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ADAPTATION OF *TRICHINELLA NATIVA* IN HOSTS

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ACADEMIC DISSERTATION

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ABSTRACT

Trichinella nativa is a member of the genus *Trichinella*, which includes nine different species and three genotypes. *Trichinella* spp. are spread worldwide and they can cause a disease called trichinellosis in both animals and humans.

In this thesis, the epidemiology of *T. nativa* and other *Trichinella* species in a boreal environment was investigated. Unusual *T. nativa* infection in a domestic cat with clinical signs was reported. Host responses induced by *T. nativa* and *Trichinella spiralis* were compared in a selective host (rat) to identify the phase of the life cycle in which the selective responses take place.

Of the 2483 carnivorous samples collected from Finland, 617 (24.8%) were positive for *Trichinella* spp. Four endemic *Trichinella* species were identified using multiplex PCR: *T. spiralis*, *T. nativa*, *Trichinella britovi*, and *Trichinella pseudospiralis*. *Trichinella nativa* was shown to be the predominant *Trichinella* species (80.1%) in all investigated host species. Red fox and raccoon dog were the most important reservoir animals when population size, estimated prevalence of *Trichinella* infection, and infection intensity were taken into account.

Trichinella infection can cause clinical manifestations; a domestic cat had an ulcer below the eyelid and a skin biopsy revealed one *Trichinella* sp. larva surrounded by inflammatory reaction and granulation tissue. Enzyme-linked immunosorbent assay and Western blotting showed a seropositive reaction against *Trichinella* spp. antigens in the cat's serum. The *Trichinella* sp. larva was identified as *T. nativa* by multiplex PCR. During the 1-year follow-up, a subcutaneous mass started to cover the previous surgical site. A biopsy sample taken from that area showed inflammatory cells and fibroblasts, with some fibrodysplasia.

To identify the phase of the life cycle in which the defense against *Trichinella* takes place, rats were infected both per orally (p.o.) and intravenously (i.v.) with *T. nativa* and *T. spiralis* larvae. After i.v. injection, 1.7% of the *T. nativa* NBL and 20% of the *T. spiralis* NBL reached the muscle tissue of the rat ($p < 0.05$). These results showed that the defense against *Trichinella* did not solely localize to the enteral or parenteral phase. The different infectivity of the two *Trichinella* species can also be partly explained by difference in reproductivity; *T. nativa* females isolated from a mouse released more NBL than those isolated from a rat. In contrast, *T. spiralis* females isolated from a mouse produced fewer NBL than those isolated from a rat.

Trichinella nativa and *T. spiralis* induced similar gene expression changes in the intestinal tissue of a selective host studied with whole-genome microarray on day five post infection (p.i.), even though the parasite burden caused by *T. spiralis* was significantly higher than that caused by *T. nativa*. When the two *Trichinella*-infected groups were pooled and compared with control animals, microarray data of the infected animals indicated nonspecific damage and an inflammatory response in the jejunal mucosa. Histopathological changes supported the microarray data.

Trichinella spp. is highly prevalent in Finland; it is a risk for domestic animals and humans. Even low infection intensity can cause clinical signs or symptoms.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to by their Roman numerals (I–IV):

- I **Airas N.**, Saari S., Mikkonen T., Virtala A-M., Pellikka J., Oksanen A., Isomursu M., Kilpelä S.-S., Lim C.W., Sukura A. 2010. Sylvatic *Trichinella* spp. infection in Finland. *The Journal of Parasitology* 96:67-76.

- II Saari S., **Airas N.**, Näreaho A., Vihma V., Autio K., Sankari S., Sukura A. 2008. A nonhealing ulcerative skin lesion associated with *Trichinella nativa* infection in a cat. *Journal of Veterinary Diagnostic Investigation* 20:839-843.

- III **Airas N.**, Näreaho A., Lindén J., Tuomola K., Sukura A. 2012. The different infectivity of *Trichinella spiralis* and *Trichinella nativa* in rat does not solely localize to enteral or parenteral phase. *Parasitology Research* 111:2281-2288.

- IV **Airas N.**, Näreaho A., Lindén J., Valo E., Hautaniemi S., Jokelainen P., Sukura A. 2012. Early *Trichinella spiralis* and *Trichinella nativa* infections induce similar gene expression profile in rat's jejunum. *Experimental Parasitology* 135:363-369.

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ABBREVIATIONS

ANOVA	analysis of variance
CI	confidence interval
CPK	creatine phosphokinase
DAB	diaminobenzidine
DAF	decay accelerating factor
DEGs	differentially expressed genes
DAB	diaminobenzidine
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
ES	excretory/secretory
Evira	Finnish Food Safety Authority
FDR	false discovery rate
FeLV	feline leukemia virus
FIV	feline immunodeficiency virus
GI	gastrointestinal
GMD	game management district
GO	gene ontology
HRP	horseradish peroxidase
IHC	immunohistochemical staining
IFN- γ	interferon gamma
IL	interleukin
iNOS	inducible nitric oxide synthase
ISS	injection site sarcoma
i.v.	intravenous
lpg	larvae per gram

MAD	median absolute deviation
ML	muscle larvae
MMC	mucosal mast cell
NBL	newborn larvae
NMRI	Naval Medical Research Institute
OD	optical density
PCR	polymerase chain reaction
p.i.	post infection
p.o.	per orally
s.c.	subcutaneously
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPIA	signaling pathway impact analysis
Stat6	signal transducer and activator of transcription factor 6
TLR	toll-like receptor

1. INTRODUCTION

Trichinella spp. belong to the genus *Trichinella* and phylum Nematoda, roundworms (Noble et al., 1989). The genus is divided into nine separate species and three genotypes (Pozio et al., 1992a; Pozio et al., 1992b; Nagano et al., 1999; Pozio et al., 2002a; Krivokapich et al., 2012). *Trichinella* spp. are unique parasites with many incomparable characteristics; they are the smallest human nematode parasites and also the largest of all intracellular parasites. *Trichinella* resides intracellularly, in both enterocytes and skeletal striated muscles cells of a host. *Trichinella* spp. males are between 0.62 mm to 1.58 mm long and 25 to 33 μm wide; females are about twice the size of the males. In male muscle larvae (ML), total length is 0.65 to 1.07 mm and width is 26 μm to 38 μm ; in female ML, total length is 0.71 mm to 1.09 mm and width is 25 μm to 40 μm .

Adult *Trichinella* worms live and reproduce in the intestine of a host and the released newborn larvae (NBL) invade into skeletal muscles (Vilella JB., 1970; Despommier DD., 1983). *Trichinella* was first discovered in 1835 (Owen, 1835). The curiosity of a young medical student James Paget to solve what caused the “sandy diaphragm” of an Italian bricklayer who had died of tuberculosis resulted in the discovery of *Trichinella*. Obviously, *Trichinella* parasite had existed much earlier; ancient prohibitions to eat pork are likely connected to *Trichinella* (Gould, 1970). The life cycle of *Trichinella* was discovered in 1850 when experimental infections were done by feeding infected human muscles to dogs (Virchow M.R., 1859; Leuckart R., 1860; Gould, 1970). *Trichinella nativa* was proposed to be a separate *Trichinella* species by Britov and Boev in 1972 (Britov and Boev 1972).

The importance of the *Trichinella* parasite is widely known. *Trichinella* causes a disease called trichinellosis, which is a zoonosis and can be fatal in case of severe infection. This disease is a public health threat because it affects human patients globally (Dupouy-Camet, 2000; Pozio, 2007; Gottstein et al., 2009). It is also an economic issue in food safety and porcine animal production.

In Finland, human *Trichinella* cases are rare. The last reported domestic human infection was in 1977, when bear meat infected three men in northern Finland (Salmi T., 1978). However, the prevalence of *Trichinella* spp. in Finnish wild animals is very high (Oksanen et al., 1998; Oivanen et al., 2002a). *Trichinella*-positive raccoon dogs and red foxes maintain the infection pressure near farms. In addition, rats can be risk factors for pig infections (Schad et al., 1987; Smith, H. J., and E. K. Kay, 1987; Leiby et al., 1990; Gamble et al., 1999; Oivanen et al., 2000; Oivanen et al., 2002b).

In this thesis, the purpose was to clarify the role of *T. nativa* in Finnish wildlife and the response it causes in a host. The focus was on *T. nativa*, which is the most common *Trichinella* species in Finland. There is not much information about *T. nativa*; earlier studies have focused mostly on *Trichinella spiralis*.

2. LITERATURE REVIEW

2.1. *Trichinella* species and life cycle

Since *Trichinella* was first discovered in 1835 until the middle of the following century, it was expected that all trichinellosis was caused by one species, *T. spiralis* (Owen, 1835). *Trichinella spiralis* was thought to have low host specificity and be capable of infecting a lot of different animal species and also humans. In the 1960s, it was noticed in Africa that different *T. spiralis* strains had different infectivity in rats and pigs (Nelson G.S. and Mukundi J., 1963). After that, experimental studies and molecular and biochemical methods have been involved in the identification of different *Trichinella* species. At present, the genus *Trichinella* includes nine different *Trichinella* species and three genotypes that are further divided into capsulated (*T. spiralis*, *T. nativa*, *Trichinella britovi*, *Trichinella murrelli*, *Trichinella nelsoni*, *Trichinella patagoniensis*, genotype T6, genotype T8, and genotype T9) and uncapsulated (*Trichinella pseudospiralis*, *Trichinella papuae*, and *Trichinella zimbabwensis*) ones (Pozio et al., 1992a; Pozio et al., 1992b; Nagano et al., 1999; Rombout et al., 2001; Pozio et al., 2002; Krivokapich et al., 2012). Despite slight interspecific size variation, there is no clear morphological difference between the species and genotypes; they are commonly identified by molecular techniques (Zarlenga and La Rosa, 2000).

Trichinella is a specific nematode because it completes all the developmental stages in a single host and the infective L1 larvae are maintained in the same host individual where they are released. The life cycle is divided into enteral and parenteral phases (Vilella JB., 1970; Despommier DD., 1983). A host becomes infected by *Trichinella* by eating raw or undercooked infected meat. In the enteral phase, the larvae are released from muscle tissue in the stomach due to low pH and digestive enzymes. The larvae migrate to the small intestine and penetrate intra-multicellularly into a row of columnar epithelial cells at the base of the villus. They go through four moltings and, after about 30 hours, they are sexually mature (Despommier DD., 1983). They mate, and after copulation the male worms die (Soulsby, 1982). Approximately from day five post infection (p.i.) onwards, the female worms start to release NBL. The length of the time period in which NBL are released is from one to several weeks and it depends on the host immunity and *Trichinella* species. The females are, however, most productive during the first week of the reproductive period (Marti and Murrell, 1986). One *Trichinella* female can release as many as 500–1500 larvae, depending on both the host species and *Trichinella* species and also the immune status of the infected host (Pozio et al., 1992a; Pozio et al., 1992b; Capo and Despommier, 1996). *Trichinella nativa* females isolated from the rat intestine produced fewer NBL in vitro during one day than *T. spiralis* females (Pozio et al., 1992a). In addition, the site in the small intestine influences

productivity; *T. spiralis* females grown in the jejunum produced more larvae than those in the ileum (Sukhdeo, 1991).

In the parenteral phase, the NBL migrate to striated muscle via lymphatic and blood vessels and actively penetrate into muscle cells. They modify the muscle cell into a nurse cell, which helps the parasite to receive nutrients and export wastes (Despommier DD., 1983). Around the nurse cells, a collagen capsule is formed for encapsulated *Trichinella* species (Despommier DD., 1990; Bruschi and Murrell, 2002). Within the nurse cell, NBL develop to the infective muscle-stage larvae without molting. This takes about 15 days. The ML can survive for years in the host muscle [up to 40 years in humans (Fröscher et al., 1988) and over 20 years, for example, in polar bears] and for weeks to some months in decaying tissues of dead hosts (Despommier, 1975; Fröscher et al., 1988; Kumar et al., 1990; Despommier et al., 1991). The survival in carcasses is enabled by anerobic metabolism of the ML and survival time depends on the temperature and moisture of the environment (Despommier, 1998). Some *Trichinella* species, *T. nativa*, *T. britovi*, and T6, can tolerate even low freezing temperatures. *Trichinella nativa* and T6 can survive and stay infective for many years in frozen carrion. Survival time depends on the host and *Trichinella* species; it is the longest in the striated muscle of carnivores and is strongly reduced, for example, in swine and rodent muscles (Kapel et al., 1999; Malakauskas and Kapel, 2003; Lacour et al., 2013). However, also non-freezing tolerant *T. spiralis* can stay infective in horse for at least four weeks at -18 °C (Hill et al., 2007a).

2.2. *Trichinella* epidemiology

2.2.1. *Trichinella* spp. worldwide

Trichinella spp. exist on all continents excluding Antarctica (Pozio and Murrell, 2006). However, reliable information on the epidemiology of *Trichinella* spp. is not gathered consistently; in many countries, reporting is founded on a voluntary basis, which results in scattered information. In domestic animals, *Trichinella* spp. have been documented in 43 countries and in 66 countries in wild animals (Pozio, 2007). Human trichinellosis has been documented globally in 55 countries (Pozio, 2007).

Some of the nine *Trichinella* species and three genotypes occur in wide geographical areas; others only have a limited patchy distribution. It can be hypothesized that the incidence of infection by different *Trichinella* species may be influenced by the ability of the ML to survive in the carcass in the environment in question and also the host species and their population density in certain areas. In total, *Trichinella* spp. have been found in more than 100 mammal species, seven avian species, and four reptile species as natural infections

(Pozio, 2005; Lee et al., 2013). However, the most important reservoirs for *Trichinella* spp. are carnivores and porcine omnivores (Pozio, 2005).

2.2.2. Sylvatic and domestic cycles

Most of the *Trichinella* species, apart from *T. spiralis*, exist predominantly in wild animals. The transmission between wildlife hosts is called the sylvatic cycle; the domestic cycle involves, for example, swine and horses. *Trichinella* infection can transmit from a sylvatic environment to a domestic cycle when wild animals are in contact with domestic animals or when domestic animals are fed, for example, undercooked feed of animal origin. *Trichinella* infection can also be transmitted via synanthropic animals, which are animals that live close to or in human habitation, like rats by swine herds or stables. The typical *Trichinella* species in the domestic cycle is *T. spiralis* (Pozio, 2001; Pozio, 2007).

2.2.3. *Trichinella* species and *Trichinella* prevalence in Finland

The four *Trichinella* species that exist in Finland are *T. spiralis*, *T. nativa*, *T. britovi*, and *T. pseudospiralis*; the most prevalent one is *T. nativa* (Kapel et al., 2001; Oivanen et al., 2002a). *Trichinella nativa* is widespread in arctic and boreal areas; it can even survive in frozen (-18°C) carnivore meat for five years (Dick and Pozio, 2001). The main hosts for *T. nativa* are both terrestrial and marine carnivores (Forbes, 2000; Kapel, 2000). It reproduces poorly in swine and rodents, so the risk for transmission from wild animals to domestic ones is low (Pozio et al., 1992b; Kapel and Gamble, 2000; Kapel, 2001). In Finland, *T. nativa* exists in wild carnivores, especially in raccoon dogs and red foxes (Oivanen et al., 2002a).

Trichinella spiralis, the domestic *Trichinella* species, is highly infective to swine and rats (Leiby et al., 1990; Kapel and Gamble, 2000). It is cosmopolitan and is present in many regions of the world. It is usually the cause of human infections. In Finland, *T. spiralis* has been found more often in domestic animals but it has also been found, for instance, in raccoon dogs and red foxes (Oivanen et al., 2002a).

Trichinella britovi is the most widespread sylvatic *Trichinella* species in temperate areas of Europe and Asia (Pozio and Murrell, 2006). It has a moderate tolerance to freezing (Pozio et al., 1992b) and can be found also in boreal areas; for example, in Finland, it has been detected in the same host with *T. nativa* as a mixed infection (Oivanen et al., 2002a). *Trichinella britovi* is prevalent in many sylvatic carnivores in Finland but it can also infect pigs (Oksanen et al., 1998; Oivanen et al., 2002a).

Trichinella pseudospiralis is the only non-encapsulated *Trichinella* species in Finland. It can infect both mammals and birds (Shaikenov B., 1980). In Finland, it has been found, for example, in raccoon dogs (Oivanen et al., 2002a).

All *Trichinella* species can exist as a single species or mixed infection, when infection with two or more species results in the finding of ML of more than one species. According to a Danish study in foxes, *T. nativa* dominated in mixed infection with *T. spiralis*; 64% of the intestinal worms and 78 % of the ML were *T. nativa* (Webster and Kapel, 2005).

In Finland, the *Trichinella* prevalence in wildlife is very high, particularly in lynxes (Oksanen et al., 1998; Oivanen et al., 2002a), raccoon dogs, red foxes and other carnivores (Freeman, 1964; Hirvelä-Koski et al., 1985; Oivanen et al., 2002a). In certain areas, the prevalence was even 70% in lynxes, 62% in red foxes, and 46% in raccoon dogs (Oivanen et al., 2002a). The prevalence of *Trichinella* infection is higher in southern Finland than in northern Finland. One explanation for this is the role of raccoon dogs as a reservoir of *Trichinella* spp. (Oksanen et al., 1998); in southern Finland, the number of raccoon dogs is higher. In addition, the prevalence of *Trichinella* has increased in Finland at the same time as the number of raccoon dogs, during the 1960s and 1970s (Helle, 1991). In an experimental infection, the raccoon dog has also been showed to be highly receptive to *Trichinella* (Näreaho et al., 2000). In addition, it is the only host species in Finland that has been infected with all of the four *Trichinella* species existing in Finland (Oivanen et al., 2002a). Another factor in the higher *Trichinella* prevalence in southern Finland is that the density of possible hosts is, in general, much higher in the south than in the north.

2.2.4. *Trichinella* infection in humans

All recognized *Trichinella* species and genotypes can infect humans. However, the pathogenicity of *T. spiralis* in humans is higher than other *Trichinella* species because of the more intense NBL production (Pozio et al., 1992b) and the stronger immune reaction they induce in humans (Pozio et al., 1993; Bruschi et al., 1999). The lowest infection dose for humans is not known. It has been estimated that from 100 to 300 *T. spiralis* larvae can cause symptoms and from 1000 to 3000 larvae can cause severe disease (Dupouy-Camet and Bruschi, 2007). However, as Gottstein et al. (2009) pointed out, these estimations are not based on scientific data.

The estimated incidence of *Trichinella* infections in humans worldwide is about 10,000 cases per year, with an approximated mortality rate of 0–0.2% (Ranque et al., 2000; Dupouy-Camet et al., 2002; Pozio, 2007; Khumjui et al., 2008; Dupouy-Camet et al., 2009; Murrell and Pozio, 2011; Hall et al., 2012). Obviously, mortality varies depending on infection dose and possibility of supportive therapy. However, because the symptoms of trichinellosis are non-pathognomonic and, in many areas, the local doctors are not familiar with this disease and its diagnostics, the incidence of trichinellosis is underreported in many countries.

The risk of trichinellosis is increased in areas where uninspected meat of different animal origins is eaten raw or undercooked. For example, in Finland, where susceptible production animals are under meat inspection and meat is usually eaten well cooked, trichinellosis in

humans is very rare even though *Trichinella* spp. prevalence in wild animals is high (Pozio, 1998). The last reported human case in Finland was diagnosed in 1977; three men were infected by *Trichinella* after eating bear meat (Salmi, 1978). Usually, the source of human trichinellosis worldwide is the domestic pig, but in Europe, infection is mainly acquired from horse or wild boar meat (Dupouy-Camet, 2000; Murrell and Pozio, 2011).

2.3. Host's response to *Trichinella* infection

The host's response to *Trichinella* infection is dependent on the host species, *Trichinella* species, and also the infection dose (Wakelin and Goyal, 1996). Different combinations are not absolutely comparable but the main features are similar. In addition, the antigens of different developmental stages are not similar. This means that different amounts of various antigens are released in different tissues of the body. This has been thought to be one reason for the survival of larvae in the host (Ortega-Pierres et al., 1996).

2.3.1. Enteral phase

As in many gastrointestinal (GI) disorders and parasite infections, in *Trichinella* infection, the noticeable features are changes in GI physiology. The amounts of fluid in the gut lumen and muscle contractility in the intestinal wall are increased (McDermott et al., 2003; Madden et al., 2004). In addition, studies in mice and rats have demonstrated that *T. spiralis* infection induces goblet cell hyperplasia and increases mucin secretion (Vermillion and Collins, 1988; Vallance et al., 1997; Khan et al., 2001; Khan and Collins, 2006; Franssen et al., 2011).

When *Trichinella* worms enter the intestine and penetrate epithelial cells, they induce an inflammation in the intestinal mucosa and activate the Th1 type immune response. A Th2 type immune response dominates later when the production of NBL has started and is responsible for the parasite's expulsion (Ishikawa et al., 1998). Thus, the immune response of the host during the intestinal phase of *Trichinella* infection is mixed Th1/Th2, but predominantly the Th2 type. Th1 is dominant in the beginning and Th2 later, after the reproduction of the worms has started (Ishikawa et al., 1998).

Parasite-specific T cells are produced during the first 2–4 days of infection (Korenaga et al., 1989). It is known that T cells mediate the host's response to *T. spiralis* infection (Wakelin and Wilson, 1979; Grecis et al., 1985; Bell et al., 1987; Korenaga et al., 1989; Ramaswamy et al., 1994). They produce mucosal cytokines that affect almost all intestinal changes during the *Trichinella* infection. These cytokines are, for example, interleukin 4 (IL-4) and IL-13, which act via the signal transducer and activator of transcription factor 6 (Stat6) (Urban et al., 2000; Kamal et al., 2001; Helmbj and Grecis, 2002; McDermott et al., 2005), and IL-5. The

activation of immune cells, especially T- cells, causes the above-mentioned changes, as well as increases in muscle function and intestinal mucin production (Marzio et al., 1990; Ishikawa et al., 1997).

The increased muscle function assists in expulsion of the worm from the gut (Vermillion and Collins, 1988; Vallance et al., 1997) and has been reported following nematode infection (Collins, 1996). The size and number of smooth muscle cells are also increased. It has been noticed in *Trichinella*-infected rats that muscle contractility is increased in the jejunum but reduced in the ileum (Marzio et al., 1990). This probably stimulates the propulsion of worms in the lumen.

Luminal epithelial cells in the intestinal wall are the first cells in host defense *Trichinella* worms deal with. One of the four types of epithelial cells is the goblet cell, which both grows in size and increases in number after the entry of *Trichinella* worms into the intestine (Garside et al., 1992). Goblet cells secrete mucins that are macromolecular components of mucus. Mucus protects the intestinal mucosa from, for example, dehydration and mechanical damage. It also provides a physical barrier between the intestinal epithelium and intestinal contents (Miller, 1987). Mucus has an important role also in host defense against *Trichinella*; it catches the worms and prevents their attachment to the intestinal epithelium (Appleton and McGregor, 1984; Bell et al., 1984). It also inhibits the motility and feeding of parasites (Miller, 1987). The second epithelial cell type, Paneth cells, is also reported to increase during *Trichinella* infection (Kamal et al., 2001). They most likely have a role in innate immunity.

In addition to goblet cell hyperplasia in the intestinal epithelium, T cells also regulate mucosal mast cell (MMC) hyperplasia and activation. The number of intraepithelial MMCs is highest approximately 14 days after the infection, when the adult worms are expelled from the intestine. After eight weeks, the MMC frequency returns to normal levels (Friend et al., 1996; Friend et al., 1998; Knight et al., 2004). According to mouse studies, MMC hyperplasia is necessary for the expulsion of adult *T. spiralis* worms from the intestine (Ha et al., 1983). Mast cells release their granule contents after activation and one function of these enzymes probably is to make the passage of antibodies easier by increasing gut permeability (Scudamore et al., 1995; Miller, 1996; Scudamore et al., 1998). Mast cells also have an important role in rapid expulsion, which is a phenomenon in rats. It has been noticed that 90–99% of *T. spiralis* first-stage larvae are expelled from the intestine during secondary infection if there is mastocytosis in the intestine during a primary infection (Bell, 1998).

Nematode infections in the intestine cause an inflammation that makes the intestinal environment hostile to the parasite and facilitates worm expulsion (Bell, 1998). In nematode infections, the inflammation is sustained mainly by eosinophils and, in *Trichinella* infection, the eosinophilia is coincident with the arrival of NBL in muscle tissue (Fabre et al., 2009b). Blood and tissue eosinophilia is regulated by Th2 cells that produce, for example, IL-5 (Coffman et al., 1989). IL-5 increases both the production and differentiation of eosinophils

in the bone marrow and also reduces the apoptosis of these cells (Gon et al., 1997; Bruschi et al., 2008). It has been thought that IL-5 also increases the contractility of intestinal muscle cells during *Trichinella* infection. This effect is probably indirect, via an increased number of eosinophils and the release of mediators like leukotrienes and other products of arachidonic acid metabolism (Vallance et al., 1999). The role of eosinophils is controversial; they induce tissue damage in the host but it is not known if they are also protective against *Trichinella* or not (Bruschi et al., 2008). When eosinophil-ablated mice were infected with *T. spiralis*, *Trichuris muris*, or *Schistosoma mansoni*, it did not successfully reveal the importance of eosinophils in the clearance of intestinal worms (Swartz et al., 2006; Fabre et al., 2009b; Svensson et al., 2011). Recently, it has been suggested that eosinophils regulate local immunity by stimulating the accumulation of Th2 cells and also by inhibiting the induction of inducible nitric oxide synthase (iNOS) in macrophages and neutrophils. Thus, eosinophils possibly support the growth and survival of the *T. spiralis* nematode (Gebreselassie et al., 2012).

The host's response against *Trichinella* at the intestinal level is seen histologically. *Trichinella* infection causes architectural changes in the small intestine, for example, villus atrophy and crypt hyperplasia (Garside et al., 1992). In addition to the increased number of goblet cells and MMCs, intestinal samples taken from rats showed increased infiltration of inflammatory cells, mainly eosinophils in the intestinal mucosa. In addition, the villi were fused and contained small hemorrhages. The changes were most prominent in the duodenum on day eight p.i. (Franssen et al., 2011).

2.3.2. Parenteral phase

When the NBL leave the small intestine, enter the circulation, and disseminate throughout the host, the parenteral phase of the life cycle of *Trichinella* begins. The host's response during the parenteral phase has not been studied as much as the response during the enteral phase, so the knowledge is not so detailed. The fine mechanisms of the myositis during *Trichinella* infection has begun to be clarified only recently, and researchers have mainly focused on infections caused by *T. spiralis* and *T. pseudospiralis*.

The enteral and parenteral phases are not separate and also overlap in time; the immunological modification of the intestinal phase also influences the following muscular phase. Interestingly, in per orally (p.o.) infected animals, the inflammation in muscle tissue is higher compared with animals infected by intravenous (i.v.) injection of NBL (Fabre et al., 2009a; Fabre et al., 2009b). This means that the host's response during muscular infection is partly regulated by the intestinal phase of infection.

When larvae reach muscle tissue, they initiate changes in the muscle cell that result in the formation of a nurse cell and induce an inflammation (myositis) around the nurse cell. The inflammation is not able to eliminate the parasite but causes the typical clinical signs of

Trichinella infection. This process is slightly different in different *Trichinella* species; for example, in *T. pseudospiralis*-infected mice, it is more diffuse and prolonged than in *T. spiralis*-infected ones (Boonmars et al., 2005). The reason for this difference is not understood. In addition, in raccoon dogs infected with *T. nativa*, the capsule of the nurse cell was thicker and the inflammatory reaction around it more intense than in the ones infected with *T. spiralis* (Sukura et al., 2002). Recently, using image analysis, it has been confirmed that the inflammatory reaction induced by non-encapsulated species (*T. pseudospiralis*) is lower than encapsulated species (*T. spiralis* and *T. britovi*), and *T. spiralis* induces a higher inflammatory reaction compared with *T. britovi* (Bruschi et al., 2009). *Trichinella nativa* was not included in that study.

It is known that T cells regulate the inflammation in muscle tissue and induce a mixed cytokine response (IL-4, IL-5, IL-10, IL-13, and interferon gamma (IFN- γ)) (Li and Ko, 2001; Morales et al., 2002; Beiting et al., 2006; Beiting et al., 2007). IL-10 is an influential regulator and limits the inflammation during the acute phase of the muscle infection. In the chronic phase, the Th2 response is induced and protects the infectious form of *Trichinella* parasite.

The local inflammatory response around the *T. spiralis* nurse cell is limited (Beiting et al., 2004). There is an infiltrate rich in macrophages (Karmanska et al., 1997b; Beiting et al., 2004), which is also seen in the cytoplasm of the nurse cell (Karmanska et al., 1997a). In addition to macrophages, CD4+ T cells, some CD8+ T cells, and rare B lymphocytes are seen in the infiltrate (Beiting et al., 2004).

2.3.3. Humoral response

All stages of *Trichinella* can activate the humoral response of the host but studies have focused mostly on the ML stage. There are three origins of antigens that can initiate the humoral response: cuticular antigens that are components of the worm cuticle, excretory/secretory (ES) antigens that are proteins produced by larvae, and somatic antigens that are internal structures, mainly somatic cell membranes (Crandall and Crandall, 1972; Dea-Ayuela and Bolas-Fernandez, 1999). The humoral response of the host can be detected by using, for example, enzyme-linked immunosorbent assay (ELISA) and Western blotting.

The antibody response and the time point of seroconversion have been observed to be dose-dependent in many host species, like rats, swine, horses, and foxes (Møller et al., 2005; Nöckler et al., 2005; Hill et al., 2007b; Franssen et al., 2011). However, antibody levels cannot be used to calculate the exact larvae per gram (lpg) values in muscle tissue, because the increase in specific antibodies is not proportional to ML doses (Salinas-Tobon Mdel et al., 2007). In addition, the antibody response also depends on the animal and *Trichinella* species

(Tomasovicova et al., 1991; Serrano et al., 1992; Soule et al., 1993; Reina et al., 1996; Møller et al., 2005; Franssen et al., 2011).

Circulating antigens are detected in the plasma or urine early during *T. spiralis* infection; in mice, this is as early as five or six days p.i. (Machnicka et al., 2001; Kolodziej-Sobocinska et al., 2006). Th2 activation during *Trichinella* infection induces an increase in host serum antibodies. These immunoglobulins (IgG, IgM, IgA, and IgE) recognize stage-specific antigens of *Trichinella* and participate in host defense by reducing worm fecundity and killing NBL (Appleton and Usack, 1993). Part of the antigens and antibodies are bound together and form immune complexes. When *Trichinella* has reached muscle tissue, the antigens and immune complexes are not detected any more, but specific antibodies remain in the host blood for a very long time. In mice, antibodies have been detected until seven months p.i. (Kolodziej-Sobocinska et al., 2006) and for several years in humans (Feldmeier et al., 1987; Bruschi et al., 1990; Bruschi et al., 2005). Specific antibodies can usually be identified starting from two or three weeks p.i.

2.4. *Trichinella* infection

2.4.1. Clinical signs

Clinical signs are best described in humans, being unspecific and related to the parasite cycle in the host. The infection dose determines the severity of the disease (Schmitt et al., 1978). In addition, the *Trichinella* species, host immunity, and general health all affect the severity of the disease (Kim, 1991; Murrell and Bruschi, 1994).

The first symptoms during the enteral phase of trichinellosis are usually general discomfort, headache, fever, and sometimes also abdominal pain and diarrhea (Murrell and Bruschi, 1994), resulting mainly from mucosal invasion of the ingested larvae. Usually, these symptoms are present during the first week of infection and are gradually abated after that. It is known also that immune complexes in the vascular system are associated with the symptoms of trichinellosis at the beginning of the infection (Feldmeier et al., 1987).

During the parenteral phase (from week two to week six p.i.), when the larvae invade skeletal muscle cells, damaging the cells, the symptoms are associated with inflammatory and allergic responses of the host (Capo and Despommier, 1996). For example, myalgia, paralysis, and periorbital or facial edema are seen (Ancelle et al., 1985; Ancelle et al., 1998). Possible complications such as encephalitis or myocarditis are usually seen within the first two weeks. Death is rare; in 1988, Ancelle et al. (1985) reported only five deaths in the past 25 years of the over 6500 infections in Europe.

When adult *Trichinella* worms stop releasing NBL and the larvae in muscle cells have completed their development, the convalescent phase of trichinellosis begins. This means that the signs of the disease have disappeared and the laboratory parameters return to normal levels. Generally, this stage initiates between the sixth and eighth weeks p.i. (Dupouy-Camet et al., 2002).

There are slightly different patterns in the disease caused by different *Trichinella* species. Symptoms relating to *T. nativa* infection are most prominent during the enteral phase. In addition, the symptoms begin later than with *T. spiralis* infection. Interestingly, even though *T. nativa* can cause the death of an individual, it usually causes almost no symptoms during the parenteral phase (Pozio et al., 1992b; Murrell and Bruschi, 1994).

Usually, *Trichinella* spp. cause clinical signs in natural host animals only when the infection dose is very large (Bruschi and Murrell, 2002). Clinical signs, when present, are similar to those in human infections including GI signs, weakness, and muscle stiffness (Bowman et al., 1993).

2.4.2. Diagnosis

Early diagnosis of trichinellosis is difficult because there are no pathognomonic signs or symptoms. In particular, during the enteral phase, the symptoms are easily misdiagnosed, for example, as bacterial or viral food poisoning.

The most frequently observed but unspecific laboratory findings in human patients are increased eosinophilia and leukocytosis in the blood sample, and increased levels of creatine phosphokinase (CPK) and other muscle enzymes (lactate dehydrogenase and aldolase) in the serum (Gould, 1970; Capo et al., 1998; Dupouy-Camet et al., 2002). Eosinophilia appears even before the clinical signs and symptoms and can remain for up to three months. The number of eosinophils correlates with the degree of myalgia (Ferraccioli et al., 1988). Leucocytosis also appears early in *Trichinella* infection and diminishes simultaneously with clinical signs and symptoms (Capo and Despommier, 1996). The level of muscle enzymes is increased in 75–90% of infected people. The increase occurs between the second and fifth weeks of infection and may stay high for over four months (Capo and Despommier, 1996; Jongwutiwes et al., 1998). A correlation between the intensity of muscular pain and increased CPK has been found (Ferraccioli et al., 1988).

The diagnosis can be confirmed by detection of the specific circulating antibodies. In animals, the antibodies can be tested in the serum or in the meat juice upon ante- or postmortem examination (Kapel et al., 1998; Møller et al., 2005). The seroconversion usually occurs from three to five weeks p.i. After the clinical symptoms have disappeared, the serum can still remain positive for over one year (Kozar and Kozar, 1968). However, antibody levels do not show a relationship with the severity of clinical disease (Boczon et al., 1981).

Muscle biopsy is recommended especially when serology is unclear. It should be taken from the predilection sites of *Trichinella* spp. to increase the sensitivity of the method. With muscle biopsy, the diagnosis can be confirmed but a negative result cannot rule out low-level infection (Murrell and Bruschi, 1994). The biopsy sample can be examined histologically or through artificial digestion. Especially in human infections, it is important to identify the source of *Trichinella* infection. Thus, other individuals exposed to *Trichinella* infection can be found and treatment can be started as soon as possible. In addition, when *Trichinella*-infected meat is identified, actions can be taken to prevent more infections. To improve the knowledge on the occurrence of *Trichinella* spp., all infections should be identified to the species or genotype level using molecular technique.

2.4.3. Treatment

In trichinellosis, the goal of treatment is to limit muscle invasion of *Trichinella* larvae and to reduce muscle damage and general symptoms (Bruschi and Murrell, 2002). The drug of choice would be effective against all developmental stages of *Trichinella* but unfortunately, that kind of drug does not exist. According to present knowledge, the best choice in the treatment of trichinellosis is the antihelminthics mebendazole and albendazole (Watt et al., 2000) and glucocorticosteroids (prednisone) (Shimoni et al., 2007). However, in the literature, there are no data that have been acquired by well-conducted case-control studies.

Antihelminthics are effective in the GI stages of the parasite but the efficacy against ML is dependent on the time between infection and treatment. When the treatment is delayed, the larvae have time to reach muscle tissue and develop a collagen capsule, which makes the larvae resistant to drugs (Pozio et al., 2001; Dupouy-Camet et al., 2002). Thus, to eliminate the infection, antihelminthics must be used in less than one week after infection, at the stage of GI invasion. However, because the diagnosis of trichinellosis is challenging, this is rarely possible.

The penetration of larvae into tissue causes cell damage and inflammation; glucocorticosteroids are used to relieve the side effects of inflammation (Dupouy-Camet et al., 2002). Because glucocorticosteroids can delay the expulsion of *Trichinella* from the intestine, they must always be used in combination with antihelminthics (Dupouy-Camet et al., 2002).

In general, medical treatment is used only in human patients; animals are rarely treated for trichinellosis.

3. AIMS OF THE STUDY

The aims of this thesis were as follows:

1. To reveal the epidemiological role of *T. nativa* in boreal environment. (I)
2. To describe the unusual clinical manifestation of *T. nativa* in a domestic cat. (II)
3. To compare host responses induced by *T. nativa* and *T. spiralis* in a selective host. (III, IV)

4. MATERIALS AND METHODS

4.1. Epidemiological studies/natural infection

4.1.1. Sylvatic samples (I)

The samples in the epidemiological study (I) were carcasses of carnivorous animals that were caught by volunteer hunters in Finland in 1999–2005. The hunters sent the animals for investigation to the Finnish Food Safety Authority (Evira) or to the Department of Veterinary Biosciences, Faculty of Veterinary Medicine, University of Helsinki, Finland. Lynx samples were collected by the Finnish Game and Fisheries Research Institute. There were, in total, 2483 samples from nine animal species including 1010 red foxes (*Vulpes vulpes*), 662 raccoon dogs (*Nyctereutes procyonoides*), 402 lynxes (*Felis lynx*), 125 brown bears (*Ursus arctos*), 102 wolves (*Canis lupus*), 75 pine martens (*Martes martes*), 53 badgers (*Meles meles*), 31 otters (*Lutra lutra*), and 23 American minks (*Mustela vison*). Samples were received from all national game management districts (GMDs) in Finland, except the archipelago between Sweden and Finland.

4.1.2. Natural infection in a domestic cat (II)

In Study II, the case was an eight-year-old, neutered Domestic Shorthair cat. The cat had a nonhealing ulcer on the skin below the left lower eyelid. The skin lesion was excised and sent for histopathological examination, where one *Trichinella* sp. larva was found in the middle of an inflammatory granulation tissue.

4.2. Experimental studies (III, IV)

The animal experiments in studies III and IV were approved by the appropriate authority (license numbers ESAVI-2010-05072/Ym-23 [III] and 17.11.2005 [IV]).

4.2.1. *Trichinella spiralis* and *Trichinella nativa*

The *Trichinella* strains used in experimental infections (III, IV) were maintained and passaged in mice. The *T. spiralis* strain was originally from a Finnish pig and the *T. nativa* strain was from a Finnish raccoon dog.

4.2.2. Experimental animals

In both experimental infections (III, IV), the mice and rats were obtained from Harlan Netherlands (Horst, the Netherlands). The mice were female and of NMRI outbred strain and the rats were male and of Wistar strain. The animals were kept at Evira under standard conditions and fed a standard laboratory mouse and rat diet. The food and fresh water were provided *ad libitum*. The condition of the animals was followed daily and the rats were weighed weekly. During study IV, the rats were housed under artificial illumination with a reversed light cycle of 12 h.

Trichinella-infected mice were used as a source of infective larvae (III, IV). *Trichinella* females for NBL production were also collected from mice (III).

Rats were infected with *T. spiralis* (ISS559) and *T. nativa* (ISS558) in studies III and IV. In study III, the rats were divided into two groups, p.o. infection and i.v. infection. At the time of infection, p.o. infected rats were nine weeks old and weighed between 248 and 297 g. Six rats were infected with *T. spiralis*, six with *T. nativa*, and three served as the uninfected control group. Six weeks after the infection, the rats were euthanized and the infection intensity (lpg) was determined.

In the i.v. infection group, six rats were infected with *T. spiralis* and six with *T. nativa* through the tail vein (III). The ages of the rats varied between 11 and 13 weeks and the weights varied between 293 and 351 g at the time of infection. I.v. infected rats were euthanized five weeks after infection and the lpg was counted.

Rats, as well as mice, were also used as a source of *Trichinella* females for the NBL production experiment (III). Those rats were infected p.o. and euthanized on day six p.i.

In the microarray study (IV), the rats were four months old and weighed approximately 400 g at the time of infection. The rats were infected p.o., six with *T. spiralis*, six with *T. nativa*, and three served as controls; thus, altogether 15 rats were studied. Five days after the infection, the rats were euthanized and samples were collected from their intestine.

Serum samples were taken from rats before the infection and at two weeks p.i. from the tail vein (III) and at the time of euthanasia, by cardiac puncture (III, IV).

7.2.2.1. Anesthesia and euthanasia

Before anesthesia, the rats were always fasted overnight in both experimental studies. In study III, the rats were anesthetized before *Trichinella* NBLs were injected into their tail vein. Anesthesia was induced by subcutaneous injection of fentanyl, 0.06 mg/kg, and fluanisone, 0.2 mg/kg (Hypnorm [0.2 ml/kg], Jansen, Oxford, UK). The anesthesia was terminated using 0.01 mg/kg naloxone (Naloxon B. Braun, B. Braun Melsungen AG, Melsungen, Germany) s.c.

In study IV, the samples were taken under terminal anesthesia, which was induced with 500 µg/kg medetomidine hydrochloride (Domitor, Orion Corporation, Espoo, Finland) and 25 mg/kg ketamine hydrochloride (Ketalar, Pfizer, Kent, UK) s.c.

All rats were euthanized with CO₂ under anesthesia, which was induced by fentanyl, 0.1 mg/kg, and fluanisone, 4 mg/kg (Hypnorm 0.4 ml/kg) in study III and in study IV, as described above.

4.2.3. Per oral infection

The rats were infected p.o. by serving *Trichinella* spp. in minced mouse meat (III, IV). First, *Trichinella*-infected mice were euthanized, skinned, and eviscerated, and the tail and distal limbs were removed. Then a homogenous mass of the meat was made with a standard kitchen blender. The infection intensity of the mouse meat (lpg) was counted (Gamble et al., 2000) and a sufficient amount of meat to achieve an infection dose of 2000 larvae/rat was given to the rats.

4.2.4. Intravenous infection

In study III, the infection doses of *Trichinella* NBL were injected into rats' tail vein under general anesthesia in order to bypass the enteral phase of *Trichinella* infection and to calculate the percentage of the NBL injected i.v. that reached muscle tissue. Due to technical reasons, the infection doses varied from 4550 to 4960 NBL, which was taken into consideration when the results were analyzed. In order to produce the infection doses, the rats were infected p.o. with 2000 ML and euthanized on day six p.i. Adult *Trichinella* females were collected for NBL production from the small intestine of the rats and cultured for two days in a 96-well plate in Dulbecco's modified Eagle's medium at 10% CO₂ and 37°C in a humid environment (Marti and Murrell 1986). The number of NBL in the infection dose was approximated by counting the mean production of NBL (NBL/♀) from the reproduction of 20 *Trichinella* females during two days. The target infection dose was 5000 NBL, so the number of females needed to obtain that number of NBL was calculated and the corresponding number of cultured wells, each containing one *Trichinella* female, was emptied. The females

were then removed from the infection dose. Before injection into the bloodstream, the infection doses were washed with saline to eliminate medium residues.

4.2.5. Newborn larvae production in vitro

To study and compare the larval production of two *Trichinella* species, *Trichinella* females were cultured in a 96-well plate, as described earlier. Every female was placed in its own well and the number of NBL produced was counted after 24 h. To determine how the host of origin influences the reproductivity of *Trichinella*, the females were collected from both rats and mice on day five p.i.

4.3. Artificial digestion

To assess the infection intensities (III, IV) of the infected animals and to identify *Trichinella* spp. larvae (I, II), artificial digestion of muscle tissue was performed with a magnetic stirrer method (Gamble et al., 2000). In p.o. infected rats and mice, a total of 1 g sample of the well-mixed meat was digested (III, IV) and in i.v. infected rats, the amount of meat digested was 100 g. In the epidemiological study (I), 10 g samples were taken from predilection sites of *Trichinella* in carnivores, e.g. the diaphragm, masticatory muscles, or forelimbs (Hermansson 1943; Kapel et al., 1994, 1995; Mikkonen et al., 2001) (I). For *Trichinella* species identification, the larvae from positive samples were collected and stored in 70% ethanol at -20°C or in 99% ethanol at 4°C (I). In study II, the digestion method was modified for small samples and the 0.13 g muscle biopsy sample was digested in 1 ml volume for 40 min at 50°C, with vigorous shaking in a vortex mixer every five min to isolate *Trichinella* larvae in the specimen.

Artificial digestion was performed at either Evira (I) or the Department of Veterinary Biosciences, Faculty of Veterinary Medicine, University of Helsinki, Finland (I, II, III, and IV).

4.4. DNA isolation and multiplex polymerase chain reaction

In study I, 3–10 *Trichinella* larvae stored in 70% ethanol were rehydrated in a decreasing ethanol series (70%, 50%, 30%, 10%, 5%, and MilliQ-water [Millipore, Billerica, Massachusetts, USA]). Larval DNA for species detection was isolated from two pools of the larvae from each host animal (I). In total, 328 larval isolates were identified using multiplex

polymerase chain reaction (PCR) according to a previously published protocol (Zarlenga et al., 1999) with slight modifications. In study II, the multiplex PCR was applied to identify the one larva in the biopsy specimen.

4.5. Serological methods

4.5.1. Enzyme-linked immunosorbent assay

ELISA was used in studies II and III to determine anti-*Trichinella* IgG antibodies. Microtiter plate wells were coated with crude larval homogenate of *T. spiralis* or *T. nativa* (II), or ES protein of *T. spiralis* (II, III) (provided by the Witold Stefanski Institute of Parasitology, Warsaw, Poland). The secondary antibody was a horseradish peroxidase-conjugated goat anti-feline IgG (II) or horseradish peroxidase (HRP)-labeled goat anti-rat IgG (III). Serum samples from raccoon dogs infected with *T. spiralis* or *T. nativa* with HRP-labeled goat anti-dog IgG (II) as a secondary antibody were used as positive controls and the sera from a young kitten and an uninfected raccoon dog served as negative controls (II). In study III, negative controls were sera from uninfected control rats; two negative controls were placed in each plate. Absorbance was measured at a wavelength of 450 nm.

4.5.2 Western blotting

In study II, the Western blot technique was performed to confirm the specificity of the cat antibodies towards *Trichinella* spp. The antigens used were crude extracts of *T. spiralis*, *T. nativa*, *T. britovi*, and *Toxocara cati*. Proteins from the antigen preparation were separated in an electric field by their molecular weight using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane. The proteins recognized by the antibodies present in the cat serum samples were visualized using an HRP-labeled secondary antibody and diaminobenzidine (DAB) as a chromogen.

4.6. Histopathology and immunohistochemical staining

To study histopathological changes in the rat intestine during *Trichinella* infection, tissue sections of the duodenum (III, IV), jejunum (IV), and ileum (IV) were taken immediately after euthanasia. In study II, the sample was a skin biopsy specimen excised from a cat, below the left lower eyelid. All samples were fixed in 10% neutral buffered formalin,

routinely processed to paraffin blocks, cut at 4 μm , and stained with hematoxylin and eosin. MMCs were stained using toluidine blue (III).

The immunoreaction against *Trichinella* spp. was assessed with immunohistochemical staining (IHC), where sera from the case cat and control cat were applied on histological sections (II). The sections were muscle samples from raccoon dogs with *T. spiralis* and *T. nativa* larvae in them. Binding of the antibodies was detected with HRP-conjugated goat anti-feline IgG (1:100) and DAB.

After taking the histological samples, the rat's intestinal tissue was screened for *Trichinella* worms (III). The intestine was cut longitudinally and incubated in Dulbecco's modified Eagle's medium at 37°C for 1 h and, after that, the fluid was examined with a stereomicroscope.

4.7. RNA isolation and microarray analysis

In study IV, a whole-genome microarray was used to analyze gene expression changes in the rat jejunum during *Trichinella* infection. The whole intestine was removed immediately after the rats were euthanized. The jejunum was opened and scrape samples from jejunal mucosa were collected with a scalpel, frozen in liquid nitrogen, and stored at -80°C. The purpose of the scrape samples was to harvest the most affected mucosa, excluding the outer submucosal and muscular layers.

Before RNA isolation, the frozen scrape samples were quickly homogenized. Total RNA was isolated according to the GenElute™ Mammalian Total RNA kit instructions (Sigma-Aldrich, MO, USA) and the samples were quantified by ultraviolet spectrophotometry. The Agilent 2100 Bioanalyzer (Agilent Technologies Santa Clara, CA, USA) was used to assess RNA integrity. Isolated RNA was stored at -80°C until further analyses.

The raw data from "Rat Genome 230 2.0 Arrays" were extracted with the default parameter settings and data analysis was accomplished with the Anduril framework (Ovaska et al., 2010). Plier (Affymetrix Inc., Santa Clara, California, USA) normalization was used to normalize the expression values and the probes were re-annotated to Ensembl (Flicek et al., 2012) gene identifiers using a custom CDF file (Dai et al., 2005) for Rat Genome 230 2.0 Array.

Genes were considered differentially expressed when they had at least a fold change of two in either direction and a False Discovery Rate (FDR) ≤ 0.001 when comparing all *Trichinella*-infected animals with the controls. A fold change limit of two and FDR ≤ 0.05 were used for *T. spiralis* vs. control and *T. nativa* vs. control comparisons to show differential expression. Fold change was calculated by taking the median expression value in the *Trichinella* and

control groups and calculating the ratio between the groups. Differential expression between the groups was tested using a t-test and the FDR multiple hypothesis correlation method (Benjamini and Hochberg, 1995) was used to correct the p-values to reduce the number of false positives.

The differentially expressed genes (DEGs) were annotated with the corresponding Gene Ontology (GO) terms using the Ensembl database to interpret the list of genes. The enriched GO terms in DEGs compared with the genome-wide reference set were computed. Fisher's exact test was used to perform GO enrichment analysis and the FDR was used to adjust all p-values. The set of DEGs was also clustered founded on their GO annotations to find genes that have GO terms alike (Ovaska et al., 2008).

Signaling Pathway Impact Analysis (SPIA) was used to conduct a pathway analysis for DEGs to recognize signaling pathways that were affected by the changes in gene expression (Tarca et al., 2009). The Ensembl database, version 68, was used to convert the Ensembl gene identifiers to Entrez gene identifiers. By taking the median, given that the median absolute deviation (MAD) of the expression values was less than or equal to one, expression values of the Ensemble gene identifiers mapping to the same Entrez gene identifier were conjoined.

4.8. Statistical analyses

All statistical analyses in study I were conducted with the analytical software package SPSS 16.0 for Windows (SPSS Inc., Chigaco, Illinois, USA). Pearson chi-square tests were used for cross-tabulated data. Associations between the variables were analyzed using Spearman's rho correlation test or a binary logistic regression procedure. Multivariate linear regression analysis was used to analyze the role of different variables in the infection density of hosts. In a single host species, the parasite burden (lpg) of different *Trichinella* species was compared using one-way analysis of variance (ANOVA).

Nonparametric matched pairs Wilcoxon test was used to compare *Trichinella* antibody levels from a 2-week time point to termination and two-sample Wilcoxon test was used to compare differences at each time point between species within infection routes (III). Two-sample Wilcoxon test was also used to test the difference between infection rates after i.v. *T. spiralis* and *T. nativa* infection, and the means and 95% confidence intervals (CIs) of infection rate, calculated with logit-transformed original data. The number of larvae *Trichinella* females produced in vitro was analyzed with pairwise t-tests with nonpooled standard deviation (SD) and Holm p-value adjustment. Larval production, infection rate, and antibody analyses were performed using the R 2.14.1 program (R Foundation for Statistical Computing, Vienna, Austria). The random effects model using the STATA 12.1 statistical package (StataCorp LP,

College Station, TX, USA) was used to evaluate weight changes over time after p.o. infection with *T. spiralis* and *T. nativa*.

In study IV, hematological values were evaluated by one-way ANOVA (PASW Statistics 18) followed by multiple comparisons between the groups with the Bonferroni post-hoc test.

5. RESULTS

5.1. Sylvatic *Trichinella* species in Finland (I)

5.1.1. Distribution in the country

Altogether, 617 animals (24.8%) of the 2483 examined animals were *Trichinella*-positive. The GMDs were used to analyze the distribution of *Trichinella* prevalence in Finland. The country is divided to 15 game administrative units, forming GMDs. The geographical distribution of *Trichinella* spp. varied; the prevalence of *Trichinella* infection was significantly higher in southern Finland than in the northern part of the country.

Molecular identification by multiplex PCR was performed for 328 larval isolates and 303 of them yielded a specific reaction. Four endemic *Trichinella* species were identified: *T. spiralis*, *T. nativa*, *T. britovi*, and *T. pseudospiralis*. The most prevalent *Trichinella* species was *T. nativa* (80.1%), followed by *T. spiralis* (12.8%), *T. britovi* (6.0%), and *T. pseudospiralis* (1.1%). In southern Finland, all four *Trichinella* species were found, but in northern Finland, only *T. spiralis* and *T. nativa* were present (Fig. 1).

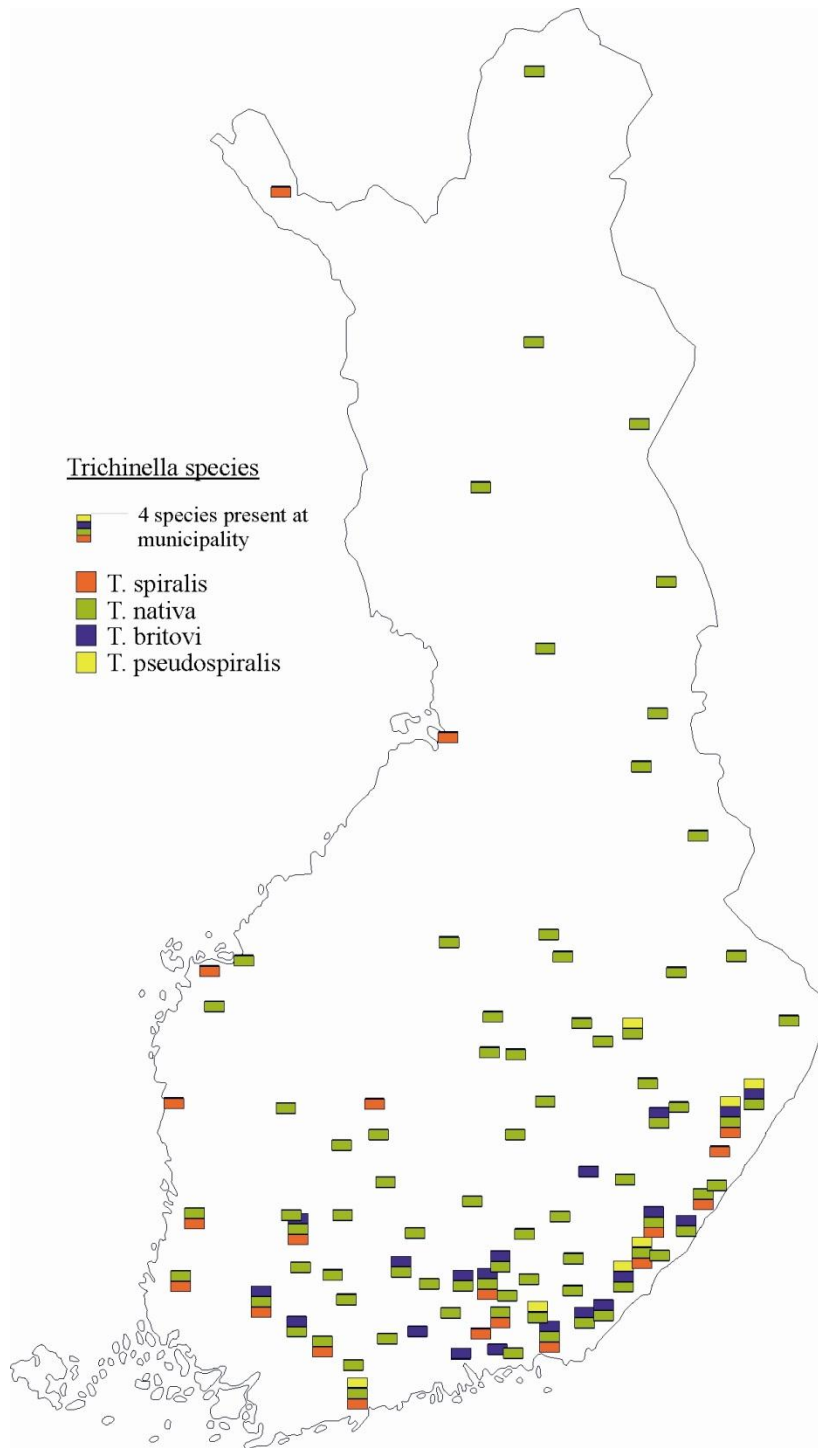


Figure 1. Distribution of different *Trichinella* species in Finland.

Most of the infections were single infections (93%); mixed infections were found in 7% of infected animals. In mixed infection, all possible two-species combinations were found; the most common one was *T. nativa* with *T. britovi* (41%). Mixed infections existed in four animal species: raccoon dog, red fox, lynx, and wolf.

5.1.2. Prevalence in different hosts

The prevalence varied from 0% to 46% in different host species. In lynx, the prevalence of *Trichinella* infection was 46%, followed by wolves (39%), raccoon dogs (28%), and red foxes (19%). In pine martens, badgers, bears, and otters, the prevalence was less than 10% (Fig. 2).

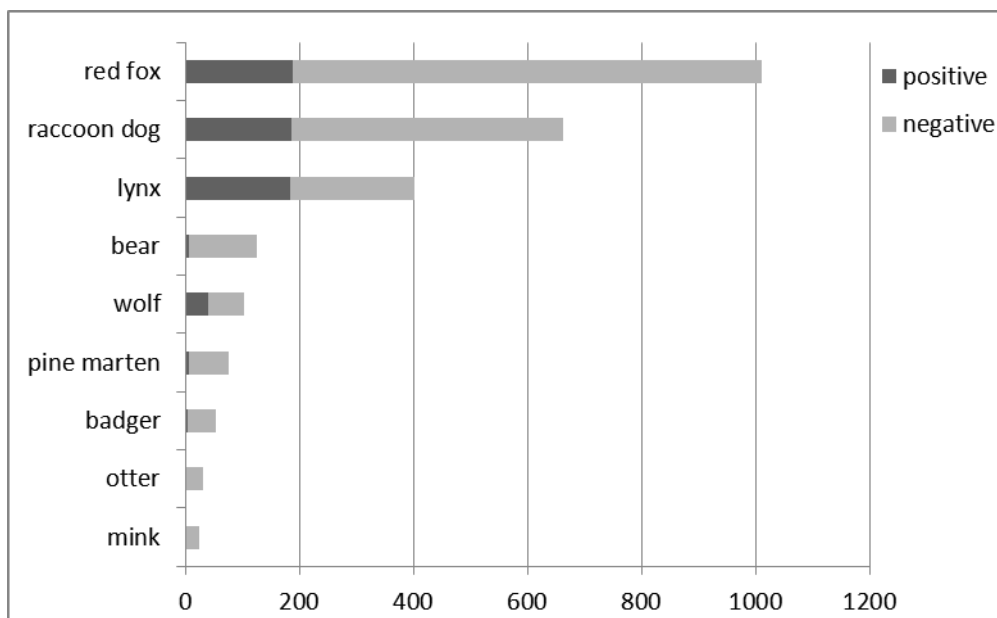


Figure 2. Sample size with positive and negative results in different host species.

The prevalence in different and also in the same host species varied a lot between GMDs; for example, from 0 to 62% in red fox and from 8 to 80% in raccoon dog. In addition, the prevalence also varied within each GMDs. Thus, *Trichinella* spp. are not equally distributed in the country. In all host species, the prevalence was higher in southern Finland; the three northernmost GMDs had a *Trichinella* infection prevalence of only 6% (Fig. 3).

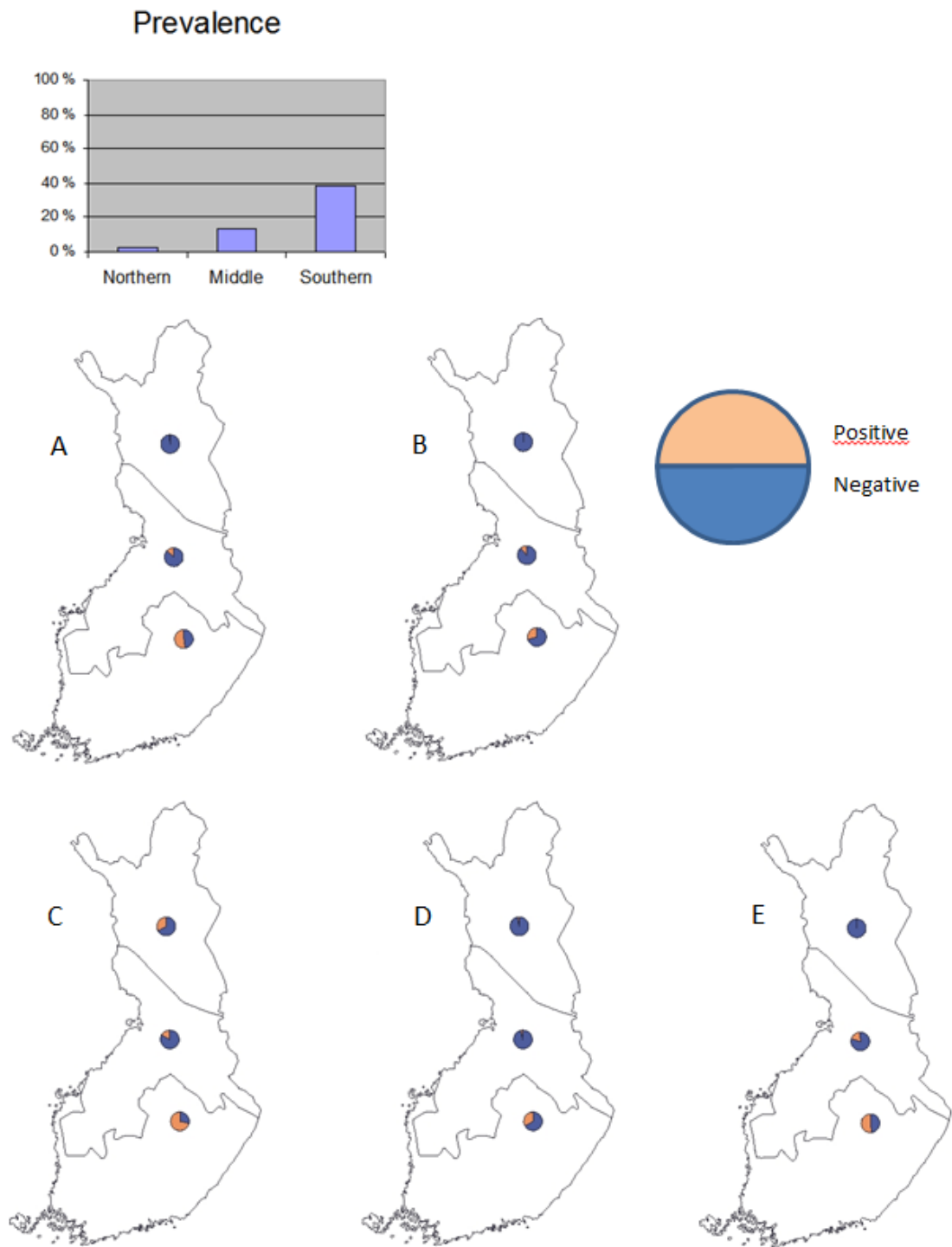


Figure 3. Finland divided to three vegetation zones shows a clear tendency of increasing *Trichinella* prevalence from north to south in five host species; A) red fox, B) raccoon dog, C) wolf, D) brown bear, and E) lynx. Original illustration: Jani Pellikka (unpublished).

Different *Trichinella* species were also unevenly spread in the different host species. In the lynx, red fox, and raccoon dog, all four *Trichinella* species were found. In all host species, *T. nativa* was the most common species; it was found in 74% of the positive samples when both single and mixed infections are taken into account. *T. spiralis* was the next prevalent (12%), followed by *T. britovi* (6%) and *T. pseudospiralis* (1%). *T. nativa* was found in four of the five *Trichinella*-positive bears. Interestingly, *T. britovi* was found more often in the lynx than other *Trichinella* species and *T. spiralis* infection was three times more likely in the raccoon dog than in the lynx (Fig. 4).

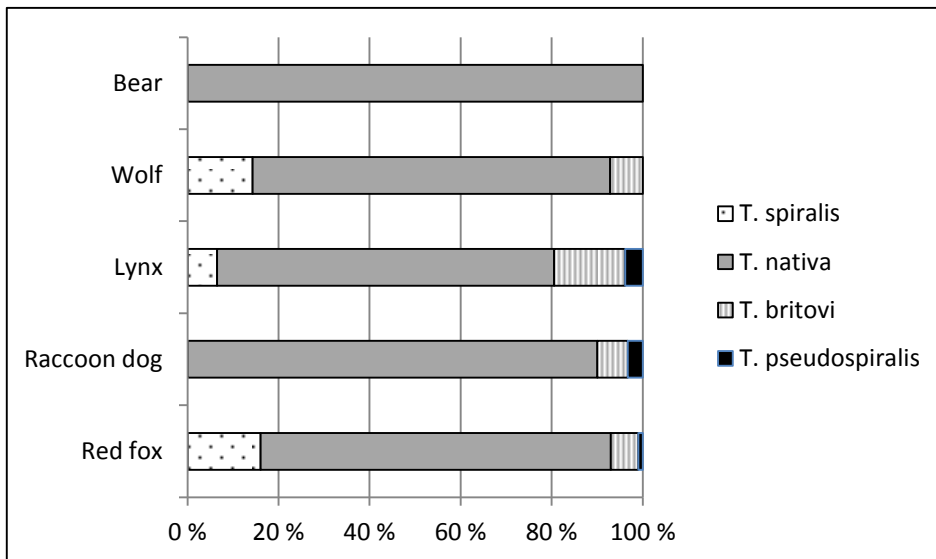


Figure 4. Distribution of different *Trichinella* species in different host species.

5.1.3. Infection intensity

Infection intensities varied between different host species varied (Fig. 5). In addition, different *Trichinella* species had different infection intensities. When the red fox, raccoon dog, and wolf were used as a host animal, linear regression analysis showed that infection intensity was dependent on both the *Trichinella* species and host species. The *Trichinella* species with the highest lpg (median 488) was *T. pseudospiralis*, but only three cases were examined. The highest individual lpg (760) was in one raccoon dog, which had mixed infection of *T. nativa* and *T. britovi*. When the population size of the host animals, the estimated number of infected individuals, and infection intensities are taken into consideration, raccoon dogs and red foxes are the most important reservoirs for *Trichinella* species, and especially for *T. nativa*, in Finland.

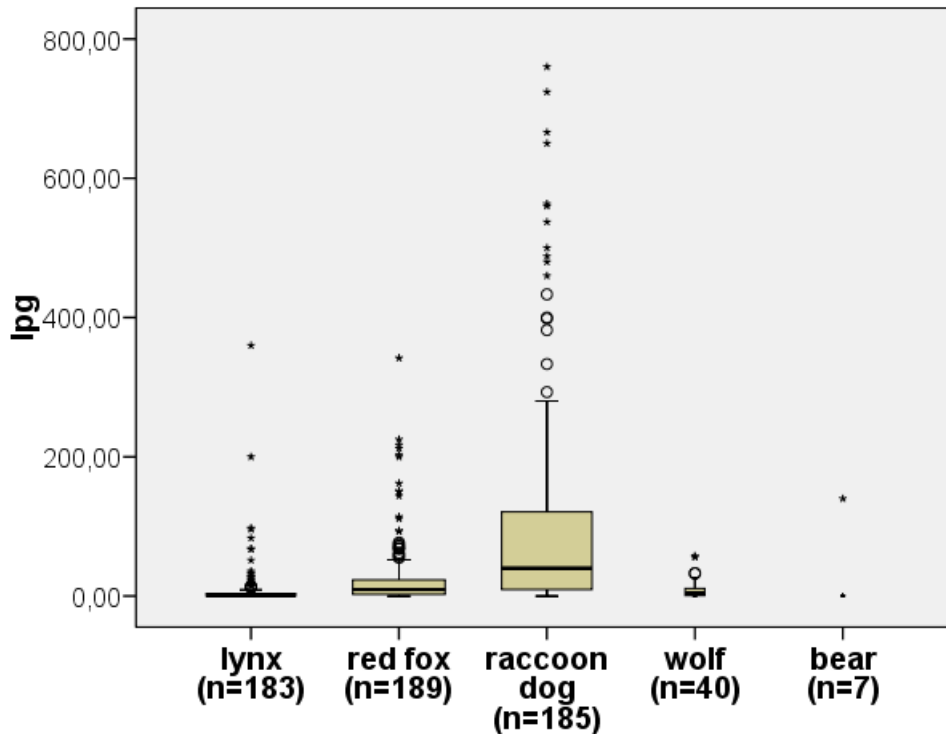


Figure 5. Box and whisker blot of overall *Trichinella* spp. intensity (larvae per gram, lpg) in different host species.

5.2. Host response

5.2.1. Clinical findings

An otherwise healthy cat had an ulcer on the skin below the left lower eyelid (II). It had been treated with oral antibiotic and topical treatment with no response. The ulcer was 4 mm x 7 mm in size and crusted, and the conjunctiva was hyperemic and swollen.

After the ulcer was removed, the surgical site healed well. However, during the 1-year follow-up, the cat began to scratch the eye again. It also squinted. The previous surgical site was alopecic and a firm, 1 cm wide, subcutaneous mass was covering the entire lower eyelid and part of the upper eyelid. There were no findings in ophthalmoscopic and fundic examinations. Contrast computed tomography showed that the muscular mass did not infiltrate the bone.

The rats and mice infected with *T. spiralis* and *T. nativa* during the experiments did not have any detectable clinical signs with the infection doses used (III, IV). However, the rats that were infected p.o. gained less weight than controls during the first two weeks after infection (III).

5.2.2. Clinical chemistry and serology

In blood samples taken from the cat when the eyelid lesion recurred (II), serum creatine kinase activity was increased (1689 U/l; reference interval: 60–350 U/l). Other values of clinical chemistry were within reference ranges. Tests for Feline leukemia virus (FeLV) and Feline immunodeficiency virus (FIV) were negative.

In rats, the white blood cell count, and the numbers of neutrophil granulocytes and lymphocytes differed significantly between the groups infected with *T. spiralis* or *T. nativa* (IV). The number of white blood cells was higher in *T. spiralis*-infected rats than in *T. nativa*-infected rats on day five p.i.

The antibody levels of the animals were measured with ELISA in all studies (II, III, IV). ELISA gave a strong positive reaction against *Trichinella* spp. in the serum of the case cat (II). To confirm the results of ELISA, Western blotting was used. The serum of the case cat was positive for all the *Trichinella* species tested and the serum of the control cat gave no reaction (II). Cross reaction with *T. cati* was excluded.

In rats, anti-*Trichinella* antibody levels were studied by ELISA at two weeks p.i. and at the time of termination (after five weeks in i.v. infected rats and after six weeks in p.o. infected rats) (III). Two weeks after the infection, the antibodies were significantly increased in p.o. infected animals. At the termination sampling point, compared with the two-week samples,

the rise in antibody levels was seen in both p.o. and i.v. infected rats in both *T. nativa*- and *T. spiralis*-infected groups. There was, however, no statistically significant difference in antibody levels between the two *Trichinella* species. The antibody titers were, altogether, higher in p.o. infected than i.v. infected rats.

5.2.3. *Trichinella* spp. in rat

5.2.3.1. Enteral vs. parenteral defense (III, IV)

Rats were infected both p.o. and i.v. with *T. nativa* and *T. spiralis* to investigate if the defense against *T. nativa* was mounted in the enteral or parenteral phase (III). The infection intensity after both infection routes was compared. All the values are presented here with medians for easier comparison

The difference in infection intensity was statistically significant in both the p.o. and i.v. infected groups between the two *Trichinella* species. In p.o. infected rats, the infection intensity of *T. nativa* was between 3 lpg and 62 lpg (median, 22) and the infection intensity of *T. spiralis* was between 680 lpg and 1760 lpg (median, 1243). In i.v. infected rats, the lpg values of *T. nativa* were between 0.08 and 1.5 (median, 0.29) and with *T. spiralis*, the lpg values were between 3.9 and 8.7 (median, 5.2). The mean infection rate (the percentage of the NBL injected i.v. that reached muscle tissue) of *T. nativa* and *T. spiralis* was 1.7% and 20%, respectively ($p < 0.05$) (III).

In study IV, the lpg values in rats infected p.o. with *T. nativa* larvae were between 0 and 2 (median, 0) and with *T. spiralis* larvae, the lpg values were between 306 and 612 (median, 590).

5.2.3.1.1. In vitro larval production (III)

The number of NBL produced during 24-h cultivation on day six p.i. was compared between *T. nativa* and *T. spiralis* females isolated from a rat or a mouse intestine. *Trichinella nativa* females isolated from a mouse produced more NBL (mean, 29; 95% CI, 26–31; $p < 0.05$) than those that were isolated from a rat (mean, 23; 95% CI, 21–24; $p < 0.05$). In contrast, *T. spiralis* females isolated from a mouse released fewer NBL (mean, 57; 95% CI, 51–62; $p < 0.05$) than those that were isolated from a rat (mean, 70; 95% CI, 65–75; $p < 0.05$) (Fig. 6).

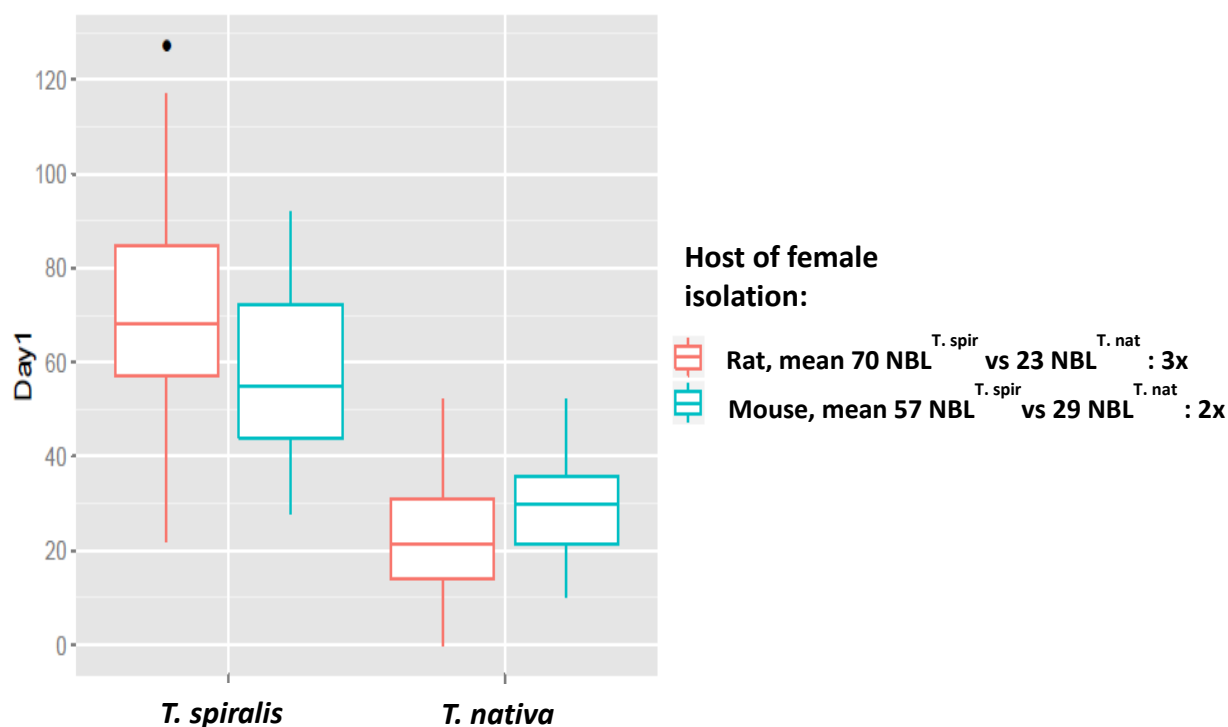


Figure 6. Mean number of newborn larvae (NBL) released during 24-h cultivation by *T. spiralis* and *T. nativa* females isolated from a rat or a mouse intestine on day six post infection.

5.2.3.2. Intestine mucosal gene expression (IV)

There was significant variation in gene expression values in the intestinal samples of both *T. nativa*- and *T. spiralis*-infected rats, compared with controls. However, the changes were not statistically different between *T. nativa*- and *T. spiralis*-infected groups, so these groups were combined into a single *Trichinella* group. This combined *Trichinella* group was then compared with controls and 551 overexpressed and 427 underexpressed genes were revealed, with a q-value of <0.001 and a fold change of at least two.

In GO analysis, these 978 over- or underexpressed genes clustered into 37 groups that were enriched in numerous “biological process” ontological categories. In the pathway analysis, seven pathways had the combined p-value of total perturbation and the number of DEGs in the pathway (pG) below 0.05. Based on the knowledge of *Trichinella* infection, four of the seven pathways showed more biological interest than others. These four pathways were “Extracellular matrix (ECM)-receptor interaction”, “Focal adhesion”, “Complement and coagulation cascades”, and “Amoebiasis”. The three omitted pathways were related to nonspecific epithelial damage or stress.

5.2.4. Histopathology and immunohistochemical staining (II, III, IV)

The histopathological changes were quite similar in *T. spiralis* and *T. nativa* infections and there was also no remarkable difference in their severity between the two *Trichinella* species (III, IV). Histopathological changes on day five after *Trichinella* infection were most severe in the jejunum (IV). The epithelium of the villi was vacuolated and degenerated (Fig. 7.). There was a moderate shortening and fusion of the villi and moderate infiltration of inflammatory cells (eosinophils, neutrophils, and lymphocytes) in the lamina propria. In addition, mild hyperemia and hemorrhage were seen in the tip of the villi. The number of intraepithelial lymphocytes and goblet cells was marginally increased. Pathological changes in the duodenum and ileum were mild or absent (IV), as in the duodenal samples taken 6 six weeks after infection (III).

In the biopsy sample taken from the cat (II), one cyst containing a *Trichinella* sp. larva was seen. The cyst was surrounded by a tissue reaction that consisted mainly of spindle cells that formed interlacing bundles. The cells were fibroblasts, which had an elongated hyperchromatic nucleus, small nucleolus, and scant eosinophilic cytoplasm. The cell boundaries were indistinct and mitotic figures were rare. Among the spindle cells, inflammatory cells (lymphocytes, histiocytes, plasma cells, and neutrophils) were also seen. The epidermis covering the lesion was ulcerated.

A new biopsy sample was taken when the clinical signs recurred. In that sample, the fibroblasts were arranged irregularly and there were moderate anisocytosis and anisokaryosis in them. Mitotic figures were rare and some leucocytes were seen all over the lesion.

In IHC staining, the *Trichinella* larva and the interior of the capsule were stained strongly positively (II). From the biopsy taken from the biceps femoris muscle, one *Trichinella* larva was isolated, which was identified as *T. nativa* using multiplex PCR (II).

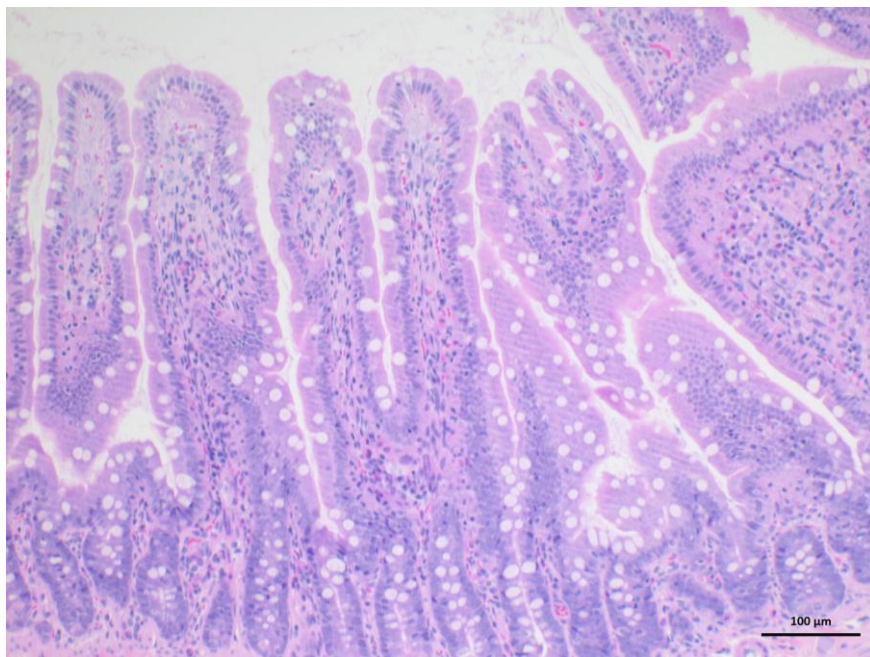
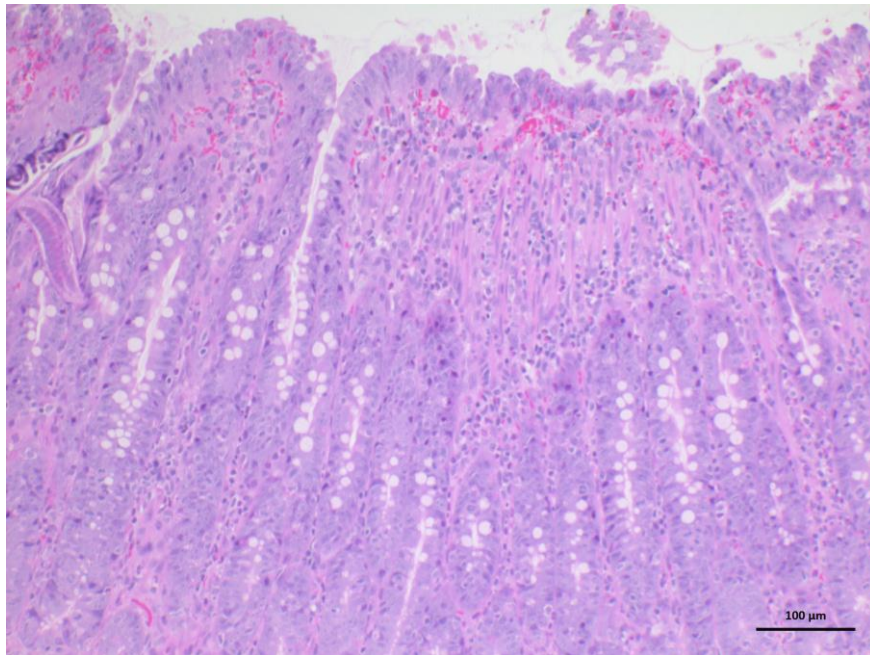


Figure 7. Inflammatory changes in rat intestine on day five after *Trichinella* infection (A) compared to control rats (B). Villi are fused, and there are hyperemia and hemorrhage in the tip of the villi. Number of inflammatory cells in lamina propria is increased.

6. DISCUSSION

6.1. *Trichinella* species in Finland

A large wildlife sample set showed *Trichinella* spp. to be common in Finland; 24.8% of all examined wild animals were *Trichinella*-positive. *Trichinella* prevalence in Finland is uniquely high in Europe and both domestic and sylvatic cycles exist (Pozio, 1998). One reason for this may be the less powerful human influence on the natural ecosystem (Pozio, 1998); in southern Europe, there are, on average, 147 inhabitants per km² and in Finland, there are 18 inhabitants per km². When wild animals live near human habitats, their feeding behavior is different. They prefer, for example, rubbish dumps instead of animal carcasses or preys (Pozio, 1998). In Sweden and Norway, *Trichinella* spp. are found in wildlife but the prevalence is lower. In Sweden, during the period of 1983–2003, the prevalence in carnivores varied between 0.05% (lynx) and 16.7% (arctic fox) (Pozio et al., 2004). In Norway, 4.8% of red foxes were positive for *Trichinella* (Davidson et al., 2006). In addition, domestic trichinellosis is rare in Sweden and Norway. Denmark is regarded as a *Trichinella*-free country, with no domestic cycle and a very rare sylvatic cycle; the prevalence in red foxes was only 0.1% in 1996 (Enemark et al., 2000). In central Europe, *Trichinella* prevalence in wildlife is also low. In Germany, the prevalence in raccoon dogs is 5% and in red foxes it ranges from 0.08%–0.22% (Littman et al., 2006). In southern Europe, particularly in Spain, trichinellosis is a public health threat; human outbreaks occur every year due to the consumption of pork products (Rodriguez de las Parras et al., 2004). However, the prevalence in red foxes is only 3% (Perez-Martin et al., 2000). In the European Union (EU), domestic trichinellosis is present, besides Finland, for example in Spain, Romania and Bulgaria (Pozio, 1998; Cuperlovic et al., 2005; Blaga et al., 2009).

In Finland, *Trichinella* has an obvious north-to-south gradient of increasing prevalence (Fig. 1). A similar north-to-south gradient of increasing prevalence has also been recognized for *Toxoplasma gondii* distribution in Finland (Jokelainen et al., 2010). In addition, a study performed in Norway, which has a similar climate to Finland, showed the latitude dependence of *Trichinella* prevalence; it was higher in red foxes in the southeastern part of the country than in the north (Davidson et al., 2006). The more moderate climate in the southern part of Finland also has an influence on wildlife density. Thus, one explanation for the higher *Trichinella* prevalence in southern Finland is the population density of the hosts, which is higher in the south compared with the northern part of the country. Thus, when animals die they are more likely to be eaten by another wild scavenger carnivore before decomposition, which facilitates the diffusion of *Trichinella* infection (Oivanen et al., 2002b). In addition, road kills increase the number of animal deaths and the carcasses to be eaten

(Fig. 8). There are higher road density and heavier traffic in southern Finland due to the distribution of inhabitants in the country.



Figure 8. Carcass of a raccoon dog on a road side scavenged by a red fox (photo: Jari Kostet).

There are many host species of *Trichinella* that are prevalent in southern Finland. The red fox has been considered to be an important reservoir host for *Trichinella*. It is widely distributed and adapted to both rural and urban surroundings. The diet of red foxes mainly involves meat, which favors the transmission of meat-borne parasites like *Trichinella*. In addition, *Trichinella* spp. are commonly found in raccoon dogs. The raccoon dog is an excellent host for *Trichinella* (Näreaho et al., 2000) and its population has been considered to be a risk factor for *Trichinella* infection (Oksanen et al., 1998; Pannwitz et al., 2010). The same was seen in our study; the combined *Trichinella* prevalence in red fox and raccoon dog was positively correlated with the population density of raccoon dog in a certain area. Raccoon dogs spread to Finland from the western regions of the former Soviet Union during the 1960s and 1970s and the *Trichinella* prevalence in Finnish wildlife has increased concurrently with the colonization of that carnivore (Oksanen et al., 1998). The lower density of raccoon dogs in Lapland may partly explain the lower prevalence of *T. nativa* and other *Trichinella* species in northern Finland. In Norway, however, where the uneven distribution pattern of *Trichinella* infection also exists, raccoon dog is not present (Davidson et al., 2006).

In addition to the difference in *Trichinella* prevalence along the north-south gradient, the prevalence in a given animal species varied a lot between GMDs and also within each GMD. In red foxes, the prevalence varied from 0 to 62% and in raccoon dogs, it varied from 0 to 55% between different GMDs. There were certain municipalities with a higher *Trichinella*

prevalence than in the areas around them. We obtained the samples from volunteer hunters and it is possible that they had used raccoon dog or red fox carcasses as bait. This could influence the local *Trichinella* prevalence by carnivore-carnivore transmission, as was seen in Russia (Pozio et al., 2001). Wolf carcasses were left in forests, which led to a prevalence of *Trichinella* infection in the wolves in that area of as high as 97.5%. The patchy distribution within GMDs may explain the varying prevalence reported, for example, earlier from Finland (Rislakki, 1956; Freeman, 1964; Hirvelä-Koski et al., 1985; Oivanen et al., 2002a). Care must be taken before any nation-level conclusions are made based on the results of *Trichinella* prevalence in certain areas. The large number of animals studied increases the reliability of the results.

Trichinella nativa is the most prevalent *Trichinella* species in Finnish wildlife; 80.1% of the positive animals were infected with *T. nativa*. In addition, in all studied host species, it was the most common *Trichinella* species. *Trichinella britovi* is common in wildlife in central and southern Europe (Pozio, 2001). In southeastern France, 2.7% of red foxes were positive for *Trichinella* and all the positive cases were identified as *T. britovi* (Aoun et al., 2012). *Trichinella nativa* and *T. britovi* live in similar ecological niches; they are both widespread in sylvatic carnivores (La Rosa et al., 1992). It has been speculated that in the north, *T. britovi* is replaced by the freezing-resistant species *T. nativa* (Oivanen et al., 2002a; Pozio and Murrell, 2006). This has been shown also in other countries: *T. nativa* is the dominant *Trichinella* species in the Palearctic and Arctic (Shaikenov, 1992; Handeland et al., 1995; Pozio et al., 1998; Pozio and Murrell, 2006). In certain host species, *T. nativa* can tolerate low temperatures and, thus, the cold climate in Finland may favor *T. nativa*; even at -18°C, the ML can remain alive in carnivores for years (Kapel et al., 1999) and are infective when the carcass is eaten by a new host. However, the complete life cycle of *Trichinella* takes place at the body temperature of a live animal; only transmission to scavengers is dependent on climate conditions.

Trichinella nativa exists in the entire Finland but the prevalence is significantly higher in the southern part of the country. The same is true with the other three Finnish *Trichinella* species. Our data also showed that *T. spiralis* had its reservoir in wildlife. *Trichinella spiralis* was found in the wolf, red fox, raccoon dog, and lynx. The identification of *T. spiralis*-infected red fox way up in Lapland was a surprise (I). It is probably the first isolation of the species so far north (Pozio and Murrell, 2006), in an area with no known domestic outbreaks. *Trichinella britovi* was found in the badger, wolf, raccoon dog, and red fox, but relatively more often in the lynx (Fig. 4).

6.2. Infection intensity

The infection intensity of *Trichinella* was dependent on both the *Trichinella* species and the host species. In raccoon dogs, the infection intensity was high with all *Trichinella* species, which indicates that the raccoon dog is an adaptive host for *Trichinella* spp. This is in accordance with earlier studies; all *Trichinella* species reproduce in raccoon dogs and infect them well (Näreaho et al., 2000; Oivanen et al., 2002a; Malakauskas et al., 2007). Raccoon dogs are commonly found in both sylvatic and domestic environments in Finland. This further stresses the importance of the raccoon dog as a reservoir for *Trichinella*. When the population size of the host animals, the estimated number of infected individuals, and infection intensities are taken into consideration, raccoon dogs and red foxes are the most important reservoirs for *Trichinella* species in Finland. The population size of red foxes is between 88,000 and 190,000 and that of raccoon dogs is between 138,000 and 278,000. When estimated prevalence was calculated based on the number of infected individuals, there were 1.3–1.5 times more infected raccoon dogs than red foxes in Finland. In addition, when the different weights of the raccoon dog (6 kg) and red fox (5.5 kg) and different infection intensities (100.3 vs. 26.4 lpg, respectively) are taken into account, the raccoon dog population in Finland carries a 5.4–6.3 times heavier *Trichinella* spp. burden than the red fox.

6.3. *Trichinella nativa* in rats

In the rat, the infection intensity with different *Trichinella* species varies a lot; the rat is a selective host for *Trichinella* (Nelson and Mukundi, 1963; Pozio et al., 1992b; Malakauskas et al., 2001; Mikkonen et al., 2005). It is known that *T. nativa*, compared with *T. spiralis*, has a limited infectivity in rats (Pozio et al., 1992a; Malakauskas et al., 2001; Mikkonen et al., 2005). This was seen also in our study; the median lpg value in rats after p.o. infection with an equal dose of *T. nativa* and *T. spiralis* was 22 and 1243, respectively (III), and in study IV, the median lpg value in *T. nativa*-infected rats was 0 and in *T. spiralis*-infected rats it was 590. When the enteral phase of the infection was bypassed and *Trichinella* larvae were injected i.v., the median lpg was 0.29 for *T. nativa* and 5.2 for *T. spiralis*. When the differences in ML production after p.o. and i.v. infection were compared between *T. nativa*- and *T. spiralis*-infected rats, it seemed that the enteral phase had a greater influence on the reduction of ML production than the parenteral phase. However, these results showed that the species-specific defense against *T. nativa* by a host occurred in both the enteral and parenteral phases.

In a previous study, rats were also infected i.v. with *T. spiralis* NBL, in order to demonstrate that only some of the NBL reached muscle tissue (Dennis et al., 1970). The results showed that 66% of the *T. spiralis* larvae injected i.v. were found in muscles. In our study, the infection rates were lower. Our *Trichinella* worms and also the rats were genetically different than those in the experiment of Dennis et al., which may have influenced the results. In addition, due to technical reasons, the infection doses were injected into the rats' tail veins an hour after the doses were prepared. This may have had an effect on the viability of the NBL. However, when nine-hour-old *T. spiralis* NBL were injected i.v. into the retro-orbital venous plexus of a mouse, 80% of the NBL were recovered as ML (Wranicz et al., 1999). The doses in our study were all handled correspondingly and were, thus, comparable.

There can be numerous reasons why some animal species, for example, the raccoon dog is an excellent host for several *Trichinella* species and why the rat is a selective host for *T. nativa*. It may be that *T. nativa* can release fewer NBL in a selective host, survives poorly in the gut lumen, or maybe it cannot invade into the intestinal epithelial cells as effectively as in an adaptive host. After the enteral phase, the defense of the host can also be directed towards the NBL or ML of *T. nativa*. Based on our results, the defense occurs in both the enteral and parenteral phases of the life cycle, so any of the previous reasons can be the explanation. When *Trichinella* enters, for example, the raccoon dog, one or more of these reasons favor the survival and reproduction of the parasite and *Trichinella* is capable of causing high infection intensity.

To compare the reproduction of *T. nativa* with *T. spiralis*, we isolated *T. nativa* and *T. spiralis* females from rat and mouse intestines on day six p.i. and cultured them in a 96-well plate for one day. The reproduction capacity of *T. nativa* was lower in those isolated from rats than those isolated from mice. The opposite was revealed for *T. spiralis*. This means that something in the gut of a selective host affects the *Trichinella* female's capacity to produce NBL. This explains, to some extent, the lower infectivity of *T. nativa* in rats, and is in accordance with earlier results (Pozio et al., 1992b). *Trichinella pseudospiralis* also produces fewer NBL in rats but it can cause a reasonable high lpg in rat muscle tissue (Pozio et al., 1992b). This is why the low production of NBL cannot explain the lower infectivity of *T. nativa* in rats entirely. In addition, all the *Trichinella* females in our study were collected on day six p.i., so the different reproduction periods of the two different *Trichinella* species are not taken into account here.

Because the enteral phase was more important in host defense against *Trichinella* than the parenteral phase, we wanted to examine more thoroughly the host response during that phase. We infected rats with *T. nativa* and *T. spiralis* and analyzed gene expression changes in the jejunum on day five p.i. (IV). Interestingly, there was a significant difference in gene expression profile between *Trichinella*-infected rats and control rats but no difference between *T. nativa*- and *T. spiralis*-infected rats. The histopathology supported these results; the histological changes were similar in both *T. nativa*- and *T. spiralis*-infected groups and there was also no difference in the severity of the changes.

It is known that *Trichinella* infection causes inflammatory changes in the intestine and that the changes are heaviest during the enteral phase of infection (Despommier DD., 1983; Bruschi and Murrell, 2002). Pathological changes were most severe in the jejunum; there was moderate infiltration of inflammatory cells (eosinophils, neutrophils, and lymphocytes) in the lamina propria, moderately shortened and fused villi with epithelial degeneration and vacuolation, and modest hyperemia and hemorrhage in the superficial lamina propria. In addition, the number of goblet cells was increased. In the duodenal and ileal samples, the pathological changes were mild. Eosinophilic inflammation and also the goblet cells are known to facilitate worm expulsion (Vallance et al., 1999).

In study III, our samples were taken from the duodenum on day 40 p.i. and no inflammatory changes were seen any more. Franssen et al. (2011) had similar findings; on day 42 after *T. spiralis* infection, there were no abnormalities seen in the rat duodenal samples, even though inflammatory changes were seen 8 days p.i. In our study, the only finding was an increase in the number of MMCs, which are considered to be important in the protective immunity in *T. spiralis* infection (Ha et al., 1983; Grecis et al., 1993; Suzuki et al., 2008). MMCs have been detected in the rat jejunum 30 days post *T. spiralis* infection (Fernandez-Blanco et al., 2011). In our study, the number of mast cells was similar in both *T. nativa*- and *T. spiralis*-infected rats, so the mast cells are considered to be important also during *T. nativa* infection in rats.

The rats in our experimental studies were without noticeable clinical signs, except that the infected rats gained less weight than controls during the first two weeks after infection. This could be due to diarrhea or reduced food intake (Auli and Fernandez, 2005). The rats did not show diarrhea and the intake of food was free. Our histological results showed that both *Trichinella* species induced a similar inflammatory reaction in the small intestine. It is possible that this inflammation reduced the absorption of nutrients in the intestine, causing weight loss. *Trichinella* can also induce nausea during the intestinal phase of infection, which can reduce appetite and, thus, the amount of food the rats eat. However, in our study, the intake of food was not monitored. In earlier studies, the body weight of the rats was reduced only when the infection dose was higher than 4000 larvae (Franssen et al., 2011). Our study showed that the effect on body weight can also be seen with a lower infection dose of 2000 larvae.

The data from microarray analysis indicated an inflammatory response and nonspecific damage in the mucosa of the rat jejunum. Because there was no significant difference in gene expression profile between *T. nativa*- and *T. spiralis*-infected rats, we combined them into one *Trichinella* infected group to examine the effects of infection in general. When this group was compared with controls, the difference was statistically different. We focused on differentially expressed genes and performed pathway analysis on them using SPIA (Tarca et al., 2009). Pathways that were biologically relevant and involved in host-parasite interaction were chosen for further examination. As a result, we suggested four pathways that were linked to *Trichinella* infection: “ECM-receptor interaction”, “Focal adhesion”, “Complement and coagulation cascades”, and “Amoebiasis”.

In these pathways, no specific gene expression changes were found. The changes were mostly related to innate immunity and inflammatory reaction which were seen also in histological samples. Innate immunity has an important role in a host's response to *Trichinella*. In our samples, the monocyte receptor CD14 and Toll-like receptors (TLR) were upregulated. Both of them mediate the immune response in innate immunity against microbes. TLR2 and TLR4 are upregulated in the rat jejunum during *Hymenolepis diminuta* infection (Kosik-Bogacka et al., 2012). In our samples, the upregulation of TLR4 was seen, but there was no overexpression of TLR2.

In addition, complement cascade-related genes C1, C8, and decay accelerating factor (DAF) were activated. The complement cascade is also linked to innate immunity. A previous study has demonstrated that complement factors C3, C8, and C9 cannot bind to *Trichinella* cuticle (Näreaho et al., 2009). In this study, C8 was downregulated, which supports the earlier results. DAF inhibits complement activation on cell surfaces (Jokiranta et al., 1995) and was upregulated in our study. This probably means that during *Trichinella* infection, the cells are more protected from the activation of complement.

6.4. *Trichinella nativa* in a domestic cat

Trichinella spiralis has been associated with domestic species and other *Trichinella* species, including *T. nativa* are thought to exist in wildlife (Kozar et al., 1965; Kapel et al., 1998; Murrell and Pozio, 2000). However, we found *T. nativa* in a Finnish domestic cat. Even though *T. nativa* is only slightly infective in swine, it can occur in domestic companion animals like cats and dogs (Lindberg et al., 1991).

Cats have been found to be susceptible hosts for *Trichinella*. Experimental feline studies have been performed but few natural infections have been reported (Schad et al., 1967; Gretillat and Vassiliades, 1968; Garkavi, 1973; Holzworth and Georgi, 1974; Bowman et al., 1993; Moisan et al., 1998; Ribicich et al., 2013).

Usually, *Trichinella* does not cause any clinical signs in cats or other animals unless they are infected with a very high infection dose (Bruschi and Murrell, 2002). Based on experimental infections, cats are reported to have only mild GI disturbances during *Trichinella* infection (Bowman et al., 1993). In a severe infection with a high infection dose, weakness, discomfort, and death have been reported (Bowman et al., 1993; Ribicich et al., 2013).

Interestingly, the cat in our study had an ulcer below the left eye and in the center of the lesion, one *T. nativa* larva was found in a biopsy sample. Even though the ulcer lesion was removed, the lesion still disturbed the cat postoperatively; it squinted and scratched the eye. A firm subcutaneous mass was beginning to grow in the periocular area. In contrast computed tomography, it was seen that the mass did not infiltrate the bone. Because it is

known that cats are susceptible to developing sarcomas secondary to chronic inflammation (Jelinek, 2003), biopsies were taken from the periocular mass of the cat. The histopathological findings were consistent with granulation tissue, inflammation, and fibrodysplasia. However, the marked proliferation of fibrous spindle cells and infiltrative growth of the lesion maintained low-grade fibrosarcoma as a differential diagnosis.

Trichinella infection is thought to act as a co-carcinogen. When *T. spiralis* and chemical carcinogens were inoculated simultaneously into rats, tumor formation was more frequent than in rats that received either of these agents alone (Gawish, 1975). In contrast, (Wang et al., 2009) *Trichinella* is also thought to be an anti-tumor agent (Wang et al., 2009); for example, it produces an anti-tumor protein that induces apoptosis (Wang et al., 2013).

In cats, *Trichinella* infection has been reported concurrent with neoplastic disease (Moisan et al., 1998) and the same phenomenon has also been seen in humans (Cheung et al., 1997; Cvorovic et al., 2005; Kristek et al., 2005). The cause for this is speculative but the most obvious reason might be the chronic inflammation induced by *Trichinella* spp. larva. After vaccination, cats are prone to develop sarcomas that are called injection site sarcomas (ISS) (Jelinek, 2003). They are thought to develop because of the irritation and inflammation induced by the vaccine (Jelinek, 2003). One non-vaccinated cat developed sarcoma after meloxicam injection (Munday et al., 2011), and ISS has also been found next to non-absorbable sutures (Buracco et al., 2002) and an implanted microchip (Daly et al., 2008). It has been speculated that even an injection through the skin can cause inflammation if, for example, hair is introduced into the subcutis (Munday et al., 2011). If even a stick of a needle can cause enough inflammation to result in the growth of cancer, it is easy to believe that *Trichinella* larvae can cause the same response.

Feline retroviruses are known to be associated with certain neoplasias in cats (Poli et al., 1994; Callanan et al., 1996). However, according to previous studies, these viruses do not stimulate ISS development (Ellis et al., 1996; Kidney et al., 2001). In humans, the malignancy induced by *Trichinella* has been linked to immunodeficiency of the patient (Segelman, 1975; Cheung et al., 1997). To analyze the possibility of simultaneous viral infections and the role of immunodeficiency in this case, the cat was tested for FeLV and FIV with negative outcome.

7. CONCLUSIONS

Sylvatic *Trichinella* infection is highly prevalent in Finnish wildlife and *T. nativa* is the most common *Trichinella* species. The prevalence is so high that *Trichinella* infection is a risk for domestic animals but also for humans. *Trichinella* infection can manifest in many ways including also a preneoplastic pattern in cats.

In the southern part of Finland, the prevalence of *Trichinella* infection is significantly higher than in the northern part. However, the prevalence in a given animal species and in a given GMD is patchy. *Trichinella* species are not equally distributed in different host species; *T. britovi* is more often found in the lynx than in other animal species. However, *T. nativa* is the most abundant *Trichinella* species among all investigated host species. The most abundant host species are the raccoon dog and red fox. The population density of raccoon dogs in a certain area is positively correlated with the prevalence of *Trichinella* infection in both raccoon dogs and red foxes. The raccoon dog carries the highest biomass of *T. nativa* among Finnish fauna.

In the host, the defense against *T. nativa* and *T. spiralis* occurs during both the enteral and parenteral phases. *Trichinella* infection alters heavily the gene expression and histology of the rat intestine, but there is no significant difference in mucosal gene expression response or intestinal histology between *T. nativa*- and *T. spiralis*-infected rats in the early phase of infection.

The reproduction of *Trichinella* females is influenced by the host animal in which *Trichinella* has reached the adult stage. *Trichinella nativa* females isolated from the rat intestine produce fewer NBL than *T. nativa* females isolated from the mouse intestine. The opposite is true for *T. spiralis*.

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