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Department of Veterinary Biosciences
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Wild and domestic animals as hosts of *Toxoplasma gondii* in Finland

Pikka Jokelainen

ACADEMIC DISSERTATION

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with the permission of the Faculty of Veterinary Medicine, University of Helsinki,
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To my favorite parasite

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Abstract

Toxoplasma gondii is a widespread protozoan parasite of humans, domestic animals, and wildlife. In this work including nationwide epidemiological cross-sectional studies and descriptive case series studies, *T. gondii* was confirmed as endemic, common, and sometimes fatal in a selection of animal hosts in Finland.

Antibodies against *T. gondii* were detected in all host species investigated, including hosts hunted or raised for human consumption. The samples were screened with a commercial direct agglutination test using a conservative cut-off for seropositivity. Specific anti-*T. gondii* IgG antibodies were detected in 9.6% of the 1215 moose (*Alces alces*), 26.7% of the 135 white-tailed deer (*Odocoileus virginianus)*, and 17.6% of the 17 roe deer (*Capreolus capreolus*) examined. Seropositive animals were found in 76.3% of 97 sheep farms and 60.0% of 25 wild boar farms, while antibodies were detected in 24.6% of the 1940 individual sheep (*Ovis aries*) and 33.0% of the 197 farmed wild boars (*Sus scrofa*) examined. Both of the possible definitive hosts present in Finland had commonly encountered the parasite: 48.4% of the 490 domestic cats (*Felis catus*) and 86.1%, a significantly higher proportion, of the 337 Eurasian lynx (*Lynx lynx*) tested seropositive. Raw meat in the diet was a major risk factor (odds ratio 2.0) for the infection in domestic cats. Evidence of exposure to *T. gondii* followed a north-south gradient in several hosts. The prevalence data indicate an environment contaminated with oocysts. PCR-confirmed *T. gondii* oocysts were found in one (0.8%) of the fecal samples from domestic cats. None of the lynx shed oocysts.

Cats also died of toxoplasmosis; the proportional mortality rate was 3.1% among the 193 domestic cats examined postmortem. The first local *T. gondii* isolates (FIN1 and FIN2) were obtained from two cats into cell culture. *Toxoplasma gondii* also caused deaths in species considered highly susceptible to clinical toxoplasmosis. The proportional mortality rates were 8.1% in 173 European brown hares (*Lepus europaeus*), 2.7% in 148 mountain hares (*L. timidus*), and 15.8% in a limited number of 19 Eurasian red squirrels (*Sciurus vulgaris*).

Direct genotyping of *T. gondii* strains was based on analysis of length polymorphism at six microsatellite markers (B18, TUB2, TgM-A, W35, B17, and M33); one fingerprinting marker (M48) was included for further characterization. Both wild and domestic animals were infected with strains belonging to genotype II, which was also the genotype of the oocysts shed. Strains of genotype II had caused all 27 fatal cases investigated in detail. The observed fatality rates among apparently immunocompetent animals challenge the prevailing assumption of nonvirulence of genotype II strains.

Toxoplasma gondii infections represent both a public health issue and a welfare issue for its hosts. The parasite thrives in Finland and should not be underestimated.

List of original publications

This thesis is based on the following original publications, referred to in the text by their Roman numerals:

I Jokelainen, P., Näreaho, A., Knaapi, S., Oksanen, A., Rikula, U. and Sukura, A. 2010. *Toxoplasma gondii* in wild cervids and sheep in Finland: North-south gradient in seroprevalence. Veterinary Parasitology. 171: 331-336.

II Jokelainen, P., Näreaho, A., Hälli, O., Heinonen, M. and Sukura, A. 2012. Farmed wild boars exposed to *Toxoplasma gondii* and *Trichinella* spp. Veterinary Parasitology. 187: 323-327.

III Jokelainen, P., Isomursu, M., Näreaho, A. and Oksanen, A. 2011. Natural *Toxoplasma gondii* infections in European brown hares and mountain hares in Finland: proportional mortality rate, antibody prevalence, and genetic characterization. Journal of Wildlife Diseases. 47: 154-163.

IV Jokelainen, P. and Nylund, M. 2012. Acute fatal toxoplasmosis in three Eurasian red squirrels (*Sciurus vulgaris*) caused by genotype II of *Toxoplasma gondii*. Journal of Wildlife Diseases. 48: 454-457.

V Jokelainen, P., Simola, O., Rantanen, E., Näreaho, A., Lohi, H. and Sukura, A. 2012. Feline toxoplasmosis in Finland: Cross-sectional epidemiological study and case series study. Journal of Veterinary Diagnostic Investigation. 24: 1115-1124.

VI Jokelainen, P., Deksne, G., Holmala, K., Näreaho, A., Laakkonen, J., Kojola, I. and Sukura, A. 2013. Free-ranging Eurasian lynx (*Lynx lynx*) as host of *Toxoplasma gondii* in Finland. Journal of Wildlife Diseases. 49: 527-534.

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Abbreviations

2-ME	2-mercaptoethanol
6-FAM	6-carboxyfluorescein
AIDS	acquired immunodeficiency syndrome
bp	base pair(s)
BSA	bovine serum albumin
CI	confidence interval
CF	complement fixation (test)
CO_2	carbon dioxide
CNS	central nervous system
DALY	disability-adjusted life year
DAT	direct agglutination test
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphates
DT	Sabin-Feldman dye test
e.g.	exempli gratia, for example
ELFA	enzyme-linked fluorescent assay
EIA	enzyme immunoassay
ext	external primer
F	forward primer
FFPE	formalin-fixed, paraffin-embedded
G	gauge
HE	hematoxylin and eosin
HEX	hexachlorofluorescein
IFAT	indirect fluorescent antibody test
IgG	immunoglobulin G
IHC	immunohistochemistry, immunohistochemical staining
int	internal primer
ITS	internal transcribed spacer
Mb	million base pairs
$MgSO_4$	magnesium sulphate
MLST	multilocus sequencing typing
MS	microsatellite(s)
ND	not detected, no data
NED	2,7′,8′-benzo-5′-fluoro-2′,4,7-trichloro-5-carboxyfluorescein
OR	odds ratio
PCR	polymerase chain reaction
PCR-RFLP	polymerase chain reaction-restriction fragment length polymorphism
QALY	quality-adjusted life year
R	reverse primer
sp., spp.	species: singular, plural
TAE	Tris-acetate-ethylenediaminetetraacetic acid buffer
Tris	tris(hydroxymethyl)aminomethane

1. Prologue

The zoonotic protozoan *Toxoplasma gondii* can kill its host
but to reach another host it desires the most

To lie dormant in a host and get it eaten by another
or shed as oocysts to be swallowed by a mother

Alas, unlucky to end up in a human it is
or in a hare that then dies and is not eaten by that pathologist

perhaps part of the game
but preventable, so whom to blame?

Pikka Jokelainen (2012)

2. Review of the literature

2.1. *Toxoplasma gondii*, a successful parasite

Toxoplasma gondii was discovered in 1908 (Nicolle and Manceaux, 1908; Splendore, 1908) in the tissues of a common gundi *(Ctenodactylus gundi*), a rodent used for *Leishmania* research at the Pasteur Institute, Tunis, and in a rabbit in Brazil. Nicolle named it based on its morphology (*toxon* = bow) and an error in the identification of the host as *C. gondi*: its correct name would have been *T. gundii* (Dubey, 2007).

Toxoplasma gondii is a zoonotic protozoan parasite that infects virtually all warm-blooded animals, including wildlife, domestic animals, and humans (Montoya and Liesenfeld, 2004; Dubey, 2010). Host can acquire the infection in numerous ways, and new ways have been suggested and confirmed even quite recently. Since its discovery, the parasite has been found in various hosts and in the environment worldwide (Dubey, 2010), and its DNA retrospectively even in mummies (Khairat et al., 2013).

Toxoplasma gondii has three stages: tachyzoite, bradyzoite, and sporozoite. It is an obligatory intracellular parasite of the nucleated cells of its hosts (Sabin and Olitsky, 1937; Jones et al., 1972; Obornik et al., 2009; Cowper et al., 2012). However, in the oocyst form that is originally produced in enterocytes, the parasite can survive for a long time outside its host (Yilmaz and Hopkins, 1972; Frenkel and Dubey, 1973; Dubey, 1998b; Dubey, 2010). The three crescent-shaped stages are all infective, and their characteristics have been described in detail (Dubey et al., 1998; Speer et al., 1998; Dubey, 2010). In acute toxoplasmosis, rapidly multiplying tachyzoites (2 x 6 µm) dominate. They are able to cross the placenta, but if ingested, they are usually destroyed in the stomach. Bradyzoites and sporozoites are the infective forms typically acquired by ingestion. Hundreds of bradyzoites (1-3 x 5-8.5 µm) are enclosed in tissue cysts in chronically infected hosts. Eight sporozoites (2 x 6-8 µm) are present in each sporulated oocyst (11 x 13 µm), millions of which are shed into the environment unsporulated by the definitive hosts of the parasite.

Toxoplasma gondii appears to be one of the most common and widespread parasites in the world. Approximately one third of the human population is infected (Birgisdottir et al., 2006; Moncada and Montoya, 2012; Flegr, 2013b). However, these estimates overlook infections in animal hosts and the environmental reservoir. In both human and animal hosts, *T. gondii* infections often go underdiagnosed, underreported (European Food Safety Authority EFSA, 2007), and underestimated. For example, chronic, latent infections in humans have been considered harmless, but new evidence suggests associations with neurological or psychiatric diseases such as schizophrenia (Torrey et al., 2007; Niebuhr et al., 2008; Miman et al., 2010a; Miman et al., 2010b; Pedersen et al., 2011; Kusbeci et al., 2011; Torrey et al., 2012; Horacek et al., 2012; Webster et al., 2013; Flegr, 2013a).

Toxoplasma gondii infections are ever common. In some countries, but not all, the prevalence of *T. gondii* infections in humans has been decreasing (Lappalainen, 1995; Kortbeek et al., 2004; Welton and Ades, 2005; Jones et al., 2007; Pappas et al., 2009; Villena et al., 2010; Hofhuis et al., 2011; Kooskora et al., 2013). In France, for example, the seroprevalence among pregnant women was 84% in the 1960s, 54% in the 1990s, and 44% in 2003 (Villena et al., 2010). In Tartu, Estonia, the seroprevalence was 61.8% in 1991-1993 (Pehk, 1994) and 54.9% in 1999-2001 (Birgisdottir et al., 2006), and a recent nationwide estimate (56.4%) indicates it is still high (Kooskora et al., 2013). The prevalence of *T. gondii* infections has decreased in some animals, such as pigs, likely due to their confinement indoors (Edelhofer, 1994; Dubey et al., 1995; Dubey, 2009b). This trend may reverse, however, due to more animal-friendly production systems, as was observed in Switzerland (Berger-Schoch et al., 2011). Nevertheless, in the larger picture encompassing wildlife, domestic animals, and humans, *T. gondii* infection appears to have remained common (Dubey, 2010).

2.1.1. Life cycle

Toxoplasma gondii is transmitted in diverse ways, and its life cycle is often described as 'complex'. However, completing the life cycle is not necessary for its existence. *Toxoplasma gondii* reproduces both asexually and sexually, lies dormant in the hosts under the control of the hosts' immune responses, and survives in the environment.

Carnivorism and vertical transmission to unborn offspring were the modes of transmission of *T. gondii* first discovered (Wolf et al., 1939; Weinman and Chandler, 1954; Dubey, 2010). As recently as only 50 years ago, researchers demonstrated the role of cat feces in the life cycle of *T. gondii* (Hutchison, 1965; Hutchison et al., 1969; Sheffield and Melton, 1969; Hutchison et al., 1970; Frenkel et al., 1970; Overdulve, 1970; Weiland and Kuhn, 1970; Witte and Piekarski, 1970; Dubey et al., 1970a; Dubey et al., 1970b; Hutchison et al., 1971; Dubey, 2009a). These three modes of transmission are usually cited as the main ones; the infection can be congenital, acquired by ingesting tissue cysts, or acquired by ingesting oocysts.

However, horizontal transmission of tachyzoite stages is also possible. Transmission assumably via bodily fluids has been reported in a herd of reindeer (*Rangifer tarandus*) in Russia (Kolychev et al., 1966; Oksanen and Buxton, 1999; Prestrud et al., 2010) and was suspected in squirrel monkeys (*Saimiri sciureus*) living in captivity (Furuta et al., 2001; Carme et al., 2009a). Various other ways of introducing *T. gondii* infection have also been reported, including iatrogenic transmission by organ transplantation or blood transfusion, laboratory accidents, and a stab wound from a butcher's knife (Siegel et al., 1971; Ferre et al., 1986; Herwaldt and Juranek, 1993; Herwaldt, 2001; Derouin et al., 2008). Studies have suggested and demonstrated venereal transmission in some hosts (Dubey and Sharma, 1980; Blewett et al., 1982; Arantes et al., 2009; Santana et al., 2010; Dass et al., 2011; Wanderley et al., 2013), and *T. gondii* has been detected in the semen of humans

(Disko et al., 1971). The milk of infected animals may contain infective *T. gondii* parasites, and any type of milk is considered a potential source of infection if consumed unpasteurized (European Food Safety Authority EFSA, 2007; Dehkordi et al., 2013).

A typical *T. gondii* life cycle illustration depicts a definitive host and an intermediate host (Figure 1), and often humans as noteworthy victims of the spillover of the cycle. A cat becomes infected after eating a rodent, and the oocysts shed by the cat are the infection source for the rodent. This resembles the simple life cycles of other parasites with predator-prey and feco-oral transmission, such as the zoonotic cestode *Echinococcus multilocularis*. This simplification fails to encompass the full complexity of known transmission routes, and that the parasite can propagate even in the absence of its definitive hosts, Felidae, and definitely without humans, who are usually dead-end hosts.

Figure 1 Simplified life cycle of *Toxoplasma gondii* showing a felid and a pregnant rodent; the feco-oral transmission route from a definitive host to another host, carnivorism, and vertical transmission to a fetus (Illustrator: Brian Lassen, 2013).

The sexual reproduction of *T. gondii* can take place only in the intestines of the definitive hosts, and the outcome is the shedding of oocysts in the feces (Hutchison et al., 1969;

Frenkel et al., 1970; Dubey et al., 1970a; Dubey et al., 1970b; Dubey, 2009a). From an epidemiological perspective, the domestic cat is considered the most important definitive host (Dabritz and Conrad, 2010). Cats typically encounter the parasite through carnivorism and require only one bradyzoite to become infected and subsequently shed millions of oocysts after three to ten days (Dubey, 1998a; Dubey, 2001; Dubey, 2009a; Dubey, 2010). The prepatent period, from infection to shedding, is longer after ingesting oocysts, suggesting adaptation to infection from tissue cysts in cats (Dubey, 1998a; Dubey, 2006; Dubey, 2010). Although the shedding time is limited, with a median of eight days yet up to three weeks, the number of oocysts produced can range from 3 to 810 million (Dubey, 2001; Dabritz and Conrad, 2010). The oocysts sporulate in one to five days depending on aeration and temperature (Dubey, 2010). The sporulated oocysts are infectious and highly resistant to environmental conditions (Yilmaz and Hopkins, 1972; Frenkel and Dubey, 1973; Dubey, 1998b; Dubey, 2010). Most cats shed only after their primary infection, but reshedding is possible (Dubey, 1976; Dubey, 1995; Dubey, 2010).

Data from studies from all parts of the world show that the majority of *T. gondii* infections in various host species are subclinical and chronic (Dubey, 2010). The parasites multiply asexually in the cells of the hosts, and the immune responses of the hosts keep the parasites under control in an almost perfect balance of co-existence (Maubon et al., 2008). Hosts carry the infection in their tissues presumably for their lifetime, and the parasites are transmitted to new hosts through carnivorism, cannibalism, and scavenging, with different hosts eating other hosts and even each other (Weinman and Chandler, 1954; Jones and Dubey, 2012). This kind of transmission alone is sufficient for many parasites (e.g. the zoonotic nematode *Trichinella* sp.).

Non-felid hosts harboring *T. gondii* are often referred to as 'intermediate hosts', but they are unnecessary for the life cycle of the parasite. At least theoretically, the parasite could survive in a host population of only definitive hosts because oocysts are also infective to them. In this work, 'intermediate hosts' are referred to simply as 'hosts'. The definitive hosts also serve as hosts, as do all the others: the parasite stays in their tissues and is infective if ingested by another host. A definitive host may also become infected by eating another definitive host harboring the parasite.

The parasite invades and multiplies in a broad spectrum of cell types, but eventually encysts in muscle tissue and the CNS (Dubey, 1997; Berenreiterova et al., 2011; Cowper et al., 2012). The CNS is a good hiding place from the host's immune responses and might also play a catalyzing role in the life cycle (Flegr, 2013a; Flegr, 2013b). The cycle from rodents to cats has been suggested to be enhanced by the parasite manipulating the behavior of rodents, as *T. gondii* infected rats appear to be attracted to the odor of cat urine (Berdoy et al., 1995; Berdoy et al., 2000; Webster, 2001; Vyas et al., 2007; House et al., 2011; Berenreiterova et al., 2011). Moreover, possible parasitic manipulation of mate choice that could further favor venereal transmission has been observed in rats: uninfected female rats preferred *T. gondii* infected male rats (Dass et al., 2011).

2.1.2. Genetic diversity

The genus *Toxoplasma* (Nicolle and Manceaux, 1909) belongs to the phylum Apicomplexa (Levine, 1970), class Sporozoasida (Leuckart 1879), sub-class Coccidiasina (Leuckart 1879), order Eimeriorina (Leger 1911), family Toxoplasmatidae (Biocca, 1956), and has only one species, *T. gondii.* The genome of *T. gondii* is about 65 Mb, not much larger than that of *Neospora caninum*, but almost three times that of *Plasmodium falciparum* and seven times that of *Cryptosporidium parvum* (Khan et al., 2007; Reid et al., 2012; TOXO DB Toxoplasma Genomics Resource, 2013).

Toxoplasma gondii is a haploid organism, whereby only the fertilized macrogamete is diploid. Fertilization is unnecessary for the parasite's progeny to have a full genome, as it comprises one set of chromosomes. Parthenogenesis appears sufficient, or even likely, for the production of oocysts due to the low numbers of microgametes formed compared with macrogametes (Ferguson, 2002).

Because *T. gondii* has no sex chromosomes, the self-fertilization of *T. gondii* macrogametocytes by clonal microgametes of the same strain is not only possible, but is apparently common (Ferguson, 2002). Clonality appears to be widespread, but is not a major disadvantage: the sexual cycle can be skipped due to the efficient asexual reproduction of *T. gondii* by endodyogeny and dispersal via carnivory (Ferguson, 2002). Moreover, the endemic parasites of low genetic and phenotypic variation are likely better tolerated by the hosts that co-evolved with them (Ajzenberg et al., 2004; Brown et al., 2005).

The clonal population structure comprising three closely related clonal lineages (genotypes I, II, and III) formed the basis for understanding genetic variation in *T. gondii* and its geographical and clinical implications (Tibayrenc et al., 1991; Sibley and Boothroyd, 1992; Howe and Sibley, 1995; Dardé, 1996; Ajzenberg et al., 2004; Dardé et al., 2007). Sequence similarity and dimorphic allelic composition among the lineages indicate a recent common ancestry; the lineages diverged within the past 10 000 years, and their widespread distribution is due to asexual transmission (Grigg et al., 2001; Su et al., 2003; Khan et al., 2006; Khan et al., 2007). More recently, a fourth clonal lineage was described and commonly found in wildlife in North America (Khan et al., 2011).

A highly clonal population structure is possible for *T. gondii* because it does not need the sexual cycle in the definitive hosts (Ferguson, 2002), nor even the definitive hosts at all (Howe and Sibley, 1995; Su et al., 2003; Khan et al., 2006). Its clonal population structure can be maintained when the definitive hosts are rarely infected simultaneously with more than one strain of the parasite (Ajzenberg et al., 2004). The occurrence of cross-fertilization depends on the prevalence of the infection, the diversity of the strains, and the occurrence of mixed infections in the prey animals and meat-producing animals. The felids, including domestic cats, are predators that hunt substantial numbers of prey of different species (Liberg, 1984; Loss et al., 2013) and may also be fed meat from several

animals, which provides opportunities for sexual recombination. In southern Sweden in the 1970s, for example, 63% of fecal samples from house cats contained the remains of a variety of vertebrate prey (Liberg, 1984). The intake of natural prey by these cats was estimated at 66 g/day, one fourth that of feral cats. Predation appears to occur regardless of whether cats receive other feed from humans (Liberg, 1984; Loss et al., 2013), and large fractions of *T. gondii* infections in domestic cats have been attributed to hunting prey as well as receiving raw meat as part of their diet (Opsteegh et al., 2012).

Recombination does occur, giving rise to genetically divergent strains. Greater diversity seems to be associated with greater host diversity (Ajzenberg et al., 2004) and less anthropization, or human influence on the environment (Mercier et al., 2011). 'Atypical' genotypes of the parasite have been linked to severe clinical manifestations in humans, especially in South America (De Salvador-Guillouet et al., 2006; Elbez-Rubinstein et al., 2009; Carme et al., 2009b; Delhaes et al., 2010; Subauste et al., 2011; Demar et al., 2012).

Multilocus typing has revealed more polymorphisms (Ajzenberg, 2010; Minot et al., 2012). Current molecular typing methods are also able to distinguish closely related isolates (Ajzenberg et al., 2010). Three types of methods are in widespread use. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods are based on variation at and distances between restriction sites: analysis of the size of DNA fragments that are produced by amplifying selected DNA sequences and digesting them subsequently with restriction enzymes (Howe et al., 1997; Khan et al., 2005; Dubey et al., 2006; Su et al., 2006; Prestrud et al., 2008). Microsatellite (MS) methods are based on analysis of the length polymorphism of microsatellite markers: variation in the numbers of short repeat motifs (Ajzenberg et al., 2005; Ajzenberg et al., 2010). Multilocus sequencing typing (MLST) is a nucleotide sequence-based approach that detects further polymorphism, but is costly for screening large sample sets (Ajzenberg et al., 2010; Su et al., 2012). Phylogenetic studies of a large dataset of combined results obtained with these three methods revealed 138 distinct genotypes, most of which could be grouped into 15 haplogroups and further into six major clades (Su et al., 2012). Representative strains of each haplogroup are included in a whole genome sequencing effort. The study provided new information on the genetic diversity and, to some extent, on geographical differences in the distribution of this diversity. Some parts of the world remain poorly represented, however: in particular, no strains from Finland, the Baltic countries, or Russia were analyzed (Su et al., 2012).

Since the beginning of the molecular typing of *T. gondii* strains, different genotypes have been linked to differences in their virulence in hosts: namely, the outcome of experimental inoculation into laboratory mice and the severity of human disease (Sibley and Boothroyd, 1992; Howe and Sibley, 1995; Dardé, 1996; Dardé et al., 1998; Ajzenberg et al., 2002; Ajzenberg, 2006; Dardé et al., 2007; Ajzenberg et al., 2009; Ajzenberg, 2010; Subauste et al., 2011; Fekkar et al., 2011; Carneiro et al., 2013). Fewer than ten tachyzoites of a virulent genotype I strain can kill a laboratory mouse, while genotypes II and III typically cause only chronic infections in mice (Maubon et al., 2008). Atypical genotypes have been

associated with severe clinical toxoplasmosis, even in immunocompetent humans (Sobanski et al., 2013). Genetic characterization of the *T. gondii* strains that cause disease and death in different host species deepens our understanding of the factors that affect the outcome of the infection, especially of the contribution of the characteristics of the parasite.

While the association between the *T. gondii* genotype and clinical outcome has received attention in human medical parasitology (Dardé et al., 2007; Ajzenberg, 2010), few similar data are available from naturally-acquired infections in non-human animal hosts. The clonal genotype II is dominant and endemic in Europe (Aubert et al., 2010; Subauste et al., 2011; Fekkar et al., 2011; Herrmann et al., 2013) and is associated mainly with mild clinical manifestations in immunocompetent humans (Sobanski et al., 2013). Human medical doctors appear to be more concerned about the imported atypical strains that cause more severe disease (Sobanski et al., 2013). From Europe, however, descriptions are available of one domestic cat, one beaver (*Castor fiber*), and four arctic foxes (*Vulpes lagopus*) that died of infections with genotype II strains without predisposing immunodeficiency (Prestrud et al., 2008; Spycher et al., 2011; Herrmann et al., 2013). Moreover, material from ovine abortion cases indicated an association of genotype II parasites, but not of genotype III parasites, with histopathological lesions consistent with toxoplasmosis (Gutierrez et al., 2012). These results are surprising because they contradict the assumption that strains belonging to genotype II would cause only mild, chronic infections (Dardé et al., 2007; Maubon et al., 2008).

2.1.3. Limits

Although the immune responses of the host fight the *T. gondii* infection, the battle is usually lost, and the attack leads to permanent occupation. Resistance is not futile, however; the host's immune response is needed to establish a balanced chronic infection that enables the host and the parasite to co-exist (Maubon et al., 2008). With no or an insufficient immune response, the parasites profilerate uncontrolled or are reactivated, and the infection may become fulminant. Instead of reaching a balanced latency, the host and its parasites may die from the resultant overwhelming infection (Maubon et al., 2008).

The infective stages differ in their resistance to external conditions. Tachyzoites are the easiest to destroy, and the conditions of the stomach are usually sufficient to kill them (Dubey, 2010). Pasteurization or boiling destroys any tachyzoites and bradyzoites present in milk. When placed in an optimal freezing medium, *T. gondii* parasites grown in cell culture can be cryopreserved (Dubey, 2010).

The tissue stages of *T. gondii* including tissue cysts can be killed by temperatures of 67°C or higher (Dubey, 2010). Cooking in a microwave oven, salting, curing, and pickling are unreliable ways of killing tissue cysts (Lundén and Uggla, 1992; Dubey, 2010). Freezing generally appears to kill tissue cysts, at least at -20°C for 54 hours, but the parasite may

survive freezing conditions (Lundén and Uggla, 1992; Dubey, 2010). A Russian study reported that *T. gondii* survived in reindeer meat at -11°C to -56°C for up to 28 days (Kolychev, 1969). At 4°C, *T. gondii* tissue cysts may remain viable for more than two months (Jacobs et al., 1960). Rotting of the tissue around the cysts appears to have little effect, as an isolate was obtained even from a bird largely consumed by maggots (Work et al., 2000).

The sporulated oocysts are highly resistant to environmental conditions (Yilmaz and Hopkins, 1972; Frenkel and Dubey, 1973; Dubey, 1998b; Dubey, 2010). In natural conditions outdoors, in shade and exposed to a temperature range of 5.5-35.5°C, they survived in cat feces for 2.5 months uncovered, and almost for a year covered (Yilmaz and Hopkins, 1972). Experimental studies have shown that oocysts remain infective for up to 54 months at 4°C and for four weeks frozen at -21°C, but were killed in one minute at 60°C (Frenkel and Dubey, 1973; Dubey, 1998b).

The effect of common disinfectants on oocysts is weak; the available reagents have no effect or require an impractically long contact time to be effective (Wainwright et al., 2007b; Dubey, 2010). Several pharmaceutical compounds have proved anti-toxoplasmic (Dubey, 2010). The aims of treatment are to control active infection and to prevent its transplacental transmission (Moncada and Montoya, 2012).

Additional studies on the survival of *T. gondii* are needed to evaluate available preventive measures and treatment options as well as to design new ones. One limitation common to many available studies is that they used few different strains of the parasite. Local strains might be more adapted to local conditions (Kolychev, 1969).

2.2. *Toxoplasma gondii* in its hosts

For *T. gondii*, any nucleated cell in a warm-blooded animal or in favorable *in vitro* circumstances is a good home. In a host, immunity should restrict the parasite to a chronic state of infection for mutual longer life (Maubon et al., 2008). A good host is one that becomes infected with a low infection dose and has several means of acquiring the infection, but is little affected by it. A host that, after infection, is likely to be eaten by another host ensures the parasite a longer life than just one host lifetime.

Toxoplasmosis is an infectious disease and upon infection, the parasite invades the tissues of its host. This should be distinguished from the contamination of food with infective stages of the parasite. Sometimes *T. gondii* infections are referred to as 'contamination', especially meat of infected animals as 'contaminated meat' (e.g. Halos et al., 2010; Pastiu et al., 2013; Centers for Disease Control and Prevention, 2013).

Most hosts assist in propagating the parasite to new hosts. Felids may contribute to the environmental oocyst reservoir by shedding, and some also support the survival of the oocysts with their habit of covering their freshly deposited feces with soil. Soil coverage likely extends the oocysts' survival (Yilmaz and Hopkins, 1972). The numbers of oocysts shed are high, making the felids highly amplifying hosts. Migrating and traveling hosts introduce the parasite to new areas (Prestrud, 2008). Chronically infected hosts serve as an amplifying reservoir: enrichment by asexual reproduction and spreading to different tissues enables the subsequent infection of even several new hosts via carnivorism and scavenging (Weinman and Chandler, 1954). The associations of chronic infection with changes in behavior, memory, and neurologic disorders (Berdoy et al., 1995; Berdoy et al., 2000; Webster, 2001; Vyas et al., 2007; Torrey et al., 2007; Pedersen et al., 2011; Dass et al., 2011; House et al., 2011; Berenreiterova et al., 2011; Pedersen et al., 2012; Torrey et al., 2012; Webster et al., 2013; Flegr, 2013a) are intriguing, especially as some of them appear to benefit the parasite in infecting new hosts.

It is most often detrimental for a parasite to kill its host. The stage of *T. gondii* dominating in acute fulminant toxoplasmosis is the tachyzoite, the most delicate and the least infective stage by ingestion to scavengers; killing the host outright usually results in the death of those tachyzoites as well (Maubon et al., 2008).

The majority of *T. gondii* infections in most host species are subclinical or asymptomatic, and chronic (Dubey, 2010); the parasite remains dormant in the tissues of the host until reactivation or until it is eaten by another host. Thus, most hosts survive the infection and acquire life-long immunity, while likely harboring the parasite in their tissues for the rest of their lives (Dubey, 2010). The immune response of the host is important for the parasite as well; without it, the infection would become fulminant (Maubon et al., 2008). Chronic, latent *T. gondii* infections can generally be detected indirectly by screening for the presence of specific antibodies produced against the parasite (Dubey, 2010). In most host

species, chronic infections are common: the seroprevalence numbers are high, and the incidence of clinical toxoplasmosis is low (Dubey, 2010).

Using serology to evaluate the prevalence of *T. gondii* infection in the hosts has its limitations. First, serology is an indirect method that detects only the humoral immune response against the pathogen: seronegative animals have been found to be infected or have tested positive for the parasite's DNA (Dubey et al., 1995; Opsteegh et al., 2011b). Serology cannot detect cross contamination, which can be regarded as a limitation of its use in monitoring at slaughterhouses. On the other hand, the immune response is systemic, whereas direct detection methods, such as PCR or bioassay, applied on small samples may miss a low or unevenly distributed parasite burden. Second, screening usually only targets IgG antibodies, methods are rarely validated or even suitable for all hosts, and cut-offs for seropositivity are often somewhat arbitrary (Dubey, 2010). Third, the assumption of the persistence of antibodies has been recently questioned in some hosts species (Opsteegh et al., 2011a; Fernandez-Aguilar et al., 2013). Regardless, advancements in serology have greatly changed our understanding of the spread, duration, origin, and dynamics of *T. gondii* infections (Sabin and Feldman, 1948; Fulton and Turk, 1959; Hedman et al., 1989; Lappalainen et al., 1993; Hill et al., 2011).

As rare exceptions, congenital toxoplasmosis has been described in previously seropositive mothers, possibly after infection with another parasite strain (Delhaes et al., 2010) or due to reactivation of a chronic infection (Andrade et al., 2010). The immunity of the host that is measurable by serology cannot thus be regarded as fully protective. This is in contrast to the common assumption behind screening programs for pregnant women.

Pregnant hosts appear to be relevant for *T. gondii*. Vertical transmission to unborn offspring can prove fatal or cause various clinical manifestations of congenital toxoplasmosis in them, but it is also a means of transmission for the parasite (Duncanson et al., 2001; Williams et al., 2005; Hide et al., 2007; Hide et al., 2009). At least in some hosts, including some strains of mice, sheep, Pallas cats, sand cats, and – at least in rare cases – humans, vertical transmission appears not to be limited to primary infection, but also occur during chronic infections, and even repeatedly (Beverley, 1959; Brown et al., 2005; Williams et al., 2005; Pas and Dubey, 2008, Andrade et al., 2010).

Severe forms of congenital toxoplasmosis are probably the most feared clinical manifestations of *Toxoplasma gondii* infection in humans, but the parasite can also cause a variety of clinical signs and symptoms after a postnatally acquired infection, and even prove fatal to its host (Montoya and Liesenfeld, 2004; McAllister, 2005; Demar et al., 2007; Dubey, 2010; Moncada and Montoya, 2012). In immunocompetent humans, the infection is asymptomatic in most cases or manifests as cervical or occipital lymphadenopathy that can last for months (Montoya and Liesenfeld, 2004). Even apparently asymptomatic or mild infections cannot be considered harmless (McAllister, 2005; Flegr, 2013b). Severe, generalized forms of the disease can also occur in the immunocompetent (Dardé et al., 1998; Montoya and Liesenfeld, 2004; McAllister, 2005;

Demar et al., 2007; Demar et al., 2012; Sobanski et al., 2013). Ocular toxoplasmosis, toxoplasmic chorioretinitis, was previously interpreted as a late manifestation of congenitally acquired infection, but can be also the clinical outcome of an acquired infection (Montoya and Liesenfeld, 2004; Subauste et al., 2011; Vasconcelos-Santos, 2012). Immunocompromised humans are at risk for toxoplasmic encephalitis, pneumonia, and other severe forms of toxoplasmosis (Lappalainen et al., 1998; Montoya and Liesenfeld, 2004; Edvinsson et al., 2009; Ajzenberg et al., 2009).

Both host and parasite factors affect the outcome of *T. gondii* infection, and the environment may also have an effect (Dardé et al., 2007; Maubon et al., 2008; Subauste et al., 2011). The immunostatus of the host is a major factor: *T. gondii* has been an important cause of morbidity and mortality in AIDS patients, especially before the introduction of highly active antiretroviral therapy (Luft and Remington, 1992; Nissapatorn, 2009), and iatrogenic immunosuppression may reactivate a latent infection in an organ recipient (Lappalainen et al., 1998).

The clinical picture of toxoplasmosis in animal hosts can resemble those described in humans (Dubey, 2010). Most infections remain subclinical, which does not necessarily mean that they are asymptomatic. Clinical signs are something others observe, whereas symptoms are subjective and can go unnoticed, yet still affect the welfare of the animal. Clinical signs are largely unspecific, and virtually all imaginable clinical signs have been listed among the possible manifestations in, for example, feline toxoplasmosis (Dubey and Lappin, 2006). Vertical transmission of the infection has been described in numerous hosts (Dubey et al., 2008; Hide et al., 2009; Dubey, 2010) and is of major veterinary importance, especially in sheep and goats (Duncanson et al., 2001; Williams et al., 2005; Hide et al., 2007; Hide et al., 2009; Dubey, 2009c; Dubey, 2010). Generalized, fatal infections have also been described in many host species (Dubey, 2010; Spycher et al., 2011). Reports of treatment success in confirmed severe clinical toxoplasmosis cases are few compared with the number of reported fatalities (Dubey, 2010).

Some host species appear to be highly susceptible to severe clinical toxoplasmosis. These species include European brown hares, mountain hares, Eurasian red squirrels, Australian marsupials, New World monkeys, Pallas cats, and sand cats (Rodhain, 1950; Christiansen and Siim, 1951; Riemann et al., 1974; Gustafsson, 1997; Ketz-Riley et al., 2003; Brown et al., 2005; Pas and Dubey, 2008; Dubey, 2010). The specific reasons behind such susceptibility remain largely unknown, but appear to include host genetics (Maubon et al., 2008; Subauste et al., 2011), differences in mounting an appropriate immune response (Gustafsson et al., 1997b; Ketz-Riley et al., 2003), and possibly a lack of co-evolution (Brown et al., 2005). Interestingly, in the hares living in Scandinavia, researchers have long observed that mortality due to toxoplasmosis is highest in the winters (Hülphers et al., 1947; Christiansen and Siim, 1951). Many other factors such as the infective stage of the parasite causing the infection, the infection dose, the infection route, and especially the characteristics of the *T. gondii s*trains causing the severe and fatal infections have seen little investigation (Prestrud et al., 2008; Spycher et al., 2011; Herrmann et al., 2013).

Genetic characterization of the *T. gondii* strains that infect different hosts in different parts of the world deepens our understanding of the molecular epidemiology of this parasite in its hosts and may reveal possible separate and overlapping cycles, reservoirs and spillovers (Dardé et al., 2007).

2.3. *Toxoplasma gondii* in the environment

Toxoplasma gondii has an environmental reservoir of durable oocysts. The parasite is present in soil and water, and the sporulated oocysts can survive the temperature ranges and other conditions of most locations (Yilmaz and Hopkins, 1972; Frenkel and Dubey, 1973; Dubey, 1998b; Dubey, 2010).

The environmental reservoir of oocysts forms an important source of *T. gondii* infection, especially for herbivorous hosts that are unlikely to eat other hosts. Such hosts include domestic animals raised and game animals hunted for human consumption. Reducing the environmental oocyst burden is considered the most effective way to reduce *T. gondii* infections in farm animals (Kijlstra and Jongert, 2008). Of the preventive measures recommended (Dubey, 1996; Tenter et al., 2000; Lehmann et al., 2003; Kijlstra and Jongert, 2009; Dubey, 2009b), the most feasible ones are keeping sties, pastures, feed, and water uncontaminated by feline feces, alongside rodent control, offal hygiene, and securing the interface with wildlife. The sporulated oocysts are environmentally resistant (Yilmaz and Hopkins, 1972; Frenkel and Dubey, 1973; Dubey, 2010) and may spread into the environment from the location where the feces were deposited (McAllister, 2005; Dabritz and Conrad, 2010). Having no cat on the farm is often mentioned as a preventive measure for the farm level, whereas preventing the infections of the cats living on the farm, using currently available preventive measure options, has received less emphasis (Kijlstra and Jongert, 2009). Preventing the infection of felids, especially of domestic cats, would likely have a detectable effect on reducing oocysts in the environment, especially in areas with no feral cats or wild felids (McAllister, 2005).

Due to the environmental oocyst reservoir, *T. gondii* can also be a waterborne pathogen (Dubey, 2004; Jones and Dubey, 2010). Moreover, coastal surface water runoff into the oceans has been linked to deaths in southern sea otters (*Enhydra lutris nereis*) (Miller et al., 2002; Kreuder et al., 2003; Miller et al., 2004; Miller et al., 2008; Shapiro et al., 2012). Considering that conventional sewage and water treatments cannot destroy the sporulated oocysts, which can survive for two years in 4°C sea water (Wainwright et al., 2007a; Wainwright et al., 2007b; Lindsay and Dubey, 2009), our blue planet is widely contaminated with infective forms of the parasite (McAllister, 2005; Dabritz and Conrad, 2010).

2.4. *Toxoplasma gondii* as a zoonotic pathogen

Toxoplasma gondii is a zoonotic parasite: infections from non-human animals to humans and vice versa are possible (Wolf et al., 1939). Humans are probably unimportant hosts from the perspective of the parasite, but human infections are a major reason for the extensive research efforts around this parasite. Due to its numerous infection routes, *T. gondii* infections belong at least to the overlapping lists of foodborne, waterborne, soil-transmitted, milk-transmitted, cat litter box -derived, iatrogenic, bloodborne, occupational, opportunistic, and zoonotic infections (Siegel et al., 1971; Cook et al., 2000; Herwaldt, 2001; Weese et al., 2002; Dubey, 2004; Derouin et al., 2008; Dubey, 2010). Eating undercooked meat of infected animals has been identified as the main risk factor for human infection (Cook et al., 2000).

The various manifestations of toxoplasmosis influence the quality of life of those affected (Kupila et al., 1999; McAllister, 2005; de-la-Torre et al., 2011). At worst, toxoplasmosis can be devastating and even fatal. Moreover, even mild chronic *T. gondii* infections cannot be considered harmless (McAllister, 2005; Torrey et al., 2007; Flegr et al., 2009; Arling et al., 2009; Pedersen et al., 2011; Pedersen et al., 2012; Kankova et al., 2012; Thomas et al., 2012; Torrey et al., 2012; Flegr, 2013b).

In the 1990s in Finland, the lifetime cost per case of congenital toxoplasmosis was estimated to be USD 0.8 million, ranging from USD 0.01 million for a subclinically infected to USD 1.5 million for a severely affected child (Lappalainen et al., 1995b). Estimates of the annual costs of congenital toxoplasmosis were USD 128 per pregnancy; lowering these costs, and thus saving USD 2.1 million per year, might have been achieved by launching a screening program (Lappalainen et al., 1995b).

The disease burden caused by pathogens that differ in the incidence of infections and the severity of their outcomes can be evaluated and compared by expressing them in disability-adjusted life years, or DALYs (Murray, 1994; Murray and Lopez, 1996; Murray and Acharya, 1997). Life years lost, time lived with a disability, and the severity of the disability are all taken into account. Calculations of the DALYs of *T. gondii* infections have emphasized that *T. gondii* belongs among the most important foodborne pathogens, together with *Salmonella* spp. and *Campylobacter* spp. (Kemmeren et al., 2006; Havelaar et al., 2007; Kortbeek et al., 2009; Havelaar et al., 2012). On the population level, the total disease burden of combined congenital and acquired toxoplasmosis (3620 DALYs per year) was higher than the burden of any of the other 13 pathogens investigated, surpassing even that of *Campylobacter s*pp. and *Salmonella* spp. (Havelaar et al., 2012). On an individual level, the disease burden of congenital toxoplasmosis ranked second, after listeriosis (Havelaar et al., 2012).

Another frequently used unit of measure is quality-adjusted life year, or QALY, which emphasize the time lived in good health. In the USA, *T. gondii* infections cost USD 3 billion and account for 11 000 QALYs lost every year (Hoffmann et al., 2012). These

numbers place *T. gondii* among the top five most important pathogens (Hoffmann et al., 2012; Batz et al., 2012). Moreover, when comparing foodborne illnesses by the mortality they cause, *T. gondii* infections ranked second after *Salmonella* spp. (Scallan et al., 2011).

The disease burden and losses caused by a zoonotic pathogen such as *T. gondii* are not limited to humans. The prevention of *T. gondii* infections in its animal hosts carries implications not only for food safety and public health (Sacks et al., 1983; McDonald et al., 1990; Cook et al., 2000; Ross et al., 2001; Kijlstra and Jongert, 2008; Kijlstra and Jongert, 2009; Veterinærinstituttet, 2009), but is also an animal health care measure that ensures the welfare of the animals and prevents the occurrence of possible clinical disease in them. The clinical signs reported from domestic pigs during *T. gondii* outbreaks include high fever, anorexia, dyspnea, vomiting, weakness, recumbency, abortions, and death (Weissenbock and Dubey, 1993; Kim et al., 2009; Dubey, 2009b). The clinical signs observed after experimental infections in sheep include not only abortions and dead lambs, which are known to cause considerable economic loss to sheep husbandry, but also invariably fever and sometimes other signs, such as respiratory distress (Dubey, 2009c). These clinical signs are surely a welfare matter for the individuals affected. In addition to animals intended for human consumption, the animal welfare aspect is also relevant for pet animals. Domestic cats obviously contaminate the environment with oocysts (Dabritz and Conrad, 2010), but may also fall ill themselves and die from the infection (Henriksen et al., 1994; Dubey, 2010): they therefore merit better protection against the infection (Opsteegh et al., 2012).

2.5. *Toxoplasma gondii* in Finland

Few studies of *T. gondii* infections in Finland are available, and most of them date to the 1990s. Estimates of *T. gondii* seroprevalences have been reported in humans and several animal host species (Table 1).

Antibodies against *T. gondii* have been reported in foxes, brown hares and mountain hares (Valtonen and Andersson, 1968), lynx (Oksanen and Lindgren, 1995), semi-domesticated reindeer (Oksanen et al., 1996; Oksanen et al., 1997), domestic pigs (Hirvelä-Koski, 1992), horses (Nieminen, 1993), cats (Näreaho, 1995; Näreaho et al., 1995), and the animals of the Helsinki Zoo (Saarhelo, 1998); the latter three were projects of undergraduate veterinary students.

In humans, overall seroprevalence was 28.7% in a study among farmers and slaughterhouse workers (Seuri and Koskela, 1992) and 20.3% among pregnant women in southern Finland (Lappalainen et al., 1992). An earlier study reported up to 66.7% of pedigree cat owners testing seropositive, a high figure compared with the 33.3% of the control group, even if having a cat in the family was not a risk factor for the infection (Ulmanen and Leinikki, 1975). The most recent estimate available suggests a continuous decrease in seroprevalence, which among participants of a national veterinary congress was 16.3% (Jokelainen et al., 2010).

In the 2000s, between 34 and 48 human toxoplasmosis cases have been reported annually (Zoonoosikeskus/Valtakunnallinen tartuntatautirekisteri, 2013). Severe, clinical cases have been described in Finland in humans (Koskiniemi et al., 1989; Lappalainen et al., 1995a; Lappalainen et al., 1998; Kupila et al., 1999) and dogs (Hollmén et al., 1995). The dogs were a litter of Newfoundland puppies with severe neurological signs, hydrocephalus revealed with diagnostic imaging, and periventricular calcifications detected postmortem. The diagnosis was confirmed with PCR targeting the B1 gene (Burg et al., 1989).

Fatal toxoplasmosis is among the frequently diagnosed infectious causes of death for many animal species in Finland, but exact numbers of cases are available only from the 1960s for nine species (Valtonen and Andersson, 1968). The proportional mortality rates could not be calculated because the total numbers of animals examined were not specified. The species in which toxoplasmosis was diagnosed were brown hare, mountain hare, dog, cat, pig, fox, squirrel, capercaillie (*Tetrao urogallus*), and black grouse (*Lyrurus tetrix*). These diagnoses were not confirmed with specific methods. Toxoplasmosis in pet animals and abortions in sheep due to *T. gondii* have been diagnosed sporadically at Finnish Food Safety Authority Evira (Finnish Food Safety Authority Evira, 2010; Finnish Food Safety Authority Evira, 2012a; Finnish Food Safety Authority Evira, 2012b). Toxoplasmosis is a notifiable disease in all animals except hares, rabbits and rodents (Eläinlääkintölainsäädäntö, 1995), but the official data available appear not to represent the true incidence (European Food Safety Authority EFSA et al., 2012).

The *T. gondii* strains causing the infections in the various hosts were characterized no further. The genotypes of *T. gondii* circulating in Finland are unknown.

An experimental study of two reindeer resulted in the death of one of them on day nine; the clinical disease responded to therapy in the other (Oksanen et al., 1996). The latter had high antibody titers, lived for nearly two years after inoculation and died in a brown bear attack. Experimental feeding of meat from infected reindeer to a seronegative lynx induced seroconversion, but no detectable oocyst shedding (Oksanen et al., 1997).

Table 1 *Toxoplasma gondii* seroprevalences in Finland

	N	n positive	Prevalence (%)	95% CI	Method	Reference
European brown hare *Lepus europaeus*	10	1	10.0	0.5-40.4	DT	(Valtonen and Andersson, 1968)
Mountain hare *Lepus timidus*	34	6	17.6	7.5-33.1	DT	(Valtonen and Andersson, 1968)
Red fox *Vulpes vulpes*	25	5	20.0	7.7-38.9	DT	(Valtonen and Andersson, 1968)
Domestic pig *Sus scrofa domesticus*	1847	47	2.5	1.9-3.3	ELISA	(Hirvelä-Koski, 1992)
Horse *Equus ferus caballus*	328	218	66.5	61.2-71.4	EIA	(Nieminen, 1993)
Reindeer *Rangifer tarandus tarandus*	2577	24	0.9	0.6-1.4	DAT	(Oksanen et al., 1997)
Domestic cat *Felis catus*	141	63	44.7	36.6-53.0	DAT	(Näreaho, 1995; Näreaho et al., 1995)

(continued)	N	n positive	Prevalence (%)	95% CI	Method	Reference
Eurasian lynx	70	51	72.9	61.6-82.3	DAT	(Oksanen and Lindgren, 1995)
Lynx lynx	8^{z}	8	100.0	68.8-100.0	DAT	(Saarhelo, 1998)
Asian lion *Panthera leo persica*	7^{z}	6	85.7	47.0-99.3	DAT	(Saarhelo, 1998)
Lion *Panthera leo*	2^{z}	2	100.0	22.4-100.0	DAT	(Saarhelo, 1998)
Amur leopard *Panthera pardus orientalis*	9^{z}	4	44.4	16.1-76.0	DAT	(Saarhelo, 1998)
Snow leopard *Uncia uncia, Panthera uncial*	36^{z}	30	83.3	68.5-93.0	DAT	(Saarhelo, 1998)
Tiger *Panthera tigris*	4^{z}	1	25.0	1.3-75.8	DAT	(Saarhelo, 1998)
Kodiak bear *Ursus arctos middendorffi*	1^{z}	0	0.0	0.0-95.0	DAT	(Saarhelo, 1998)
Red panda *Ailurus fulgens*	2^{z}	2	100.0	22.4-100.0	DAT	(Saarhelo, 1998)

(continued)	N	n positive	Prevalence (%)	95% CI	Method	Reference
Wolverine *Gulo gulo*	3 [z]	2	66.7	13.2-98.3	DAT	(Saarhelo, 1998)
Alpine ibex *Capra ibex*	31 [z]	4	12.9	4.2-28.3	DAT	(Saarhelo, 1998)
Blackbuck *Antilope cervicapra*	4 [z]	0	0.0	0.0-52.7	DAT	(Saarhelo, 1998)
Chamois *Rupicapra rupicapra*	18 [z]	4	22.2	7.5-45.3	DAT	(Saarhelo, 1998)
Guanaco *Lama guanicoe*	5 [z]	2	40.0	7.3-81.8	DAT	(Saarhelo, 1998)
Barbary sheep *Ammotragus lervia*	6 [z]	0	0.0	0.0-39.3	DAT	(Saarhelo, 1998)
Moose *Alces alces*	6 [z]	6	100.0	60.7-100.0	DAT	(Saarhelo, 1998)
Yak *Bos mutus, Bos grunniens*	3 [z]	3	100.0	36.8-100.0	DAT	(Saarhelo, 1998)

(continued)	N	n positive	Prevalence (%)	95% CI	Method	Reference
Fallow deer *Dama dama*	19 [z]	19	100.0	85.4-100.0	DAT	(Saarhelo, 1998)
Mountain goat *Oreamnos americanus*	25 [z]	10	40.0	22.4-59.8	DAT	(Saarhelo, 1998)
Markhor *Capra falconeri heptneri*	36 [z]	22	61.1	44.6-75.9	DAT	(Saarhelo, 1998)
European forest reindeer *Rangifer tarandus fennicus*	25 [z]	5	20.0	7.7-38.9	DAT	(Saarhelo, 1998)
Musk ox *Ovibos moschatus*	3 [z]	3	100.0	36.8-100.0	DAT	(Saarhelo, 1998)
Himalayan tahr *Hemitragus jemlahicus*	23 [z]	4	17.4	5.8-36.8	DAT	(Saarhelo, 1998)
White-tailed deer *Odocoileus virginianus*	11 [z]	3	27.3	7.5-57.8	DAT	(Saarhelo, 1998)
Wapiti *Cervus Canadensis*	11 [z]	8	72.7	42.2-92.6	DAT	(Saarhelo, 1998)

(continued)	N	n positive	Prevalence (%)	95% CI	Method	Reference
Wisent, European bison *Bison bonasus*	10 [z]	5	50.0	21.2-78.8	DAT	(Saarhelo, 1998)
Kulan *Equus hemionus kulan*	5 [z]	5	100.0	54.9-100.0	DAT	(Saarhelo, 1998)
Plains zebra *Equus quagga*	2 [z]	0	0.0	0.0-77.6	DAT	(Saarhelo, 1998)
Przewalski's wild horse *Equus przewalskii*	4 [z]	0	0.0	0.0-52.7	DAT	(Saarhelo, 1998)
Hamadryas baboon *Papio hamadryas*	1 [z]	0	0.0	0.0-95.0	DAT	(Saarhelo, 1998)
Common marmoset *Callithrix jacchus*	3 [z]	0	0.0	0.0-63.2	DAT	(Saarhelo, 1998)
Grivet *Chlorocebus aethiops*	9 [z]	1	11.1	0.6-43.9	DAT	(Saarhelo, 1998)
Red wallaby *Macropus rufogriseus*	3 [z]	2	66.7	13.2-98.3	DAT	(Saarhelo, 1998)

(continued)	N	n positive	Prevalence (%)	95% CI	Method	Reference
Barnacle goose *Branta leucopsis*	10 [z]	2	20.0	3.5-52.0	DAT	(Saarhelo, 1998)
Human, veterinarians	317	51	16.1	12.4-20.4	ELFA	(Jokelainen et al., 2010)
Human, pregnant women	16733	ND	20.3	19.6-21.1*	Indirect ELISA	(Lappalainen et al., 1992)
Human, slaughterhouse workers	159	40	25.2	18.9-32.3	ELISA	(Seuri and Koskela, 1992)
Human, pig farmers	142	53	37.3	29.7-45.5	ELISA	(Seuri and Koskela, 1992)
Human, grain or berry farmers	106	24	22.6	15.4-31.3	ELISA	(Seuri and Koskela, 1992)
Human, pedigree cat owners	123	67	54.5	45.6-63.1	CF	(Ulmanen and Leinikki, 1975)
	120	80	66.7	57.9-74.7	IFAT	(Ulmanen and Leinikki, 1975)
Human, medical students	223	51	22.9	17.7-28.7	CF	(Ulmanen and Leinikki, 1975)
	223	93	41.7	35.4-48.3	IFAT	(Ulmanen and Leinikki, 1975)
Human, controls	123	43	35.0	26.9-43.7	CF	(Ulmanen and Leinikki, 1975)
Homo sapiens	96	32	33.3	24.5-43.2	IFAT	(Ulmanen and Leinikki, 1975)
	69	12	17.4	9.8-27.7	ELFA	(Jokelainen et al., 2010)

CI = confidence interval, Mid-P Exact
ND = no data
* Confidence interval from the original publication.
[z] = Animals from the Helsinki Zoo.

Toxoplasma gondii appears to be largely unchallenged in Finland. The infections are not monitored at slaughterhouses (Eläinlääkintölainsäädäntö, 2011), and the health care programs of farm animals appear to place little emphasis on preventing them (Eläintautien torjuntayhdistys ETT ry, 2013; Finnish Food Safety Authority Evira, 2013b). No vaccine is commercially available against toxoplasmosis for any host species, and no pharmaceutical product is specifically registered for its treatment in any animal host species (Kariaho, 2013). Pregnant women receive some advice on how to avoid *T. gondii* infection (National Institute for Health and Welfare, 2013a; National Institute for Health and Welfare, 2013b), and toxoplasmosis is acknowledged as an occupational hazard for veterinarians during pregnancy (Reijula et al., 2005), but no systematic screening of pregnant women has been launched despite the calculations and recommendations of the 1990s (Lappalainen et al., 1995b). Toxoplasmosis is missing from the list of zoonoses of the National Institute for Health and Welfare (National Institute for Health and Welfare, 2013c). *Toxoplasma gondii* was not even mentioned in the risk analysis report on raw milk produced in Finland (Perkiömäki et al., 2012), nor included on the list of 11 main zoonotic pathogens in the national zoonoses strategy for 2013-2017 (Ministry of Agriculture and Forestry and Ministry of Social Affairs and Health, 2013).

3. Aims of the study

The general aim of this study was to clarify the molecular epidemiology of *T. gondii* in Finland, especially for possible evidence of separate domestic and sylvatic cycles. The specific aims were:

1. to estimate the prevalence of specific anti-*Toxoplasma gondii* IgG antibodies (I, II, III, V, VI) and the incidence of naturally acquired fatal toxoplasmosis (III, IV, V, VI) in selected wild and domestic host species, as well as the percentage of possible definitive hosts shedding *T. gondii* oocysts (V, VI) in Finland

2. to determine and evaluate risk factors for *T. gondii* infections in these hosts (I, II, III, IV, V, VI)

3. to investigate the toxoplasmosis cases in detail from the point of view of both the host, by describing the pathological changes, and the parasite, by genetically characterizing the causative *T. gondii* strains (III, IV, V) in order to unveil possible associations between the genotype of the parasites and the outcome of the infections.

4. Materials and methods

4.1. Study design and ethics

The studies were nationwide epidemiological cross-sectional studies (I, II, III, V, VI) and descriptive case series studies (III, IV, V) of naturally-acquired *T. gondii* infections in non-human animal hosts. The study area was Finland, located in northeastern Europe between the 60th and 70th parallels. The studies were carried out in 2008-2012, but also included retrospective material (II, III, IV, VI).

No animal was killed and no non-human blood was drawn solely for these studies. The blood samples from sheep and wild boars were taken primarily for another disease surveillance program and project (I, II). The blood samples from purebred pet cats were sampled with the owners' consent for a feline DNA bank and for parasitological and virological research use (ethical permit ESAVI-2010-0392/Ym-23), and the blood samples from shelter cats were leftover routine diagnostic samples taken by shelter veterinarians (V). Other samples were taken after the animals were killed either by legal hunting (I, III, VI) or slaughtering (II), or had either died (III, IV, V) or had been euthanized by a veterinarian (V) and subsequently submitted for postmortem examination.

No bioassays in laboratory animals were used in these studies, and the parasite isolation was performed into cell cultures.

The persons whose samples were used in the blind experiment to evaluate the effect of hemolysis on the serology method provided their oral informed consent. All information obtained from the hunters, farmers, and cat owners in the questionnaires (I, II, V, VI) are stored securely and treated confidentially. The samples are stored coded.

4.2. Hosts included in the studies

Toxoplasma gondii has a wide variety of host species, including practically all warm-blooded animals (Dubey, 2010). The inclusion of hosts for these studies was based on relevance from several viewpoints, including assumed importance for the success of the parasite in Finland, potential risks for human infections, as well as veterinary clinical relevance and animal welfare issues. These studies included both wild, free-ranging animals and domestic animals.

4.2.1. Hosts intended for human consumption

The prevalence of chronic *T. gondii* infections was investigated in selected hosts that were either raised or hunted for human consumption. Moose (*Alces alces*), white-tailed deer (*Odocoileus virginianus*) and roe deer (*Capreolus capreolus*) are free-ranging wild ruminants that are important game animals (I). In 2005-2010, the annual game bag was 57 097-75 587 moose, 21 900-25 694 white-tailed deer, and 3165-4182 roe deer (Finnish Game and Fisheries Research Institute, 2013). Harvested moose meat comprises 75% of the entire game meat yield of 10 million kilograms (Finnish Game and Fisheries Research Institute, 2009). Sheep (*Ovis aries*) was selected as a domestic ruminant host (I), and farmed wild boar (*Sus scrofa*) falls in-between: a game animal farmed for slaughter (II). The domestic sheep population is 130 000, and the meat production is 95 000 kg per year (Agricultural Statistics, 2013a; Agricultural Statistics, 2013b). The annual game bag of free-ranging wild boars is 75-110 (Finnish Game and Fisheries Research Institute, 2013), whereas 150-466 farmed wild boars are slaughtered for meat each year (Finnish Food Safety Authority Evira, 2013a).

The European brown hare (*Lepus europaeus*) and mountain hare (*Lepus timidus*) were included mainly due to their suggested high susceptibility to fatal toxoplasmosis, but they are also important game animals hunted for their meat (III; 4.2.2), and Eurasian lynx (*Lynx lynx*) as a potential definitive host (VI; 4.2.3), but their meat is also occasionally prepared as food in Finland.

All the moose and deer were hunted, and all the sheep and wild boars were raised for human consumption (I, II). All the wild boars were slaughtered, passed meat inspection, and used for human consumption (II).

4.2.2. Hosts suggested as highly susceptible to severe clinical toxoplasmosis

Fatal cases of toxoplasmosis among European brown hares, mountain hares (III), and Eurasian red squirrels (*Sciurus vulgaris*) (IV) were selected for investigation in detail, as these host species have previously been proposed as exceptionally susceptible to severe clinical toxoplasmosis (Rodhain, 1950; Christiansen and Siim, 1951; Gustafsson, 1997).

In 2005-2010, the annual game bag was 159 200-209 900 mountain hares, 65 900-89 000 brown hares, and 4400-10 200 squirrels (Finnish Game and Fisheries Research Institute, 2013).

4.2.3. Possible definitive hosts

Both felids living in Finland, the domestic cat (*Felis catus*) (V) and the Eurasian lynx (VI), were investigated for various aspects of toxoplasmosis and for their role in the epidemiology of *T. gondii* infections.

The domestic cat population in Finland comprises approximately 800 000 pet cats. Pet cats are legally permitted to roam unleashed (Public Order Act, 2003). Feral cats are practically non-existent.

The Eurasian lynx is the only wild felid living in Fennoscandia. The lynx population has doubled in the past decade and currently numbers about 2340-2610 adult lynx (Finnish Game and Fisheries Research Institute, 2008). Although protected (Council Directive, 1992), lynx are hunted with special permits granted by the Ministry of Agriculture and Forestry.

4.3. Sampling and sample storage

4.3.1. Sample size calculations

The sample sizes for the seroprevalence studies were calculated beforehand to obtain estimates at an acceptable precision (L = 0.03-0.05) and type I error rate (5%; Z = 1.96) (Dohoo et al., 2003; Dean et al., 2013). The calculations were based on expected seroprevalences of 10-20% for moose, 30-40% for deer, 10-45% for sheep (I), 10% for farmed wild boars (II), < 5% for hares (III), 45% for domestic cats (V), and 70-75% for lynx (VI). The sample size calculation for sheep (I) was revised with an intra-cluster correlation coefficient (0.2) and a sampling plan of 20 samples per flock to account for the clustering of sheep into flocks where they are more likely to be similarly exposed to *T. gondii* (Dohoo et al., 2003). The overall sample size for moose (I) was tripled to allow for comparison between data from different geographical areas. The target sample sizes were 700 moose, 350 white-tailed deer and 350 roe deer, 1920 sheep (I), 150 farmed wild boars (II), 100 brown hares and 100 mountain hares (III), 400 cats (V), and 300 lynx (VI).

The expected proportional mortality rates used in sample size planning (Dohoo et al., 2003; Dean et al., 2013) were 10% for European brown hares (III), 5% for mountain hares (III), and 3% for domestic cats and lynx (V, VI). The target sample sizes therefore comprised at least 150, 75, 50 and 50 necropsied animals, respectively. For the squirrels (IV), no previous estimates of mortality rate were available to serve as the basis for a calculation, so the sample was a convenience sample.

4.3.2. Samples for serology

4.3.2.1. Moose and deer (I)

The 1215 moose samples originated from all 15 game management districts (61-114 samples from each). The 135 white-tailed deer samples and 17 roe deer samples originated from the south-western game management districts where their populations are the largest.

Participating hunters voluntarily collected the samples from wild cervids during the 2008-2009 hunting season. The sampling packages (n = 2917) were sent to the 15 game management districts for subsequent distribution to the hunters. Each package contained two plastic blood sample tubes (VACUETTE® Serum Clot Activator, Greiner Bio-One GmbH, Kremsmünster, Austria) to be filled with blood. The participation rate was 47%; during the study period, 1367 sample packages arrived at the laboratory by mail within four days of each kill. Upon arrival, the sera were separated by centrifugation, divided into aliquots, and stored at -20°C until analyzed.

4.3.2.2. Sheep (I)

The 1940 individual sheep samples originated from 97 separate flocks: 20 samples per flock, 10-22 flocks from each of the six provinces.

The Finnish Food Safety Authority Evira provided these aliquots of sheep sera, which local municipal veterinarians drew from sheep over one year of age for a disease surveillance program in 2008. The serum aliquots were stored frozen at -20°C until analyzed.

4.3.2.3. Farmed wild boars (II)

The 197 farmed wild boar sera selected for this study were collected in 2007-2008. They originated from 25 separate farms, each with 6-150 animals, and each providing 1-43 samples.

This survey was part of a nationwide epidemiological study with voluntary sampling by wild boar farmers in 2005-2008. The animals were stunned and bled on the farms, and the farmers collected the blood samples. The samples were then sent to the laboratory within three days, and the sera were separated and stored frozen at -20°C until analyzed.

4.3.2.4. Hares (III)

From May 2006 to April 2009, veterinary pathologists at the Finnish Food Safety Authority Evira collected sera from 116 European brown hares and 99 mountain hares, which private citizens had voluntarily submitted for post-mortem examination. The sera were stored frozen at -20°C.

4.3.2.5. Domestic cats (V)

The DNA bank plasma samples were from 445 purebred cats from across Finland, whereas the plasmas or sera from 45 shelter cats came from a shelter located in the capital city of Helsinki in southern Finland. The samples were collected between September 2008 and August 2009, and the sera or plasmas were separated and stored at -20°C until analyzed.

4.3.2.6. Lynx (VI)

The 337 lynx samples originated from across the mainland of Finland based on granted hunting permits: more lynx were hunted in areas with a high lynx population.

The lynx samples for serology were serosanguineus fluid samples collected from thawed hearts. During the hunting season from December 2010 to February 2011, hunters sent lynx carcasses to a field station of the Finnish Game and Fisheries Research Institute, where the hearts were removed, sealed in individual plastic bags, and shipped frozen to the laboratory.

4.3.3. Fecal samples

4.3.3.1. Cats (V)

Fresh fecal samples from litter boxes were collected in 2009 from 131 shelter cats.

Individual fecal samples were collected from 76 cats, and pooled samples were collected from 55 cats kept in groups. The majority (n = 117) of the cats sampled were impounded at two cat shelters located in the capital city of Helsinki in southern Finland, 12 in Vaasa, and two in Rovaniemi.

The samples were stored at +4°C for a maximum of seven days and analyzed.

4.3.3.2. Lynx (VI)

Individual fecal samples were collected directly from the rectum of 332 lynx at the field station of the Finnish Game and Fisheries Research Institute in 2010-2011. For logistical reasons, these samples had to be shipped and stored frozen until analyzed.

4.3.4. Animals submitted for postmortem examination

4.3.4.1. Hares (III)

Private citizens voluntarily submitted the hares to the Finnish Food Safety Authority Evira for postmortem examination. The hares included in the study (173 European brown hares and 148 mountain hares) were examined by the veterinary pathologists over a three-year period from May 2006 to April 2009; the length of the study period was based on the sample size calculations.

4.3.4.2. Squirrels (IV)

Between May 2006 and April 2009, private citizens submitted the squirrels included in the study to the Finnish Food Safety Authority Evira for postmortem examination. Due to the lack of previous estimates, this time period was designated to be the same as for the hares with no specific sample size calculation. The convenience sample comprised 19 squirrels.

4.3.4.3. Domestic cats (V)

The database search was carried out among all domestic cats examined postmortem at the Veterinary Pathology Department, Faculty of Veterinary Medicine, University of Helsinki, between January 2008 and June 2011 (n = 193).

4.3.4.4. Lynx (VI)

The database search of the wildlife pathology laboratory of the Finnish Food Safety Authority Evira included all lynx submitted for postmortem examination between January 2000 and May 2010 (n = 167).

4.3.5. Fresh and frozen tissue samples

4.3.5.1. Domestic cats (V)

Since 2008, the veterinary pathologists at the Faculty of Veterinary Medicine, University of Helsinki, informed of all feline cases in which toxoplasmosis was suspected based on clinical history or suggestive gross findings observed during necropsy. Fresh samples of different tissues (brain, heart, liver, lung, lymph node) from all such cats were collected with sterile instruments for *T. gondii* isolation. In addition, selected tissue samples of the same cats were stored frozen.

4.4. Controls and reference samples

Control samples were included in all analyses. In the methods with several steps, they were introduced at different steps in order to control for them separately. For the molecular methods in particular, this entailed several controls in the final analyses, but this practice ensured that the methods worked without problems and ruled out contamination.

For serology, all plates included the negative and positive controls provided in the kit at two dilutions: 1:40 and 1:4000. The antigen control (all reagents but the serum) was carried out in two wells on each plate.

For immunohistochemical staining, sections with no primary antibody and tissue sections known to be positive or negative for *T. gondii* served as controls. The negative control tissue sections came from the same species: formalin-fixed, paraffin-embedded (FFPE) liver tissue from a European brown hare that had died of listeriosis (III), FFPE tissues from a Eurasian red squirrel that had died of head trauma (IV), and liver tissue from a cat euthanized due to a *Pasteurella multocida* infection (V). The positive control tissue was liver tissue from a cat that had died of generalized toxoplasmosis, confirmed with all methods available in the laboratory.

Alongside DNA extraction from the samples, DNA was extracted from at least one negative control (nuclease-free water) and at least one positive control (*T. gondii* tachyzoites cultivated in cell cultures). Tissues from the same host species as the samples were included, if available, from individuals confirmed to be free of *T. gondii* or confirmed to be infected with *T. gondii*. The DNA extracts from the controls served as controls in all subsequent molecular methods.

For the molecular methods, more references and controls were included as DNA, whether frozen DNA from previous rounds of DNA extraction in our lab or obtained from other laboratories. DNA from the *Neospora caninum* strain NC1 (provided by Dr. Katarzyna Gozdzik of the Witold Stefański Institute of Parasitology in Warsaw, Poland) and nuclease-free water always served as negative controls. In addition, DNA extracted from uninfected cell cultures and from tissue samples of known *T. gondii*-negative animals of the same species were included as controls. DNA samples from selected *T. gondii* parasite strains served as positive controls and references. These included the strains RH (genotype I; provided by Eva-Britt Jakubek of the National Veterinary Institute in Uppsala, Sweden), GIL (genotype I), PSP-2007-TON (genotype II), PRU (genotype II), TOU-2004-FEU (genotype III), and NED (genotype III; provided by Dr. Daniel Ajzenberg and Professor Marie-Laure Dardé of the Toxoplasma Biological Resource Centre in Limoges, France), as well as FIN1 (genotype II) and FIN2 (genotype II; the local strains isolated in these studies (V; Table 7)). Controls were also routinely added after the PCR step as products from earlier PCR runs to control for the quality and repeatability of the last steps of the method.

In case a new strain yielded a genotyping result identical to one of the references used, the analysis was repeated from the original material with the reference strain removed from the controls in order to rule out cross contamination. In addition, analysis was carried out separately at each marker (not as a multiplex PCR) to confirm the results when needed; and more fingerprinting markers could have been used, if necessary.

Strains RH, PSP-2007-TON, FIN1, and FIN2 were cultivated in cell cultures and cryopreserved to provide reference material. The RH strain was obtained from cell cultures of National Veterinary Institute, Sweden. PSP-2007-TON was obtained from the Toxoplasma Biological Resource Centre, Limoges, France, as a suspension of the CNS of an infected laboratory mouse. FIN1 and FIN2 are local strains isolated from domestic cats with fatal generalized toxoplasmosis (V). The reference cultures were regularly checked with diagnostic PCR and MS genotyping to confirm that no cross-contamination occurred between the strains or in the uninfected cell lines.

4.5. Serology

4.5.1. Direct agglutination test

A commercial direct agglutination test (DAT; Toxo-screen DA, bioMérieux, Marcy-L'Étoile, France), a screening test for specific anti- *Toxoplasma gondii* IgG antibodies, was chosen as the serology method for these studies. The principle of the method is the agglutination of formalin-treated *Toxoplasma* parasites if the sample contains specific IgG antibodies. Possible IgM is denatured by 2-mercaptoethanol (2-ME) (Johnson et al., 1989).

The method was performed according to the manufacturer's instructions. The results were read after 18 hours, and a light table providing even illumination from below or a spot light source served to assist with reading and to eliminate problems caused by background color in the hemolyzed samples.

We used a four-point scale to record the results:

3 – positive – an unshrunken mat covering the majority of the bottom of the well
2 – positive – a large mat covering at least half of the bottom of the well
1 – negative – a ring or mat covering less than half of the bottom of the well
0 – negative – a button.

These results were further interpreted as a dichotomous outcome: seropositive = 1 or seronegative = 0. That is, instead of including borderline results (result '1' on our four-point scale), as the manufacturer suggests, only clear positives were considered positive; all the rest, including those showing a borderline reaction, were interpreted as negative.

The cut-off for seropositivity was 1:40 for all samples. Most samples were diluted to only 1:40; any samples that tested positive at this dilution were defined as seropositive (I, II, III, V, VI). Only the samples of purebred cats (V) were diluted to 1:40 and 1:4000; samples that tested positive at either or both of these dilutions were defined as seropositive.

A subsample of 80 feline samples (V) and 9 sera from hares that had died of generalized toxoplasmosis (III) that were available in sufficient quantities were further diluted to 1:60, 1:180, 1:540, 1:1620, 1:6000, 1:18 000, 1:54 000, and 1:162 000 to determine the titers.

4.5.2. Blind experiment to evaluate the effect of hemolysis

In 2008, we performed a blind experiment to test whether hemolysis affected the results of the DAT. The details of this experiment have not been published previously.

For this test, we used blood samples from one *T. gondii* seropositive and one seronegative person. One sample from both persons each was processed routinely, while hemolysis was introduced to the others in selected ways: one sample was frozen, one was shaken vigorously, one was mixed with a small amount of tap water, and one was permitted to rot (left uncapped at room temperature). The sera were separated by centrifugation and divided into two aliquots. One of the aliquots served directly as a sample for DAT, and the other to test a commercially available method for clearing hemolyzed samples (HemogloBind, Biotech Support Group, Monmouth JCT, NJ, USA) prior to DAT. The removal of hemoglobin was carried out according to the manufacturer's instructions.

The sera were coded to conceal their identity during the DAT and when reading the results. The hemolysis was naturally visible.

The coded sera were stored frozen until analyzed, as were the research samples, and DAT was performed according to the manufacturer's instructions at two dilutions: 1:40 and 1:4000. The controls provided in the kit were included on the plate. The results were recorded using the four-point scale above.

4.6. Coprological investigations

Fecal samples from possible definitive hosts of *T. gondii w*ere screened for the presence of *T. gondii*-like oocysts using flotation techniques. The technique selected as the main method is based on the use of a special FLOTAC apparatus (University of Naples Federico II, Naples, Italy) for flotation with saturated $MgSO_4$ (magnesium sulphate; VWR International, Leuven, Belgium) solution, centrifugation, and transferring the top portion of the suspension aside for light microscopy. This was carried out as described in the instructions of the manufacturer of the FLOTAC apparatus. The screening was performed at 100X and 200X magnifications, and further examination at 400X.

Of each sample from domestic cats, two parallel five-gram samples were examined, whereas only one five-gram sample was examined from each lynx. When *T. gondii*-like oocysts were detected, the oocyst-rich top portion was collected and then frozen and thawed three times prior to DNA extraction (QIAamp DNA Stool Mini Kit, Qiagen GmBH, Hilden, Germany). DNA was extracted according to the manufacturer's instructions, and stored frozen until analysed with diagnostic PCR and direct genotyping.

4.7. Questionnaires

The questionnaires for the hunters of moose, deer, and lynx inquired about the animal's species (I, in areas where both deer and moose were sampled), age-group (calf or adult) (I), sex (I, VI), the geographical location of the kill (I, VI), additional information about the hunt (VI), and the hunter's contact information. The questionnaires for wild cervids were color-coded: one color for areas where only moose were sampled and another for areas where all three cervid species were sampled. The personnel of the Taivalkoski field station of the Game and Fisheries Research Institute provided detailed measurements of the lynx.

The questionnaire for the wild boar farmers (II) inquired about background information on the farms and animals, including the age and sex of the animals, the type and size of the herd, and the size and location of the farm.

The questionnaire for the cat owners (V) included the cat's signalment (e.g. date of birth, date of death, sex, breed, blood group), lifestyle (diet, outdoor access, international activities), and disease history (also of the cat's close relatives).

4.8. Clinical data

All clinical data available for the domestic cats that died of toxoplasmosis (V) were re-evaluated and summarized. The data included observed clinical signs and their duration, the laboratory results of clinical chemistry analyses and hematology, and the results of examinations of fecal samples.

Clinical data from the other hosts examined were unavailable. The hunted animals (I, VI) as well as the animals raised for human consumption (I, II) presumably appeared to be healthy. The animals submitted for postmortem examination (III, IV, V, VI) were found dead or euthanized.

The disease history of the cats (V) whose owners completed the questionnaires provided only limited information with apparent relevance to *T. gondii* infections. The diseases or disease groups mentioned were included in the premilinary analyses of associations, and in particular, any associations of the infection with reproduction or behavioral problems warranted further study.

4.9. Pathology and histopathology

The postmortem diagnoses were made at the Veterinary Pathology Department, University of Helsinki (V) and the Finnish Food Safety Authority Evira (III, IV, VI) and were re-evaluated in these studies (III, IV, V, VI). The original necropsy reports and histological tissue sections of the domestic cats that died of generalized toxoplasmosis (V) were re-evaluated in detail, together with one specialist in veterinary pathology (Outi Simola, DVM, DipECVP), to obtain comparable data from the cases.

The diagnoses were based on necropsy findings and the routine histologic examination of tissue samples. In addition, special stainings, bacterial cultures and other investigatons were carried out as needed.

The necropsies were performed according to standard protocols, and morphological diagnoses were recorded. The tissue samples routinely taken from the main organs, as well as the additional samples taken as needed, were fixed in 10% neutral buffered formalin and processed into paraffin-embedded tissue blocks. The samples were sectioned and stained with hematoxylin and eosin (HE) for microscopic evaluation. The diagnoses were then entered into the databases of the Veterinary Pathology, University of Helsinki (V) and the Finnish Food Safety Authority Evira (III, IV, VI).

4.10. Immunohistochemistry

Immunohistochemical staining (IHC) for *T. gondii* was perfomed to confirm the diagnoses of toxoplasmosis (III, IV, V) and to evaluate the burden and spread of the parasites in different tissues (IV, V). The 4-μm sections of liver (III, IV, V) and other available tissues (IV, V) were routinely processed (Lab Vision PT Module, Lab Vision Corporation, Fremont, CA, USA) for automated IHC staining (Lab Vision Autostainer 480, Lab Vision Corporation).

The primary antibody was selected from three commercial antibodies, the performances of which were tested in both manual and automated immunohistochemical staining. Citrate buffer (pH 6.0) was the solution used for heat-induced antigen retrieval, breaking the protein cross-links that might mask the antigenic sites.

The preferred antibody selected for further optimization of the IHC method was *Toxoplasma gondii* epitope-specific rabbit antibody (Thermo Fisher Scientific, Runcorn, UK). The protocol optimized for automatic staining entailed a 1:500 dilution of the primary antibody and reagents from a labeled streptavidin-biotin immunoenzymatic antigen detection system (UltraVision Detection System, Anti-Polyvalent, HRP/DAB kit; Lab Vision Corporation).

4.11. Isolation, maintenance and cryopreservation of *T. gondii* in cell cultures

In 2007, cell culture techniques for maintaining *T. gondii* strains were adapted to the laboratory (biosafety level 2) at the Department of Veterinary Biosciences, University of Helsinki, from the National Veterinary Institute i Sweden. The adapted protocols included the cultivation and quantification as well as the freezing and thawing of cells and *T. gondii* parasites. The main changes to the original protocols included simplifying the reagent list with commercial products, donning more personal protective equipment, and employing a specific freezing container to achieve more constant and optimal cooling rates.

African green monkey (*Chlorocebus* sp.) kidney epithelial cells (Vero cells, European Collection of Cell Cultures, Salisbury, UK) were grown in T25 flasks (Easy Flask 25 V/C, NUNC A/S, Roskilde, Denmark) in Dulbecco's modified Eagle medium (DMEM+GlutaMAX-I, Life Technologies Ltd., Paisley, UK) and 10% fetal bovine serum (Thermo Fischer Scientific, Cramlington, Northumberland, UK). Sub-culturing at optimal confluency was carried out with a recombinant enzyme, an animal origin-free alternative for trypsin, for detaching the cells (TrypLE Express, Life Technologies).

The incubator (Serie CB Incubator, Binder GmbH, Tuttlingen, Germany) maintained the temperature at 37°C and the CO_2 concentration at 5%. Its water bath was cleaned weekly with 70% ethanol and refilled with one liter of ultrapure water (MilliQ, EMD Millipore Corporation, Billerica, MA, USA).

The cryopreservation of cells and parasites was further optimized by including a freezing container that promises an optimal rate of cooling for cell preservation, close to -1°C/min (Mr. Frosty Cryo 1°C Freezing Container, Nalgene, Thermo Scientific). The freezing medium was a fully supplemented commercial medium (Recovery cell culture freezing medium, Life Technologies), and an electric freezer was used for ultra-low temperature storage (Sanyo Ultra Low MDF-C2156VAN, Sanyo Electric Co. Ltd., Osaka, Japan).

The isolation of *T. gondii* parasite strains from the tissues of animals with a suspected toxoplasmosis diagnosis was attempted directly into cell cultures. Small samples of the tissues (CNS, heart, liver, lung, lymph node) were first aseptically cut into pieces with sterile instruments, then further homogenized with a syringe by injecting them through a hypodermic cannula (18G), and finally poured onto fresh cell cultures.

Penicillin and streptomycin (PenStrepGlutamine, Life Technologies) were added to the flasks for the first two passages when attempting a new isolate. All other cultures were antibiotics free, and the protocols were strictly aseptic.

All the cultures were regularly examined for cell and parasite growth and monitored for other possible changes with an inverted contrasting microscope (Leica DM IL, Leica

Microsystems GmbH, Wetzlar, Germany). Any flasks with color changes reflecting unexpected changes in pH or showing signs of microbial contamination were discarded.

A digital microscope camera (Leica DFC425 with Leica Application Suite (LAS) software, Leica Microsystems) served to document growth in the flasks. In addition, thin pieces of glass were placed on the bottom of selected flasks and removed for cytological staining (modified Giemsa) after fixation with methanol or 10% neutral buffered formalin.

4.12. DNA extraction

The tissues of the animals that died of generalized toxoplasmosis (III, IV, V) were rich in *T. gondii* parasites in IHC, and DNA was extracted directly from them for further investigations with molecular methods. Both FFPE and frozen tissues were used when available. In addition, DNA was routinely extracted from frozen samples of cell cultures to confirm successful isolations (V) and the integrity of the strains maintained in the cell cultures. DNA was also extracted from fecal samples that tested positive for *T. gondii*-like oocysts (V).

The main DNA extraction kit (the DNA IQ™ System and Tissue and Hair Extraction Kit; Promega, Madison, WI, USA) was selected based on previous experience, as well as for its applicability to the FFPE samples. This method uses paramagnetic resin to bind the DNA and thus requires less centrifugation and washing. The extractions were performed according to the manufacturer's instructions. The FFPE samples were incubated in the Incubation Buffer/Proteinase K solution overnight; the other samples, for two hours. The amount of resin per sample was increased to 10 µl, and the final elution volume was adjusted to 30 µl in order to strike a good compromise between DNA yield and final volume.

Three freeze-thaw cycles and a special DNA extraction method for fecal samples (QIAamp DNA Stool Mini Kit, Qiagen) were used to extract DNA from flotation-enriched fecal samples with *T. gondii*-like oocysts (V).

When necessary, the DNA samples were stored frozen at -20°C until analyzed. Archives of these DNA remain frozen in storage.

4.13. Detection of *T. gondii* DNA with PCR

The diagnostic PCR protocol established targets the 529-bp fragment that is repeated 200- to 300-fold in the *T. gondii* genome (Homan et al., 2000).

For this PCR, the total volume of 50 µl comprised a commercial master mix (2x PCR Mastermix, Promega) at a final concentration of 1x, 0.5 µM of primers (Oligomer, Helsinki, Finland; Table 2), 7 µl of DNA, and nuclease-free water (Nuclease-free water, Promega). The PCR conditions in the PTC-200 Peltier Thermal cycler (MJ Research Inc., Waltham, MA, USA) were 94°C for seven minutes followed by 35 cycles of 94°C, 55°C, and 72°C for one minute each, and a final ten-minute extension at 72°C. The results were visualized under ultraviolet light after electrophoresis on 2% Tris-Acetate-EDTA (TAE) agarose gel (NuSieve 3:1 Agarose, Cambrex BioScience Rockland Inc., Rockland, ME, USA) with ethidium bromide.

Table 2 The PCR primers used for the diagnostic PCR for *Toxoplasma gondii*.

	Primer sequence	Reference
TOX4	5' CGCTGCAGGGAGGAAGACGAAAGTTG 3'	(Homan et al., 2000)
TOX5	5' CGCTGCAGACACAGTGCATCTGGATT 3'	(Homan et al., 2000)

4.14. Direct genetic characterization of *T. gondii* parasites

A genotyping method based on microsatellite markers was adapted from the laboratory of the University of Limoges, France. The major changes made to the original protocol (Ajzenberg et al., 2005) included a different fluorescent label for the marker W35 in order to improve the resolution between it and M48 (same change in the updated method described in Ajzenberg et al., 2010), as well as the number of cycles, and the temperature and time of the annealing step of the PCR.

The method is based on length polymorphism analysis of microsatellite markers after multiplex PCR. Six of the markers we used (B18, TUB2, TgM-A, W35, B17, and M33) are well described for genotyping (Blackston et al., 2001; Ajzenberg et al., 2005), and additional markers have been described for fingerprinting (Blackston et al., 2001; Ajzenberg et al., 2010), that is, for distinguishing closely related isolates. In our method, a seventh marker, fingerprinting marker M48, was included for further characterization. The results obtained with the genotyping markers are identical to those of the reference strain of the same genotype, while the fingerprinting marker M48 can show variation beyond the genotype level. In case of a mixed infection with two strains belonging to the same genotype, this marker could show the mixed infection (two peaks). Another reason for keeping this seventh marker in the analysis was to rule out contamination. If a result from a sample was fully identical to a reference strain, the analysis was repeated from the very beginning, from the original material, without that particular reference strain in the same run. If necessary, further analysis with more fingerprinting markers was available.

The total volume of 25 µl for the multiplex PCR comprised a commercial master mix (2x QIAGEN Multiplex PCR Master Mix; Qiagen) at a final concentration of 1x, 0.04 µM of each primer (Applied Biosystems, Warrington, UK; Table 3), 5 µl of nuclease-free water (Nuclease-free water, Promega), and 4 µl of DNA. The forward primers were 5'-end labeled with 6-carboxyfluorescein (B18, TUB2, and M48), hexachlorofluorescein (TgM-A, W35, and B17), or 2,7',8'-benzo-5'-fluoro-2',4,7-trichloro-5-carboxyfluorescein (M33). The PCR conditions in the PTC-200 Peltier Thermal cycler (MJ Research) were 95°C for 15 minutes followed by 40 cycles of 94°C for 30 seconds, 61°C for four minutes, and 72°C for one minute, and a final thirty-minute extension at 60°C. The PCR products from DNA extracted from cell culture were diluted 1+30, and the DNA products of the *T. gondii* reference strains were diluted 1+20 in deionized formamide (Sigma-Aldrich Co, St. Louis, MO, USA). One microliter of each diluted or undiluted product was then mixed with 23.5 µl of deionized formamide and 0.5 µl of size standard (GeneScan 500 ROX Size Standard, Applied Biosystems) before being taken to the Institute of Biotechnology for automated capillary electrophoresis (3130xl Genetic Analyzer, Applied Biosystems). The products were separated based on size, and identified based on the fluorescent labels. The data were analysed, and the sizes of the alleles estimated by comparing with the sizing curve using a free software (PeakScanner Software version 1.0, Applied Biosystems; Figure 5). A variation of the method, in which each primer pair was used separately, enabled further resolution and confirmation of the identity of the markers, when necessary.

Table 3 The PCR primers of the MS genotyping method.

	Primer sequence	Reference
Genotyping markers*		
B18 [TG/AC]$_n$		
FB18	5’ 6-FAM-TGGTCTTCACCCTTTCATCC 3’	(Ajzenberg et al., 2005; Ajzenberg et al., 2010)
RB18	5’ AGGGATAAGTTTCTTCACAACGA 3’	
TgM-A [TG/AC]$_n$		
FTgM-A	5’ HEX-GGCGTCGACATGAGTTTCTC 3’	(Ajzenberg et al., 2005; Ajzenberg et al., 2010)
RTgM-A	5’ TGGGCATGTAAATGTAGAGATG 3’	
W35 [TC/AG]$_n$, [TG/AC]$_n$		
FW35	5’ HEX-GGTTCACTGGATCTTCTCCAA 3’	(Ajzenberg et al., 2005; Ajzenberg et al., 2010)
RW35	5’ AATGAACGTCGCTTGTTTCC 3’	
B17 [TC/AG]$_n$		
FB17	5’ HEX-AACAGACACCCGATGCCTAC 3’	(Ajzenberg et al., 2005; Ajzenberg et al., 2010)
RB17	5’ GGCAACAGGAGGTAGAGGAG 3’	
TUB2 [TG/AC]$_n$		
FTUB2	5’ 6-FAM-GTCCGGGTGTTCCTACAAAA 3’	(Ajzenberg et al., 2005; Ajzenberg et al., 2010)
RTUB2	5’ TTGGCCAAAGACGAAGTTGT 3’	
M33 [TC/AG]$_n$		
FM33	5’ NED-TACGCTTCGCATTGTACCAG 3’	(Blackston et al., 2001; Ajzenberg et al., 2010)
RM33	5’ TCTTTTCTCCCCTTCGCTCT 3’	
Fingerprinting marker		
M48 [TA/AT]$_n$		
FM48	5’ 6-FAM-AACATGTCGCGTAAGATTCG 3’	(Blackston et al., 2001; Ajzenberg et al., 2010)
RM48	5’ CTCTTCACTGAGCGCCTTTC 3’	

* The repeat motif(s) in square brackets; F = forward primer, R = reverse primer, 6-FAM = 6-carboxyfluorescein, HEX = hexachlorofluorescein, NED = 2,7′,8′-benzo-5′-fluoro-2′,4,7-trichloro-5-carboxyfluorescein.

4.15. Statistical analyses

Two-by-two tables and test statistics (mainly chi-square and mid-P exact) of open source software for epidemiological statistics served to evaluate simple associations (Dohoo et al., 2003; Dean et al., 2013) and to make preliminary comparisons (I, II, III, IV, V, VI). Confidence intervals were computed using mid-P exact (Lydersen et al., 2009; Dean et al., 2013). P values < 0.05 were considered statistically significant.

Logistic regression analyses with Stata 9.2 (I) and 11.0 (II, V, VI) software (StataCorp, College Station, TX, USA) were used to evaluate the combined effect of selected variables. The variables were selected not only based on their presumed biological relevance; we also tested the fit of other available variables to the models. The multivariable models were built stepwise using forward selection and backward elimination, as well as testing for interaction and confounding. For the wild boar data, further analysis included building a random effects logistic model to account for herd effects (II).

5. Results

5.1. Anti-*Toxoplasma gondii* IgG antibodies in all host species investigated

Anti-*Toxoplasma gondii* IgG antibodies were detected in some animals of all the host species investigated (I, II, III, V, VI; Table 4) at the sample dilution of 1:40. The highest seroprevalence estimate was for lynx (VI), and the lowest ones were for the hares (III).

Table 4 *Toxoplasma gondii* seroprevalences in Finland (I, II, III, V, VI).

	N	n positive	Prevalence (%)	95% CI
Domestic sheep	1940	477	24.6	22.7-26.5
Moose	1215	116	9.6	8.0-11.3
White-tailed deer	135	36	26.7	19.7-34.6
Roe deer	17	3	17.6	4.7-40.9
Farmed wild boar	197	65	33.0	26.7-39.8
European brown hare	116	9	7.8	3.9-13.8
Mountain hare	99	7	7.1	3.1-13.5
Domestic cat	490	237	48.4	44.0-52.8
Eurasian lynx	337	290	86.1	82.0-89.5

CI = 95% confidence interval

Veterinary pathologists collected the sera from European brown hares and mountain hares during necropsies. The sample included sera from nine European brown hares and three mountain hares that died of acute generalized toxoplasmosis (confirmed in III). There were no seropositives among the 203 brown hares that died of other causes (0.0%; 95% CI

0.0-2.8), as opposed to four seropositives among the 96 mountain hares that died of other causes (4.2%; 95% CI 1.3-9.7; $P < 0.05$).

Seroprevalence among female cats with reported abortions, stillbirths, or other reproduction problems (66.7%) was higher than the overall seroprevalence ($P < 0.05$). The seroprevalence in cats with reported behavioral problems (53.2%) was similar to the overall seroprevalence.

5.1.2. Hemolysis has no effect on the results of the serology method

In our blind experiment, hemolysis had no effect on the DAT results (Table 5). The only false-negative result was from the positive sample that was experimentally hemolyzed by adding a small amount of tap water, at a dilution of 1:4000. By contrast, applying the method to remove hemoglobin affected the results (Table 5). Three aliquots of the positive sample yielded a false negative result at a dilution of 1:40, and all did so at a dilution of 1:4000.

Table 5 The results of the blind experiment show the effect of experimental hemolysis on the results of the serology method at the two dilutions. The results appear on a four-point scale: 0 and 1 are negative; 2 and 3 are positive.

	Known negative sample		Known positive sample	
Dilution	1:40	1:4000	1:40	1:4000
Non-hemolyzed	0	0	3	2
Non-hemolyzed, hemoglobin removed	0	0	2	0*
Frozen	0	0	3	2
Frozen, hemoglobin removed	0	0	3	0*
Shaken	0	0	3	2
Shaken, hemoglobin removed	0	0	0*	0*
Mixed with water	0	0	3	1*
Mixed with water, hemoglobin removed	0	0	1*	0*
Rotten	0	0	3	3
Rotten, hemoglobin removed	0	0	1*	0*

* False negative result

5.2. Risk factors for *Toxoplasma gondii* infections

5.2.1. Age

Adult age was a significant risk factor in moose (I), white-tailed deer (I), farmed wild boars (II), domestic cats (V), and lynx (VI). In moose, white-tailed deer, and cats, the odds of testing seropositive were about three times higher in adults than in those under one year old. In the wild boar, animals older than 24 months had two times higher odds for seropositivity than did the younger age group. Lynx presumably over one year old had 14.3 (95% CI 5.0-41.0) times higher odds for testing seropositive than did lynx of a presumed age of 7-10 months. The odds of testing seropositive increased by 20% for every year increase in the cat's age (V). Although the exact ages of the lynx were unknown, their odds for testing seropositive increased by 1.5 for each kilogram gained in weight (VI).

Age was a confounder in the simple logistic regression models for moose and farmed wild boar, whereas no interaction was evident between age and sex. Weight was confirmed as a confounder in lynx: it was related to seropositivity and sex.

5.2.2. Geographic location

Especially in moose, the latitude gradient observed in seroprevalence was striking (I). Seroprevalence in the south was over 15 times higher than in the north, with no explaining age distribution difference in the samples from these locations. A similar geographical north-south gradient in seroprevalence was also evident in domestic sheep (I), farmed wild boars (II), and lynx (VI).

5.2.3. Winter

Most of the fatal cases (79%) in brown hares were diagnosed during the coldest winter months. The proportional mortality rate from toxoplasmosis was eight times higher ($P < 0.001$) during the coldest winter months than in the other months.

5.2.4. Human actions

Detailed information about possible sources of *T. gondii* infection or preventive measures implemented on the sheep and wild boar farms was unavailable (I, II). Outdoor access was a significant risk factor for *T. gondii* infections of domestic cats in univariable analysis with an odds ratio of 1.6 (V). More than half of the cats (58.2%) had outdoor access.

Providing the cat with raw meat in its diet was a common practice (78.4%) and a significant risk factor with an odds ratio of 2.0 (V).

5.3. Contribution of the definitive hosts: ongoing shedding

Toxoplasma gondii-like oocysts were detected in the fecal samples of two domestic cats, one of which was confirmed with PCR to be shedding *T. gondii* at the time of sampling. None of the fecal samples from lynx had detectable *T. gondii*-like oocysts. The PCR-confirmed *T. gondii* oocyst shedding prevalence was thus 0.8% (95% CI 0.04-3.7) in domestic cats, which did not differ significantly from the 0.0% (95% CI 0.0-0.9) found in lynx.

5.4. Naturally-acquired fatal infections with endemic genotype II

5.4.1. Proportional mortality rates from toxoplasmosis

In the material examined postmortem, toxoplasmosis was detected and confirmed to be among the causes of death in brown hares (III), mountain hares (III), squirrels (IV) and domestic cats (V), but not among the lynx (VI; Table 6).

Table 6 Proportional mortality rates from toxoplasmosis in Finland (III, IV, V, VI).

	N	n died of toxoplasmosis	Proportional mortality rate (%)	95% CI
Brown hare	173	14	8.1	4.7-12.9
Mountain hare	148	4	2.7	0.9-6.4
Squirrel	19	3	15.8	4.2-37.2
Domestic cat	193	6	3.1	1.3-6.4
Lynx*	167	0	0.0	0.0-1.8

CI = 95% confidence interval
* Proportional mortality rate expresses mortality due to a specific cause in dead animal material (Dohoo et al., 2003) and is inherently subject to variation in the material included. A major portion of these lynx was submitted to postmortem examination after traffic accidents and by police authorities, so this rate estimate is not comparable with those of other species.

All the brown hares and mountain hares that died of toxoplasmosis and from which serum was available tested seropositive (III). All the brown hares that tested seropositive had died of toxoplasmosis (III). The proportional mortality rate was significantly higher in brown hares than in mountain hares ($P < 0.04$).

The course of toxoplasmosis appeared to be acute in all cases. No concurrent, possibly predisposing other diseases or conditions were diagnosed. The hares that died of toxoplasmosis were heavier than those that died of other causes (III). The domestic cats all had a short history of illness and exhibited unspecific clinical signs for approximately one week (V). The observed clinical signs included apathy, inappetence, dehydration, fever,

respiratory signs, incoordination, and icterus. The most striking laboratory result was markedly elevated levels of the liver enzyme alanine aminotransferase.

None of the cats had an antemortem diagnosis of toxoplasmosis (V), and in many of the cases, the diagnosis was evident no sooner than upon the histopathological evaluation of tissue samples. Gross postmortem findings in acute toxoplasmosis were unspecific and often scarce, with the exception of the enormous enlargement of the spleen (splenomegaly) which was confirmed in this study to be a typical finding in affected hares (III).

The cases were all generalized, and the liver and lungs were the organs most consistently affected. The diagnoses were confirmed (III, IV, V), and the burden of *T. gondii* parasites in different tissues were evaluated (IV, V) with immunohistochemistry (Figure 2-4). Large to massive numbers of parasites were seen in nearly all tissue samples examined (III, IV, V).

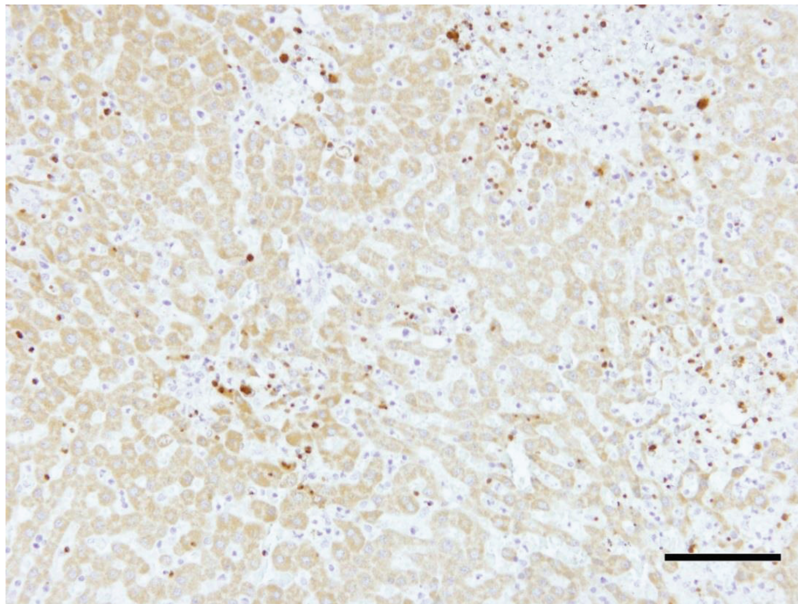

Figure 2 Immunohistochemical (IHC) staining of the liver tissue of a squirrel (case 3) that died of toxoplasmosis, showing numerous positively stained *Toxoplasma gondii* parasites. Scale bar 100 µm.

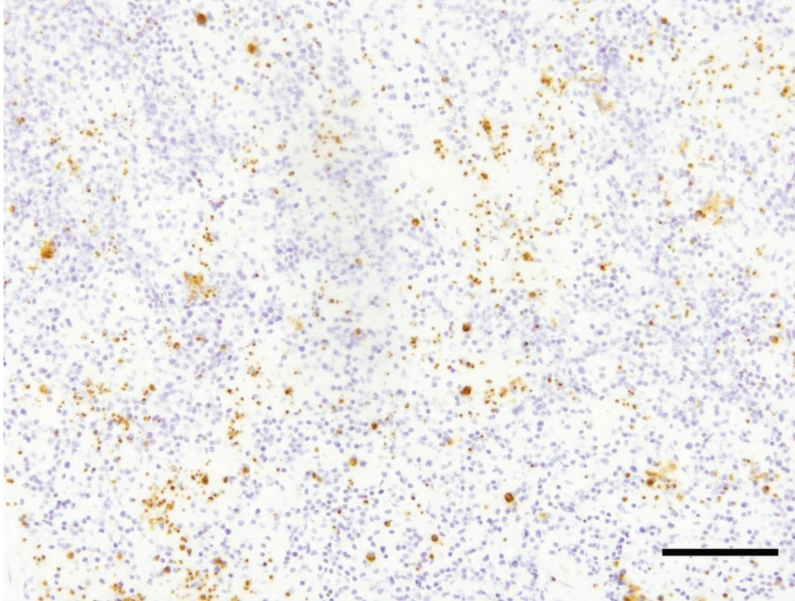

Figure 3 Immunohistochemical (IHC) staining of the spleen of a squirrel (case 1) that died of toxoplasmosis, showing numerous positively stained *Toxoplasma gondii* parasites. Scale bar 100 µm.

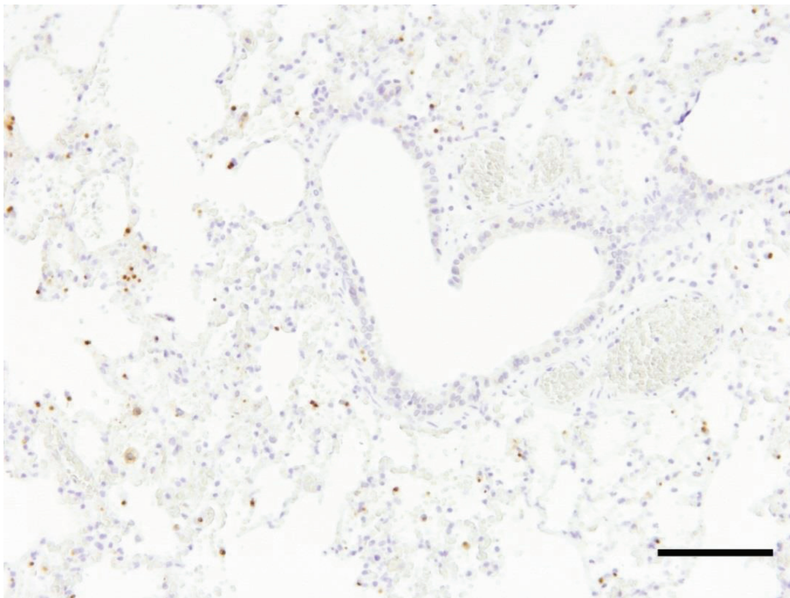

Figure 4 Immunohistochemical (IHC) staining of the lung tissue of a squirrel (case 2) that died of toxoplasmosis, showing numerous positively stained *Toxoplasma gondii* parasites. Scale bar 100 µm.

5.4.2. Endemic genotype II infecting both domestic and wild hosts

Toxoplasma gondii strains belonging to genotype II, which is endemic in Europe, was confirmed to be present and endemic in Finland also (III, IV, V). Genotype II was the only genotype detected, and no signs of mixed infections were evident (Figure 5, Table 7). *Toxoplasma gondii* strains belonging to this genotype caused all the fatal cases (III, IV, V). Genotyping proved successful from archived FFPE material as well (III, IV, Table 7).

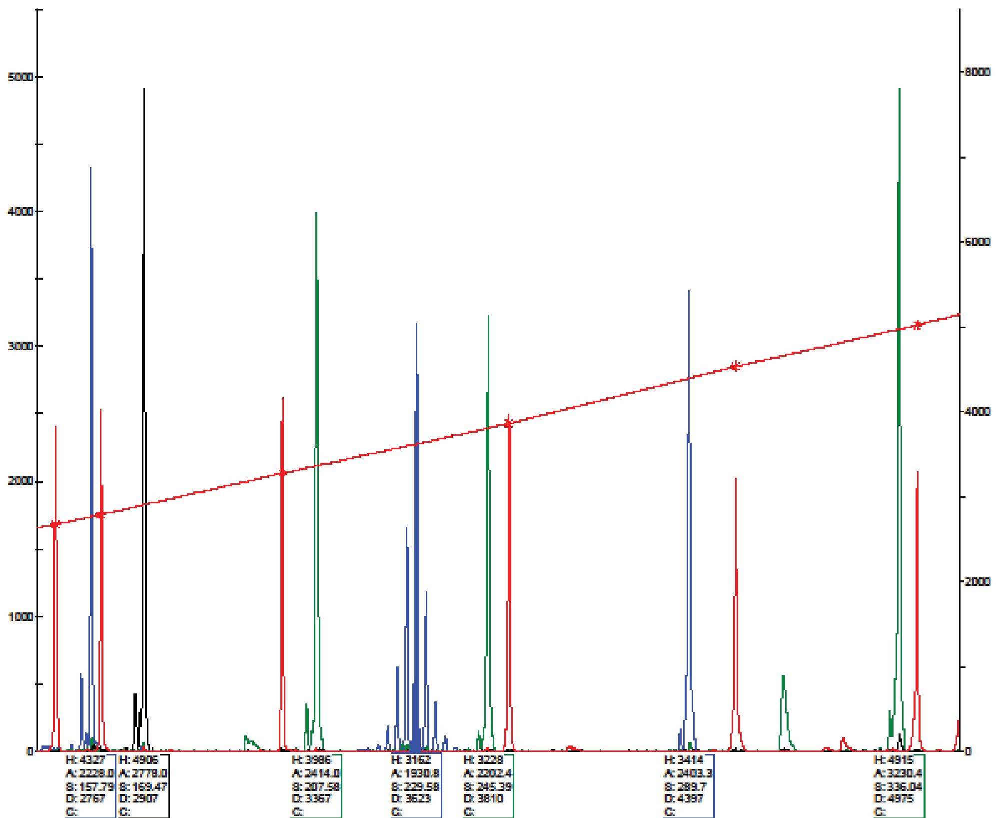

Figure 5 Direct genetic characterization of the local *Toxoplasma gondii* strain FIN1 from the fifth passage in the cell cultures. FIN1 was isolated from a domestic cat (case 5) that died of toxoplasmosis. Electropherogram from the analysis performed with PeakScanner software, showing the color-coded peaks together with the sizing standard (red) overlaid. The x-axis shows the sizes in basepairs; the y-axis, the peak heights in relative fluorescence units.

Table 7 Results of direct genetic characterization of *Toxoplasma gondii* from Finland. Six microsatellite markers (B18, TUB2, TgM-A, W35, B17, and M33) were used for genotyping, and M48was included as a fingerprinting marker.

	B18[a]	B18	TUB2[a]	TUB2[b]	TgM-A[a]	TgM-A	W35[a]	W35[b]	B17[a]	B17[b]	M33[a]	M33[b]	M48[a]	**Genotype**
European brown hare (III)														
case 1	158	2	ND[c]	-	207	2	(>)245	2(.3)	ND	-	169	1.2	213	**II[d]**
case 2	158	2	ND	-	207	2	(>)245	2(.3)	ND	-	169	1.2	215	**II[d]**
case 3	158	2	289	2.3	207	2	(>)245	2(.3)	336	2.3	169	1.2	215	**II**
case 4	158	2	289	2.3	207	2	(>)245	2(.3)	336	2.3	169	1.2	215	**II**
case 5	158	2	289	2.3	207	2	(>)245	2(.3)	336	2.3	169	1.2	217	**II**
case 6	158	2	289	2.3	207	2	(>)245	2(.3)	336	2.3	169	1.2	223	**II**
case 7	158	2	289	2.3	207	2	(>)245	2(.3)	336	2.3	169	1.2	215	**II**
case 8	158	2	289	2.3	207	2	(>)245	2(.3)	336	2.3	169	1.2	215	**II**
case 9	158	2	289	2.3	207	2	(>)245	2(.3)	336	2.3	169	1.2	215	**II**
case 10	158	2	289	2.3	207	2	(>)245	2(.3)	336	2.3	169	1.2	215	**II**
case 11	158	2	289	2.3	207	2	(>)245	2(.3)	336	2.3	169	1.2	219	**II**
case 12	158	2	ND	-	207	2	(>)245	2(.3)	ND	-	169	1.2	225	**II[d]**
case 13	158	2	289	2.3	207	2	(>)245	2(.3)	336	2.3	169	1.2	229	**II**
case 14	158	2	289	2.3	207	2	(>)245	2(.3)	336	2.3	169	1.2	217	**II**
Mountain hare (III)														
case 1	158	2	289	2.3	207	2	(>)245	2(.3)	336	2.3	169	1.2	217	**II**
case 2	158	2	289	2.3	207	2	(>)245	2(.3)	336	2.3	169	1.2	227	**II**
case 3	158	2	289	2.3	207	2	(>)245	2(.3)	336	2.3	169	1.2	213	**II**
case 4	158	2	289	2.3	207	2	(>)245	2(.3)	336	2.3	169	1.2	225	**II**

	B18[a]	B18	TUB2[a]	TUB2[b]	TgM-A[a]	TgM-A	W35[a]	W35[b]	B17[a]	B17[b]	M33[a]	M33[b]	M48[a]	**Genotype**
Eurasian red squirrel (IV)														
case 1	158	2	289	2.3	207	2	(>)245	2(.3)	336	2.3	169	1.2	233	**II**
case 2	158	2	289	2.3	207	2	(>)245	2(.3)	336	2.3	169	1.2	233	**II**
case 3	158	2	289	2.3	207	2	(>)245	2(.3)	336	2.3	169	1.2	215	**II**
Domestic cat (V)														
case 1	158	2	289	2.3	207	2	(>)245	2(.3)	336	2.3	169	1.2	223	**II**
case 2	158	2	289	2.3	207	2	(>)245	2(.3)	336	2.3	169	1.2	215	**II**
case 3	158	2	289	2.3	207	2	(>)245	2(.3)	336	2.3	169	1.2	231	**II**
case 4	158	2	289	2.3	207	2	(>)245	2(.3)	336	2.3	169	1.2	235	**II**
case 5 (FIN1)	158	2	289	2.3	207	2	(>)245	2(.3)	336	2.3	169	1.2	229	**II**
case 6 (FIN2)	158	2	289	2.3	207	2	(>)245	2(.3)	336	2.3	169	1.2	215	**II**

[a] Allelic polymorphism is expressed as the size (in base pairs) of the PCR product.

[b] 1.2 = the allele is shared by genotypes I and II; 2.3 = the allele is shared by genotypes II and III.

[c] ND = not detected.

[d] Results not obtained from all six genotyping markers.

Despite the allele being shared by genotypes II and III, the estimated length of the PCR-product at marker W35 fell consistently between 245 and 246 bp from the reference and local strains belonging to genotype II, and between 244 and 245 bp from the genotype III reference strains (Table 7).
Two of the squirrels, those found dead together, yielded identical genotyping results at all markers, pointing to a common source of infection (Table 7).

5.4.3. Local isolates

Two *T. gondii* strains were isolated directly from the tissues of two local cats with generalized fatal toxoplasmosis (V). Rampant infection was evident by the third passage from the CNS and the myocardium samples from cat 5, and from the CNS and the lung tissue samples from cat 6. The first isolate was obtained in 2009 (FIN 1, from cat 5), and the second in 2011 (FIN 2, from cat 6). Their growth rate and pattern in the cell cultures resembled those of the genotype II reference strain PSP-2007-TON: multifocal coalescent disruption of the cell layer, ready for passage to a fresh cell flask approximately every 8-10 days.

6. Discussion

6.1. Strengths and limitations of the study

6.1.1. Sample quality

The blood samples from the sheep (I) and domestic cats (V) were taken, centrifuged, and stored according to routine protocols. The farmers collected the blood samples from farmed wild boars upon slaughter (II), and the hunters collected the blood samples from wild cervids after the kill (I). Many of the latter samples were exposed to temperatures below zero upon transportation to the laboratory via mail, and hemolysis was commonly observed in the sera. The sera from the blood samples collected from necropsied hares (III) and the serosanguineous fluid samples from the thawed lynx hearts (VI) were collected after a longer postmortem period, which likely affected their quality. Storing biological fluid samples at room temperature for weeks has been shown to decrease the proportion of them identified as seropositive with DAT (Jakubek et al., 2012); moreover, the degradation of antibodies and changes in the body fluid compartment after death affected the serological test results from dead animals stored at +10 °C within days (Tryland et al., 2006).

Hemolysis due to freezing, prolonged time before sampling, or other reasons likely did not affect the results obtained. According to the manufacturer of the serology method, the DAT has been validated for use with good quality human sera, but not for hemolyzed, lipemic or icteric sera, or for biological fluids other than sera. However, hemolysis has been mentioned not to interfere with the test that has been used for a multitude of animal species (Dubey, 2010). Moreover, we found no effect when testing known anti-*T. gondii* antibody-positive and -negative samples hemolyzed in various ways, including freezing. The one false negative result observed in the positive sample hemolyzed by tap water was likely due to dilution. Interestingly, numerous false negative results were seen after attempting to clear the sera by removing hemoglobin (Table 5). A light table was used for reading the plates, which were also read from below against a light source; the background color present in some samples due to hemolysis did not impair the reading of the plates. This contrasts with the difficulties that others encountered in interpreting strongly hemolyzed samples at low dilution (De Craeye et al., 2011); worth noting is that the study made no mention of the use of a light table or other exogenous light source.

The fecal samples from domestic cats (V) were collected fresh and analyzed within a week, a time period considered acceptable. For logistic reasons, the fecal samples from lynx (VI) had to be frozen, which did affect their quality, namely their consistency and the morphological appearance of the helminth eggs. Although we did not expect this to have influenced the detection of *T. gondii*-like oocysts, false negativity cannot be fully

excluded. Of note, other parasites were identifiable in the same lynx samples, including protozoans such as *Eimeria* sp. (from their prey) and *Isospora* sp. (Deksne et al., 2013).

Overall, the lynx samples were the most problematic in terms of quality, due to the mid-winter hunting season and the need to store and transport them to the field station before the laboratory. Consequently, some of the samples might actually have been exposed to freezing and thawing twice before analysis.

6.1.2. Sample representativity

The host species included in these studies are restricted and serve as examples of host species for *T. gondii* present in Finland. Some species not included in these studies might be even more relevant for the local epidemiology of this parasite or for its zoonotic risk to humans. Nevertheless, domestic cat is surely in the centre of *T. gondii* epidemiology (Dabritz and Conrad, 2010), and these studies cover a representative selection of both wild and domestic hosts.

Hunting seasons were conveniently limited time periods to permit a cross-sectional study design, and organized cooperation with hunters made intensive, nationwide sampling possible (I, VI). Yet winter hunting seasons were challenging for the transport of samples, and very young animals were not sampled in these hunts. The inclusion of archived materials and databases, as well as cooperation with other ongoing research efforts (the feline DNA bank (V), disease surveillance programs and the projects of other groups at the University of Helsinki (II) and Evira (I, III, IV), as well as the game animal research of Finnish Game and Fisheries Research Institute (VI)) made it possible to achieve large sample sizes during limited periods of time (I, II, V, VI), and longer sampling periods to encompass seasonal variation (II, III, IV, V, VI).

The hunter-harvested samples of moose (I) and lynx (VI) represent their respective populations well. More animals were hunted and sampled in areas with larger populations. A slight dominance of samples from males (56.6%, range 43.2.-71.3 in the game management districts) and adults (61.6%, range 51.1-75.0%) was evident in moose (I). The gender balance was surprisingly even in the lynx, given that female lynx accompanied by cubs under one year of age are protected from hunting (Hunting Act, 1993). Most sheep sera were from ewes, and the sampling frame covered only sheep over one year of age (I); most sheep are slaughtered for human consumption at a younger age than that. The majority of domestic cats (V) sampled for serology were purebred, pedigreed cats, whereas the majority of cats living in Finland are non-purebred. The sample thus represents mainly purebred pedigreed cats and was likely affected by the participatory activity of their owners. Four of the five breeds with the highest sample size in the study have been in the top ten most common cat breeds in Finland in recent years (Suomen Kissaliitto, 2011; Suomen Kissaliitto, 2013). However, the most common breed in the sample, Korat, is not among the most common breeds in the register. The seroprevalence

among Korat cats was the lowest of the five breeds, lowering the overall seroprevalence estimate.

The sample sizes obtained exceeded the calculated sample sizes for all the host species and investigations, except for the deer for serology. The squirrels can be considered a convenience sample. Our hunter-harvested lynx sample size was exceptionally large compared with several other studies in this and other lynx species (Oksanen and Lindgren, 1995; Ryser-Degiorgis et al., 2006; Garcia-Bocanegra et al., 2010; Dubey, 2010): 91% of all lynx legally hunted during the season and approximately 14% of the entire estimated population. Regardless, the actual number of animals examined remains more relevant than the proportion of the population sampled because a larger sample size (n) means narrower confidence intervals and diminishes the effect of any sampling bias.

In the sampling from sheep for serology (I), the clustering of the sheep into flocks was taken into account, which dramatically increased the sample size needed. This is a relevant point to consider when sampling animals and evaluating prevalence estimates from animals kept in groups, as they are likely to have been similarly exposed. The presence and level, or lack, of oocyst contamination likely varies by farm. In addition, vertical transmission might be relevant in maintaining *T. gondii* infection in sheep (Duncanson et al., 2001; Williams et al., 2005; Hide et al., 2007; Hide et al., 2009).

Most compromises had to be accepted with regard to the fecal samples. While the ages of most of the cats sampled were unknown, cats of different ages were included (V). Pooling the samples could not be avoided when sampling at the cat shelters, because many of the cats are kept in small groups, and we aimed to sample without causing the cats unnecessary distress (V). Individual samples would have been better than pooled samples to fully ensure that all the cats in the group were included. Due to the time of the hunting season, the fecal samples from lynx included no samples from very young cubs (VI). If shedding is expected to occur mainly after the primary infection, as in cats (Dabritz and Conrad, 2010), this could explain why no lynx were found to shed *T. gondii*.

The animals submitted for postmortem examination (III, IV, V, VI) cannot be regarded as representing the populations well, and the results from different species are not fully comparable due to different motivation to submit animals of different species. The wild animals examined (III, IV, VI) included all animals of those species examined postmortem during the study periods because the Finnish Food Safery Authority Evira is the only laboratory that performs wildlife pathology in Finland, whereas domestic cats can be submitted for postmortem examination at places outside the University of Helsinki. Most domestic cats do not undergo postmortem examination after death or euthanasia, and the cost of the examination may have a selective effect. Hares and squirrels are typically submitted by individual citizens who want to know the cause of death of animals they have found dead, whereas the lynx material includes more animals that died in traffic accidents and were subsequently submitted for examination by the police. Overall, in the wildlife material submitted for pathology, animals found dead near human settlements and

roads are likely overrepresented because these animals are the most likely to be found dead by humans. Animals dying in more sparsely populated areas are more likely to be found by scavengers than by active citizens, and are thus likely underrepresented. To find cases of toxoplasmosis, however, samples originating from the vicinity of humans may be optimal, thus overestimating their number, if the infection affects the behavior of animals that would normally avoid humans. Infection might predispose them to being killed by hunting or in a traffic accident. These cases may also represent the sentinels of pathogen spillover from or into domestic circles, and the parasite strains humans could become infected with as well.

6.1.3. Methodology

The serology method (DAT) has been validated for human sera only. Nevertheless, DAT and its in-house version, the modified agglutination test (MAT), have both been widely used for screening the sera of different animals species (European Food Safety Authority EFSA, 2007; Dubey, 2010), and bodily fluids when sera are unavailable (Tryland et al., 2006; Ryser-Degiorgis et al., 2006; Jakubek et al., 2012), for anti-*Toxoplasma gondii* IgG antibodies. We chose the commercial DAT rather than an in-house version for these studies in order to obtain more accurate comparability of the results.

One major advantage of DAT is that it requires no species-specific reagents. The most important reagents are the antigen (formalin-treated parasites) and 2-ME that denatures IgM (Pike and Chandler, 1972; Johnson et al., 1989). Preservatives other than formalin have also been used for antigen preparation (Oksanen et al., 1996; Montoya et al., 2007; Dubey, 2010). According to the manufacturer, DAT specifically detects only one antibody class: IgG. These antibodies are relevant for epidemiological studies, because they are specific and presumably long-lasting (Dubey, 2010). However, results contradicting this assumption have emerged: the persistence of antibodies may be related to the host species or to other factors (Opsteegh et al., 2011a; Opsteegh et al., 2011b; Fernandez-Aguilar et al., 2013). Moreover, negative DAT results indicate the absence of a detectable level of IgG antibodies, but do not rule out other types of immune response.

The manufacturer suggests using two dilutions (1:40 and 1:4000), with 1:40 serving as the cut-off for positivity. We chose to use only one dilution for most of the samples due to the cost of the kits and the little extra benefit expected from two dilutions. Some false negative results were expected due to the prozone phenomenon. In the cat study (V), the prozone phenomenon was observed in 1.3% of the samples: 6 of the 445 samples diluted to both 1:40 and 1:4000 yielded a negative result at 1:40 and a seemingly paradoxical positive result at 1:4000. This prozone phenomenon occurs due to an excessively high concentration of antibodies in relation to the antigen for agglutination to occur; few or no antibodies bind more than one antigenic particle, forming a bridge between them. As a result, the observed result at too low a dilution is a false-negative, and positivity is seen ony when the sample is sufficiently diluted.

The samples that tested positive at a dilution of 1:40 or 1:4000 (the samples exhibiting the prozone phenomenon detected by using two dilutions) or both were defined as seropositive. This threshold for seropositivity is the one recommended by the manufacturer and the one used in several studies with which we wanted our results to be comparable (Näreaho et al., 1995; Oksanen and Lindgren, 1995; Oksanen et al., 1997; Saarhelo, 1998; Vikøren et al., 2004; Ryser-Degiorgis et al., 2006). Many other studies use a lower titer (reciprocal of the highest dilution that yields a positive result), such as 16 or 25, as the cut-off, so our 40 is high in comparison (Gamarra et al., 2008; Dubey, 2009b; Dubey, 2009c; Dubey, 2010). Thus, our estimates of seroprevalences can be considered conservative.

The reading of the DAT results is simple and fast. We used a four-point scale to record the results for initial comparison and evaluation, and a dichotomous interpretation as the final result. The instructions of the manufacturer state that a positive reaction is "a mat covering *about half* of the well base" and the shrinking of its borders is acceptable, whereas all smaller mats are borderline reactions. In our interpretation, the mat was evaluated as covering at least half of the bottom of the well (interpreted as positive) or less than half of the bottom of the well (interpreted as negative). Our interpretation may in certain individual cases be stricter than the manufacturer's recommendation, further making our estimates conservative.

According to the manufacturer, the sensitivity of DAT is 96.2% (95% CI 94.55-97.39) and its specificity is 98.8% (95% CI 96.46-99.60) compared with the dye test, which is considered the gold-standard in human serology. The sensitivity and specificity of this method for use in different animal species is largely unknown. A recent study showed that ELISA and MAT did not differ significantly when used to detect *T. gondii* antibodies in cats (Zhu et al., 2012). The manufacturer of DAT also reports high precision when retesting the same samples in the same and different runs, and by different operators: the maximum deviation observed was a difference of one dilution within the recommended 10 dilutions. The positive predictive value of this test in a population with low seroprevalence, however, is only moderate (Sukthana et al., 2001). Using a high cut-off and explicit criteria for defining a sample as positive minimized this problem.

Hemolysis was not expected to affect the results obtained even though the method has not been validated for hemolyzed sera. The manufacturer recommends collecting a new sample in case of a hemolyzed sample, which is impossible from wildlife sampled by hunters. It has been noted that hemolysis would not affect the results of DAT/MAT (Dubey, 2010), but no studies have earlier confirmed this. We found no effect on the DAT results when testing samples hemolyzed in a variety of ways, whereas clarifying the samples with Hemoglobind (Biotech Support Group) prior to testing yielded false-negative results.

Sera are naturally better material for serology than tissue fluid samples, but obtaining high numbers of good quality serum samples from wildlife is challenging. Body fluids, such as serosanguineous fluid collected from the heart or thoracic cavity, are often used (Ferroglio et al., 2000; Ryser-Degiorgis et al., 2006; Jakubek et al., 2012). The seroprevalence estimates obtained from such material are likely underestimates due to lower antibody levels than in serum, and postmortem sampling (Ferroglio et al., 2000; Tryland et al., 2006; Jakubek et al., 2012).

The method used for oocyst detection, FLOTAC, is sensitive and has minimal background visibility blocking problems because the top portion of the flotation solution is transferred aside before microscopy. This also allowed for easy collection of the top portion for the necessary PCR confirmation of the *T. gondii*-like oocysts detected as well as for their further characterization (V).

More detailed clinical data were available from only some of the cats (V); clinical chemistry and hematological parameters, for example, were examined in only four of the six cats. Comparing detailed clinical data with postmortem findings in a larger number of toxoplasmosis cases would be of veterinary relevance: toxoplasmosis is a challenging clinical diagnosis, but should be on the differential diagnosis list, especially for young cats with acute pneumonia and hepatitis (V).

Diagnosing toxoplasmosis postmortem can be challenging as well, due mainly to unspecific gross findings. In a Swedish study, several previously missed toxoplasmosis cases were found in hares retrospectively by using IHC (Gustafsson et al., 1988). IHC has been shown to be a good method for evaluating the spread and burden of parasites in tissues (Uggla et al., 1987; Gustafsson et al., 1988), a finding that our studies (III, IV, V) also confirmed. Without this technique, the spread and numbers of parasites would have been greatly underestimated.

Isolating *T. gondii* strains directly into cell cultures is simple, but requires aseptically collected samples. A positive result can be detected faster than with bioassay in mice or cats (Derouin et al., 1989; Miller et al., 2000).

For the molecular methods, the DNA concentrations of the DNA extracts were not routinely measured. The methods were not quantitative, and the samples were not uniform. The possibility of false negatives was accepted, and the sensitivity of the molecular methods has not been determined in detail. In these studies, the molecular methods served for confirmation and for further characterization. Specific DNA was successfully amplified from many sample types, including various FFPE and frozen tissues, feces, and cell culture samples. All these were rich in parasites by microscopy.

The parasites may be unevenly distributed in the sample, so even a sensitive method may fail to detect the parasites or their DNA. In these studies, flotation methods served as pre-enrichment methods for fecal samples and centrifugations for cell culture samples. More

simple methods for enriching the parasites from various sample types (e.g. meat, milk, semen, feces, and soil) would broaden the reseach possibilities.

Six MS markers for genotyping appeared to be enough for these studies, and the seventh fingerprinting marker proved valuable in providing further characterization (Table 7). Genotyping with fewer than four or five markers should be interpreted cautiously because more markers could reveal more polymorphisms (Ajzenberg, 2010; Subauste et al., 2011). The updated version of the MS genotyping method (Ajzenberg et al., 2010), with its 15 MS markers, would be an effective tool for investigating outbreaks.

The MS genotyping method yielded consistent results from different samples from the same individuals (IV, V), and the analysis almost always proved successful at all markers (Table 7), even if its sensitivity is lower than that of methods based on nested-PCR (Ajzenberg et al., 2010). We also tested a nested-PCR-RFLP-method for genotyping (Howe et al., 1997; Khan et al., 2005; Dubey et al., 2006; Su et al., 2006), but the results were occasionally inconsistent and showed low reproducibility even from reference material. The genotyping method as well as the number of markers used for genotyping are relevant in evaluating any genotyping results. The combined use of several methods is optimal in investigating genetic diversity (Su et al., 2012).

In these studies (III, IV, V), the parasites present in the sample material were characterized directly, without a bioassay step that could have had a selective effect. This is another relevant point to consider when evaluating genotyping results. For example, mixed infections with more than one strain might be missed if isolation into cell cultures or especially bioassay in laboratory animals were performed first.

Formalin fixation adversely affects PCR amplification (Ben-Ezra et al., 1991), but the genetic analysis of *T. gondii* was also successful from DNA extracted from archived FFPE material (III, IV, Table 7). It is thus possible to re-investigate old cases with new molecular methods, to carry out retrospective molecular epidemiological studies, and to utilize the practical storage and transport as FFPE samples in long-term prospective studies.

6.2. Major findings and their relevance

6.2.1. Exposure to *Toxoplasma gondii* is common

Of all the host species examined, some animals had mounted a detectable IgG antibody response against *T. gondii*. The infections were naturally acquired, most likely within Finland: *T. gondii* is endemic. Wild animals do not respect international borders, and the estimates can be regarded as estimates of the area. Some domestic cats could have encountered the parasite during travels or via imported feed.

Based on the serology results, the animals intended for human consumption had commonly encountered *T. gondii*. The seroprevalence in sheep (24.6%; I) was similar to previously reported estimates from Scandinavia and elsewhere (Lundén et al., 1992; Skjerve et al., 1998; Dubey, 2009c). The seroprevalence in moose (9.6%; I) was lower than estimates of 12.6% from Norway ($P < 0.01$) and 20.4% from Sweden ($P < 0.0001$), which were obtained with the same method and used the same cut-off for seropositivity as we did (Vikøren et al., 2004). The seroprevalence in white-tailed deer (26.7%; I) was higher than the overall seroprevalence in moose ($P < 0.0001$), but the difference was nonsignificant in comparison to moose originating from the same southwestern game management districts where the deer were sampled. Interestingly, the seroprevalence in white-tailed deer was lower than the estimates of 30.0-64.2% reported in the United States (Lindsay et al., 1991; Vanek et al., 1996; Dubey, 2010), which were obtained using MAT and 1:25 or 1:50 as the cut-off for seropositivity. Despite a higher cut-off for defining a sample as seropositive, the *T. gondii* seroprevalence in Finnish farmed wild boars (33.0%; II) was seven times higher than that in captive wild boars in Brazil (Fornazari et al., 2009). The seroprevalence in Finnish farmed wild boars was also higher than an ELISA-derived seroprevalence (20.3%; $P < 0.05$) in Latvian farmed wild boars (Deksne and Kirjusina, 2013). The free-ranging wild boars of Estonia had a lower seroprevalence (24.0%; $P < 0.05$) with the same method and cut-off we used (Velström et al., 2013), whereas ELISA yielded a seroprevalence similar to ours (35.1%) in free-ranging wild boars in Latvia (Deksne and Kirjusina, 2013).

All the seropositive brown hares died of the *T. gondii* infection, whereas no seropositives were detected among the hares that had died of other causes (III). In contrast, a seroprevalence of 4.2% (4/96) was found among mountain hares that had succumbed to causes other than toxoplasmosis (III). If the latter results are true positives, these animals might have had a mild, chronic infection without parasites in the organs examined. Chronic infections thus appeared to be relatively rare to nonexistent among hares, while toxoplasmosis is confirmed to be a common infectious cause of death in both species of hare (III). This observation is similar to those of Swedish studies from the 1980s; toxoplasmosis was the cause of death in 10% of brown hares and 4% of mountain hares, while no seropositives were found among 176 apparently healthy brown hares despite the use of several serology methods (Gustafsson et al., 1988; Gustafsson and Uggla, 1994).

Together, these Nordic findings strongly support the hypothesis that these species of hare are highly susceptible to severe clinical toxoplasmosis. However, other studies have identified more seropositive hares, leaving the hypothesis debatable (Valtonen and Andersson, 1968; Hejlicek et al., 1997; Frölich et al., 2003; Aubert et al., 2010). Of note, very recent observations suggest that antibodies might not persist in some species of hare, making this question even more intriguing (Fernandez-Aguilar et al., 2013). On the other hand, experimental studies have also demonstrated toxoplasmosis to be a severe and fatal infection among hares (Gustafsson et al., 1997a; Gustafsson et al., 1997b; Sedlak et al., 2000).

Almost half the domestic cats (V) and a majority of the lynx (VI) had antibodies against *T. gondii*. The seroprevalence in lynx can be considered an underestimate, since it was obtained from serosanguineus fluid that likely contained lower antibody levels than serum (Ferroglio et al., 2000; Jakubek et al., 2012).

In domestic cats, the seroprevalence had not changed from the previous estimate (Näreaho et al., 1995; Näreaho, 1995) obtained with the same method and cut-off for seropositivity – but from shelter cats only, whereas ours is mainly from pedigreed, purebred pets. In contrast, the seroprevalence in lynx was significantly higher than the previous estimates from Finland and Sweden, which also used the same method and cut-off (Oksanen and Lindgren, 1995; Ryser-Degiorgis et al., 2006). The proportion of samples from young animals and of samples from the northern parts of the countries may partly explain the difference between the Swedish estimate and ours. Nevertheless, the lynx population of Finland has more than doubled since those estimates (Finnish Game and Fisheries Research Institute, 2008), and more lynx could now be exposed to possible pathogen spillover to and from domestic animals (Power and Mitchell, 2004).

The north-south gradient in the evidence of exposure to *T. gondii* was present in a wild and a domestic herbivore, a farmed omnivore, and a wild, free-ranging carnivore (I, II, III, IV, VI), thus exemplifying the importance of a sampling plan that geographically covers the area of interest. A similar gradient has been observed previously in Finland in reindeer: the highest *T. gondii* seroprevalences were detected in the southernmost herding districts and were attributed to corral feeding, which leads to more contact with domestic cats (Oksanen et al., 1997). Several other studies have also reported geographical differences in *T. gondii s*eroprevalences (Uggla and Hjort, 1984; Vikøren et al., 2004; Ryser-Degiorgis et al., 2006; Malmsten et al., 2011; Beral et al., 2012), and infection kinetics over the lifetime of animals may also differ by region (Halos et al., 2010).

Climatic factors may partly explain the geographical gradients. The northern temperate climate and mean annual temperature of 5.5 °C in southern Finland certainly differs from the subarctic climate and below zero mean temperature of the north (Finnish Meteorological Institute, 2013). Herbivorous animals, such as moose, sheep, and hares most likely encounter *T. gondii* via oocysts in their feed or water (Skjerve et al., 1998; Tenter et al., 2000; Kijlstra and Jongert, 2008). The harsh northern winters may affect

sporulation, but likely not the survival of sporulated oocysts (Frenkel and Dubey, 1973; Hill and Dubey, 2002; European Food Safety Authority EFSA, 2007; Dubey, 2010). A more likely explanation is the concentration of the human population in the southern and southwestern parts of Finland, with its domestic cats. Moreover, our findings (III, IV) as well as others' (Hülphers et al., 1947; Christiansen and Siim, 1951) indirectly confirm the survival of oocysts: hosts that likely become infected from oocysts died of acute toxoplasmosis in the winter. However, carnivorism may also be possible in these hosts. Squirrels are omnivorous, and evidence suggests that herbivorous hares might sometimes eat the tissues of other animals (Rausch et al., 1956). Overall higher host population density might be another local explanatory factor, similarly to *Trichinella* spp. infections transmitted by carnivorism and scavenging (Airas et al., 2010). For the lynx in particular, the main prey species in the south differ from those in the north, which may partly explain the gradient (VI).

Geographical variation in the prevalence of *T. gondii* infection is relevant for risk assessment and recommendations. Seropositive animals have at least encountered the parasite and can therefore be considered sentinels, indicating that humans may also encounter the parasite in these same areas. Seropositivity correlates with the chronic presence of *T. gondii* in many hosts (Lindsay et al., 1991; Richomme et al., 2009; Halos et al., 2010), but not well in others (Opsteegh et al., 2011b). Animals carrying *T. gondii* may pose a risk for human infection when humans handle their carcasses (Sacks et al., 1983; McDonald et al., 1990; Ross et al., 2001; Vikøren et al., 2004) or eat their insufficiently cooked meat (Cook et al., 2000). The meat of hunter-harvested wildlife is mainly consumed locally. Privately consumed game is not subject to meat inspection, and the current inspection does not even attempt to detect *T. gondii* infections.

Seropositive animals were found throughout Finland, including the northern regions, thus showing that cold climate and remote locations pose no barrier to this parasite. The hosts of *T. gondii*, undoubtedly including humans, may encounter the parasite even in the northernmost parts of Europe.

6.2.2. Risk factors and actions

As expected, the seroprevalences were higher in older animals (I, II, V, VI) with a longer time during which to encounter the parasite. The hosts investigated typically appeared to have encountered the parasite after birth, as an acquired infection. No signs of antibody waning were noted. *Toxoplasma gondii* infections were common, especially in the southern part of Finland (I, II, III, IV, VI).

The risk analysis of feline infections identified several important points for cat owners to consider (V). Cats typically become infected by ingesting raw meat, either in the form of prey they catch or meat their owners feed to them. Raw meat was part of the diet of 78.4% of the cats (V) and a major risk factor for seropositivity. Cats do need meat in their diet,

but should receive it after sufficient cooking or at least freezing to avoid *T. gondii* infection. Allowing cats to roam outdoors freely gives them opportunities not only to hunt infected animals, but also to defecate in the environment – both to become infected and to spread the infection causing risk to other hosts. Access outdoors was common and a risk factor for seropositivity even though it did not fit the final multivariable models (V). Cats that have opportunities to become infected and then to defecate into the environment may contribute to the oocyst burden of the environment.

The observed differences between seroprevalences in different cat breeds (V) were also likely associated with their lifestyles: over 80% of Birmans, a breed with the highest seroprevalence (65.3%), received raw meat in their diets and roamed outdoors. Korats had a significantly lower seroprevalence (40.2%), and fewer than half of them received raw meat in their diets, and 80% were kept exclusively indoors.

Cat owners could do more to protect their pets from encountering *T. gondii*, as well as to ensure that their cats do not fall ill from it or spread it. Furthermore, the infection was associated with reproduction problems (V), which are of particular concern to cat breeders. No vaccine is currently commercially available, yet the known risk factors are the points where preventive action could be taken (Elmore et al., 2010; Dabritz and Conrad, 2010; Opsteegh et al., 2012).

Proper disposal of cat litter, whether in communal waste or by burning, hampers the spread of the parasite from infected cats (Dabritz and Conrad, 2010). The feces of domestic cats should not be disposed of via toilets, as *T. gondii* oocysts are not destroyed by chemical and physical sewage water treatments even at levels up to six times higher than routinely used for raw sewage (Wainwright et al., 2007a; Wainwright et al., 2007b). Because of this, the manufacturers of cat litter in California advise against flushing cat feces down toilets (Dabritz and Conrad, 2010).

For the parasite, the environmental reservoir of oocysts is an important source of infection for many hosts, including domestic and game animals intended for human consumption. Interestingly, it is often referred to as contamination instead of a reservoir (McAllister, 2005; Robert-Gangneux and Dardé, 2012). Human actions that allow or even enhance the shedding of the oocysts into the environment by the definitive hosts kept as pets can be considered contamination of the environment.

6.2.3. Domestic cats have the major definitive host role in Finland

When discussing the environmental oocyst burden in 2010 (I), placing the blame on domestic cats for contaminating the environment while describing the contribution of the local wild felids as “unknown” felt only partly reasoned. Speculations about the importance of lynx were based on limited data (Oksanen and Lindgren, 1995) showing that lynx commonly encounter the parasite. The shedding of *T. gondii* oocysts by this lynx species has never actually been reported (Ryser-Degiorgis et al., 2006; Dubey, 2009a).

Our serology results indicate that both cats and lynx often encounter *T. gondii* in Finland (V, VI). Using the same method and cut-off, the seroprevalence in lynx (86.1%) was significantly higher than that in cats (48.4%; $P < 0.0001$). The seroprevalence estimate in lynx may be an underestimate due to the available sample material, which consisted of serosanguineus fluid samples collected postmortem instead of sera (Ferroglio et al., 2000; Tryland et al., 2006; Jakubek et al., 2012).

Infection was more common in lynx than before (Oksanen and Lindgren, 1995; Ryser-Degiorgis et al., 2006), and ever common in cats (Näreaho, 1995; Näreaho et al., 1995). The proportional mortality rate from toxoplasmosis in cats (V) did not differ significantly from a Danish estimate (Henriksen et al., 1994).

The odds of seropositivity in cats increased with age (V) and in lynx, with weight gained (VI). Older lynx are likely heavier, but males are almost always heavier than females of the same age. In the multivariable models, which included weight and sex, weight can be cautiously interpreted to represent age, as sexual dimorphism was accounted for by including the sex of each animal in the models (VI).

One cat was confirmed to be contributing to the environmental oocyst burden by shedding oocysts of the endemic genotype II (V). In contrast, no *T. gondii*-like oocysts were detected in the fecal samples from the lynx (VI). Seropositive domestic cats are regarded as cats that have previously shed oocysts (Dubey, 2010), and most of the lynx examined were seropositive (VI). Similarily to cats (Dabritz and Conrad, 2010), the shedding of oocysts by lynx might occur mainly soon after primary infection. However, this extrapolated assumption was not supported by the experimental infection of a lynx: no shedding was detectable despite seroconversion (Oksanen et al., 1997). Moreover, only a small proportion of the population of domestic cats sheds oocysts at any given moment (Dabritz and Conrad, 2010), as this study (V) also showed. The shedding of oocysts lasts for a limited period and for most cats occurs only once (Dabritz and Conrad, 2010; Dubey, 2010). Obtaining a sample size of lynx sufficient to find shedders can be challenging. Our study and a Swedish one (Ryser-Degiorgis et al., 2006) investigated fecal samples from altogether 68 young lynx, 34 of which tested seronegative, and detected no shedding. Sampling younger lynx might be the way to show that lynx do indeed shed oocysts after naturally acquired infection or to confirm that they do not.

If lynx do not shed oocysts, their role in the epidemiology of *T. gondii* is limited to that of infected individuals that are eaten by other hosts, mainly scavengers, and an unknown contribution through other infection routes. The human consumption of lynx meat is not unknown in Finland, and carcass handling might pose another gateway to humans (Sacks et al., 1983; McDonald et al., 1990; Ross et al., 2001; Vikøren et al., 2004). Cats are only rarely eaten by predators (including lynx) in Finland, so they serve almost exclusively as a source of oocysts.

Domestic cats, with a population of approximately 800 000, clearly outnumber the fewer than 3000 lynx, and thus play the major definitive host role in the epidemiology of *T. gondii* in Finland. Preventing the infections of pet animals is far easier than applying preventive measures to free-ranging, protected wildlife.

6.2.4. No signs of separate domestic and sylvatic cycles

Wildlife, semidomesticated, domestic and pet animals, and humans can encounter *T. gondii* in Finland. This has been shown by serological methods in these studies (I, II, III, V, VI) and previous ones (Valtonen and Andersson, 1968; Lappalainen et al., 1992; Hirvelä-Koski, 1992; Nieminen, 1993; Oksanen and Lindgren, 1995; Näreaho, 1995; Näreaho et al., 1995; Oksanen et al., 1997; Saarhelo, 1998). Moreover, *T. gondii* strains belonging to the endemic genotype II have now been shown to be an occasional cause of death of both wild and domestic hosts (III, IV, V; Table 7), and one domestic cat was also shown to shed parasites of this genotype. This same genotype infecting both wild and domestic animals suggests that the infections in the two host groups do not follow separate cycles.

Free-ranging wild animals are part of the host range of *T. gondii* in Finland and contribute to its success. Because numerous host species eat each other, wild hosts are unlikely to be dead-end hosts. Carnivorism and scavenging in a suitable host density ensures the parasite a steady supply of new hosts. Although by no means the only way, humans may become infected when eating undercooked meat or handing the carcasses of infected game. Domestic cats may become infected by eating small wildlife prey. If the lynx shed oocysts, they may provide a source of infection for other hosts, including domestic animals. Wildlife in the southern part of Finland, where the human population is concentrated, appears to be infected more often than the wildlife in the north. This suggests that domestic cats living with humans play a crucial role in the epidemiology of *T. gondii* by shedding oocysts that serve as a source of infection for both wild and domestic hosts. Among the wildlife, obvious victims of this spillover, such as brown hares and mountain hares (III), and possibly red squirrels (IV), often die from the infection.

6.2.5. Some hosts appear to be highly susceptible to severe clinical toxoplasmosis

Our results (III) support the hypothesis that brown hares and mountain hares are extremely susceptible to death from toxoplasmosis. Toxoplasmosis was confirmed as a common infectious cause of death for both hare species, and all the brown hares that encountered *T. gondii* and mounted a measurable antibody response against it died of it. These infections were naturally acquired; the infective stage of the parasite causing the infection, the infection dose, and the infection route were likely ones to which these animals are “best” adapted. The parasite strains that caused the deaths were all of genotype II, which is generally considered a genotype of low virulence and causing mainly chronic, subclinical infections.

The Swedish studies (Gustafsson et al., 1988; Gustafsson and Uggla, 1994; Gustafsson et al., 1997a; Gustafsson et al., 1997b) reached a similar conclusion: zero prevalence of antibodies, a common cause of death, extensive tissue damage after experimental infection, and the lack of a proper cellular response. The parasites used in the experimental infections were reportedly of low virulence in mice, but the genotype of the strain used is unknown. Overall, limited data have been available on the characterization of *T. gondii* strains that cause fatal disease in hares. An Italian isolate caused chronic infection when inoculated into mice (Olliaro et al., 1993), whereas Danish and Japanese isolates were as highly virulent in mice as the genotype I strain RH (Christiansen and Siim, 1951; Shimizu, 1958; Shimizu and Takagaki, 1959).

Red squirrels might also be a host species that is very susceptible to severe, clinical toxoplasmosis, even when infected with parasite strains of low virulence to other hosts. Despite the very limited sample size, the proportional mortality rate in our study was high, and the three fatal cases were all caused by *T. gondii* strains of genotype II (IV). Similar observations have been reported in squirrels kept in a zoo: the parasites that caused three deaths caused chronic infection when inoculated into mice, but killed an inoculated red squirrel within eight days (Rodhain, 1950). The genotype of those parasites is unknown, but their virulence in mice resembled that of genotype II.

The hosts that are highly susceptible to death from this common parasitic infection should be protected from it when possible. Investigating the infections in these hosts in detail could reveal key information and deepen our understanding of the parasite-host interaction and the factors affecting the outcome of the infections. At least in these cases, the deaths cannot be attributed to a particularly virulent causative strain, and no immunosuppression was evident either.

6.2.6. Underestimated genotype II can kill

All the *T. gondii* strains that had killed the hosts in our studies belonged to genotype II, and the genetic diversity was low (III, IV, V, Table 7). These infections were naturally acquired and thus support the endemic presence and dominant status of this genotype in Europe – and now confirmedly in Finland also. Thus far, strains belonging to other genotypes have not been detected in Finland.

The result seems surprising because genotype II strains typically cause only subclinical, chronic infections in immunocompetent hosts and are thus considered nonvirulent (Maubon et al., 2008). Recently, however, genotype II strains have also been found to cause death in a variety of host species elsewhere in Europe (Prestrud et al., 2008; Spycher et al., 2011; Herrmann et al., 2013). The victims were arctic foxes, a cat, and a beaver; ours included brown hares, mountain hares, red squirrels, and cats (III, IV, V). None of these altogether 33 animals had apparent signs of immunodeficiency or any concurrent, possibly predisposing disease. The infections were naturally acquired, so the infective stages of the parasite, infection doses, and infection routes were likely nothing out of the ordinary natural environment, but something to which these hosts should be adapted.

Genetic characterization of the parasite strains that cause severe and even fatal toxoplasmosis in different host species helps to deepen our understanding of one of the major current topics in toxoplasmosis research: evaluating the effect of the genotype of the parasite on the outcome of infection. Too little is known of *T. gondii* genotype distribution and effect in the infections of animal hosts: such data could provide relevant insight into this interesting topic in human medical parasitology (Ajzenberg et al., 2002; Ajzenberg et al., 2009; Subauste et al., 2011; Fekkar et al., 2011; Carneiro et al., 2013). Animal hosts outnumber human hosts, which permits the collection of more data with reasonable time and effort. Moreover, animals can be regarded as sentinels for the *T. gondii* strains present in an area. The strains the animals harbor and the possibly more virulent strains that cause disease in the animal hosts are the ones humans may encounter as well, so their characterization is also relevant from the perspective of public health.

Genotype II *T. gondii* parasites should not be underestimated, as natural infections with strains belonging to this genotype can be fatal. The endemic, common presence of parasites of this potentially dangerous genotype in the environment and in animal hosts is both a risk factor and a welfare issue for them as well as for other hosts, including humans.

7. Conclusions

Toxoplasma gondii thrives in Finland. It is both common and endemic, has infected a wide variety of host species, and is shed into the environment.

Toxoplasma gondii appears to have no separate domestic and sylvatic cycles in Finland. Strains belonging to genotype II were found infecting both domestic and wild hosts, and the genetic diversity was low.

Toxoplasma gondii genotype II is thus endemic in Finland. The virulence of *T. gondii* strains of this genotype should not be underestimated. Such strains had killed six cats, fourteen brown hares, four mountain hares, and three squirrels.

Known risk factors for *T. gondii* infections show where preventive measures could prove most effective. Domestic cats in particular should be better protected from encountering this parasite.

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