible to overlook some of the infections with low worm burden as the sensitivity of this method has been estimated to be 60–80% (Conraths and Deplazes, 2015). In addition, it is recommended to reanalyse the *Echinococcus*-positive samples with a subsequent PCR-based assay as cross reactivity can occur with antigens from *Taenia*

species, therefore providing a possibly large number of false-positive results (Torgerson and Deplazes, 2009). As a result, molecular techniques should be preferred instead of immunological techniques.

Molecular methods based on PCR can be used to detect parasite specific DNA in both faecal and environmental samples. Although PCR-based identification methods are potentially highly specific and sensitive, they are also laborious and expensive (Conraths and Deplazes, 2015). In general, parasite DNA is first extracted and amplified with species or genus specific primers, and then sequenced. As the parasite eggs are rarely evenly distributed in the faecal sample and only a small amount of material is analysed, probable infection can remain undetected. Therefore, *Echinococcus* eggs have frequently been concentrated in the sample prior to DNA extraction by using saturated salt solution as described in Mathis et al. (1996). On its own, this concentration method is not suitable for identifying the *Echinococcus* species, since the eggs of these tapeworms are morphologically indistinguishable from those of other species in the family Taeniidae. Therefore, subsequent molecular method is always required. In order to isolate DNA from the extremely resistant *Echinococcus* eggs, boiling in potassium hydroxide (KOH; Bretagne et al., 1993; Mathis et al., 1996; Dinkel et al., 1998; Stefanic et al., 2004; Al-Sabi et al., 2007; Dyachenko et al., 2008) or in sodium dodecyl sulfate (SDS; Van der Giessen et al., 1999) have previously performed on the samples. On the other hand, Cabrera et al. (2002) and Klein et al. (2014) have showed that sequential freezing and heating of samples already favours the disruption of the keratin layer of the *Echinococcus* egg and extraction of the DNA.

In general, there are genetic methods (Trachsel et al., 2007; Boubaker et al., 2013; Dinkel et al., 2011) available for identifying *Echinococcus* spp. parasites. Some of these methods only detect the tapeworm species (Trachsel et al., 2007; Boubaker et al., 2013), but molecular identification of the host species responsible for contaminating the environment is also important, since the food habits of urban foxes are anthropogenic and the excrements can be morphologically indistinguishable from those of domestic dogs. Host species of *Echinococcus* tapeworms have previously successfully identified in few studies, using relatively long sequences of DNA (160–478 base pairs; Nonaka et al., 2009; Dinkel et al., 2011; Jiang et al., 2011). However, readiness to analyse older samples with degraded DNA is needed. Moreover, Dinkel et al. (2011) and Jiang et al. (2011) used universal carnivore primers that need additional sequencing of the product in order to determine the exact host species, which makes the analysis more expensive. Alternatively, quantitative real-time PCR techniques to detect and quantify *E. multilocularis* DNA in fox faeces also exist (Dinkel et al., 2011; Knapp et al., 2014), but for many laboratories these can be too expensive or difficult to apply. Therefore, a highly specific and sensitive PCR-based diagnostic method that can detect tapeworms and identify their host species, also from degraded faecal samples, on the basis of DNA fragment size would be useful.

2. OBJECTIVES OF THE STUDY

Although, the first finding of *E. granulosus* in Estonia dates back to the beginning of 20th century (Marcinkute et al., 2015), *E. multilocularis* was identified for the first time in Estonia only in 2003 (Moks et al., 2005). In this study the authors reported of four infected red foxes shot in south-eastern counties and one from the second biggest island Hiiumaa. It is highly likely that *E. multilocularis* has been present in Estonian wildlife for longer, but remained unnoticed. While the prevalence of *E. granulosus* in wildlife is currently low (4% in wolf and 0.8% in moose; Moks et al., 2006; 2008), Moks et al. (2005) demonstrated a relatively high *E. multilocularis* infection rate (29.4%) among local red foxes. This, in turn, represents a considerable risk for environmental contamination with the life-threatening parasite eggs.

Unlike the native red fox, raccoon dog is an alien species in Estonia that was first introduced to the area in 1950, though the very first animal was recorded already in 1938 (Aul et al., 1957). The latter could have originated from the Staraya Russa region in Novgorod oblast (Russia), where 50 animals were introduced in 1935 (Aul et al., 1957; Pavlov et al., 1974). A similar dispersal pattern was described for Pskov oblast (Russia), where the first animals seen in 1930s had migrated from the neighbouring Novgorod oblast and first animals officially released only in 1947 (Pavlov et al., 1974). Alternatively, the first animals recorded in Estonia could have derived from those 50 individuals, which were introduced to Leningrad oblast (Russia) in 1936 (Pavlov et al., 1974). The officially introduced raccoon dogs (n=88) to Estonian territory in 1950 were not brought directly from the species native range in the Far East, but originated from Kalinin oblast (current Tver oblast) in European part of Russia (Pavlov et al., 1974). The animals used for translocation were probably free of parasites, as they were held in separate cages and regularly treated with anthelminthics (Skorodumov, 1937). According to Skorodumov (1937) anti-parasitic medicine was administered to adult raccoon dogs once a year (in autumn) and when parasite eggs were detected in faeces. Shortly, if the animals were infected with hookworms and ascarids, they were treated with carbon tetrachloride or etylene chloride four; fern *Dryopteris filix-mas* extract was used against tapeworm infection (Skorodumov, 1937).

During the last decades the population size of both red foxes and raccoon dogs in Estonia has been affected mainly by infectious diseases such as rabies and sarcoptic mange, and to a lesser degree by hunting. Shortly after the oral vaccination campaign against rabies was imposed to Estonian wildlife in autumn 2005, the number of foxes and raccoon dogs started to increase rapidly. Judging by hunting bags recorded during the period 2005–2009 (growth from approximately 4,000 to 9,000 red fox individuals and from approximately 6,000 to 12,000 raccoon dog individuals) the number of red foxes and raccoon dogs has increased considerably (Veeroja and Männil, 2015). Local Veterinary and Food Board could also have affected the number of hunted animals, as there

was a monetary reward system implemented for rabies monitoring programme among Estonian red fox and raccoon dog populations during that time. For each animal head sent to the laboratory authorised by Veterinary and Food Board the hunters were paid 80 EEK (~5 EUR). However, after the harsh winter of 2010/2011 sarcoptic mange started to spread extensively and the trends of foxes and raccoon dogs have since been continuously decreasing (Veeroja and Männil, 2015).

The parasite fauna of Estonian red foxes and raccoon dogs was investigated about a decade ago, when a pilot study revealed 16 and 5 helminth taxa, respectively (Moks, 2008). Considering that only a small number of animals from both species (17 red foxes and 21 raccoon dogs) were examined, it is important to evaluate the red fox and raccoon dog parasite fauna in Estonia by including significantly larger set of samples. Moreover, as the known endoparasite fauna of both red foxes and raccoon dogs in Europe is significantly larger than shown by Moks (2008), e.g. consisting of 32 and 25 different parasite taxa in red foxes and raccoon dogs in Belarus, respectively (Shimalov and Shimalov, 2002, 2003), it seems likely that both canid species in Estonia harbour more parasite taxa than indicated by the previous study. In addition, since approximately a decade has passed from the identification of *E. multilocularis* in red foxes by Moks et al. (2005), it would be of considerable interest to evaluate the changes in the prevalence of *E. multilocularis*.

Since the parasite fauna of an animal is intimately related to its feeding habits (majority of parasitic infections are acquired orally), it is important to study the relationships between these two factors. Moreover, it has been shown that the animals infected with sarcoptic mange are frequently undernourished (Newman et al., 2002), and are therefore constantly in search of food, encountering potentially a wider range of different parasites than healthy animals. Therefore, it is also relevant to analyse differences in the parasite fauna of healthy and mangy animals.

After the increase in Estonian red fox numbers, they have been reported in 33 out of 47 towns nationwide (Plumer et al., 2014). Since about 30% of foxes are known to be infected with *E. multilocularis* in natural habitats in Estonia (Moks et al., 2005), and cases of human echinococcosis in the Baltic region are in rise (Marcinkute et al., 2015), it is of great significance to monitor parasite spillover into urban areas, where it presents a particular public health risk. Furthermore, as there are many dog owners, who do not clean up after their dog, it is essential to estimate the extent of environmental contamination with zoonotic parasite eggs in urban settlements. Although there are various invasive and non-invasive techniques available for detecting *E. multilocularis* infection in definitive host, there is an urgent need for highly specific and sensitive non-invasive molecular diagnostic methods that can detect tapeworms and identify their host species even from degraded faecal samples collected in urban areas.

The present study had the following aims:

1) To describe and compare the endoparasite fauna of Estonian red foxes and raccoon dogs to determine the species potential for environmental contamination with *Echinococcus* eggs and other zoonotic agents (**I, II, III**);
2) To assess the co-occurrence of parasite species and food categories, since the parasite fauna of an animal is closely related to its feeding habits (**II, III**);
3) To develop a highly sensitive and specific PCR method coupled with non-invasive sampling to identify both the host species and *Echinococcus* tapeworm species from faeces (**IV**);
4) To collect carnivore faeces in an urban area in Estonia to identify *Echinococcus* infection in urban canids (**IV, V**).

3. MATERIALS AND METHODS

3.1. Methods used to study parasites of rural raccoon dogs and red foxes

Sample collection

255 raccoon dog and 111 red fox carcasses were collected from animals legally harvested by hunters, and examined for internal parasites (**I, II, III**). Samples were collected between autumn 2010 and spring 2012 from different parts of Estonia. While all the foxes originated from the mainland, raccoon dogs were also collected from the Estonia's biggest island Saaremaa (Figure 2).

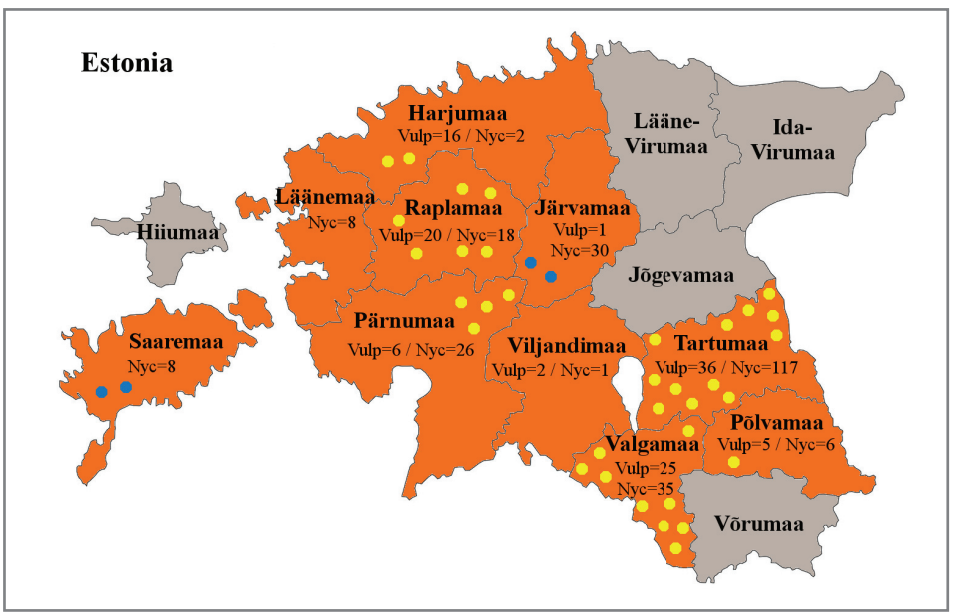

Figure 2. Map of red fox (Vulp) and raccoon dog (Nyc) carcasses collected in Estonia. The number of animals examined is shown below the county name. Blue and yellow dots indicate the *Echinococcus multilocularis*-positive raccoon dogs and red foxes, respectively.

All animals collected with fur (227 raccoon dogs and 99 red foxes) were examined for sores and patches of thick crusty skin as signs of sarcoptic mange. After weighing the carcasses, internal organs were removed and kept at –80 ºC for at least 5 days before parasitological examination as a safety precaution (Eckert et al., 2001). Lungs, gall bladder and urinary bladder were studied using washing and sieving technique for helminth detection. Briefly, the respective organ was cut open and the lumen was washed with tap water through 200 µm mesh sieve to reveal helminth infection. The small and large intestines were separated and examined by the sedimentation and counting technique. Up to 200 specimens

were counted per helminth species, since continuing to very large numbers (often thousands) would have been too laborious. Parasites were preserved in 95% ethanol.

Additionally, muscle samples were taken from the diaphragm and forearm of the carcasses to identify *Trichinella* larvae. However, these were not subjected to parasitological examination during this study.

Parasite identification

Trematodes, cestodes and nematodes were identified according to their morphology after Kozlov (1977). Cestodes from the genera *Echinococcus, Taenia* and *Mesocestoides* were further identified after Abuladze (1964), Loos-Frank (2000) and Hrčkova et al. (2011), respectively (**I, II, III**).

As the scoleces of tapeworms from the genus *Taenia* were deformed and lacking some of the features required for morphological identification (e.g. hooks), these samples were submitted to genetic identification. Genomic DNA was extracted from the scoleces and a 506 bp (base pair) fragment of *cox1* gene in mtDNA was amplified as described in **II**. Eventually, the PCR products were purified and sequenced (**II, III**).

Statistical analyses

For statistical analysis, collected animals were divided into two seasons reflecting the availability of natural food resources: 1) autumn (August–November); and 2) winter and early spring (December–April). Nonparametric Mann-Whitney U test was used to analyse intensity of infection and variation in the number of helminth species between the sexes as well as between the seasons (**II, III**). The same analysis was used to determine whether scabied animals harboured more helminth species, and if sarcoptic mange influenced the intensity of helminth infection (**III**). Mann-Whitney U test was also used to measure if males weighed more than females (**II**, **III**), to detect differences in animal weight between the two seasons and to compare parasite infections between the two host species. Intensity of parasitic infection in each animal was determined as sum of the number of observed parasite specimens from different species (1–784 in red foxes and 0–450 in raccoon dogs). However, as the upper limit in counting the parasite specimens from one species was set to 200, this should be considered as relative intensity. Chi-square test was used to detect variations in infection rates of different parasite species between the two canid species. Nonparametric tests were performed using software STATISTICA (StatSoft Inc.).

For comparative analyses of parasites and consumed food items, we used the host species dietary data from Süld et al. (2014) and Soe et al. (unpublished), originating from the same animals used in this study. To assess the co-occurrence of parasite species and food categories, we calculated the C-score (Stone and Roberts, 1990) for all pairs of parasite species, and for parasite species and

food types as described by Süld et al. (2014) (**II, III**). Essentially, we generated 999 random matrices with fixed row and column occurrence (e.g. if parasite A has a prevalence of 20% in the raw data, this level of prevalence will remain the same in all randomised data sets) and recalculated all pairwise C-scores for each matrix. Observed C-scores were standardised and the significance of effect was estimated from the number of randomised C-scores more extreme than the observed value. The co-occurrence analyses were carried out using package "vegan" (Oksanen et al., 2015) in software R (R Development Core Team, 2014).

To evaluate the parasite overlap between raccoon dogs and red foxes Pianka's index (Pianka, 1973) was calculated. Values of this index can vary between 0 (no overlap) and 1 (complete overlap).

3.2. Developing a non-invasive molecular method for detection of *Echinococcus* tapeworms

The aim was to develop a highly sensitive and specific PCR method to detect both *Echinococcus* parasite and its host species from degraded faecal samples collected in an urban area. Therefore, species-specific primers (specific to both *Echinococcus* species present in Estonia, red fox and domestic dog) amplifying short sequences of mitochondrial DNA (mtDNA) were designed (Technical Appendix Table in **IV**). Primers were designed to guarantee a difference in the length of the PCR product in order to determine the species on the basis of amplicon size when separated by electrophoresis. Adult *E. multilocularis* specimens and faecal samples used in developing the new method were obtained from the intestine of hunted red foxes. DNA samples of *E. granulosus* were from the sample collection of our workgroup and sequenced earlier by Moks et al. (2008) and Saarma et al. (2009). Genomic DNA was extracted from the faecal samples of approximately 250 mg using QIAamp DNA Stool Mini Kit (Qiagen).

PCR reactions were performed in a volume of 20 μL containing 1× Advantage 2 SA PCR Buffer, 1× Advantage 2 Polymerase Mix (Clontech), 0.2 μM dNTP (Fermentas), 0.25 μM of each primer, and different amount of purified DNA.

To estimate the sensitivity of the new method, the number of *Echinococcus* tapeworm eggs necessary to obtain a positive PCR result was determined. Specifically, 1, 3, 5, 7, 10, 15, and 20 *E. multilocularis* eggs were added to fox faecal samples that were previously known to be uninfected with the tapeworm (**IV**).

3.3. Methods used to detect *Echinococcus* infection in urban canids in Estonia

Canine faecal samples were collected from January to March in 2012 and 2013 from the streets and parks of Tartu, Estonia. During the study period 14 transects were surveyed weekly. We collected 239 scat samples that were deep frozen at −80 °C for safety precautions (**IV**, **V**). Small amount (~250 mg) of stool from each sample was first heated at 65 °C for 15 min and then stored at −80 °C until subsequent DNA extraction (**IV, V**). PCR was performed twice for each sample using the primers described in the Technical Appendix in **IV**.

On the basis of amplicon size we could determine the parasite and its host species. However, to verify parasite identification, DNA from 2 *E. multilocularis*-positive samples and 2 *E. granulosus*-positive samples was sequenced using the same primers as used in the primary PCR (**IV, V**). To verify host species, part of mtDNA was sequenced for 5 presumed fox samples and 5 presumed dog samples, using primers that produced longer amplification products: 327 bp fragment of cytochrome c oxidase II (COII) gene in red fox mtDNA was amplified with primers VN1f (5’ – TACTAACTACCAAGCTAACCCAC) and VN2r (5’ – TAAGTATTCGGACAGTTATTTCC); and a 197 bp fragment of control region in dog mtDNA was amplified with primers Canis1F (5’ – CGTCGTGCATTAATGGTTTG) and Canis2R (5’ – GCGAGAAGAGGGACATTACG) (**IV**).

4. RESULTS

4.1. Parasites of rural raccoon dogs and red foxes

E. multilocularis infections

Due to decomposition or hunting damage, some samples were excluded from the analyses. In total, 249 and 108 small intestines were examined for raccoon dogs and red foxes, respectively (**I, II, III**). Zoonotic tapeworm *E. multilocularis* was found in 1.6% (n=4) of raccoon dogs and in 31.5% (n=34) of red foxes (**I, II, III**). The four tapeworm positive raccoon dogs originated from counties Saaremaa (n = 2) and Järvamaa (n = 2) (**I**; Figure 2). Both animals from Saaremaa harboured high tapeworm burdens with >200 *E. multilocularis* specimens in their small intestines; the animals from Järvamaa were infected with fewer specimens (**I**). *Echinococcus multilocularis*-positive red foxes originated from 6 counties located in the mainland (Harjumaa, Pärnumaa, Põlvamaa, Raplamaa, Tartumaa, Valgamaa; Figure 2). Half (17/34) of the infected red foxes harboured less than 50 *E. multilocularis* specimens (Table 1). Although we stopped parasite counting at 200, two analysed foxes likely harboured more than one thousand *E. multilocularis* specimens.

Table 1. Infection intensity of *E. multilocularis* in examined red foxes (n=108) and raccoon dogs (n=249).

E. multilocularis	**1–10**	**11–50**	**51–100**	**101–150**	**151–200**	**>200**	**Total**
Red foxes	8	9	2	2	2	11	**34**
Raccoon dogs	1		1			2	**4**

General parasite fauna

We identified a total of 17 helminth taxa from the internal organs of both species in Estonia, raccoon dogs and red foxes (**II, III**; Table 2). While all the analysed red foxes were infected, two raccoon dogs were not parasitised. Raccoon dogs were most often infected with *Uncinaria stenocephala* (97.6%) and *Alaria alata* (68.3%) (**II**). Examined red foxes were highly infected with *Pearsonema plica* (91.5%), *A. alata* (90.7%), *Eucoleus aerophilus* (87.6%), *U. stenocephala* (84.3%) and *Mesocestoides* spp. (77.8%) (**III**). Infection with other helminth species was considerably lower (Table 2). Genetic identification of tapeworms confirmed the presence of *Taenia polyacantha* in both canid species. In addition to the summarised parasite taxa shown in Table 2, we also found tapeworms that remained unidentified from the small intestine of 7 raccoon dogs.

Table 2. Prevalence of different parasite taxa identified from Estonian red foxes and raccoon dogs. Asterisks (*) mark the parasite species of zoonotic potential.

Parasite species	Prevalence (%)		χ^2	P-value
	Red fox	Raccoon dog		
Trematoda	90.7	69.1	19.18	**<0.01**
*Alaria alata**	90.7	68.3	20.32	**<0.01**
*Metorchis bilis**	60.0	19.5	8.52	**<0.01**
Isthmiophora melis	0.9	6.0	4.57	**0.03**
Plagiorchis elegans	–	0.8	–	–
Cestoda	90.7	30.5	109.33	**<0.01**
Mesocestoides spp. (*M. lineatus** and *M. litteratus*)	77.8	21.3	101.66	**<0.01**
Taenia spp. (*T. polyacantha**)	70.4	8.4	146.03	**<0.01**
*Echinococcus multilocularis**	31.5	1.6	70.69	**<0.01**
Diphyllobothrium sp.*	1.9	–	–	–
Nematoda	100	98.8	3.13	0.08
Pearsonema plica	91.5	10.8	201.47	**<0.01**
*Eucoleus aerophilus**	87.6	30.0	97.24	**<0.01**
*Uncinaria stenocephala**	84.3	97.6	26.92	**<0.01**
Crenosoma vulpis	53.3	15.0	54.89	**<0.01**
Ascarids (*Toxascaris leonina** and *Toxocara canis**)	32.4	8.0	34.34	**<0.01**
Molineus patens	8.3	13.7	2.01	0.16
*Aonchotheca putorii**	2.8	3.6	0.16	0.69
Angiostrongylus vasorum	2.9	1.3	1.10	0.29
Sarcoptic mange (*Sarcopes scabiei**)	21.2	15.0	1.33	0.25

Considering individuals for whom all organs were examined (intestines, lungs and urinary bladder), most (84.9% out of 205) of the raccoon dogs were infected with one to four species, while the majority of red foxes (75.5% out of 98) were infected with five to eight species (**II, III**). Red foxes were infected with significantly higher number of parasite species than raccoon dogs (mean species richness 6.34 vs. 2.86) (Mann-Whitney U test: $z=-11.47$; $p<0.01$). The parasite infection was similarly more intense in red foxes (mean number of parasite specimens 290 vs. 143) (Mann-Whitney U test: $z=6.65$; $p<0.01$). When parasite taxa were analysed separately, prevalence of many parasites differed significantly between raccoon dogs and red foxes (Table 2). Specifically, while both host species harboured similar number of nematode species ($\chi^2=3.13$; $p=0.08$), the prevalence of Trematoda ($\chi^2=19.18$; $p<0.01$) and Cestoda ($\chi^2=109.33$; $p<0.01$) was significantly higher for red foxes. The only parasite species, which infection was significantly higher in raccoon dogs, were *I. melis* ($\chi^2=4.57$; $p=0.03$) and *U. stenocephala* ($\chi^2=26.92$; $p<0.01$). Parasite overlap index (Pianka's index) between the two host species was 0.78.

Ectoparasitic *Sarcoptes scabiei* infection was detected in 21.2% (n = 21) of the foxes and 15.0% (n = 34) of the raccoon dogs. Scabied foxes were infected with higher number of parasite species and specimens than scabies-free foxes (**III**). Although the mean numbers of helminth species and parasite specimens were higher for the scabied raccoon dogs (3.15 vs. 2.81 species, and 183 vs. 135 specimens), the difference was statistically not significant (Mann-Whitney U test: $z=-0.73$; $p=0.46$ and $z=-1.95$; $p=0.051$).

Variation between the sexes and seasons

Male red foxes weighed significantly more than females (**III**). Mean weights were 5.26 and 4.52 kilograms for males and females, respectively. In contrast, we did not find any statistically significant differences between raccoon dog sex and weight (mean weight for males 4.81 vs. 4.84 for females; **II**). On the other hand, heavier raccoon dogs were infected with higher number of parasite specimens (**II**). There was also no significant relationship between animal sex and the number of helminth species it harboured in neither red foxes (**III**) nor raccoon dogs (**II**). While we did not find any significant difference between raccoon dog sex and infection with any identified helminth species (**II**), infection with nematode *M. patens* occurred more often in female foxes, and *T. canis* infection was more prevalent in male foxes (**III**).

The number of helminth species detected in raccoon dogs varied between seasons (Mann-Whitney U test: $z=-5.91$; $p<0.01$): animals sampled from the autumn period were infected with more helminth species than animals collected from winter and early spring (mean number of species 3.57 vs. 2.28). As expected, raccoon dogs weighed significantly more in autumn (Mann-Whitney U test: $z=-3.99$; $p<0.01$). Moreover, foxes similarly harboured on average more helminth species in autumn (Mann-Whitney U test: $z=-2.36$; $p=0.02$) (**III**), however, their weight did not depend on the season (M-W-U: $z=-0.38$; $p=0.70$).

Co-occurrence analyses

Analysis of co-occurrence between raccoon dog parasite species and different food categories detected four significant relationships: 1) *A. alata* & Invertebrates (co-occurrence), 2) Trematoda & Natural plants (co-occurrence), 3) Trematoda & Invertebrates (co-occurrence), and 4) Trematoda & Carrion (separation) (**II**). However, no significant relationships were detected between different parasite species of raccoon dogs (**II**). The co-occurrence analyses of red fox parasite species as well as parasite species and consumed food items did not reveal any significant relationships (**III**).

4.2. Non-invasive molecular method for detecting *Echinococcus* tapeworms

It appeared that the fox- and parasite-specific primers worked best under different PCR conditions than dog primers. The PCR protocol producing the strongest signal with red fox and parasite primers was: 95 °C for 1 min; 10 cycles at 95 °C for 20 s, annealing at 67 °C for 20 s (temperature reduced by 1 °C in each cycle) and extension at 68 °C for 30 s; followed by 35 cycles under the same conditions, except that the annealing temperature was 57 °C; and a final extension at 68 °C for 3 min. The best PCR protocol with dog primers required similar touchdown procedure with the exception of higher annealing temperature (reduced from 71 °C to 66 °C in each cycle).

The assay for detecting parasite DNA showed very high sensitivity: 1 egg was sufficient to give an *E. multilocularis*-specific result, and all primer pairs were specific to the relevant parasite and host species, except for the one developed for dogs, which in our laboratory experiments sometimes amplified also wolf mtDNA (**IV**).

4.3. Infection of urban canids with *Echinococcus* tapeworms

DNA was successfully extracted and amplified from 209 (87.4%) of 239 faecal samples collected (**IV, V**). On the basis of primer specificity and amplicon size, we determined host and parasite species. Of samples producing positive results, 28 (13.4%) were from red foxes and 181 (86.6%) from dogs. Two (7.1%) fox faecal samples were infected with *E. multilocularis* tapeworms (**IV**; Table 3). While none of the analysed dog samples were positive for *E. multilocularis*, *E. granulosus* was detected in four (2.2%) dog faecal samples (**V**; Table 3).

Table 3. Summary table of collected urban faecal samples.

Parasite species	**Fox samples** No. of positive/analysed (%)	**Dog samples** No. of positive/analysed (%)
E. multilocularis	2/28 (7.1%)	0/181
E. granulosus	0/28	4/181 (2.2%)

DNA sequences obtained from both *E. multilocularis*-positive red foxes showed 100% identity with an *E. multilocularis* tapeworm sequence AB018440 in GenBank (**IV**). Sequence analysis of *E. granulosus* demonstrated that the tapeworm-positive urban dogs in Tartu were infected with genotype G1, being 100% homologous to *E. granulosus* genotype 1 sequence AF297617 in GenBank (**V**). All sequenced fox and dog samples also belonged to the corresponding species (**IV**).

5. DISCUSSION

5.1. Parasites of rural raccoon dogs and red foxes

E. multilocularis infections

Our results indicate that both raccoon dogs and red foxes are important definitive host species for *E. multilocularis* in Estonia. While the share of infected foxes was rather similar to the results from a previous study (29.4% vs. 31.5%; Moks et al., 2005; **III**), this investigation provides more reliable data about wildlife echinococcosis in Estonia due to considerably larger sample size. Furthermore, *E. multilocularis* was identified for the first time in Estonian raccoon dogs (**I**, **II**). As a result, Estonia is now the 6th country in Europe after Germany, Poland, Latvia, Slovakia and Lithuania (Thiess et al., 2001; Machnicka-Rowinska et al., 2002; Bagrade et al., 2008; Hurnikova et al., 2009; Bružinskaite-Schmidhalter et al., 2012), where *E. multilocularis*-infected raccoon dogs have been reported. The observed difference in the prevalence between the two canid species (31.5% in foxes vs. 1.6% in raccoon dogs) can be explained by differences in their food preferences, especially in consumption of rodents that serve as the main intermediate host for *E. multilocularis*. While Estonian raccoon dogs mostly feed on anthropogenic plants (FO=56%) and carrion (FO=48%), and less on rodents (FO=19%), red foxes, on the contrast, are effective hunters and consume small mammals considerably more frequently (FO=53%; Süld et al., 2014). The same phenomenon of higher prevalence rates of *E. multilocularis* in red foxes than in raccoon dogs from the same area was observed in Belarus (7.5% vs. 0%; Shimalov and Shimalov, 2002; 2003), Latvia (36% vs. 21%; Bagrade et al., 2008) and in Japan (56.7% vs. 23.1%; Yimam et al., 2002). Therefore, it can be suggested that although raccoon dogs are susceptible for *E. multilocularis*, they may not play as important role in the parasite epidemiology as red foxes do. Nevertheless, raccoon dogs can play considerable role in sustaining the parasite life cycle by contributing in the environmental contamination with infective parasite eggs, especially in areas where raccoon dog numbers are high.

Tapeworm *E. multilocularis* seems to exist all over Estonia: during this study infected red foxes were found from six counties located in the mainland (**III**; Figure 2), whereas infected raccoon dogs were identified in two additional counties, including the Estonia's biggest island Saaremaa (**I**, **II**; Figure 2). The parasite distribution area could be even larger including counties Läänemaa and Viljandimaa, but probably due to the low sample size we failed to identify infected animals from these areas. It is somewhat surprising that none of the examined raccoon dog carcasses from the southern counties (Tartumaa, Põlvamaa, Valgamaa) were infected, despite the large proportion of infected red foxes and the large number of raccoon dog samples available from the area. However, the situation is similar to that in Brandenburg, Germany, where no

infected raccoon dogs were found in the southern part of the state, despite infected red foxes being abundant in the area (Schwarz et al., 2011).

One of the reasons why the proportion of infected foxes has remained high and infected raccoon dogs have been identified for the first time in Estonia could be related to supplementary feeding sites. While these sites are intended for wild boars, they are also known to attract many non-target species, e.g. rodents, red foxes and raccoon dogs (Oja, 2011). Rodents as intermediate host species for *E. multilocularis* can easily become infected with the tapeworm by eating contaminated substances at the supplementary feeding sites. Despite the difference in the amount of consumed rodents, yet this food category constitutes a substantial proportion in both red fox and raccoon dog diet in Estonia (Süld et al., 2014). Supplementary feeding sites therefore represent potential hot-spots for the spread of *E. multilocularis* and other pathogens in Estonian wildlife.

General parasite fauna

The examined red foxes and raccoon dogs were infected with the same number (n=17) of endoparasite taxa (**II**, **III**; Table 2). In comparison, the previous study conducted about a decade ago reported a rather different number of parasite taxa in raccoon dogs (n=5) compared to that found in red foxes (n=16; Moks, 2008). This could be seen as a result of low sample size (21 raccoon dog carcasses; Moks, 2008). Most of the parasite taxa identified in this study are common to both canids, with only two species being different: *Plagiorchis elegans* occurred only in raccoon dogs and *Diphyllobothrium* sp. occurring only in red foxes (Table 2). Despite the similar number of helminths detected in red foxes during these two studies, there are also some differences in the species composition. Notably, we found five helminth species previously not identified in Estonian foxes (*Mesocestoides litteratus*, *Toxascaris leonina, Molineus patens, Angiostrongylus vasorum* and *Aoncotheca putorii*), and Moks (2008) identified four species of helminths not found in the current study (*Taenia pisiformis*, *T. serialis*, *Spirocerca lupi* and one unidentified acanthocephalan). Taking into account the results of these two studies, Estonian red foxes seem to harbour a minimum of 21 helminth species, which is very similar to the results from a Danish study (n=21; Saeed et al., 2006), but considerably less than found in Iberia (n=34; Segovia et al., 2004) and Belarus (n=32; Shimalov and Shimalov, 2003). In comparison with Moks (2008), we found 13 additional species in raccoon dogs during this study: 2 new trematode species (*Metorchis bilis* and *Plagiorchis elegans*), 3 new cestode species (*Mesocestoides litteratus*, *Taenia polyacantha* and *E. multilocularis*), and 8 new nematode species (*Eucoleus aerophilus*, *Crenosoma vulpis*, *Molineus patens*, *Pearsonema plica*, *Toxocara canis*, *Toxascaris leonina*, *Aoncotheca putorii* and *Angiostrongylus vasorum*) (**II**). Moreover, *A. vasorum* was identified for the first time in Estonian canids, although infected foxes have been reported elsewhere in Europe (Segovia et al., 2004; Saeed et al., 2006; Magi et al., 2009; Franssen et al., 2014; Tolnai et al., 2015).

The foxes and raccoon dogs examined in this study were highly parasitised. While all the examined red foxes were infected (100% infection rate) with at least one helminth species (**III**), there were two raccoon dogs not parasitised (**II**). Difference was detected in the number of parasite species infecting the two host species and infection intensity. The reason why red foxes harboured significantly higher number of helminth species (mean species richness 6.34 vs. 2.86) and specimens (mean number of parasite specimens 163 vs. 121) could be that they probably encounter a wider variety of foodstuffs, especially during the coldest period of year, when raccoon dogs usually hibernate. Moreover, it has been shown that Estonian red foxes consume significantly more food categories (e.g. small mammals, carrion) associated with parasitic infections than raccoon dogs (Süld et al., 2014). However, global climate warming may increase the parasite load in Estonian raccoon dogs in the future.

When parasite taxa were analysed separately, prevalence of 11 parasite taxa differed significantly between raccoon dogs and red foxes (Table 2). In contrast, Al-Sabi et al. (2013) detected only 7 significant relationships between the two host species, but they also identified fewer parasite species. Probably due to the fact that Estonian red foxes are more successful predators than raccoon dogs, the prevalence of Trematoda and Cestoda was significantly higher for red foxes, whereas infection with Nematoda did not differ between the two host species. The winter hibernation strategy of raccoon dogs may also contribute to lower parasite prevalence, because of the reduced food consumption during that period. In general, we detected more parasite species, in which the infection was significantly more prevalent in red foxes compared to raccoon dogs (e.g. *A. alata*, *M. bilis*, *Mesocestoides* spp., *Taenia* spp., *E. multilocularis*, *P. plica*, *E. aerophilus*, *C. vulpis*, and ascarids; Table 2). The only parasite species, in which raccoon dogs were significantly more infected, were *I. melis* and *U. stenocephala* (Table 2). Trematode *I. melis* is indeed more likely to infect raccoon dogs since it is transmitted via amphibians and freshwater fish (Table S1 in **II**), which are found near the preferred habitat of raccoon dogs (shores of water bodies and floodplains; Heptner and Naumov, 1998). Infective stages of *U. stenocephala*, on the other hand, are most probably acquired directly from soil, making supplementary feeding sites, to which raccoon dogs are probably more attracted for the opportunity to feed on grain, a likely place for parasite transmission. Despite the differences in the prevalence of parasite species between the two hosts in Estonia, parasite overlap between red foxes and raccoon dogs was still estimated to be high (0.78; Pianka's index). This could be seen due to the fact that both canids are medium-sized carnivores that predominantly consume same food items but in varying amounts.

Variation between the sexes and seasons

We detected sexual dimorphism, with males weighing more than females, in Estonian red foxes (**III**), but not in raccoon dogs (**II**). In addition, there was no significant relationship between animal sex and the number of helminth species

it harboured in both canid species. However, it is interesting to note that all the raccoon dogs infected with more than five helminth species were males (**II**). Raccoon dogs are monogamous, and pairs tend to den and forage together most of the time (Kauhala et al., 1993; Drygala et al., 2008). Therefore one raccoon dog couple probably encounter similar variety of food items and parasites. This may explain why we did not detect significant differences in helminth infection between the sexes, though in many vertebrate species, males are more frequently parasitised than females (Poulin, 1996; Zuk and McKean, 1996). On the other hand, we showed that infection was more intense (higher numbers of parasite specimens) in heavier animals (**II**). Thus, helminth infection intensity in raccoon dogs seems to be determined by weight, not by sex.

When analysing differences in infection with different parasite species between the sexes, we detected no significant relationship in monogamous raccoon dogs (**II**), however, infection with nematode *M. patens* occurred more often in female foxes, and *T. canis* infection was more prevalent in male foxes (**III**). This could be a result of different feeding habits of female and male foxes: females consume more rodents, while the remains of larger mammals are more prevalent in the stomach of male foxes (E. Soe, pers. comm.).

Both canid species sampled from the autumn (August–November) period were infected with more helminth species than animals collected from winter and early spring (December–April). This finding is supported by the fact that the availability of different food categories is higher in autumn: all the crops ripen during that period, amphibians are in move to find hibernating places and the abundance of small rodents is the highest. Thus, all these food categories represent potential sources of different helminth infections. Furthermore, red foxes and especially raccoon dogs forage intensively and widely in the autumn in order to accumulate subcutaneous fat to survive the cold winter (Drygala et al., 2008). Additionally, in case of extreme winter weather with deep snow (>35cm) and freezing temperatures, as in the winters of this study (Estonian Weather Service, 2012–2013), it becomes difficult for raccoon dogs to forage (Kauhala et al., 2007) and they tend to stay in their dens for several days or even weeks without feeding, which leads to fewer feeding opportunities and less contact with parasites.

Co-occurrence analyses

The significant co-occurrence between *A. alata* and "Invertebrates" observed in Estonian raccoon dogs is difficult to explain. It could just be a feeding habit of infected animals to compensate for a deficiency of certain nutrients that are present in invertebrates. Since *A. alata* constitutes the majority in the group Trematoda, the same co-occurrence was apparent between Trematoda and "Invertebrates" (**II**). In addition, we found that raccoon dogs infected with trematodes fed significantly more on "Natural plants", whereas the opposite was found for "Carrion" (**II**). The larvae of one of the most frequent trematode parasites detected in this study – *A. alata*, have been increasingly detected in

wild boar tissues during the official *Trichinella* inspections (Riehn et al., 2010; Portier et al., 2011). Moreover, wild boar remains constituted the largest share (frequency of occurrence, FO = 18%) of the "Carrion" consumed by raccoon dogs originating from the same animals as in this study (Süld et al., 2014). On one hand this fact appears to be in discord with the negative relationship observed for "Carrion" in the co-occurrence analysis; however, raccoon dogs that were already infected with a high burden of *Alaria* flukes (often seen >200 specimens) might have preferred to feed more on natural plants (e.g. different grasses) and less on carrion. Intentional consumption of grass has been documented among many carnivore species, e.g., dogs, wolves and civet (*Viverricula indica*), and it has been hypothesised that plant consumption occurs more often in animals exhibiting signs of illness (Stahler et al., 2006; Sueda et al., 2008; Su et al., 2013). Furthermore, in paper **II** we found that animals, which harboured more helminth species, fed significantly more on "Natural plants". Given this, we suggest that these data indicate self-medicating behaviour among Estonian raccoon dogs.

While the co-occurrence analysis between consumed food items and parasite species detected four significant relationships in raccoon dog, none of the relationships was significant for red fox data (**III**), although we expected to find some, e.g. between "Small mammals" and *E. multilocularis*. One of the reasons for not detecting any significant effects in red foxes could be due to the low sample size. Moreover, there is also a time lag between the consumption of infected prey and the presence of adult helminth specimens in the definitive host. Therefore, to investigate the relationships between consumed food items and acquired parasites, a considerably larger sample size covering the whole year is required.

In principle, a parasite species could facilitate or prevent the infection of another parasite species. However, we did not detect any significant competitive or facilitative interaction between the parasites in neither host species, regardless that the most prevalent parasite species (*A. alata, U. stenocephala, E. aerophilus* and *P. plica* for red fox; *U. stenocephala* and *A. alata* for raccoon dog) frequently appeared together in one host (**II**, **III**). It is important to note, however, that as the analysis determines whether the level of co-occurrence is higher or lower than what would be expected at random, abundant species will also occur with high co-occurrence at random.

5.2. Red foxes and raccoon dogs as vectors for zoonotic parasites in Estonia

A parasite identified in red foxes and raccoon dogs was considered zoonotic, if it had previously been described as an agent of human infection. The number of zoonotic species found in red foxes (n=10; **III**) was very similar to that found in raccoon dogs (n=9; **II**), with the exception of *Diphyllobothrium* sp. that was

recorded only in foxes. Probably the most important parasite found in this study and causing life-threatening disease in humans was *E. multilocularis*. Although this tapeworm was already recorded in a previous parasitological study of red foxes, it was found in this study for the first time in Estonian raccoon dogs (**I, II**). Despite the almost 20-times lower prevalence found in raccoon dogs compared to red foxes, raccoon dogs can still contribute significantly to the environmental contamination risk with *E. multilocularis* eggs due to the relatively high numbers. Moreover, two of the four infected raccoon dogs harboured more than 200 *E. multilocularis* specimens (**I**; Table 1), indicating its high suitability to act as a definitive host for *E. multilocularis*. In Europe the prevalence of *E. multilocularis* in red foxes can vary on a large scale from around 0% in Denmark, Belgium, Italian Alps and Sweden (Brochier et al., 2007; Di Cerbo et al., 2008; Osterman Lind et al., 2011; Al-Sabi et al., 2013) to 58.7% in Lithuania (Bružinskaite-Schmidhalter et al., 2012). While susceptible to *E. multilocularis*, the red fox seems "immune" to another species of this genus, namely the *Echinococcus granulosus*, although the parasite can infect the red fox in nature (Jenkins and Craig, 1992; Segovia et al. 2004; Keidans et al. 2005). To lower the infection risk of *E. multilocularis* to humans in Central Europe, anthelmintic baits have been distributed to foxes (Hegglin et al., 2003; Romig et al., 2007; König et al., 2008). Significant reduction in parasite prevalence, however, can be achieved only if the baiting is continued for a long period. Major drawback of this approach is that the dewormed foxes are not immune to *E. multilocularis* and can be re-infected shortly after ingesting the bait, because the larval stage in the intermediate host is not affected by the anthelmintic. The bait could also be eaten by non-target animals such as a wild boar (Antolova et al., 2006) or a hedgehog (*Erinaceus europaeus*; Hegglin et al., 2004). Furthermore, anthelmintic drug included to the bait has no ovicidal effect, meaning that the excreted *E. multilocularis* eggs can survive for up to one year in the environment (Veit et al., 1995; Eckert et al., 2001), and thus infect new intermediate hosts.

There were two overlapping helminth species highly prevalent in both canid species: *U. stenocephala* and *A. alata* (**II**, **III**; Table 2). Larvae of both parasite species can occasionally infect humans and cause severe disease. *Uncinaria stenocephala* larvae can pass through a person's skin and migrate sub-cutaneously, causing a painful itchy rash called cutaneous larva migrans (Tamminga et al., 2009). *Alaria alata* infection can be acquired by humans after eating inadequately cooked wild boar or frog meat (Möhl et al., 2009). Upon infection with *A. alata* larvae, the parasite can migrate to various organs (e.g. the eye) or muscle tissue and cause severe illness (Wasiluk, 2009). Unlike raccoon dogs, which were mostly infected with the previously mentioned species, there were two additional parasite species detected in a large proportion of foxes: *E. aerophilus* and *P. plica*. While nematode *P. plica* was the most abundant species found in Estonian red foxes, it is not infective to humans. Lung worm *E. aerophilus*, which is mostly transmitted via earthworms, prob-ably infects humans directly by ingesting the parasite eggs with inadequately

washed vegetables. The clinical symptoms of human infection with *E. aerophilus* are mainly pulmonary, e.g. bronchitis, coughing, blood in the sputum, fever, and shortness of breath (Laloševic et al., 2008). Infectivity to humans and the observed high prevalence rates in Estonia make *E. aerophilus*, *U. stenocephala* and *A. alata* together with *E. multilocularis* the pathogens representing a considerable public health risk. Lithuania is the only country, where *A. alata*, *E. aerophilus*, *U. stenocephala* and *E. multilocularis* are even more abundant among red foxes and raccoon dogs than in Estonia (Bružinskaite-Schmidhalter et al., 2012; Marcinkute et al., 2015), making the Baltic region hyperendemic for these four parasite species.

Distribution pattern of *T. canis*, one of the most abundant zoonotic parasites worldwide transmitted by canids (Despommier, 2003), is somewhat similar to the one of *E. multilocularis* in Estonia. Briefly, in a previous parasitological study conducted about a decade ago, this ascarid was found only in red foxes and probably as a result of small sample size not in raccoon dogs (Moks, 2008). Whereas in this study, *T. canis* was found in both red foxes and raccoon dogs (**II, III**). Furthermore, the prevalence of *T. canis* found in red foxes by Moks (2008) is exactly the same as reported for *E. multilocularis* (29.4%), and has remained almost the same (29.6% reported in **III**). Although transmitted by wider number of intermediate hosts (Table S1 in **II**) than *E. multilocularis*, canids can be infected with *T. canis* among others by eating infected rodents, which also serve as intermediate host for *E. multilocularis*. In order to investigate the similarities found during this study in more detail future studies are needed.

As the disease caused by the ectoparasitic itch mite *S. scabiei* is known to impose considerable nutritional stress on individual animals (Newman et al., 2002) and as the animals collected during this study were from the period when the sarcoptic mange started to spread extensively in Estonia (2010/2011), it was of considerable interest to compare the parasite fauna of healthy and infected individuals. As a result, we found that scabied animals were indeed infected with higher number of parasite species and specimens than scabies-free animals. In addition, we found three parasite species in which the infection was more intense among scabied foxes: *U. stenocephala*, *T. canis* and *E. aerophilus* (**III**). One of the reasons for seeing such relationship with *U. stenocephala* and *T. canis* could be that oral infection with the eggs of these geohelminths may have occurred by simply cleaning the fur, which is already in bad condition due to the sarcoptic mange. It is not clear, however, why such relationship was found with *E. aerophilus*. This nematode is located in respiratory organs of the definitive host, suggesting that there might be an association with scabies and lung parasites. On the other hand, it is likely that the infection occurred more frequently in scabied animals, because earthworms, the intermediate hosts for *E. aerophilus*, are relatively easy prey for a hungry and nearly hairless fox.

5.3. Non-invasive method detects *Echinococcus* infection in urban canids in Estonia

The molecular diagnostics we developed proved to be highly sensitive, being able to detect the presence of a single *Echinococcus* egg in a scat sample (**IV**). As the method is based on regular PCR, it is also relatively low-cost. Other similar studies have used flotation method to concentrate parasite eggs and alkaline lysis of the egg shell prior PCR analysis (Mathis et al., 1996; Al-Sabi et al., 2007). In this study we demonstrated that concentrating eggs is not necessary and sequential heating and cooling of samples can be used instead of lysis. Moreover, the flotation solution can act as a PCR inhibitor, making the analysis more time-consuming. As the described molecular method enables identification of species on the basis of amplicon size, subsequent sequencing is not necessary, making this method cost-effective. The method is especially suitable for analysing faecal samples since it amplifies relatively short sequences of mtDNA, allowing species-specific signals to be detected also from samples where DNA may be highly degraded. In other similar studies, much longer sequences (e.g. 395 bp for *E. multilocularis* in Trachsel et al., 2007, and 355 bp for dog in Nonaka et al., 2009) have been amplified, rendering analysis of degraded samples likely less sensitive. As the dog primers used in this study can potentially also amplify grey wolf DNA, it would be of significant importance to develop new dog-specific primers in further studies, especially when dealing with rural areas. Recently, such primers were developed, based on a notion that three nucleotide positions in the mtDNA control region enable differentiation between wolves and dogs in Estonia (U. Saarma, pers. comm.). However, this analysis requires sequencing of the control region fragment. Moreover, taking into account that wolf-dog hybrids occur in Estonia (Hindrikson et al., 2012); it may not always be possible to distinguish between dogs, wolves and hybrids using only mtDNA. In the future studies, the corresponding mtDNA sequence of golden jackal, a new carnivore species in Estonia, should also be taken into account, if this method is used on faecal samples collected from rural areas in Estonia.

Of the 239 faecal samples collected from the urban area in Estonia, we managed to successfully extract and amplify DNA from 209 samples (**IV, V**). The remaining samples were either too degraded or contained large amount of PCR inhibitors that could not be removed by the commercial DNA extraction kit used. Of the positive samples we could allocate 28 to red foxes and 181 to dogs (**IV, V**). As a result, the new molecular method enabled to detect *E. multilocularis* in urban fox scats and *E. granulosus* in dog scats in an urban area (Tartu) in Estonia. Molecular analysis demonstrated that all the sequenced host and parasite samples belonged to the corresponding species.

While *E. multilocularis* has been reported in urban areas of the European Union in earlier studies (e.g. Hofer et al., 2000; Deplazes et al., 2004), this is the first such record of *E. granulosus* G1 (sheep strain) (**V**). It is possible that

dogs may have carried the parasite into urban environment following a hunting trip in rural area, since feeding the viscera of wild and domesticated animals to dogs is commonly practiced in the region. On the other hand, free-roaming stray dogs that move between rural and urban areas could also have been the source of contamination. It is most unlikely that the genotype (G1) we detected origins from the sylvatic cycle, since completely different genotypes have so far been recorded from wild mammals in Estonia: genotypes G8 and G10 have been demonstrated to infect Estonian moose (*Alces alces*) and grey wolves (Moks et al., 2006, 2008). Therefore, genotype G1 is most probably transmitted via the domestic/synanthropic cycle, and further sampling of production animals is necessary to determine the transmission path of *E. granulosus* G1 in more detail.

Since a large proportion of rural foxes are known to harbour *E. multilocularis* in Estonia (Moks et al., 2005), we also expected to find infected dogs from the urban area. Moreover, such cases have previously been reported in Slovakia and Lithuania (Antolova et al., 2009; Bružinskaite et al., 2009). However, none of the dogs analysed in Tartu were identified as *E. multilocularis*-positive (**IV, V**). Since dogs remain highly susceptible hosts to both *Echinococcus* tapeworms and they have a close contact with humans, it would be of considerable importance to increase the public knowledge about the potential health risks caused by the dog parasites. The impolite habit of dog owners of not removing faeces of their pet after taking it for a walk represents a significant health risk for both animals and humans, especially when pets are not regularly dewormed. In order to control *Echinococcus* tapeworm infection in urban environment and avoid human infections, dog owners should always pick up and properly dispose their pet faeces. Moreover, as proposed by European Scientific Counsel Companion Animal Parasites dogs with access to rodents or raw offal and carcasses should be regularly (every 4–6 weeks) administered effective anthelmintics (ESCCAP, 2010). The red fox is the most important canine species responsible for environmental contamination with *E. multilocularis* egg. In order to avoid further increase in fox densities people should not leave anthropogenic food items (e.g. pet food or compost) accessible to urban foxes (Contesse et al., 2004). Most importantly, public knowledge on the risks imposed by *Echinococcus* tapeworms should be increased.

SUMMARY

Zoonoses are infectious diseases that can be transmitted from animals to humans and vice versa. Zoonotic diseases and their agents (helminth parasites, viruses, etc.) have been an important subject of research for both veterinarians and medical doctors for decades. It has been estimated that approximately 60% of infectious agents known to be pathogenic to humans are zoonotic, representing a major threat to public health not only in developing countries, but also in industrialised countries in Europe. In general, humans can acquire zoonotic parasites from a direct contact with an infected animal or by consuming water and foodstuffs contaminated with the infective stages of a parasite. One of the most dangerous helminth parasite species that can be transmitted from animals to humans is *Echinococcus multilocularis*. In Europe, the red fox (*Vulpes vulpes*) and to a lesser extent also the raccoon dog (*Nyctereutes procyonoides*) are the main definitive host species for this cestode. However, adult worms can also develop in domestic dogs and cats. During the last decades, the number of red foxes appears to have increased rapidly and they have colonised urban areas, bringing zoonotic pathogens to the immediate neighbourhood of humans and their companion animals. According to this, urban foxes may represent even greater danger to human health than rural foxes. To estimate the role of red foxes and other canids in contaminating the environment with eggs of zoonotic parasites, the prevalence of the parasite should be regularly monitored in both urban and rural areas.

The aims of this study were to describe and compare the parasite fauna of rural red foxes and raccoon dogs in Estonia in order to determine the species potential as the source of environmental contamination with eggs of *Echinococcus* and other zoonotic parasites (**I, II, III**). Furthermore, we wanted to study the co-occurrence of parasite species and consumed food categories (**II, III**). Another important aim was to develop and apply a new non-invasive molecular method to identify both the host (fox or dog) and *Echinococcus* species from faecal samples collected in the urban environment (**IV, V**).

Parasite studies revealed that both red foxes and raccoon dogs in rural areas of Estonia are infected with the same number of endoparasite species (n=17). Moreover, the species composition of these two canid species was also similar (**II, III**). Raccoon dogs were most often infected with *Uncinaria stenocephala* and *Alaria alata* (**II**), red foxes were additionally highly infected with *Eucoleus aerophilus* and *Pearsonema plica* (**III**), the first three being zoonotic species, with only the latter not infective to humans. Infection with life-threatening *E. multilocularis* was found in 31.5% and 1.6% of the red foxes and raccoon dogs, respectively (**I, II, III**). During this study *E. multilocularis* was reported for the first time in Estonian raccoon dogs (**I**). The almost 20-fold difference in prevalence between the two canid species can be explained by the difference in food preferences, especially the much larger frequency of rodents, which serve

as the main intermediate host for *E. multilocularis*, in red fox diet. Given the results of current study, the tapeworm probably exists all over Estonia.

In addition to endoparasites, we also studied ectoparasitic *Sarcoptes scabiei* infection in Estonian red foxes and raccoon dogs and found relatively large number of infected animals (**II, III**). As the disease is known to impose considerable nutritional stress on infected animals and as the animals collected during this study were from the period when the sarcoptic mange started to spread extensively in Estonia, it was of considerable interest to compare the parasite fauna of scabied and healthy individuals. This study demonstrated that scabied animals were more infected with endoparasites than their healthy conspecifics (**II, III**).

Since the parasite fauna of an animal is closely related to its feeding habits, we studied the relationships between parasitic infection and consumed food categories in both red foxes and raccoon dogs (**II, III**). We found that heavily infected raccoon dogs consumed plant material significantly more than less-infected individuals (**II**). Such intentional consumption of grass in raccoon dogs could indicate self-medicating behaviour, which has been documented among other carnivore species with signs of illness.

In order to study infection with *Echinococcus* species in urban carnivores we developed a sensitive and non-invasive molecular method to identify both parasite and host species from faecal samples. The method enables to detect the presence of *E. multilocularis* from red fox scats when only a single parasite egg is present in the sample (**IV**). The PCR based method was used to detect *Echinococcus* infection in faecal samples collected in Tartu, Estonia. As a result, we found that 7.1% of red fox samples tested positive for *E. multilocularis* (**IV**) and 2.2% of dog samples for *E. granulosus*, another cestode species from the genus *Echinococcus* that can infect humans (**V**). While it was expected to find infected urban foxes, the infection with *E. granulosus* in dog samples was somewhat surprising as the genotype we identified (G1) is responsible for synanthropic cycle, which has not been reported in Estonia for several decades.

In conclusion, due to the high number of red foxes and raccoon dogs, both canid species can be considered as important sources of zoonotic parasites in Estonia. Furthermore, urban foxes that live in close proximity with humans and contaminate the urban environment with parasite eggs that cause life-threatening alveolar echinococcosis can play major role in terms of public health. Therefore, public knowledge on the risks imposed by *Echinococcus* tapeworms should be increased.

SUMMARY IN ESTONIAN

Echinococcus multilocularis ja teised zoonootilised parasiidid Eesti koerlastel

Zoonoosid on nakkushaigused, mis levivad loomadelt inimestele ning vastupidi, olles juba aastakümneid oluliseks uurimisteemaks nii veterinaaridele kui ka arstidele. Kuna umbes 60% inimesel diagnoositud nakkushaigustest on zoonootilised, ei kujuta need loomade kaudu levivad haigused ohtu inimeste tervisele mitte ainult arengumaades vaid ka Euroopa riikides. Üldiselt võib inimene selliste haigustekitajatega nakatuda otsese kokkupuute teel nakatunud loomaga või tarbides parasiidi nakkusvõimeliste arengujärkudega saastunud vett ja toiduaineid. Üheks kõige ohtlikumaks loomadelt inimestele kanduvaks parasiidiks võib pidada alveokokk-paelussi (*Echinococcus multilocularis*). Selle paelussi lõpp-peremeesteks on Euroopas põhiliselt punarebane (*Vulpes vulpes*) ja väiksemal määral ka kährikkoer (*Nyctereutes procyonoides*), kuid nakatuda võivad ka koerad ja kassid. Viimastel aastakümnetel järsult tõusnud arvukuse tõttu on rebased asunud elama ka inimasulatesse, tuues sinna kaasa ka ohtlikud parasiidid. Inimesega lähestikku elavad linnarebased kujutavad seetõttu veelgi suuremat ohtu inimeste tervisele kui maapiirkonna rebased. Et hinnata millist rolli mängivad rebased ja teised koerlased keskkonna saastamisel zoonootiliste parasiitide munadega, tuleks neid nakkuste suhtes regulaarselt kontrollida nii linna- kui ka maapiirkondades.

Käesoleva töö eesmärkideks oli kirjeldada ja võrrelda Eesti maapiirkondades esinevate punarebaste ja kährikkoerte parasiite, et hinnata kuivõrd olulised on need koerlased keskkonna saastamisel alveokokk-paelussi ning teiste zoonootiliste parasiitide munadega (**I, II, III**). Lisaks sellele uurisime punarebastelt ja kährikkoertelt leitud parasiidiliikide ja tarbitud toidukategooriate koosesinemist (**II, III**). Viimase eesmärgina soovisime välja töötada ja ka kasutada uut mitte-invasiivset molekulaarset meetodit, mis suudaks identifitseerida nii peremehe (rebane või koer) kui ka ehhinokoki liigi väljaheiteproovist, linnakeskkonnast kogutud proovidel (**IV, V**).

Eesti maapiirkondades elavate punarebaste ja kährikkoerte parasitofauna uuringu tulemustest selgus, et mõlemad koerlased on nakatunud sama arvu parasiitidega (n=17) ning identifitseeritud liigid on samuti sarnased (**II, III**). Arvukamateks parasiidiliikideks olid kährikkoera puhul *Uncinaria stenocephala* ning *Alaria alata* (**II**), rebasel nendele lisaks veel ka *Eucoleus aerophilus* ning *Pearsonema plica* (**III**), kusjuures loetletud neljast liigist vaid viimane ei kujuta ohtu inimese tervisele. Inimesele eluohtliku alveokokk-paelussi nakkus leiti 31,5% rebastel (**III**) ja 1,6% kährikkoertel (**I, II**), kellel diagnoositi see paeluss Eestis esmakordselt. Ligi 20-kordne erinevus kahe peremeesliigi nakatumises on tõenäoliselt tingitud nende erinevatest toidueelistustest, eelkõige nende võimest püüda närilisi, kes on alveokokk-paelussi

vaheperemeesteks. Käesoleva töö tulemustest selgus, et tõenäoliselt on alveokokk-paeluss levinud üle kogu Eesti.

Lisaks siseparasiitidele uurisime ka ühe ektoparasiidi, süüdiklesta (*Sarcoptes scabiei*), esinemist Eesti koerlastel ning leidsime, et märkimisväärne hulk rebaseid ja kährikkoeri on nakatunud parasiidi poolt põhjustatava kärntõvega (**II, III**). Kuna varasemast on teada, et kärntõbistel loomadel on suurenenud energiavajadus ning töösse kaasatud loomad olid pärit ajaperioodist, mil kärntõbi oli Eestis laialt levinud, oli võimalik võrrelda nakatunud ja tervete loomade parasitofaunat. Käesoleva töö tulemusena leidsime, et kärntõbised loomad on tõepoolest endoparasiitidega rohkem nakatunud kui nende terved liigikaaslased (**II, III**).

Kuna looma parasitofauna on suurel määral seotud tema toitumisharjumustega, siis uurisime ka seoseid rebaste ja kährikkoerte parasiteerituse ja toitumise vahel (**II, III**). Ühe tulemusena leidsime, et siseparasiitidega enim nakatunud kährikkoerad olid söönud taimset materjali oluliselt rohkem kui vähem nakatunud isendid (**II**). Rohttaimede eelistamine võib viidata varasemalt ka teistel kiskjatel kirjeldatud ravimis-käitumisele, mida on seostatud kõrgema parasiteeritusega.

Uurimaks linnaloomade nakatumist ehhinokokk-paelussidega töötasime välja tundliku mitte-invasiivse molekulaarse metoodika, mis võimaldab väljaheiteproovist määrata nii parasiidi kui ka lõpp-peremehe liigi. Veelgi enam, alveokokk-paelussiga nakatunud rebase saab antud metoodikat kasutades kindlaks teha ka siis, kui proov sisaldab ainult ühte parasiidi muna (**IV**). Kasutasime seda PCR'il põhinevat metoodikat Tartu linnast korjatud proovide peal ning leidsime, et 7,1% rebaselt pärinevatest proovidest olid nakatunud alveokokk-paelussi munadega (**IV**) ning 2,2% koeralt pärinevatest proovidest sama perekonna teise inimest nakatava liigi, põistang-paelussi (*E. granulosus*), munadega (**V**). Kui nakatunud linnarebaste leidmine oli oodatav tulemus, siis ehhinokokk-paelussiga nakatunud koerte leidmine oli üllatav, sest leitud genotüüp (G1) levib koduloomade tsüklis, mida pole Eestis aastakümneid kirjeldatud.

Kokkuvõttes võib öelda, et oma kõrge arvukuse tõttu võib nii rebaseid kui ka kährikkoeri pidada olulisteks zoonootiliste parasiitide levitajateks Eestis. Eriti suurt rolli inimese tervise seisukohalt kujutavad siinkohal linnarebased, kes elavad inimeste vahetus läheduses ja saastavad linnakeskkonda eluohtlike parasiidimunadega. Seetõttu peaks tõstma inimeste teadlikkust ehhinokokk-paelusside poolt põhjustatavate terviseriskide osas.

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Leidi Laurimaa, John Davison, Urmas Saarma. "Infestation of urban foxes with life-threatening tapeworm *Echinococcus multilocularis* in Tartu, Estonia." 16.–17.05.2013/ Tallinn, Eesti/ 2nd Conference of Doctoral School of Earth Sciences and Ecology "Down to Earth". Suuline ettekanne.

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