Trichinella in wildlife and pork production: evaluation of risk-based monitoring

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Trichinella in wildlife and pork production: evaluation of risk-based monitoring

Trichinella in wild en varkensvleesproductie:
Evaluatie van risico-gestuurde monitoring

(met een samenvatting in het Nederlands)

Proefschrift

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Nowhere is it more true that
‘Prevention is better than Cure’
than in the case of Parasitic Diseases

– Rudolf Leuckart –

1886

Chapter 1

Introduction

Nematodes of the genus *Trichinella* are parasites of many different species of mammals, birds and reptiles. Six encapsulated *Trichinella* species (*T. spiralis, T. britovi, T. nativa, T. murelli, T. patagoniensis* and *T. nelsoni*) and three encapsulated genotypes (*Trichinella* T6, T8 and T9) have been recognized, which infect carnivorous and omnivorous mammals (Pozio, 2005; Pozio et al., 2009b). Three non-encapsulated *Trichinella* species infect birds (*T. pseudospiralis*) and crocodiles (*T. zimbabwensis* and *T. papuae*). No *Trichinella* infections have been demonstrated in amphibians or fish (Pozio, 2005). *T. spiralis* and *T. pseudospiralis* have a cosmopolitan distribution, due to dispersion by humans and birds respectively, whereas all other species have a more restricted geographical distribution (Pozio et al., 2009a) (Table 1).

The *Trichinella* lifecycle

The infective stage of encapsulated *Trichinella* species is a first stage larva that resides inside striated muscle tissue of an infected host within a so-called nurse cell (Figure 1 C), consisting of a collagen capsule (mainly collagen type IV and VI) inside an infected myocyte (Despommier, 1998). *Trichinella* larvae (Figure 1D) are liberated in the acidic pepsin environment of the stomach after ingestion of infected meat. In the mucosa of the small intestine, the liberated larvae moult four times within 30 - 36 hours and develop into adult male and female worms (Figure 1A and 1B). After mating, fertilized females produce eggs which embryonate *in utero*, resulting in shedding of variable numbers of newborne larvae per female from 6-7 days (pi) to 6-7 weeks post infection (pi), depending on the *Trichinella* species and age, sex and species of the host. Ultimately, the intestinal adult worms are expelled by the host (Despommiers, 2005; Pozio et al., 2003). In vitro, the newborne larval production of *T. spiralis* females is about 110 per 72 hours, whereas all other species produce far less, at <50 per 72 hours (Murrell et al., 2000). Newborne larvae penetrate the intestinal wall and migrate via the lymph and blood vessels to striated muscle cells, which they invade. Once inside a muscle cell, the *Trichinella* newborne larva takes control of its new environment by down-regulation of muscle contractile proteins, such as actin and myosin, followed by enlargement of the invaded myocyte and host cell nucleus multiplication to 40 – 60 nuclei per cell. The *Trichinella* larva further modifies its niche through excretion of tyvelosylated peptides that localize to the nucleoplasm of the enlarged myocyte and upregulate collagen synthesis, to build the collagen capsule of the nurse cell within approximately 26 days. A constant supply of nutrients to nourish the larva inside the nurse cells is provided by a network of small blood vessels that are attached to the outer layer of the collagen capsule, initiated by the *Trichinella* larva, possibly after an initial hypoxic event within the nurse cell. Default reaction of the vertebrate host to a hypoxic event is upregulation of vascular endothelial growth factor (VEGF) which in turn triggers angiogenesis (Despommier, 1998). Thus, inside its nurse cell, the mobile and metabolically active *Trichinella* muscle larva awaits the death of its present host and ingestion by the next,

producing tyvelosylated proteins to control its environment throughout the whole infection period (Despommier, 1998). Once a host has died, the *Trichinella* muscle larvae (ML) are perfectly able to survive in decaying meat for long periods of time. *T. spiralis* ML in artificially infected pork meat that had been buried in the ground at a depth of 30-100 cm, remained infective for rats for more than 91 days (Jovic et al., 2001; Oivanen et al., 2002). *T. britovi* and *T. nelsoni* in decaying mouse carcasses remained infective to laboratory mice after storage at room temperature for up to 45 days (Gottstein et al., 2009). *Trichinella* ML are susceptible to inactivation by freezing, the extent of which

varies with *Trichinella* species and the host species in which the larvae developed. After freezing *T. spiralis* in infected wild boar meat for one week at -20 °C, all muscle larvae were inactivated, whereas it took eight weeks at the same temperature and in the same host to inactivate all *T. britovi* (Lacour et al., 2013).

In a study conducted with pork infected with *T. spiralis*, *Trichinella murrelli*, *Trichinella pseudospiralis* and *Trichinella nativa*, no live larvae were obtained after 82 hours at -20 °C (Hill et al., 2009), whereas in another study, *T. nativa* larvae in rat muscle survived one week of freezing at -18 °C, but not four weeks (Malakauskas and Kapel, 2003a). *T. spiralis* in infected horsemeat is not inactivated within one week at -18 °C , and even after four weeks at -18 °C, few *T. spiralis* larvae were demonstrated (Hill et al., 2007). *T. nativa* and

Table 1. Geographical distribution of *Trichinella* species and genotypes. Adapted from (Pozio et al., 2009a).

Encapsulated species and genotypes			
***Trichinella* spp.**	**Distribution**	**Cycle**	**Host range**
T. spiralis	Cosmopolitan, except arctic regions	Domestic and sylvatic	Swine, rats, seldom carnivores
T. nativa	Arctic and subarctic areas of Holarctic region	Sylvatic	Terrestrial and marine carnivores
T. britovi	Temperate areas of Palearctic region, northern and western Africa	Sylvatic, seldom domestic	Carnivores, seldom swine
T. murrelli	Temperate areas of Nearctic region	Sylvatic	Carnivores
T. nelsoni	Ethiopic region	Sylvatic	Carnivores, seldom swine
T. patagoniensis	Argentina	Sylvatic	Carnivores
Trichinella T6	Canada and United States	Sylvatic	Carnivores
Trichinella T8	South Africa and Namibia	Sylvatic	Carnivores
Trichinella T9	Japan	Sylvatic	Carnivores
Non-encapsulated species			
***Trichinella* spp.**	**Distribution**	**Cycle**	**Host range**
T. pseudospiralis	Cosmopolitan	Sylvatic, seldom domestic	Mammals and birds
T. papuae	Papua New Guinea, Thailand	Sylvatic, seldom domestic	Swine, saltwater crocodiles
T. zimbabwensis	Ethiopia, Mozambique, South Africa, Zimbabwe	Sylvatic and domestic	Nile crocodiles, Nile monitor lizards, lion

Trichinella T6 tolerate temperatures as low as -15 °C for one or more years in wolf, polar bear and arctic fox muscle. *T. britovi* remains infective in frozen red fox muscle for up to three months at -15 °C (Pozio et al., 1994b).
Infection with non-encapsulated *Trichinella* species leads to developments comparable to those described for the encapsulated species, up to the moment where encapsulated species orchestrate the formation of a nurse cell capsule, which increases over time to 18.7 – 33.5 µm (Sacchi et al., 2001). In contrast, a nurse cell capsule is absent in muscle cells that had been invaded by *T. pseudospiralis* and *T. papuae*, two non-encapsulated species (Sacchi et al., 2001; Xu et al., 1997). Upon *T. spiralis* infection of the muscle cell, the parasite is protected by its thick capsule against inflammatory reactions of the host, whereas *T. pseudospiralis* downregulates the immune reactions of the host, thus preventing an intensive cellular response (Lee and Ko, 2006). Also, the network of small blood vessels that is typical for nurse cells of encapsulated species, is absent from nurse cells of non-encapsulated species, which are surrounded by blood vessels that do not appear to be different from those surrounding uninfected muscle cells (Khositharattanakool et al., 2013).

Sylvatic cycle of *Trichinella*

The sylvatic or wildlife cycle refers to infection of wild animals with species of the genus *Trichinella* for which the main reservoirs are carnivores (Pozio, 2005). The sylvatic cycle is present all around the globe and natural infections with encapsulated *Trichinella* spp. have been detected in 150 mammalian species, whereas non-encapsulated species have been detected in 14 species of mammals and 13 bird species (Pozio, 2005). The main reservoir for *T. spiralis*, a cosmopolitan species most infective to humans, are wild boar, although wild carnivores also play a role as reservoir (Pozio and Murrell, 2006). The other cosmopolitan, but non-encapsulated species is *T. pseudospiralis*, which infects mammals and birds. *T. britovi* circulates predominantly in carnivores (e.g. foxes, wolves, raccoon dogs, jackals) and mustelids and to a lesser extent domestic pigs and wild boar of the Eurasia-Palearctic region (Pozio et al., 2009a; Zarlenga et al., 2006). *T. nativa* circulates in terrestrial carnivores such as lynx, bear and red fox and marine carnivores such as walrus of the Holarctic region (Pozio et al., 2009a; Zarlenga et al., 2006). *Trichinella* T6 and *T. murrelli* infect carnivores of the North-American continent, whereas *T. patagoniensis* occurs in carnivores of Argentina (Pozio et al., 2009a). *T. nelsoni*, *Trichinella* T8 and *T. zimbabwensis* infect carnivores of the African continent, although for the latter species, reptiles are the predominant hosts.

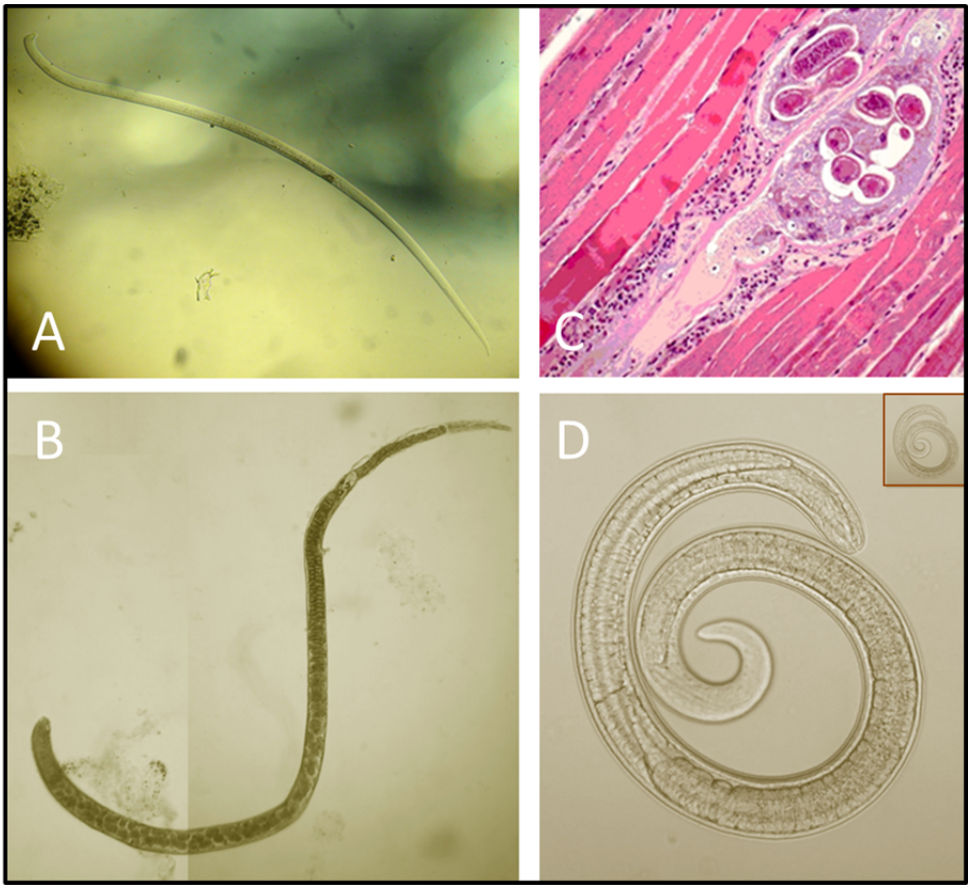


Figure 1. Developmental stages of *Trichinella* spp. Adult male (1.4 - 1.6 mm long, A) and female (3 - 4 mm, B) *Trichinella* mate in the small intestine of the host, after which females produce newborne larvae that migrate to striated muscle, where they encapsulate (C). After ingestion by a subsequent host, muscle larvae (0.8 – 1 mm in length, D) are liberated from their capsule and moult four times to develop into adult worms. See chapter 8 for detailed explanation of panel C; panel D, insert: approximate size in comparison to adults. Photography by Frits Franssen (A, B, D) and Marianne Koedam (C), RIVM.

On the Asian continent, *Trichinella* T9 infects carnivores in Japan and *T. papuae* circulates in swine and salt water crocodiles of Papua New Guinea and Thailand (Pozio et al., 2009a).

Domestic cycle of *Trichinella*

The domestic cycle refers to *Trichinella* infections in domestic animals such as pigs and horses. Under open housing conditions, *T. spiralis* can be maintained in a pig herd after anthropogenic introduction by improper farm management (e.g. feeding non-cooked scraps, offal from slaughter or wildlife remains), by tail or ear biting, and cannibalism in case dead animals are not removed promptly. Additionally, *T. spiralis* can circulate between pigs and rodents or mustelids, at or around farms (Pozio, 2001). The role of rats in the transmission of *Trichinella* spp. has for long been under debate. *T. spiralis* may persist in rats through scavenging and cannibalism (Leiby et al., 1990; Mikkonen et al., 2005), following exposure to improper pig slaughter procedures and poor hygiene at farms (Stojcevic et al., 2004). On the other hand, *T. spiralis* could persist in rats in the absence of a known source of infection (Leiby et al., 1990; Takumi et al., 2010). At farms with open housing conditions, pigs can acquire *Trichinella* infections via rats entering stables (Schad et al., 1987), but domestic pigs may also become infected by eating *Trichinella* infected rat carcasses in feed (Oivanen et al., 2002).

Requirements for controlled housing explicitly exclude these risks: for recognition as controlled housing, food business operators must fulfil a number of conditions. They have to assure that construction and maintenance of buildings are sufficient to prevent entrance of animals other than pigs. Additionally, they must have a rodent control programme in place. Feed must be acquired from certified producers and all feeds must be stored in closed rodent-proof containers. Furthermore, food business operators must ensure that dead animals are removed instantly and report the proximity of a rubbish dump to the competent authorities in order to perform a risk assessment (European-Commission, 2014; OIE-Manual, 2012). As a result, *Trichinella* is virtually absent from pigs raised under controlled housing in the European Union (Alban et al., 2011; Pozio, 2014).

Human trichinellosis

Human trichinellosis has been reported in 55 countries on five continents, strongly correlating with cultural and culinary habits of eating raw or undercooked meat from back-yard or free-ranging pig, horse, dog, wild boar (*T. spiralis* and *T. britovi*), bear and walrus (*T. nativa*) (Dupouy-Camet, 2000). *Trichinella* outbreaks in non-endemic areas result from non-intentional transfer of *Trichinella* e.g. with meat products that are brought home from endemic regions (Pozio and Marucci, 2003). Also tourism from non-

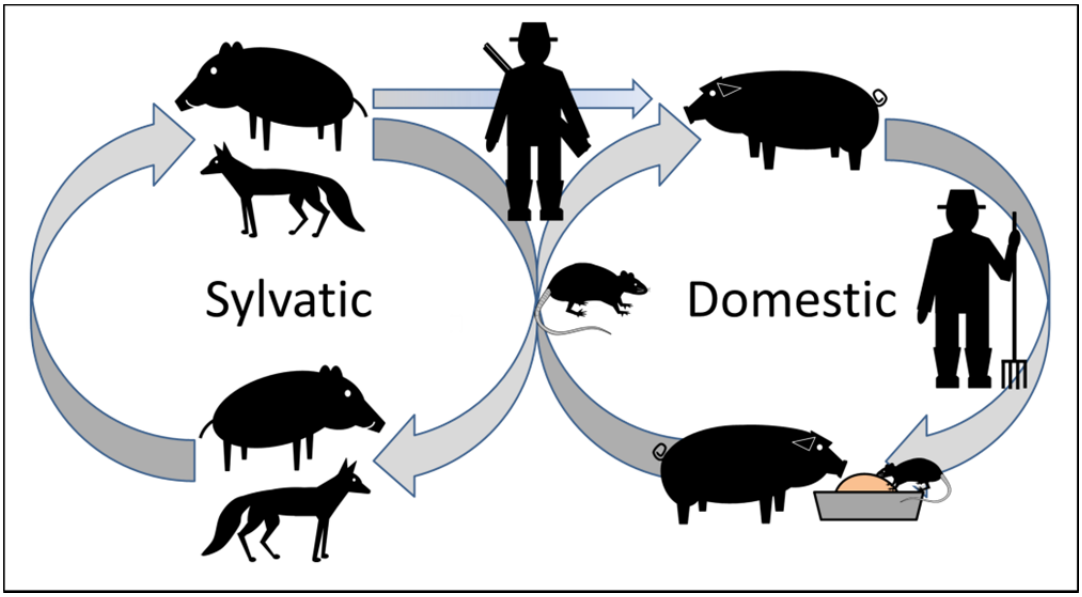


Figure 2. *T. spiralis* and *T. britovi* sylvatic cycle. Red fox is the main reservoir for *T. britovi*, whereas wild boar is the main reservoir for *T. spiralis*, although both *Trichinella* species infect both host species. Small mammals, such as rats, may serve as transport species for *T. britovi* and *T. spiralis* in the wild, but also to domestic pigs under non-bio-secure conditions. At this point, *Trichinella* spp. enter the domestic cycle, although introduction by feeding scraps of slaughtered pigs or wildlife is a far more frequent, which also is a source of infection for rats that are living on or near the farm. Animation drawn by the author of this thesis.

endemic areas to endemic countries may play a role, when travellers eat not properly cooked or cured meat or meat products, after which the infection often manifests itself when they have returned to their homes (Cuperlovic et al., 2005; Mikkonen et al., 2005; Neghina, 2010; Neghina et al., 2010b; Nockler et al., 2007; Pinelli et al., 2004; Pozio, 2007).

Humans, unlike other mammals, develop clinical trichinellosis after ingestion of *Trichinella* spp. muscle larvae. Following the lifecycle of the parasite, trichinellosis is characterised by three stages: the intestinal phase, in which *Trichinella* muscle larvae penetrate the mucosa of the small intestines to moult and mature. After mating, females produce newborne larvae that penetrate the gut wall and migrate to the lymph. Depending on the dose, this phase can start from 20 hrs pi onwards and is characterized by loose stools or diarrhoea. The next, acute phase, starting from 4-6 days pi, is characterised by general weakness, chills, headache, fever, excessive sweating and

tachycardia (Pozio et al., 2003) and coincides with *Trichinella* new born larvae migrating from the lymph nodes through the blood vessels to the muscle cells. The blood vessels of the conjunctiva may become inflamed, causing allergic reactions such as facial and periocular oedema, petechiae and subcutaneous bleeding of the nailbeds. Invasion of the muscle cells and nurse cell formation are characterised by muscle pain and may be accompanied by difficulties with motility, breathing and even heart failure. The last phase is the convalescent stage, in which signs and symptoms disappear between 6 and 8 weeks pi (without treatment), when mature female *Trichinella* cease giving birth to new borne larvae and the latter have completed invasion of muscle cells (Pozio et al., 2003). The severity of symptoms varies with the level and duration of larval uptake and *Trichinella* species. *T. spiralis* and *T. pseudospiralis* are most pathogenic to man, the former causing (< 0.2%) lethality. *T. britovi* is moderately pathogenic, *T. murrelli* and *T. nelsoni* are moderately to highly pathogenic and deaths have been reported for these species (Pozio et al., 2003). Anthelmintic treatment (e.g. Albendazol or Mebendazol) is only possible in the intestinal phase during the first days after infection, since anthelmintics generally do not reach muscle tissues, due to poor absorption in the gut (Dupouy-Camet et al., 2002; Pozio et al., 2003).

Worldwide, around 11 million people have been infected with *Trichinella spiralis*, based on serological evidence (Dupouy-Camet, 2000). Anti-*Trichinella* antibodies have been shown to persist 2-8 years after an acute infection in 10 out of 10 patients (Bruschi et al., 2005) and 38% of 128 patients still had antibodies ten years after an acute infection (Harms et al., 1993). A far lower number of 65,818 confirmed human cases and 42 deaths have been recorded from outbreak data during the period 1986 – 2009 (Murrell and Pozio, 2011). The majority of outbreaks was reported from Europe (87%), of which 50% were from Romania (Murrell and Pozio, 2011).

The global health impact of trichinellosis has been estimated at 76 Disability-Adjusted Life Years per billion persons per year, which is low compared to other parasitoses, but underreporting may play a role (Devleesschauwer et al., 2014). In a multi-criteria risk based ranking of foodborne parasites, *T. spiralis* ranked the seventh important parasitic pathogen, behind *Taenia solium*, *Echinococcus granulosus* and *E. multilocularis*, *Toxoplasma gondii*, *Cryptosporidium* spp. and *Entamoeba histolytica* (FAO-WHO, 2014a). All other *Trichinella* species combined ranked 16th in that multi-criteria comparison. While the socio-economic importance of *T. spiralis* was judged low in comparison to other foodborne parasites, its importance for international trade was perceived the highest of all (FAO-WHO, 2014a). Nevertheless, despite the relatively low burden of *Trichinella* spp. and easily applicable inactivation method such as freezing (except for *T. nativa* and to

some extend *T. britovi*) and cooking, this pathogen is still considered emerging or re-emerging in Europe and Asia (Liu and Boireau, 2002; Murrell and Pozio, 2011). In Europe, *Trichinella* infections re-emerged due to political instability and coinciding failure of veterinary inspection in the Balkan countries (Cuperlovic et al., 2005). The growing popularity of organic pig producing systems, driven by increased consumer interest in traditional or more animal- and environment friendly production systems (Kijlstra et al., 2009), may increase the risk of exposure to *Trichinella* spp. (Boutsini et al., 2014; Oivanen et al., 2000). Also traditions and habits drive persistence of *Trichinella* spp., such as feeding scraps of home-slaughtered pigs or wildlife remains (Neghina, 2010), tradition of hunters leaving skinned carcasses or animal entrails in the field (Pozio et al., 2001a) and consumer food preferences involving raw meat dishes and products (Liu and Boireau, 2002; Neghina, 2010). Also illegal practices, such as mixing of *Trichinella* infected pork with beef (Akkoc et al., 2009) facilitate outbreaks in humans.

Detection of *Trichinella* at meat inspection

To prevent human trichinellosis, *Trichinella* susceptible animals that are used for meat production, such as domestic pigs and horses, but also wildlife such as wild boar and bear, are tested for *Trichinella* at slaughter. Predilection sites for *Trichinella* spp. differ per animal species, which must be taken into account when selecting sampling sites for detection of *Trichinella*. Predilection sites for *Trichinella* are diaphragm, tongue and masseter in domestic pig, tongue and masseter in horses and tongue and diaphragm in wild boar (Gamble, 1996)(Forbes, 1999)(Pozio, 1998)(Kapel, 2000), reviewed in (Noeckler, 2000). The method of choice for detection of *Trichinella* ML in muscle tissue is the artificial digestion method for pooled samples. For this method, 100 samples of 1 gram diaphragm from domestic pigs are pooled or 20 samples of 5 gram from wild boar and horse (European-Commission, 2005). In 2006, the artificial digestion method has been described as the reference method in Europe (European Reference Method, EU-RM). This method has been validated for the detection of live *Trichinella* muscle larvae (ML) and critical control points are well described (Forbes and Gajadhar, 1999; Gamble, 1999; Gamble et al., 2000; Rossi and Pozio, 2008).

The EU-RM is obligatory for the control of meat within the European Union. Since 2006, derogations have been described with amendments in 2014 for pig farms or holdings fulfilling the criteria of controlled housing (European-Commission, 2014). Pork that is intended for export to countries outside the EU however, is subjected to regulations that are defined by the food safety authorities of those countries.

Detection of *Trichinella* antibodies

Serology using excretory/secretory (ES) proteins derived from *Trichinella* ML as antigen, is a useful surveillance instrument to detect IgG antibodies directed against *Trichinella* spp. However, infection dynamics of *Trichinella* do not allow identification of infected individuals, as the antibody level depends on the level of infection and persistence of different *Trichinella* spp. This has been extensively shown for *Trichinella* infections in domestic pigs and in wild boar. In infection experiments with domestic pigs and wild boar, serological responses measured by an in-house *Trichinella* ELISA reflected infection and persistence characteristics of *Trichinella* species (Kapel, 2001; Kapel and Gamble, 2000). The most used assay is ELISA based on ES antigen, which allows high throughput sample processing; alternatively, immunofluorescence antibody tests are used. The former test, however, is prone to a certain degree of false positive reactions, despite reported high sensitivity and specificity. To circumvent this drawback, highly specific Western blot

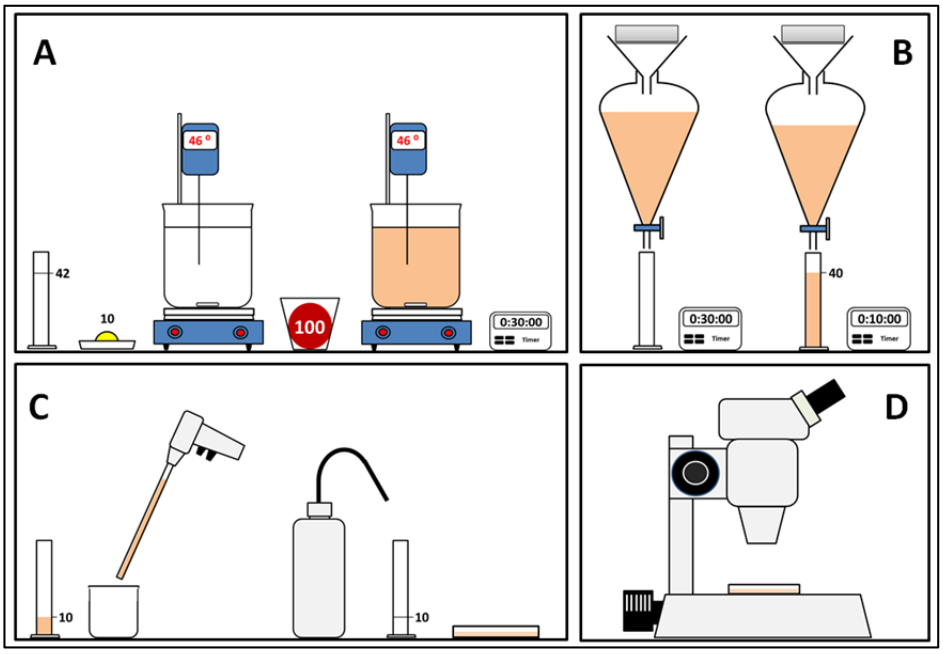


Figure 3. Artificial digestion method for pooled samples. The EU-RM uses an aqueous pepsin / HCl solution for artificial digestion of 100 g meat, consisting of 100 pooled muscle samples of 1 g (domestic pig) or 20 samples of 5 g (wild boar, horse). The method consists of four steps: meat digestion to liberate *Trichinella* ML (A), two sedimentation steps (B), isolation of sedimented *Trichinella* larvae (C) and finally, microscopic enumeration of *Trichinella* ML at 10-20x magnification (D). The first sedimentation step of the EU-RM includes filtration of the meat digest through a stainless steel sieve with mesh size 180 µm, to retain crude debris and undigested small meat particles. Animation drawn by the author of this thesis.

assays have been developed for conformation purposes (Blaga et al., 2009; Gomez-Morales et al., 2014).

Trichinella species identification

Trichinella ML that are isolated during meat inspection, may be identified using morphological and biological features (Murrell et al., 2000), but reliable species identification is only possible using molecular techniques. These facilitated rapid and unambiguous identification of *Trichinella* ML for routine and epidemiological analyses. *Trichinella* species determination relies on banding pattern using gel electrophoresis following PCR of the mitochondrial CO1 gene alone (Nagano et al., 1999) or in combination with nuclear markers when using multiplex PCR (Zarlenga et al., 1999). The latter is considered the reference molecular diagnostic method by the EU Reference Laboratory for Parasites (Istituto Superiore di Sanitá). Alternatively, PCR of the 5S intergenic spacer region followed by sequencing (Rombout et al., 2001b) or restriction fragment length polymorphism (RFLP) analysis (Rombout et al., 2001a) may be used for *Trichinella* species identification. According to EU Regulation, the national reference laboratories (NRLs) throughout the European Union are responsible for confirmation by species identification, aided by the European Reference Laboratory for Parasites (EURL, ISS, Rome, Italy).

Economic aspects of *Trichinella* control at slaughter

Twelve to fifteen million pigs are slaughtered annually in the Netherlands and 691,276 tons of pork are annually exported to countries within the European Union and 43 third countries, among which Japan, South-Korea and Russia (data of 2006 and 2007 (Statistics-Netherlands, 2015)). Over the period 2008 – 2013, between 0.99 and 1.3 million live domestic pigs and 210,766 free-ranging pigs were imported annually into the Netherlands from other European countries, (Statistics-Netherlands, 2015) (Table 2). Additionally, 178 – 237 million kg of fresh and cooled pork from domestic pigs and 35.5 – 67.5 million kg of fresh and cooled meat of free roaming pigs and wild boar were imported annually into the Netherlands from other European countries, (Statistics-Netherlands, 2015). Roughly 200 Million pigs that presently go to slaughter annually in the European Union, are tested individually at a cost of 0.12 to 3 US dollar per pig, mounting to an estimated 24 – 600 million US dollar (Kapel, 2005; Pozio, 1998), but in small slaughterhouses the cost for *Trichinella* control can increase up to 10 – 15 US

Table 1.2. Import of live pigs into the Netherlands during the period 2007 - 2013.

Import of domestic pigs

country	2008	2009	2010	2011	2012	2013
Belgium	607,991	717,916	718,675	716,890	613,153	652,042
Bulgaria	0	0	0	0	0	60
Denmark	260,193	236,524	155,184	29,514	39,278	115,196
Germany	316,691	245,519	228,629	445,880	191,414	103,542
France	13,715	36,582	11,711	11,687	13,263	11,029
Hungaria	9,186	5,313	6,366	16,884	93,168	187,440
Italy	738	0	50	1,202	553	2,725
Croatia	0	0	0	0	0	17,771
Luxemburg	0	0	265	0	0	0
Poland	2,474	4,429	2,329	3,815	1,734	4,158
Portugal	0	0	0	0	0	607
Slovenia	0	0	0	0	881	222
Slovakia	5,793	1,054	0	468	2,591	559
Spain	5,531	21,150	137	3,644	6,519	4,074
Tchech Republic	27,979	34,414	33,421	14,229	29,783	11,940
United Kingdom	186	6	7	0	0	0
totals:	1,250,477	1,302,907	1,156,774	1,244,213	992,337	1,111,365

Import of free-ranging pigs

country	2008	2009	2010	2011	2012	2013
Belgium	675	562	5	0	0	0
Bulgaria	0	0	0	0	0	0
Denmark	54,522	0	867	0	0	0
Germany	21,280	88,929	24,675	0	0	0
France	2,865	0	6,581	0	0	0
Hungaria	1,594	17	0	0	0	0
Italy	2,450	0	0	0	0	0
Spain	0	0	1,248	0	0	0
Tchech Republic	4496	0	0	0	0	0
totals:	87,882	89,508	33,376	0	0	0

dollars per pig (Pozio, 2014). In view of high cost and effective freedom of trichinellosis of domestic pigs from controlled housing (Pozio, 2015), European and also worldwide support for a risk based *Trichinella* control increased (Alban et al., 2008; Alban et al., 2011; FAO-WHO, 2014a). In 2006, European legislation (EU 2075/2005) was introduced to relieve the mandatory individual carcass control for *Trichinella* spp. for countries keeping pigs under controlled housing and no *Trichinella* detection in pigs or humans for at least 10 succeeding years. A constraint for acceptance of a country as a region of negligible risk was inclusion of wild life survey, and a maximum *Trichinella* prevalence of 0.5% in wildlife. This 0.5% prevalence was however not scientifically supported and needed to be better evaluated.

The Directive towards a risk based *Trichinella* control was adapted in June 2014 (European-Commission, 2014). From that moment on, countries were no longer under exemption, but instead farms or holdings had to show freedom of *Trichinella* in their pig population by surveillance. Meat control for *Trichinella* remained mandatory for horses, sows, boars and susceptible wildlife. However globally, meat industries may continue testing individual pig carcasses to protect economic interests regarding international trade to third countries.

Risk-based surveillance

The husbandry systems at risk, where most *Trichinella* infections occur, are free ranging and back yard pig farms (Alban et al., 2011). Thus, risk-based surveillance depends on two pillars:

1. Exemption from *Trichinella* examination of domestic pigs from controlled housing with a proven absence of *Trichinella* spp. for three years and a *Trichinella* prevalence at country level lower than 1 per million at 95% confidence (European-Commission, 2014);
2. Mandatory testing of domestic animals at risk (pigs from non-controlled housing, free-ranging and back yard pigs, long lived pigs (sows, boars), horses and game) (European-Commission, 2014).

Wildlife surveillance to determine presence of *Trichinella* spp. and possible exposure of domestic pigs to *Trichinella* spp. is no longer included in the EU regulation of 2014. However, evaluation of the attribution of *Trichinella* transmission from wildlife and pigs under different housing conditions to humans, needs further examination to identify risk levels of these components. Subsequently, control measures that address the most important transmission risk may be identified.

Recently, the Codex Alimentarius Commission, responsible for preparing guidelines for food safety on a global level, developed a spreadsheet model to evaluate residual risks of infection with *Trichinella* spp. from *Trichinella*-tested domestic pigs under different hypothetical scenarios, resulting in quantitative risk-based information that allows evaluation of different meat hygiene programmes for *Trichinella* spp. in meat (FAO-WHO, 2014b). Although this model can be of benefit to *Trichinella* control, the model in its present form only generates an estimate of the number of infected portions of pork, but no risk estimates, as it does not account for *Trichinella* spp. distribution in meat and temperature inactivation of *Trichinella* ML by the consumer. Moreover, it does not include dose-response relationships for *Trichinella* spp. infections in humans.

Aims and outline of this thesis

The overall aim of this thesis was to study the presence and prevalence of *Trichinella* spp. in wildlife host species that play a role in the ecology of the parasite and its presence in the food chain, which could be used to develop a quantitative microbial risk analysis (QMRA) model for *Trichinella*. Such a QMRA model may be used to identify hazards in the chain of events from the presence of *Trichinella* parasites in domestic life stock or wildlife, to *Trichinella* infection of humans. Additionally, a QMRA model could be used to quantify attributions of factors such as husbandry system (e.g. controlled housing, free ranging), types of meat and meat products (e.g. different pork cuts, fresh meat), or carcass control (e.g. introduction of alternative equipment).

The overall aim resulted in the following research questions in order to

1. Develop and validate methods to detect and characterize *Trichinella* spp. in wildlife;
2. Study the seroprevalence of *Trichinella* in wild boar and the prevalence of *Trichinella* spp. in red foxes to follow trends in time, which might provide insight in the natural fluctuations of *Trichinella* spp. in wildlife;
3. Study the risk of *Trichinella* infection in humans through consumption of wild boar meat or pork from pigs raised under controlled or non-controlled housing conditions, using a quantitative microbial risk analysis (QMRA) model for *Trichinella* spp.

We developed detection methods and evaluated the *Trichinella* prevalence in Dutch wild boar (Chapter 2) and red fox (Chapter 3). In Chapter 4, we quantified the effect of an alternative sieve size in the artificial digestion method that is mandatory according to a

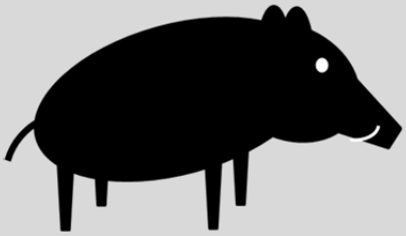
third countries' specific regulation regarding *Trichinella* control in pork, but deviates from EU regulation EC 2075/2005.

Various *Trichinella* isolates from individual *T. spiralis* and *T. britovi* larvae isolated from wild boar and red fox were analysed, using the mitochondrial cytochrome c oxidase 1 gene and the nuclear 5S intergenic spacer region. Subsequently, sequence variation was analysed, resulting in the finding of hybrids between *T. spiralis* and *T. britovi* (chapter 5).

We studied long-term data of parasitic infections other than *Trichinella* spp. in red fox, to investigate whether characteristics of *Trichinella* infection dynamics in wildlife in the Netherlands fit into a general pattern (chapter 6).

We studied the serological response to a wide range of *Trichinella* infection doses in experimentally infected laboratory rats (Chapter 7) to evaluate the dose dependency in rats. In Chapter 8, we studied parasitic infections including *Trichinella* in wild rats that were caught in different environments, such as farms, suburban environments and rural environments. Finally, we developed a quantitative microbial risk assessment (QMRA) model to assess the actual consumer risk of infection and disease, following consumption of pork or wild boar meat. We included the whole chain of events, from distribution of *Trichinella* larvae in and among pigs and wild boar, test characteristics using artificial digestion, meat inactivation by cooking, consumer preference data concerning eating undercooked meat and the dose response relation of *Trichinella* infections in humans (Chapter 9).

Chapter 2



Analysis of long-term serological *Trichinella* surveillance data of wild boar from a non-endemic area.

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Parasites & Vectors - in revision.

Meat originating from wild boar and pigs under non-controlled housing conditions is the most important source for trichinellosis outbreaks in humans. Artificial digestion, which is used for routine meat inspection at the slaughterhouse to identify *Trichinella* positive animals, is a less suitable instrument to monitor *Trichinella* spp. at population level, since it is labour intensive and less sensitive than serological techniques.

We included previously unpublished serological data of wild boar during the period 1999 – 2005, and we tested 3778 wild boar sera collected during the period 2007 – 2015, in a *Trichinella* in-house ELISA. Since validation of serological assays for wildlife surveillance is often hampered by a lack of well-defined field sera, we included wild boar sera originating from Poland and Romania, which are regarded endemic for *Trichinella* spp., to determine an appropriate cut-off value. The aim of the present study was to evaluate serological response of Dutch wild boar to *Trichinella* spp. during the last two decades.

Over the years 2007 – 2015, six wild boar sera tested positive by ELISA, of which three were confirmed by Western blot, resulting in an overall seroprevalence of 0.079%, which was low compared to 2% seroprevalence in 2005 ($p = 0.0473$). The overall seroprevalence in the preceding period (1999 – 2004) was 5.7%.

The present study shows that *Trichinella* seroprevalence in Dutch wild boar over the periods 2004 – 2005 and 2007 – 2015 is very low, with only four confirmed positive animals out of 3944 animals tested – one in 2004 by artificial digestion and three in 2014 – 2015 by Western blot.

Key words: Trichinella, wild boar, ELISA, Western blot.

Background

In Europe, four out of twelve recognised species and genotypes of *Trichinella* (*T. spiralis*, *T. britovi*, *T. nativa* and *T. pseudospiralis*) circulate in carnivores, such as red foxes, wolves, lynxes and bears, and in omnivores such as wild boar and raccoon dogs (Pozio et al., 2009a). Through improper farm management, poor hygienic standards (Neghina, 2010; Stojcevic et al., 2004) or illegal practices (Akkoc et al., 2009), domestic pigs can become infected with *T. spiralis*, *T. pseudospiralis* and to a lesser extent *T. britovi* (Kapel, 2001).

Ingestion of *Trichinella* muscle larvae (ML) in improperly cooked meat from wild boar and pigs kept under non-controlled housing conditions, is the most important source for trichinellosis outbreaks in humans in Europe (Angheben et al., 2008; Arévalo et al., 2009; Bartuliene et al., 2009; Gallardo et al., 2007; Golab et al., 2007a; Neghina et al., 2010a; Nockler et al., 2007; Reiterova et al., 2007; Stensvold et al., 2007). Also illegal practices, such as mixing meat from different animal species may cause large outbreaks (Akkoc et al., 2009).

To prevent human trichinellosis in the European Union, individual wild boar carcasses intended for commercial use, have to be tested at the meat inspection using artificial digestion in pooled samples (EU 2015/1375) (European-Commission, 2015). Artificial digestion is a good method to prevent human trichinellosis and a sensitive technique to detect *Trichinella* spp. infection in meat at a detection limit of 1-3 *Trichinella* spp. muscle larvae (ML) per gram (LPG). However, it is a less suitable instrument to monitor low infection levels of *Trichinella* spp. at the population level, since artificial digestion is labour intensive and less sensitive than serological techniques for the purpose of prevalence determination (Gomez-Morales et al., 2014; Nöckler et al., 1995; Venturiello et al., 1998).

After experimental infection of domestic pigs and wild boar with *T. spiralis* and *T. britovi*, a serological response measured by an in-house *Trichinella* ELISA mounted within three weeks post infection and lasted more than 37 weeks (Kapel, 2001; Kapel and Gamble, 2000). In contrast, the serological response to infection with *T. nativa* and *T. pseudospiralis* declined from 15 – 20 weeks onward, after an initial seroconversion at three weeks post infection (Kapel, 2001; Kapel and Gamble, 2000).

The ELISA to detect anti-*Trichinella* IgG has been validated to evaluate present or past infection of domestic pigs (Gomez-Morales et al., 2009; Teunis et al., 2009). This ELISA in combination with Western blot has been used to determine seroprevalence of *Trichinella* species in wild boar in endemic and non-endemic regions (Cuttell et al., 2014; Gamble et al., 1983; Gomez-Morales et al., 2014).

In the Netherlands, a prevalence of 1.8% in wild boar has been demonstrated during the period 1976 – 1980 (Franchimont et al., 1993). A seroprevalence of 3.1 – 9.8% was estimated in wild boar during the period 1996 – 1998 (van der Giessen et al., 2001), using a serum dilution of 1:20 in an in-house ELISA (van Knapen et al., 1986). In the same period in 1998, *Trichinella spiralis* muscle larvae were found in the diaphragm of two out of

eleven wild boar (18%) originating from the southeast of the country (0.2 and 0.3 LPG). Nonetheless, the Netherlands is a non-endemic country, since no Trichinella has been found in domestic pigs for the past fourteen years and no autochthonous human cases have been seen for decades.
The aim of the present study was to evaluate exposure of Dutch wild boar (*Sus scrofa*) to *Trichinella* spp. during the last 15 years. We used a validated in-house *Trichinella* ELISA (Teunis et al., 2012) and a panel of known negative and positive pig sera as controls. We describe two datasets: wild boar samples collected and tested during the period 1999 – 2005 (Dataset I) and wild boar samples collected and tested during the period 2005 – 2007 (Dataset II). Wild boar sera originating from Poland and Romania, both regarded endemic for *Trichinella* spp. (Bilska-Zajac et al., 2013; Nicorescu et al., 2015), were anticipated to provide enough positive samples to aid in determining an appropriate cut-off value.

Materials and Methods

Wild boar tested in the period 1999 – 2004 (Dataset I)

In the Netherlands, wild boar are tolerated only in two natural reserves, where the populations are restricted to 600 – 800 individuals in reserve 'Veluwe' and 60 - 70 animals in reserve 'Meinweg'. Outside these areas, wild boar are hunted year round in an effort to control damage to crops and to prevent traffic accidents (Guldemond et al., 2015).
During the period 1999 – 2004, 1,748 sera of wild boar originating from the Central and South-Eastern part of the Netherlands were collected by Dutch hunters as part of an ongoing surveillance of animal diseases coordinated by GD Animal Health (GD, Deventer, the Netherlands) (Figure 2.1). The age of these wild boar is estimated and recorded by hunters, based on dental pattern and wear (personal communication Leo Dekkers, GD Animal Health). Sera were sent to the National Institute for Public Health and the Environment (RIVM, Bilthoven, the Netherlands) to test for antibodies against *Trichinella* spp.
Additionally, during the period autumn 2003 – spring 2005, paired serum and diaphragm samples of 118 wild boar from the central Dutch region Veluwe and 48 paired wild boar samples of the south-eastern province of Limburg, were collected with the objective to compare serological testing and artificial digestion.

Artificial digestion of muscle samples collected in 2004

Diaphragm samples (45 g) of in total 166 wild boar from the region Veluwe and the province of Limburg were tested with artificial digestion using the semi-automated Trichomatic[35] method (Annex I, Chapter IIC, (European-Commission, 2005)). Species identification of *Trichinella* ML that were isolated from diaphragm samples following artificial digestion, was performed by PCR and sequencing of the 5S rDNA intergenic spacer region (Rombout et al., 2001b).

Serological testing of wild boar sera using in-house RIVM ELISA

The 1,748 wild boar sera (period 1999 – 2004) were tested in a serum dilution of 1:20. The serum samples of in total 166 wild boar from the region Veluwe and the province of Limburg, were tested with an in-house RIVM *Trichinella* ELISA at a serum dilution of 1:20 and/or 1:100 as described below. Out of the 166 additional sera from Veluwe and Limburg, 13 sera that tested positive at serum dilution 1:20 and /or 1:100 and two negative sera were verified at the German National Institute for Risk Analysis BfR (Bundesinstitut für Risikobewertung, Berlin, Germany).

T. spiralis antigen consisting of excretory/secretory (ES) proteins was produced as described previously (Gamble, 1985). An indirect ELISA using *Trichinella* ES antigen was used as described previously (van Knapen et al., 1986). Briefly, polystyrene flat-bottom ELISA plates were coated with 0.15 µg *T. spiralis* ES proteins per well for 1 h at 37°C and saturated with 1% BSA in PBS/0.05% Tween 20 (PBS-T)). Sera (100 µl per well, 1:20 or 1:100 dilutions in PBS-T were applied and incubated for 1 h at 37°C. Rabbit anti-porcine IgG conjugated to Horse Radish Peroxidase (Pasteur Institute, Paris, 100 µl 1:8000 in 1% BSA/PBS-T) was added and plates were incubated for 1 h at 37 °C. Finally, antigen-antibody interaction was visualized by adding 100 µl freshly prepared H_2O_2 (1.5 mmol/l) / AHB (5-amino-2-hydroxybenzoic acid, 4.7 mmol/l) substrate solution per well and incubation for 1 h at room temperature. The reaction was stopped with 25 µl 1M NaOH and optical density (OD) values were determined with a spectrophotometer at 449 nm. In between steps, wells were washed with PBS-T.

Determination of the ELISA cut-off value

Sera of two *Trichinella* positive (infected) and two negative pigs (non-infected) were used as internal controls (van Knapen et al., 1986; van Knapen et al., 1980). The average and standard deviation of blank-corrected OD-values of 144 non-infected negative control pig sera were determined and the cut-off value was calculated as $CO = mean + 3 \times SD$ (van Knapen et al., 1986).

Wild boar tested in the period 2007-2015 (Dataset II)

Animals

Blood samples of 3,823 Dutch wild boar were collected in the Central and South-Eastern part of the Netherlands (Figure 1) as described above, and sent to RIVM (Bilthoven, the Netherlands).

Polish wild boar sera (n = 556) were collected in 2012 in ten different voivodeships (provinces), with varying prevalences of *Trichinella* spp. as demonstrated by artificial digestion results at the Polish slaughterhouse laboratories. Romanian sera of wild boar (n= 101) were collected at random. It was anticipated that these cohorts would provide a positive component for cut-off determination.

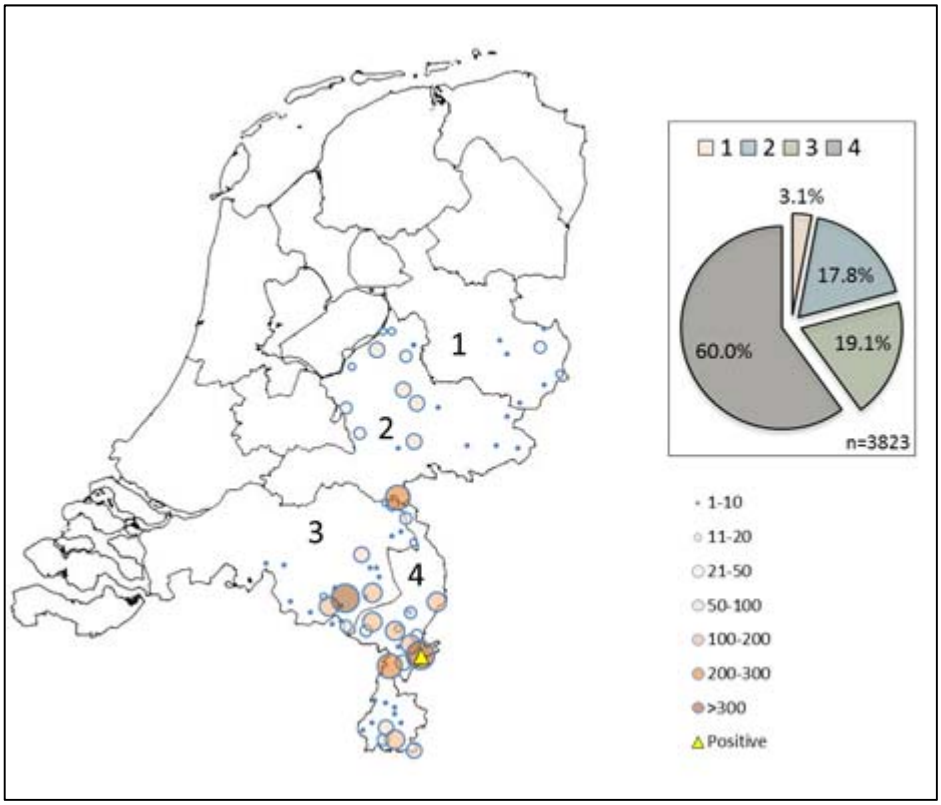

Figure 1. Geographical distribution of wild boar sampled during the period 2008 – 2015 (Dataset II). The pie chart represents the percentage of wild boar originating from each of four provinces. 1: Overijssel, 2: Gelderland, 3: Brabant, 4: Limburg.

Trichinella control at the meat inspection

Trichinella test results of Dutch hunted wild boar that had been tested according to EU Regulation 2075/2005 (European-Commission, 2005) at the meat inspection over the period 2007 – 2012, were collected from the data reported by the Netherlands Food and Product Safety Authority to EFSA (EFSA, 2011; EFSA and ECDC, 2014). Data covering the years 2013 and 2014 were obtained from Dutch slaughterhouse laboratories performing the *Trichinella* meat control according to EU Regulation 2075/2005.

Test results of Polish and Romanian hunted wild boar that had been tested for *Trichinella* according to EU Regulation (European-Commission, 2005, 2015) at the meat inspection over the period 2007 – 2012, were extracted from EFSA / ECDC reports (EFSA, 2011; EFSA and ECDC, 2014).

Serological testing of wild boar sera using RIVM in-house ELISA

In total 3778 collected wild boar sera were tested in the in-house RIVM ELISA. The RIVM in-house ELISA was performed as described above, with some modifications. After coating and saturation of flat-bottom ELISA plates (Nunc 66904), incubation with diluted sera (100 µl per well, 1:100 dilution in PBS-T for 1 h at 37°C) and washing, each well was incubated for 1 h at 37 °C with 100 µl Rabbit anti-swine IgG conjugated to Horse Radish Peroxidase (Dako, Glostrup, Denmark, 1:8000 in 1% BSA/PBS-T). After washing, antigen-antibody interaction was visualized by adding 100 µl Sure Blue^{TM} TMB substrate solution (KPL, Gaithersburg, USA) per well and incubation for ten minutes at room temperature. The reaction was stopped with 100 µl 2M H_2SO_4 and optical density (OD) values were determined with an ELISA reader (Bio-Tek EL808 Ultra Microplate Reader, Bio-Tek Instruments, Inc.) at 450 nm.

Sera that performed above cut-off value were tested by Western blot to confirm their positivity by reaction to specific bands of *Trichinella* ES antigen as described before (Gomez-Morales et al., 2014).

Control sera for RIVM in-house ELISA

A panel consisting of one high and one low positive pig control serum and one low, three medium and one high negative control sera (according to their OD's) was included on each ELISA test plate during the period 2009 - 2015. During the period 2004 – 2008, only high positive and low negative control sera were used. The low positive control serum (SCP+114), which was collected from an experimentally infected pig, two days after seroconversion, was generated at the European Reference Laboratory (EURL, Istituto Superiore di Sanitá, Rome, Italy) (Gomez-Morales et al., 2015). The other negative and positive control sera were used earlier to validate our in-house ELISA (Teunis et al., 2009). Mean OD minus Blank values of control sera on 88 ELISA plates were used to calculate average long-term control values to enable correction for day-to-day and plate-to-plate variation. For each control serum, mean ± SD and mean ± 2SD were calculated; control sera of 10 ELISA plates performed outside mean ± 2SD and were therefore not included to calculate a long-term average value. However, normalised OD values (ODn, see next paragraph) of wild boar sera on these plates were all in a reasonably low range (ODn < 0.23), except one sample (ODn = 0.46). Analysis performed with- (n = 3778) and without (n = 3459) these outlier plates led to the same cut-off value.

Calculation of normalized OD values and cut-off determination

Measured (OD - Blank) values of the panel of negative and positive control sera on each test plate were plotted against their long-term average values as described earlier (Opsteegh et al., 2010). A straight line was fitted with slope *a* and intercept *b*, for which goodness of fit (R^2) was determined using the linear regression analysis tool in Microsoft Excel. R^2 values > 0.95 were considered to correspond to a good fit; control sera in the vast majority of test plates yielded R^2 values >0.98.

Wild boar sera were tested in duplicate, and normalized OD minus blank (ODn) values were calculated using the formula ODn = ((OD - Blank) – *b) /a*. The 99.75 percentile of the Log(*ODn)* values of in total 3839 Dutch, Polish and Romanian serum samples was identified to obtain the cut-off value.

Table 1. Results of serological monitoring of wild boar during the period 1999 – 2004 (A), and paired results of digestion (B) and ELISA (C and D) of Dutch wild boar in the period between autumn 2003 and spring 2005 (Dataset I). ND: not determined.

A				
Region	Year	OD above cut-off / total tested 1:20	Prevalence	95%CI
Veluwe & Limburg	1999	9 / 163	5.50%	2.9 - 10.1
Veluwe & Limburg	2000	26 / 414	6.20%	4.5 - 9.3
Veluwe & Limburg	2001	12 / 382	3.10%	1.8 - 5.4
Veluwe & Limburg	2002	12 / 278	4.30%	2.7 - 7.3
Veluwe & Limburg	2003	23 / 234	9.80%	6.7 - 14.4
Veluwe & Limburg	2004	17 / 277	6.10%	3.8 - 9.6
B				
Region	Year	digestion positive / total tested	Prevalence	95%CI
Veluwe	2004	0 / 54	0	0.05 – 6.5
Veluwe	2005	0 / 54	0	0.05 – 6.5
Limburg	2004	1 / 47	2.10%	0.5 – 11.0
C				
Region	Year	OD above cut-off / total tested 1:20	Prevalence	95%CI
Veluwe	2004	12 / 59	20.30%	12.1 – 32.3
Veluwe	2005	ND	-	
Limburg	2004	7 / 45	15.60%	7.8 – 28.8
D				
Region	Year	OD above cut-off / total tested 1:100	Prevalence	95%CI
Veluwe	2004	0 / 59	0	0.04 – 5.9
Veluwe	2005	0 / 59	0	0.04 – 5.9
Limburg	2004	1 / 45	2.20%	0.5 – 11.5

Western blot

Serum samples that tested positive by ELISA were diluted 1:100 and then tested by highly sensitive western blotting according to a previously validated published protocol (Gomez-Morales et al., 2014). Excretory/secretory proteins obtained from *T. spiralis* (Gamble et al., 1988), were electrophoretically separated by SDS–PAGE and transferred to nitrocellulose membrane. The nitrocellulose filters were blocked with 5% skimmed milk in 1X Tris–Borate Saline–Tween (TBST) for 1 h. An experiment was considered to be valid when all of the pre-stained protein standards (250, 150, 100, 75, 50, 37, 25 and 20 kD) were separated and transferred onto the nitrocellulose membrane, and the relative

mobility of each standard was within the standard range previously established by 3 independent experiments. Each nitrocellulose filter was cut into strips, each of which was then incubated with 1:100 pig serum with 3% w/v skimmed milk in 1 X TBST at RT for 1 h and immunoreactions were visualized using goat anti-swine IgG conjugated with horseradish peroxidase. To reveal proteins with high efficiency, the LiteAblotW Plus chemiluminiscence system (Euroclone, Pero, Milan, Italy) was added to the strips for 5 min. Finally, the proteins were then visualized on a ChemiDoc™ XRS System (Bio-Rad) and images were analyzed using the Image Lab™ software version 4.0 (Bio-Rad). The positivity/negativity of each serum sample was then determined by comparing the relative migration value (Rf) of each sample with the positive control on the same blot.

Statistical analysis

Serological, geographical and temporal data of Dutch wild boar were evaluated using a generalised linear model approach in the statistical software package 'R', version 3.01 (R-Team, 2008). Log(ODn) values were analysed in relation to the categorical predictors: 'year' and 'area' (Brabant, Limburg, Veluwe and Other), and 'age' in months as numerical predictor as well as 'Age' in years as categorical predictor. In formula: $Log(ODn) \sim year + age + area$.

Results

Wild boar tested in the period 1999 – 2004 (Dataset I).

During the period 1999 – 2004, 1,748 wild boar were tested using the in-house ELISA at a serum dilution of 1:20, which revealed a seroprevalence between 3.1% and 9.8% (Table 1A). During the period autumn 2003 – spring 2005, paired serum and diaphragm muscle samples of 118 wild boar from the central Dutch region Veluwe and 48 wild boar of the south-eastern province of Limburg were tested using both artificial digestion and serology. Artificial digestion of 45 g diaphragm samples revealed a *Trichinella* prevalence of 2.1% (n = 42) in Limburg (Table 1,B-D), whereas none of the animals from Veluwe was positive for *Trichinella*. Fifty-nine *Trichinella* ML were isolated from 45 g of diaphragm (1.3 LPG) of one wild boar originating from Limburg. PCR and sequencing of the *Trichinella* ML identified the larvae as *Trichinella pseudospiralis*. Using the *Trichinella* ELISA, one wild boar serum sample was seropositive (seroprevalence 2.2% in Limburg). However, the *T. pseudospiralis* positive animal was serologically negative, at both a 1:20 and a 1:100 dilution. A panel of thirteen positive and two negative serum samples at dilution 1:20, was retested at serum dilution 1:100 in our lab. At the 1:100 dilution, one out of these 15 sera tested was positive. The serum panel was sent to BfR for testing with ELISA in serial dilution, whereby a titre of 1:80 was considered positive. Only the serum that was positive at RIVM at dilution 1:100 was confirmed as positive at BfR.Wild boar tested in the period 2007-2015 (Dataset II)

Table 2. Overview of *Trichinella* results at meat inspection of wild boar Serosurveillance results of *Trichinella* using in-house ELISA (RIVM) on Dutch wild boar during the period 2007 – 2015 (Dataset II) and.

Year	Digestion positives / total tested (Zomer et al., 2015)	95% CI	ODn above cut-off / total tested	prevalence	95% CI
2007	0 / 881	0 – 0.43%	0 / 201	0	0 – 1.88%
2008	0 / 3154	0 – 0.12%	0 / 216	0	0 – 1.75%
2009	0 / 2010	0 – 0.19%	0 / 599	0	0 – 0.64%
2010	0 / 2504	0 – 0.15%	0 / 441	0	0 – 0.86%
2011	0 / 1332	0 – 0.29%	0 / 384	0	0 – 0.94%
2012	0 / 3238	0 – 0.12%	0 / 518	0	0 – 0.74%
2013	0 / 1835	0 – 0.21%	0 / 611	0	0 – 0.62%
2014	0 / 1960	0 – 0.20%	3 / 528[b]	0.6%	0.19 – 1.66%
2015	NA	NA	3 / 280[c]	1.07%	0.36 – 3.1%
Totals:	18088		3778	0.16%	0.00 – 0.30

NA: data for 2015 were not yet available at the moment of writing the manuscript.
[b] Western blot confirmed one positive sample (seroprevalence 0.19%) from the province of Limburg. [c] Western blot confirmed two positive samples (seroprevalence 0.71%) from the province of Limburg.

Animals

The sex distribution of 3,823 collected Dutch wild boar was 51.1% male (n = 1955) and 48.5% female (n = 1854); the sex of 14 animals (0.4%) was not recorded. Ten percent of tested Dutch wild boar collected during the period 2007 – 2015 originated from Veluwe (Figure 1, central part of region 2). Sixty percent of tested wild boar originated from the province of Limburg (Figure 1, region 4) and 21.5% from the adjacent province of Noord-Brabant ('Brabant', Figure 1, region 3).

Trichinella control at the meat inspection

During the period 2007 - 2014, all 18,088 Dutch wild boar tested negative for *Trichinella* ML, using artificial digestion according to EU Regulation 2075/2005 (European-Commission, 2005) at slaughterhouse laboratories (Table 2). In Poland, 2832 out of 685,595 tested wild boar were *Trichinella* positive (overall prevalence 0.42% (95% CI 0.41% – 0.44%) and in Romania, 378 out of 95,990 wild boar tested, were positive for *Trichinella* spp. (overall prevalence 0.39%, 95% CI 0.35% – 0.43%) at the meat control using artificial digestion according to EU Regulation 2075/2005 (European-Commission, 2005) during the period 2007 – 2012.

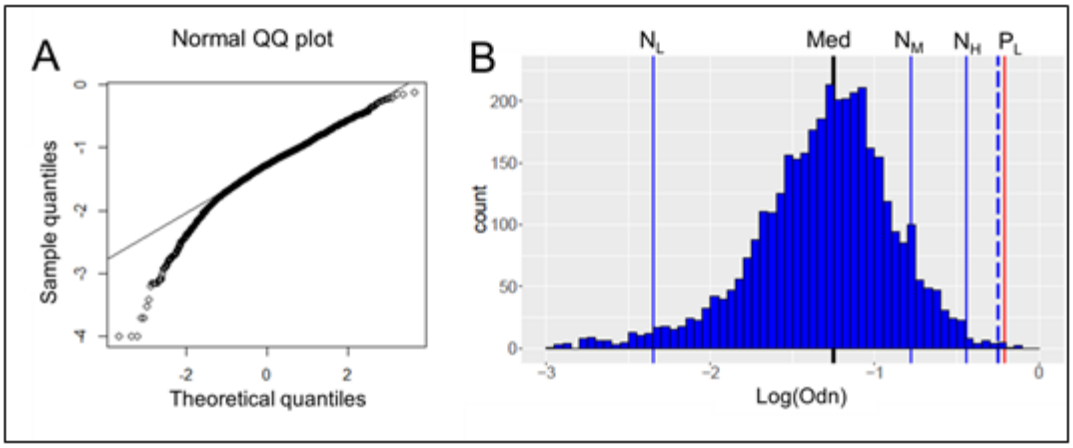

Figure 2. Frequency distribution of ODn values of wild boar sera and cut off determination.
A. Q-Q (comparison of quantiles) plot of the data shows that ODn values are non-normally distributed. Ideally, normally distributed data follow a linear pattern characterised by $y = x$.
B. Frequency distribution of Log(ODn) values of Dutch (n = 3778), Polish (n = 556) and Romanian (n = 101). ODn values of in total 636 sera (439 Dutch sera, 140 Polish and 16 Romanian sera) were lower than the blank ODn value, resulting in negative ODn; these sera were not included, since it is not possible to calculate a Log(ODn) of these samples. A cut-off value was determined at the 99.75 percentile (ODn = 0.5623, dashed line). Med: median, N_L: low negative control (long-term (lt)OD: 0.0045 ± 0.0099), N_M: medium negative control (ltOD: 0.1656 ± 0.0722), N_H: high negative control (ltOD: 0.3620 ± 0.0619), P_L: low positive control (ltOD: 0.6205 ± 0.2694). Dashed blue line: cut-off at 99.75 percentile.

Cut-off determination

In total 3839 Dutch, Polish and Romanian serum samples combined, were plotted in a frequency distribution, but no positive component was recognised by binary mixture modelling (Opsteegh et al., 2010), which would otherwise allow cut-off determination. Instead, we determined the 99.75 percentile of the Log(ODn) values as cut-off value, corresponding to ODn 0.5623, which was slightly lower than the ODn of the low positive

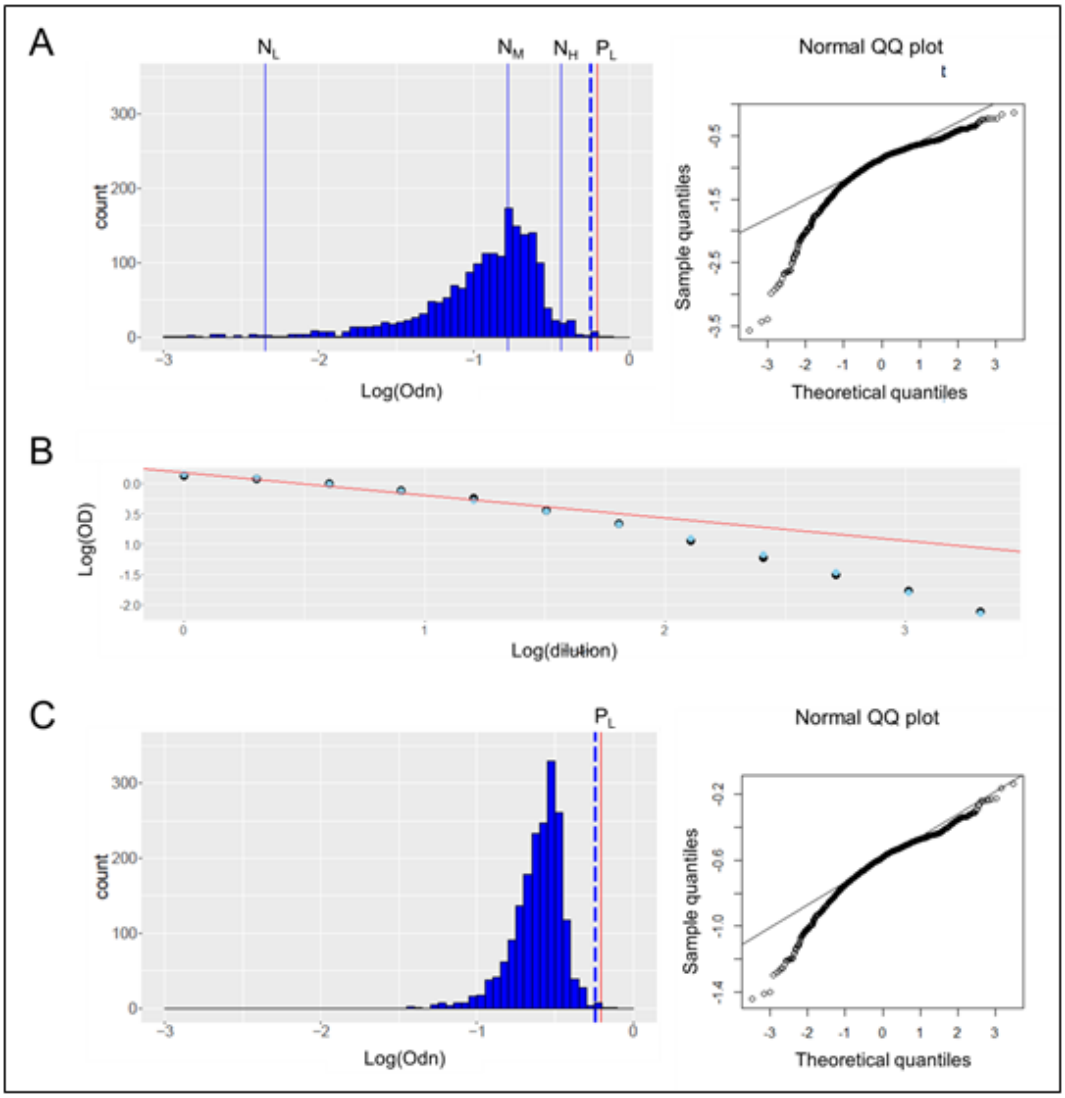

Figure 3. Detailed analysis of Dutch wild boar sera to explore skewed distribution.
A. Frequency distribution of Log(ODn) of Dutch wild boar that were corrected for individual control serum outliers per test plate, which did not alleviate the non-normal distribution as shown in the QQ plot. The cut-off value is the same as determined from combined Dutch, Polish and Romanian sera.

B. The high positive control serum was diluted 1:100 and from this, a serial 1:1 dilution was prepared and tested by RIVM ELISA, following normal procedures. Measurements are shown in double logarithmic scale. A linear fit through the first six data points (red line) showed that data deviated increasingly with further dilution. A quadratic function (blue filled circles) was fitted to observed measurements (black filled circles), to quantify deviation from linearity.
C. Parameters from the analysis under B were used for further correction of ODn values shown in panel A, which improved things considerably, according to the Q-Q plot, but corrected Log(ODn) values still were non-normally distributed. Note that the Q-Q plot scale differs from the one used in 3A. The resulting cut-of value was slightly higher than previously obtained (Log(ODn) = -0.243, ODn = 0.5714).
N_L: low negative control (long-term (lt)OD: 0.0045 ± 0.0099), N_M: medium negative control (ltOD: 0.1656 ± 0.0722), N_H: high negative control (ltOD: 0.3620 ± 0.0619), P_L: low positive control (ltOD: 0.6205 ± 0.2694). Dashed blue line: cut-off at 99.75 percentile.

control serum (taken 2 days after seroconversion) and higher than the ODn of the high negative (non-infected) control (Figure 2).
The Log(ODn) values were non-normally distributed and left skewed (skewedness -1.0626). To explore the reason for this skewedness in ODn distribution of the wild boar samples, further analysis was undertaken with the Dutch sera from the period 2009 – 2014, since these data were all generated using the panel of control sera including the low positive control, and geographic origin, hunting date and age of the hunted animal were known for these sera. For the year 2015, comprehensive information concerning year, area and age was not yet available for all serum samples. Outlier control sera were identified and removed, and long-term average control values were recalculated, to fine tune calculated ODn values. However, this did not correct the distribution skewedness (Figure 3A).
We then tested the linearity of measurements at low OD, by performing ELISA with two-fold dilution series of the high positive control serum according to standard procedure. Blank-corrected OD's of the first six data points were linear, whereas measurements of further dilutions were progressively non-linear and followed a quadratic function (Figure 3B). We derived an equation, allowing us to correct for the non-linearity: $Log(ODn) = \alpha \times (Log(dilution))^2 + \beta \times Log(dilution) + \gamma$, with α = -1.127539, β = 0.8710373 and γ = 0.030853, obtained by fitting calculated to observed data (Figure 3B). We transformed Log(ODn) values below -0.25 (chosen from the Q-Q plot of Figure 3A). The resulting Log(ODn) distribution improved towards a normal distribution, but still was somewhat left-skewed, possibly reflecting biological effects (Figure 2.3C).
Corrected Log(ODn) values correlated significantly with factor 'year' ($p = 2\times 10^{-16}$) and 'area' ($p < 0.01$); factor 'age' did not contribute significantly to the model ($p = 0.4171$). Subsequently, factors 'year' and 'area' were tested including interaction between both factors, using the formula $Log(ODn) \sim (year + area)^2$. The results in Table 3 show that in relation to year 2009 and area 'Brabant' (included in intercept), Log(ODn) values of

2010 are significantly lower ($p = 4.1\times10^{-4}$) than in 2009. Log(Odn) values in 2011 are higher than in 2009 ($p = 1.7\times10^{-2}$), whereas in 2012, Log(ODn) values are significantly lower than in 2011 ($p = 6.6\times10^{-4}$), although Log(ODn)values of areas 'Limburg' ($p = 7.3\times10^{-5}$) and 'Veluwe' ($p = 6.5\times10^{-3}$) are significantly higher. In 2013, Log(ODn) values of 'area Limburg' ($p = 5.8\times10^{-4}$) and 'area Other' ($p = 1.7\times10^{-2}$) are significantly higher (Figure 4).The cut-off value, taken from the 99.75 percentile of the Log(ODn) values for 2009 – 2014 after exclusion of outlier control values (-0.243, corresponding to ODn 0.5714), was only slightly higher than that obtained with all Dutch, Polish and Romanian sera taken together (ODn 0.5623).

Trichinella seroprevalence

Using the RIVM ELISA, six out of 3778 tested Dutch wild boar sera reacted above cut-off 0.5714 (Table 2), which represents an observed over-all seroprevalence of 0.16% (95%CI0.00 – 0.30%) and year-prevalences of 0.6% (95%CI 0.21 – 1.64%) in 2014 and 1.1% (95%CI 0.39 – 3.09%) in the first quarter of 2015 (Table 2). During the period 2007 – 2013, all wild boar sera were negative.

Five wild boar sera from Poland (n = 556) tested above the cut-off value using the rivm ELISA (seroprevalence 0.90%, 95% CI 0.1% – 1.7%). All wild boar sera from Romania (n = 101) were negative in the RIVM ELISA.

Six Dutch samples that exhibited above cut-off ODn values and six Dutch samples showing near cut-off or below cut-off values were sent to the European Reference Laboratory for Parasites (ISS, Rome Italy) for Western blot analysis. Three Polish serum samples showing above cut-off values and two samples around or below cut-off were available for confirmation by Western blot.

Confirmation by Western blot

In total twelve Dutch and five Polish serum samples were selected for Western blot analysis at the European Reference Laboratory for Parasites (ISS, Rome Italy). Two out of six Dutch wild boar samples from 2007 - 2015 that were positive in the RIVM ELISA (one in 2014 and one in 2015) were confirmed by Western blot (Figure 5). One serum from 2015 that tested negative in RIVM ELISA at dilution 1:100, tested positive in ELISA at dilution 1:50 at ISS and was confirmed by Western blot. This resulted in an overall seroprevalence in Dutch wild boar of 0.079% (95%CI 0 – 0.2%); the seroprevalence for 2014 was 0.13% (n = 528). When only taking into account the province of Limburg, where the seropositive wild boar originated, the seroprevalence would be 0.28% (95%CI 0 – 0.8%) in 2014, which is not significantly different (p = 0.21) from the seroprevalence of 2.2% (95%CI 0.5 – 11.5%) that was demonstrated in the same province in 2004 – 2005, using the same serum dilution.

Table 3. Results of linear model analysis of Log(ODn) in relation to factors 'year' and 'area'.

	Estimate	p-value	
(Intercept)	-0.61311	2.00×10^{-16}	*
year2010	-0.28598	0.000408	*
year2011	0.1149	0.016672	*
year2012	-0.12268	0.000661	*
year2013	-0.04173	0.320255	
year2014	0.05551	0.105223	
areaOther	-0.0481	0.242244	
areaLimburg	-0.05167	0.124729	
areaVeluwe	0.07777	0.076147	
year2010:areaOther	0.15482	0.150922	
year2011:areaOther	0.04993	0.561586	
year2012:areaOther	0.05204	0.312003	
year2013:areaOther	0.18661	0.017232	*
year2014:areaOther	0.08908	0.098937	
year2010:areaLimburg	0.12459	0.138699	
year2011:areaLimburg	0.05237	0.343735	
year2012:areaLimburg	0.15492	7.29×10^{-5}	*
year2013:areaLimburg	0.1578	0.000581	*
year2014:areaLimburg	0.05803	0.126011	
year2010:areaVeluwe	-0.15336	0.158879	
year2011:areaVeluwe	-0.12052	0.167727	
year2012:areaVeluwe	0.15144	0.006543	*
year2013:areaVeluwe	0.10338	0.161046	
year2014:areaVeluwe	-0.08973	0.079558	

Estimates of Log(ODn) compared to baseline (2009, Noord-Brabant). In 2010, the average Log(ODn) was 0.47 lower. In 2012, the average Log(ODn) was 0.12 lower, but on top of this, area Limburg was 0.26 higher (so net 0.06 higher) and area Veluwe was 0.15 higher (net 0.03 higher). In 2013 the average Log(ODn) was 0.04 lower, but on top of this, area Other was 0.18 higher (net 0.14 higher) and area Limburg was 0.15 higher (net 0.11 higher). Adjusted R-squared: 0.2336. Asterisks indicate significance at $p < 0.05$.

Figure 4. Factor analysis to explore left-skewed distribution of ODn values that were corrected for control serum outliers and apparatus measurements.
A pairs-plot stratifies the log(ODn) values according to year and area. Boxplots show 25th and 75th percentiles; thick horizontal lines represent median values. Red line represents cut-off value (ODn 0.5714). Sig: significance at $p < 0.05$.

Discussion

The aim of the present study was to evaluate long-term exposure of Dutch wild boar (*Sus scrofa*) to *Trichinella* spp. during the last two decades and to compare these findings with the preceding period 1996 – 1998, when seroprevalence was 3.1 – 9.8% (van der Giessen et al., 2001).

Two wild boar out of eleven tested (18%), originating from the province of Limburg (the same region), were demonstrated positive for *T. spiralis* muscle larvae by artificial digestion during the period 1996 – 1998, which may corroborate the high recorded seroprevalence (van der Giessen et al., 2001). It should be noted that for these wild boar, nine times higher weight samples (45g) were tested than is currently used for meat inspection (5g) and these wild boar were tested negative during routine testing at meat inspection. Initially, a serum dilution of 1:20 was used before 2004, following a previously optimised ELISA protocol for pigs and humans (van Knapen et al., 1986; van Knapen et al., 1980). However, this may have rendered non-specific reactions in our in house ELISA, since only ELISA test results using a 1:100 serum dilution were confirmed by interlab comparison with BfR (Berlin, Germany) at the same serum dilution.

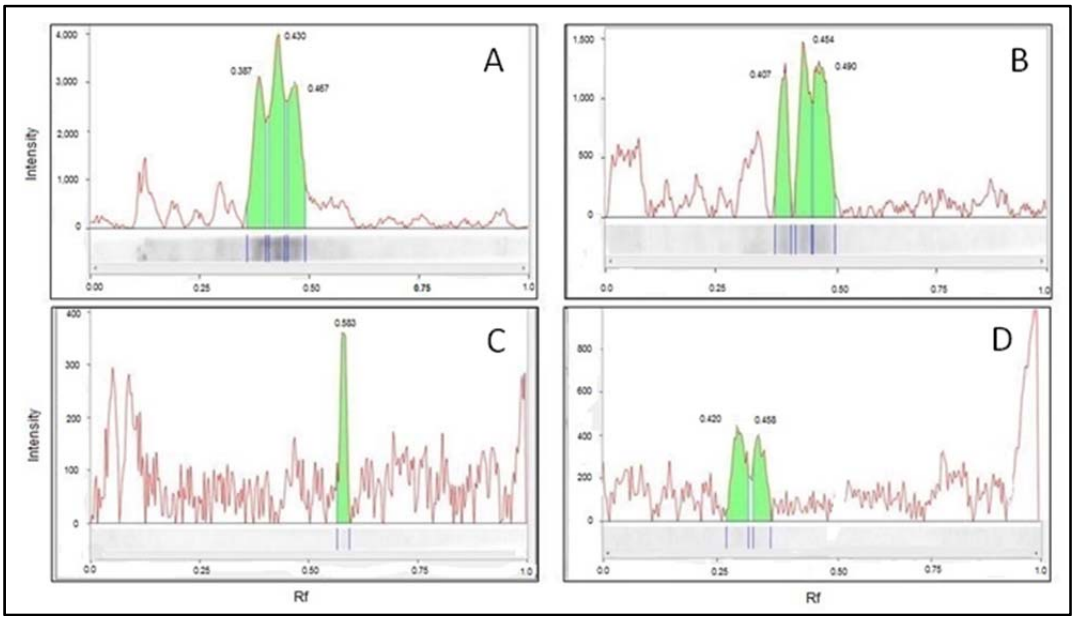

Figure 5. Seropositive wild boar samples recognized *Trichinella spiralis* excretory / secretory antigens by Western blotting.
Signal intensities and relative migration values (Rf) of the recognized proteins.
A. Serum samples from a naturally infected Polish *Trichinella* seropositive wild boar (PL 498).
B. a Dutch *Trichinella* seropositive wild boar (NL 1457-1) reacted specifically to three *Trichinella* spp. bands on Western blot.
C. A serum sample of a Dutch *Trichinella* wild boar that was seropositive by ELISA, showed only non-specific reactions by Western blot (NL 2685-5).
D. A serum sample from a seronegative Dutch wild boar (NL 2683-2).

Therefore, harmonisation and validation of the ELISA in 2005, led to an adapted serum dilution of 1:100. Alternatively, cut-off determination based on average OD + 3SD before 2005, may have overestimated positivity, due to right tailed and therefore non-normally distributed non-Log-transformed OD values (Jacobson, 1988).
Defining a cut-off value for wildlife serum samples is challenging, since appropriate negative or positive control animals are usually not available. Therefore, serum samples from endemic regions were used in the present study. However, the seroprevalence in this sample set was too low to allow binary mixture model analysis to determine a cut-off value. Given the absence of *Trichinella* larvae at wild boar meat inspection in the Netherlands, we anticipated that the wild boar of the studied cohort harboured negligible to very low levels of *Trichinella*, with Log(ODn) distribution representing variation in baseline level. Given the low correlation coefficient between low level larval count and

OD-value for *Trichinella* in individual pigs (Teunis et al., 2009), above cut-off results in ELISA are unfit to predict actual infection levels in meat of individual animals.
The average *Trichinella* prevalence determined at meat inspection in Poland was 0.42% during the period 2007 – 2012 (EFSA, 2011; EFSA and ECDC, 2014), and a survey performed in Poland in 2006 revealed a *Trichinella* seroprevalence of 0.68 – 0.74% in wild boar, using an in-house ELISA (Bien, 2006, 2007). The seroprevalence in a selected sample set (n = 556) from voivodeships (provinces) where *Trichinella* occurs at varying levels, was 0.90% in our ELISA, which is not significantly different from the seroprevalence determined in the Polish survey (p = 0.7834, Fisher's Exact test). Note that these seroprevalence data are not confirmed by Western blot. The prevalence in Romanian wild boar at meat inspection was 0.39% (EFSA, 2011; EFSA and ECDC, 2014), and no positive samples were identified in our ELISA, which is likely due to the limited sample size (n = 101).
Using a 1:100 serum dilution in the *Trichinella* ELISA during the period 2007 – 2013, no positive Dutch wild boar was found out of 3,011 tested, and no *Trichinella* ML were found at the Dutch slaughterhouse laboratories after artificial digestion of 16,509 wild boar samples during that period. During the period 2014 – 2015, six Dutch wild boar sera out of 808 tested, exhibited ODn values above cut-off value. During that period, no wild boar out of 1,579 tested positive by artificial digestion at the meat inspection. Other studies have shown that ELISA sensitivity may be 30 – 100 times higher than that of artificial digestion (Gamble et al., 1983; Gomez-Morales et al., 2014). At an observed upper prevalence limit of 0.0055% (= 1 / in total 18,088 tested) for Dutch wild boar, this would indicate an upper seroprevalence range of 0.16 – 0.55, corroborating the low observed seroprevalence level in the present study.
The Log transformed ODn values (period 2007 – 2015) showed a non-normal, left tailed distribution. Re-analysis of ELISA data after correction for control serum outliers and measurement deviations at very low OD, did not completely resolve the non-normal distribution of Log(ODn) values. Linear model analysis showed that wild boar age did not correlate with Log(ODn), whereas hunting year and area correlated significantly with Log(ODn). Ultimately, we were not able to identify what caused the variation between and within years.
Wild boar may be exposed to many more pathogens than *Trichinella* spp. alone, possibly resulting in serological cross-reactions. Using the ELISA, we found six positive samples out of 3778 Dutch wild boar tested over the period 2007 - 2012, of which three sera were confirmed positive using Western blot. The resulting overall seroprevalence of 0.079%, is considerably lower than overall seroprevalence reported from Italy (2.2%), using a similar method (Gomez-Morales et al., 2014).
The Western blot has been well characterised and a validation study using wild boar sera collected in Italy, showed that only 10% of ELISA positive wild boar that were tested at a 1:50 dilution were confirmed by Western blot (Gomez-Morales et al., 2014). In that study, a seroprevalence of 2.2% was determined, whereas the prevalence based on artificial

digestion was 31.4 times lower (0.07%). A drawback of Western blot confirmation is its high cost and labour intensive procedure. Since we have only three positive serum samples in our study, this indicates that our ELISA seems more specific and suitable to study seroprevalence.

All three *Trichinella* seropositive animals belong to two subpopulations, adjacent to natural reserve Meinweg, which consists of marshland and extends into Germany. In addition, the *Trichinella* larvae positive wild boar originated from this same area. Increasing numbers of wild boar in the northern part of the Province of Limburg and the province of Noord-Brabant, originate from wild boar expansion from the Dutch and German Meinweg area, influx from other German populations and influx from the Belgian province of North-Limburg in Flanders, Belgium (Jansman et al., 2013). Area 'Veluwe' harbours a separate and more isolated population of wild boar (Jansman et al., 2013).

In conclusion, the present study shows that evaluation of serological results using wildlife samples is often hampered by absence of well-defined field sera. We show that *Trichinella* seroprevalence in Dutch wild boar over the periods 2004 – 2005 and 2007 – 2015 is very low, with only four confirmed positive animals out of 3,944 tested – one in 2004 by artificial digestion and three in 2014 – 2015 by western blot. Higher seroprevalence measured during the period 1999 – 2004 likely resulted from non-specific reactions in the ELISA at serum dilutions of 1:20 or differences in cut-off calculation that were used at that time.

Competing interests

The authors declare that they do not have competing interests.

Author contributions

FF wrote the study design, generated and analysed serological data and wrote the manuscript. AS and MO helped with statistical analysis and contributed to the manuscript. MAGM confirmed and analysed samples by Western blot. MR provided sera and prevalence data from Poland. AG provided sera from Romania. JvdG wrote the project, coordinated and supervised the study in the Netherlands and contributed to the manuscript. All authors read and approved the final version of the manuscript.

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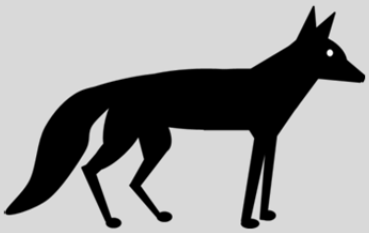
Chapter 3



Trend analysis of *Trichinella* in a red fox population from a low endemic area using a validated artificial digestion and sequential sieving technique

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Freezing of fox carcasses to minimize professional hazard of infection with *Echinococcus multilocularis* is recommended in endemic areas, but this could influence the detection of *Trichinella* larvae in the same host species. A method based on artificial digestion of frozen fox muscle in combination with larva isolation by a sequential sieving method (SSM), was validated using naturally infected foxes from Latvia.

The validated SSM was used to detect dead *Trichinella* muscle larvae (ML) in frozen muscle samples of 369 red foxes from the Netherlands, of which one fox was positive (0.067 larvae per gram, LPG). This result was compared with historical *Trichinella* findings in Dutch red foxes of more than 15 years ago.

Molecular analysis using 5S PCR showed that both *T. britovi* and *T. nativa* were present in the Latvian foxes; no mixed infection was found. Of 96 non-frozen *T. britovi* ML, 94% was successfully sequenced, whereas this was the case for only 9% of 72 frozen *T. britovi* ML. The single *Trichinella* sp. larva that was recovered from a frozen foreleg of a Dutch fox did not yield PCR product, probably due to severe freeze-damage.

In conclusion, the SSM presented in this study is a fast and effective method to detect dead *Trichinella* larvae in frozen meat. We showed that the *Trichinella* prevalence in Dutch red fox was 0.27% (95% CI 0.065-1.5%), in contrast to 3.9% in the same study area fifteen years ago. Moreover, this study demonstrated that the efficacy of 5S PCR for identification of *Trichinella britovi* single larvae from meat that had been deep-frozen is not more than 8.3%. This is the first time that the effect of deep freezing on *Trichinella* species identification was quantified. To increase species identification sensitivity and at the same time generate DNA sequence information for molecular epidemiology, a combination of methods may be considered.

Keywords: *Trichinella*, sequential sieving, sequencing, frozen fox muscle, Netherlands, Latvia

Introduction

Trichinella species infect a wide range of mammals, including humans (Pozio, 2005; Pozio et al., 2009b). In the European Union, the magnetic stirrer method (EU reference method, EU-RM) according to European regulation EC 2075/2005 (European-Commission, 2005) is used for individual carcass control of *Trichinella* susceptible animals intended for human consumption and for surveillance of *Trichinella* infections in wildlife. This method includes two consecutive sedimentation steps to isolate *Trichinella* muscle larvae (ML) and has been validated for the detection of live larvae, for which critical control points are well described (Rossi and Pozio, 2008). To analyse *Trichinella* in wildlife, some adjustments to the magnetic stirrer method are necessary to improve efficiency, like prolongation of digestion time, since meat of wildlife is more difficult to digest. In Europe, the red fox is considered an indicator species for *Trichinella* infections in wildlife and many studies are being carried out to determine the prevalence and infection rate of *Trichinella* in red fox populations (Claes, 2013; Clausen and Henriksen, 1976; Enemark et al., 2000; Hurnikova and Dubinsky, 2009; Malakauskas et al., 2007; Sluiters et al., 1972; van der Giessen et al., 1998; van Knapen et al., 1993; Zimmer et al., 2009). Since in Europe the red fox is also a final host for *Echinococcus multilocularis*, a zoonotic parasite and causative agent of alveolar echinococcosis in humans, fox carcasses are deep frozen at -80 °C for minimally one week, to inactivate the infective stage of this fox tapeworm prior to post mortem examination, according to WHO biosafety instructions (WHO, 1984). Already between -18 and -30 °C, freezing kills most *Trichinella* ML within one week (Lacour et al., 2013; Malakauskas and Kapel; Pozio et al., 1994a; Randazzo et al., 2011), thereby altering their sedimentation characteristics (Rossi and Pozio, 2008), which is a key factor in the analysis with EU-RM. Gamble (Gamble, 1999) showed that live larvae settled with a sedimentation speed of about 2 cm/min in meat digest at 40 °C. This is enough to pass through 2 litres of meat digest in a separatory funnel within 16 minutes. At 4 °C the sedimentation speed was less, which would prolong the sedimentation time to 24 – 28 minutes. In contrast, Dyer and Evje (Dyer and Evje, 1971) recovered only 80% of spiked dead *Trichinella* ML spiked in 2 litres *Trichinella*-free meat digest after one hour of sedimentation (twice the time routinely used in EU-RM).

Well before the EU-RM was established, Henriksen (Henriksen, 1978) successfully used a filtration method to isolate dead *Trichinella* ML from experimentally infected rabbits. Enemark *et al*. (Enemark et al., 2000) used 22 µm disposable filters to retain ML after artificial digestion of fox fore legs that had been kept at -20°C for three to ten months prior to analysis. Retained ML were visualized by subsequent iodine/hypochlorite staining, which renders these larvae unsuitable for molecular species identification.

Van der Giessen *et al*. (van der Giessen et al., 1998) used the Trichomatic^{35} method, an automated system by which naked *Trichinella* larvae were isolated on a 14 µm mesh size nylon filter for subsequent microscopical examination. Subsequently, isolated individual larvae were identified as *Trichinella britovi*, using a single larva PCR and reversed line blot analysis as described by Rombout *et al.* (Rombout et al., 2001b).

In this study, we describe validation of an artificial digestion method using the magnetic stirrer method, followed by a sequential sieving step to isolate dead *Trichinella* larvae from naturally infected fox muscle samples. We show that the recovery rate of spiked dead *Trichinella* larvae in meat digest is 60% using EU-RM, while the recovery rate using SSM is 92%, making SSM the technique of choice to detect dead *Trichinella* larvae in frozen meat. Consequently, the most sensitive technique was used to analyse the recovery rate of *Trichinella* larvae before (EU-RM, live larvae) and after (SSM, dead larvae) freezing of naturally infected fox samples. Moreover, the efficacy of molecular identification was studied on isolated ML originating from foxes from an endemic area, before and after freezing. The validated sequential sieving method was used to study *Trichinella* prevalence in the red fox population in the eastern border region of the Netherlands. Obtained *Trichinella* prevalence was compared to historical data to analyse trends in time.

Materials and methods

Animals and *Trichinella* larvae

The left Foreleg of 35 *Trichinella* positive (EU-RM) (European-Commission, 2005) non-frozen red foxes from Latvia were collected during routine inspection at the Institute of Food Safety, Animal Health and Environment BIOR (Riga, Latvia). These animals originated from all four Latvian regions (Vidzeme 6, Zemgale 7, Latgale 9 and Kurzeme 11 individuals, 2 not specified). After primary analysis of the muscle samples by EU-RM without freezing, the forelegs were frozen and kept at -80 °C for one to two weeks, after which a second muscle sample from the same foreleg was tested with SSM at BIOR. A digestion time of 30 - 40 minutes was used for artificial digestion as described (European-Commission, 2005; Rossi and Pozio, 2008). After detection of ML, isolated *Trichinella* larvae were kept in 96% ethanol at room temperature until further use. For analysis with multiplex PCR (Zarlenga et al., 2001), pools of five *Trichinella* ML were isolated from 30 foxes from all four regions of Latvia (Vidzeme 6, Zemgale 7, Latgale 8 and Kurzeme 9 individuals). For single larva PCR, individual *Trichinella* ML from the same 30 foxes that were found positive both before and after freezing, were transferred to 5 µl of DNAse free water and stored at -20 °C until further use.

Live *Trichinella britovi* larvae for the validation of detection by sequential sieving were obtained from a farmed wild boar, which tested positive during regular meat inspection in Latvia (Zemgale region, Latvia, Institute of Food Safety, Animal Health and Environment BIOR). *Trichinella spiralis* (ISS 14) larvae for use in spike experiments were obtained from experimentally infected mice by the EU-RM. This work was approved by the Ethical Committee of the Dutch National Institute for Public Health and the Environment (RIVM) (DEC permit number 201200223).

For *Trichinella* survey in the Netherlands from October 2010 - April 2013, 369 Dutch foxes

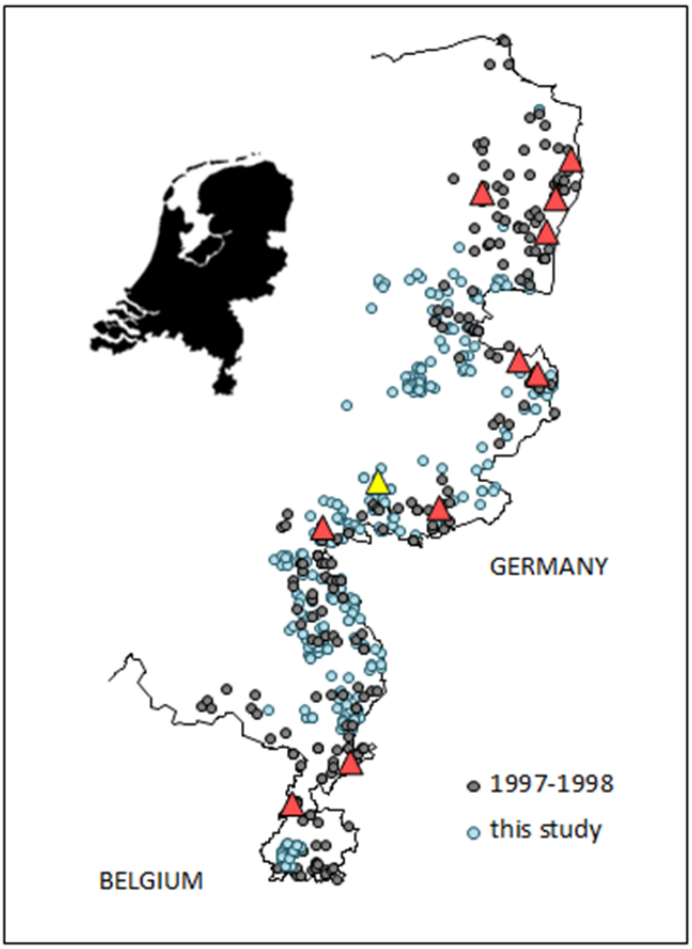

Figure 1 - Geographical origin of Dutch red foxes
At the eastern border of the Netherlands (outline) 369 foxes were collected during the period 2010-2013 (blue circles), of which one fox was positive for *Trichinella* (yellow triangle). In contrast, in a similar study in 1997-1998 (grey circles), eleven *Trichinella* positive foxes (red triangles) were found in a collection sample of 276 red foxes, ten of which in the same study area [21].

were collected by hunters from the border region with Germany in the east and Belgium in the south (Figure 1). The majority of foxes (287) was collected during the hunting season November 2010 - April 2011. Collected foxes were sent to RIVM, Bilthoven, the Netherlands. Upon arrival, fox carcasses were stored at -80 ˚C to inactivate the eggs of possibly present *E. multilocularis* (Veit et al., 1995) according to WHO guidelines (WHO, 1984). After a minimum period of one week, carcasses were thawed at approximately 10 °C and dissected. Muscles of both lower forelegs of each fox were collected and 15 g of muscle tissue was analysed for *Trichinella*, using the validated SSM.

Validation experiments

Crucial steps of the EU-RM for the detection of *Trichinella* larvae are complete digestion of muscle tissue and high effectivity of the procedure to isolate *Trichinella* ML. To validate the method for detection of dead *Trichinella* larvae in frozen meat samples, the process was separated into three stages.

1. Isolation and detection of dead larvae. The efficacy of dead *Trichinella* ML isolation using EU-RM and SSM was compared by spiking dead Trichinella ML in meat digest and subsequent recovery of ML. The sequential sieving method to detect *Trichinella* larvae was further validated by adding live or dead larvae to water or *Trichinella*-free meat digest and recovery by SSM.
2. Feasibility of the use of *Trichinella* spiked frozen samples. Minced pork meat was spiked with live *Trichinella* larvae and subsequently, the spiked samples were frozen, to evaluate the possible effect of freezing on the recoverability of these larvae. The spiked and frozen samples were subjected to artificial digestion during 30 min according to EU 2075/2005 and subsequent detection of larvae by sequential sieving.
3. Validation of sequential sieving in relation to EU-RM. The sequential sieving method was validated by comparison of data obtained by analysis of fox forelegs using the EU-RM before freezing and data from analysis by SSM after freezing at -80 °C.

Validation of larva detection

Stainless steel sieves, approximately 18 centimetres in diameter, with mesh size 300 µm, 63 µm and 38 µm were stacked in decreasing mesh size order. A mesh size of 300 µm was used to retain undigested particles, instead of 180 µm, which is used in the EU-RM for the detection of live *Trichinella* ML. This reduced the risk of losing dead, comma shaped ML, which have typical measurements of 745-975 µm length by a width of 36 µm (Anderson, 1992). To validate the efficacy of the smaller mesh size sieves to retain *Trichinella* larvae, 1-39 live naked ML in tap water (BIOR, Latvia) or 1-134 dead naked ML in *Trichinella*-free fox meat digest (RIVM, Netherlands) were poured into the upper, larger mesh size sieve. Subsequently, the ML were carefully washed off the sieves with tap water using a laboratory squeeze bottle, under an angle of approximately 45 degrees. ML that concentrated in the lower rim of the sieves after washing were collected in Petri dishes in approximately 20 ml of rinse water. The number of larvae per sieve was determined microscopically. The experiments were conducted by two researchers per location (BIOR and RIVM), the first author being one of them on both locations.

This sequential sieving method to isolate dead *Trichinella* larvae was compared to sedimentation as used in EU-RM. For this purpose, *Trichinella*-free meat digest was spiked with ten dead, 6-shape to comma-shaped *Trichinella* larvae, which were picked randomly and transferred to approximately 2 ml tap water. Subsequently, the larvae were rinsed into 2 liter of meat digest fluid, in twenty replicate tests. The spiked fluid was either transferred to a separatory funnel and left to sediment for 30 min, after which the lower 40 ml were sedimented again for 10 min in a glass cylinder according to EU-RM, or passed through a stack of stainless steel sieves according to SSM. Residual fluids from EU-RM

were passed through a 38 µm mesh size sieve, to isolate ML that did not sediment within the given time.

Feasibility of *Trichinella* spiked frozen samples

Six minced pork samples (100 g) were spiked with 10 live naked *T. spiralis* ML (RIVM strain, ISS14) and were frozen for two weeks at -80 °C. Three control samples spiked with 10 *Trichinella* ML were kept at +4°C.

Validation of sequential sieving method

To evaluate possible loss of *Trichinella* ML by freezing fox carcasses, the number of *Trichinella* ML was determined in unfrozen muscle samples of individual fox upper forelegs, originating from 35 foxes collected in Latvia as described above. Briefly, 15 gram of muscle tissue per fox leg was digested according to the EU-RM, with adaptation of the digestion fluid volume to 250 ml and the use of a 1-litre separation funnel to sediment possibly present live *Trichinella* larvae.

Thirty-five *Trichinella* positive forelegs (9 - 169 ML per 15 g muscle tissue) were frozen and kept at -80 °C for one to two weeks. Following this period, deep frozen fox legs were thawed at approximately 18 °C and kept at 8 °C until analysis within 24 hours and artificial digestion was performed as described above, during 40 minutes, to guarantee complete matrix digestion. Liberated, naked 6-shaped to comma-shaped *Trichinella* ML were isolated by sequential sieving through a stack of 300, 63 and 38 µm mesh size sieves.

Trichinella monitoring in the Netherlands

Fox carcasses were thawed at approximately 10 °C. Per individual fox, 15 g lower foreleg muscle tissue sample was isolated and pools of 4-7 foxes were digested for 40 minutes in 2 litre tap water of 46°C, containing 0,5 % (w/v) pepsin and 0,2% HCl (v/v) according to the EU-RM. After artificial digestion, sequential sieving through stacked stainless steel sieves with mesh size 300 µm and 63 µm was used, to isolate naked *Trichinella* ML. Foxes of pools that tested positive for *Trichinella* were re-tested individually using the same method.

Statistical analysis

Validation of larva detection

Trichinella ML recovery data of liquid samples that were spiked with either live or dead free ML are assumed randomly distributed. Therefore, a generalized linear model approach with Poisson link function was used to fit data with and without the factor 'live/dead'. Subsequently, both models were compared by likelihood ratio test to select the model with the lowest AIC-value (Akaike's Information Criterion).

The ability of EU-RM and SSM to recover dead *Trichinella* ML from spiked meat digest was compared with Fisher's Exact test.

Validation of sequential sieving method

Isolated *Trichinella* ML were counted independently by two researchers and for each fox, the average value of these two counts was used. The data were plotted and outliers were identified using Grubb's analysis of residuals for best linear fit. Identified outliers were excluded from further analysis. Average parasite numbers before and after freezing were analysed by generalized linear model approach, with negative binomial link function. This distribution allows for overdispersion, and is therefore suitable for parasite count data that typically have a contagious distribution in host tissues (Alexander, 2012). We checked the prerequisite of equal variances by means of the non-parametric Bartlett test of homogeneity of variances (Bartlett, 1973). We built a model with variate 'count', dependent on covariate 'freezing status' with levels 'frozen' or 'fresh'. A p-value below 0.05 for this covariate indicates a significant effect of freezing. Statistical analyses were performed using the software package 'R', version 3.0.1 (R-Team, 2008).

Study in a low-endemic area in the Netherlands

Lower foreleg muscles of 369 Dutch foxes were examined in pools of 4-7 animals using artificial digestion and sequential sieving through 300 and 63 µm. One single *Trichinella* sp. ML was recovered, which was stored in 5 µl sterile DNAse free water and kept at -20 °C until further use.

DNA isolation and molecular confirmation of *Trichinella* ML by Multiplex PCR

DNA was isolated using QIAGEN® QIAamp DNA Mini Kit Tissue Protocol. Of thirty foxes, a pool of five *Trichinella* ML was analysed per animal before freezing. The concentrations of extracted DNA in samples were measured with ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE 19810, USA). The Multiplex PCR was directed at the ITS1, ITS2 and ESV genes as described by Zarlenga *et al.* (Zarlenga et al., 1999). PCR reactions were performed in a total volume of 30 µl, containing 15 µl 2x Master mix (PROMEGA M7505, USA), 1 µl of 10 pmol/µl oligonucleotide mixture, 4 µl of RNAse-free water and 10 µl of DNA. As positive control, *T. spiralis*, *T. britovi* and *T. nativa* DNA was used. The PCR conditions were 95 °C for 4 min followed by 35 cycles of 95 °C for 10 s, 55 °C for 30 s, 72 °C for 30 s. PCR products were analysed by QIAxcel ScreenGel 1.1.0 (Qiagen, Hilden, Germany) and identified according to banding pattern as described earlier (Zarlenga et al., 1999, 2001). This work was performed at BIOR (Riga, Latvia).

DNA isolation and molecular confirmation of *Trichinella* ML by single larvae PCR

DNA was isolated from 3-4 individual ML per Latvian red fox before and after freezing, from three individual larvae from the Latvian wild boar and from the single isolated larva from Dutch red fox according to the protocol described by Pozio *et al*. (Pozio et al., 2001a). Briefly, 2 µl of 0.05 M TRIS-HCL pH 7.6 was added to each larva in 5 µl H2O, which

Table 1. Recovery of dead *Trichinella* larvae spiked in meat digest.

		EC 2075/2005		SSM	
	spike	**sedimentation**	**residual fluids***	**63 µm**	**38 µm**
1	10	4	6	8	0
2	10	7	3	8	1
3	10	2	8	9	0
4	10	4	5	10	0
5	10	6	3	10	0
6	10	9	1	10	0
7	10	6	3	8	0
8	10	8	2	10	0
9	10	8	2	10	0
10	10	6	4	9	0
sum:	100	60	37	92	1

Ten dead, 6-shape to comma-shaped *Trichinella* larvae were picked randomly and transferred to approximately 2 ml tap water. Subsequently, the larvae were rinsed into 2 liter of meat digest fluid. The spiked fluid was either transferred to a separatory funnel and left to sediment for 30 min according to EU 2075/2005, or passed through a stack of stainless steel sieves according to SSM. SSM performed significantly better than EU-RM for detection of dead larvae in meat digest ($p = 6 \cdot 10^{-12}$, Fisher's Exact test).
* number of larvae found after sieving the residual fluids through 38 µm sieve following sedimentation

was overlaid with mineral oil and heated to 90 °C for 10 min. Subsequently, 0.4 µl proteinase K and 2.6 µl H2O was added, followed by incubation at 48 °C for 3 hours and finally a 10 min proteinase K inactivation step at 90 °C. A single larvae PCR directed at the 5S ribosomal rDNA intergenic region was used as described earlier (Bandi et al., 1993; Liu et al., 1996), to determine the species of isolated *Trichinella* ML by DNA sequence analysis, to investigate possible occurrence of simultaneous mixed *Trichinella* infections and to evaluate the influence of freezing on DNA sequencing efficacy. 5S PCR test sensitivity was determined by PCR and agarose gel analysis of four repetitive dilution series with a range of 5 ng to 1 pg *T. britovi* control DNA. PCR amplicons were purified using standard procedures (ExoSAP-IT®, Affymetrix, Cleveland, Ohio, USA). Sequence PCR reactions were carried out on both DNA strands in 20 µl final volume containing 3 µl of amplicate, 7 µl sequence buffer, 1 µl of Big Dye Terminator and 1 µl of forward or reverse PCR primer. Sequence PCR was performed under the following conditions: 95 °C for 1 min, followed by 25 cycles of 96 °C for 10 min, 50 °C for 5 min and finally 60 °C for 4 min. Trace files of the obtained sequences were generated on an automated ABI sequencer. DNA sequences were assembled, edited manually, and analysed with BioNumerics

version 7.1 (Applied Maths NV, Sint-Martens-Latem, Belgium). Cluster analysis of the sequences was conducted using BioNumerics 7.1 with Jukes-Kantor correction setting and bootstrap analysis of 2500 replicates. Sequence homology ≥99 % was considered proof of identity between isolates and available 5S rDNA sequences of *Trichinella* species from Genbank. This work was performed at RIVM (Bilthoven, Netherlands).

Results

Validation of larva detection

The sensitivity to detect dead *Trichinella* ML in meat digest of the EU-RM was 60% (n=100), whereas the SSM performed significantly better with 92% (n=100) sensitivity ($p = 6 \cdot 10^{-12}$, Fisher's Exact test) (Table 1). Overall sensitivity of the sequential sieving to detect *Trichinella* ML was 92.9% when using dead ML (n = 451) and 88.9% (n = 280) for samples spiked with live ML. Using the recovery data of the spiked samples, a Poisson generalized linear model was fitted with and without the factor 'live/dead'. Comparing both models, the model without 'live/dead' factor was favoured resulting from lower AIC-value (Akaike's Information Criterion) and a p-value of 0.58 after likelihood ratio testing. The best fitting model to describe the relationship between the number of spiked and counted larvae was $count = 0.91 spike$, the slope of which is close to, but significantly different from 1 (p = 0.0198) (Figure 2A). In total 2833 dead *Trichinella* ML were isolated from 31 frozen Latvian fox forelegs by sequential sieving, of which 0.4% (12 ML) passed through the 63 µm mesh size and were retrieved from the 38 µm mesh size sieve. Of live larvae, 5.8% (14 out of 243) passed through the 63 µm sieve and were collected from the underlying 38 µm sieve. From these results, it was decided to use a combination of sieves with mesh size 300 µm and 63 µm to study *Trichinella* prevalence in deep-frozen foxes from a low-endemic area (the Netherlands).

Effect of freezing on *Trichinella* larvae

Minced pork samples were spiked with free larvae (without nurse cell), to increase the precision of recovery evaluation. Detection of *T. spiralis* (RIVM strain, ISS14) ML in frozen pork samples spiked with 10 ML using artificial digestion according to EU-2075/2005 with 30 min digestion time and subsequent detection of larvae by sequential sieving, showed a sensitivity of only 48.3% (n = 60), whereas the recovery from control samples stored at 4 °C was 80% (n = 30) (data not shown). It was then decided to abandon this artificial line of evaluation and to continue the validation with naturally infected fox forelegs before and after freezing, since the latter was to be used for the prevalence study in a low-endemic area.

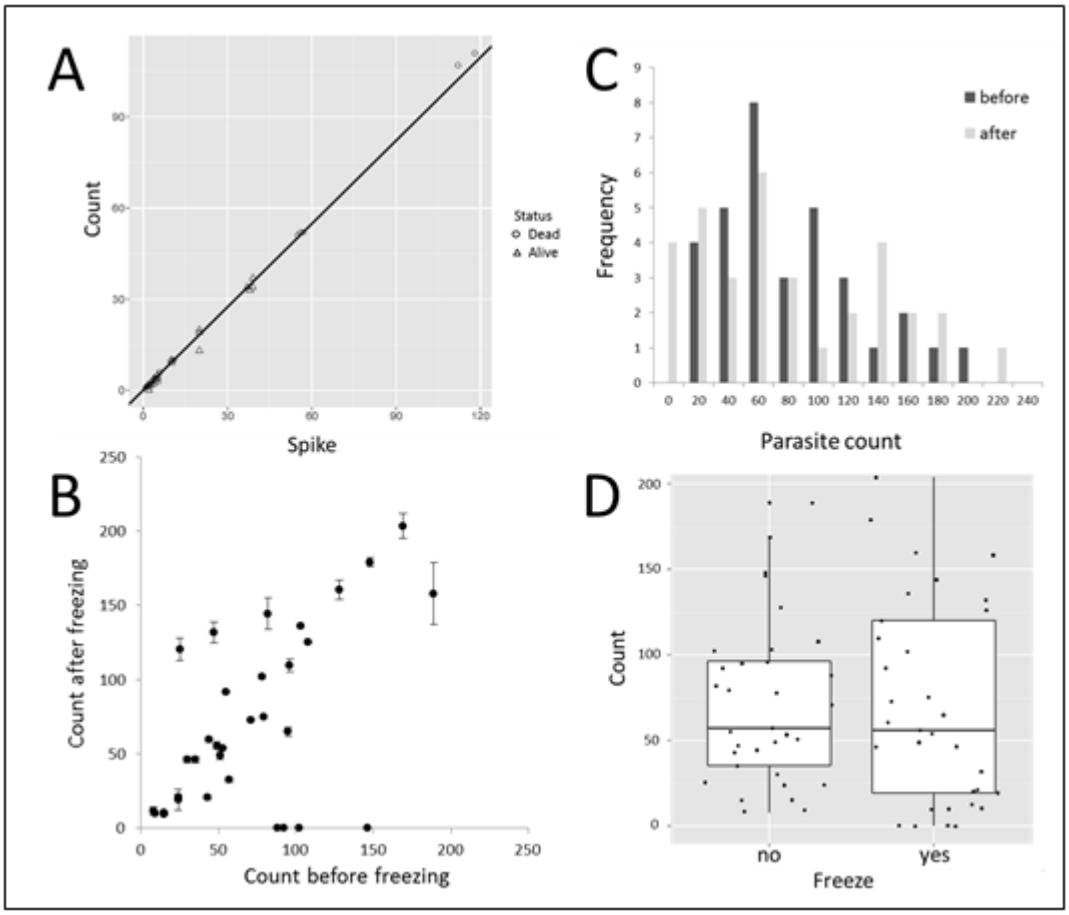

Figure 2 - Recovery of dead or live *Trichinella* larvae

A - Fourty-one data points of two combined experiments using the SSM are shown: single to fourfold spikes and counts of dead larva (20 samples, RIVM), and triplicate spikes and counts of live ML (21 samples, BIOR). Identical data points from the same experiment appear as one single data point in the graph.

B - *Trichinella* larvae were isolated using the EU-RM for live larvae (before freezing) and by the SSM for dead larvae (after freezing). Individual data points represent average values of duplicate counts by two researchers; error bars represent counts range. One identified outlier is omitted here.

C - Parasite counts mentioned under A display a negative binomial distribution.

D - Parasite counts before freezing (freeze no) and after freezing (freeze yes) overlap and median values before (57) and after (56) freezing were comparable. Top and bottom of the boxes represent 25th and 75th percentiles respectively.

Validation of sequential sieving method

Given the poor performance of EU-RM to detect dead *Trichinella* ML in meat digest, and the fact that about 6% of live ML actively pass the 63 µm sieve with SSM, it was decided to compare the most efficient method to detect live *Trichinella* ML in non-frozen meat (EU-RM) with the best method to detect dead *Trichinella* ML in frozen meat (SSM). In most cases, parasite counts in 35 Latvian fox forelegs before and after freezing were comparable (Figure 2B); in one occasion 575 *T. britovi* ML were found after freezing, against 150 prior to freezing (data not shown). This count was identified as a significant outlier in Grubb's test and therefore excluded from further analysis (G = 4.5713, U = 0.3476, $p = 1.3 \cdot 10^{-7}$). In four samples, no ML were found after freezing against 88-146 ML before freezing, which might be related to the highly uneven distribution of *Trichinella* in host muscle tissue and the dispersed count data. Indeed, parasite counts showed a skewed frequency distribution consistent with a negative binomial distribution (Figure 2C). This was confirmed by testing these data for overdispersion (Z = 6.5193, $p = 3.5 \cdot 10^{-11}$) (R-Team, 2008). Median parasite counts of the fox legs before and after freezing were highly similar with 57 and 56 ML respectively (Figure 2D). Variances were not significantly different (K-squared = 1.6677, df = 1, p = 0.1966, non-parametric Bartlett test of homogeneity of variances) and GLM analysis of parasite counts with the variable 'freeze' as factor revealed no significant difference (Z = -0.068, p = 0.946).

Study in a low-endemic area in the Netherlands

One fox out of 369 tested positive for *Trichinella*, with one larva (Figure 3) found in a pool of six foxes. Analysis of the individual foxes that were included in the positive pool did not lead to further findings. Assuming constant prevalence over the study period, we may combine all study years, to arrive at a prevalence of 0.27% (95% CI 0.065-1.5%). Prevalence calculated only from the 287 foxes collected from November 2010 – April 2011 reached 0.35%. In contrast, analysis of 276 foxes from a previous study at the eastern border region of the Netherlands (the same region as in this present study), collected from December 1997 - March 1998 (van der Giessen et al., 1998), revealed a significantly higher *T. britovi* prevalence of 3.9% (p = 0.0006, Fisher's Exact Test) at a density of 0.04 - 0.71 LPG (van der Giessen et al., 1998). Also in the period 1969 - 1971, a significantly higher prevalence (2.8%, n = 106) compared to this present study, was found in foxes from the same border region by digestion and subsequent sieving through sterile gauze (Sluiters et al., 1972), (p = 0.036, Fisher's Exact Test).

Molecular characterization of *Trichinella* ML

Multiplex PCR

Multiplex PCR on five isolated ML each of 30 individual Latvian foxes at BIOR (Riga, Latvia) showed that 28 animals were infected with *T. britovi* and two with *T. nativa*. It is not possible however, to detect simultaneous *T. britovi* and *T. nativa* infections by multiplex

Table 2 - Species identification of Trichinella larvae

#	animal	Multiplex PCR before freezing	single larva 5S PCR before freezing				single larva 5S PCR after freezing		
1	67038	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	NP	NP	NP
2	70414	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	NP	NP	NS
3	72119	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	NP	NP	NP
4	72407	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	NP	NP	NP
5	74391	*T. britovi*	*T. britovi*	*T. britovi*	NP	NP	NP	NS	NP
6	75633	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	NP	NP	NP
7	75068	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	NP	*T. britovi*	*T. britovi*
8	74497	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	NP	NP	NP
9	75475	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	NS	NS
10	75748	*T. nativa*	*T. nativa*	*T. nativa*	*T. nativa*	ND	NP	NP	NP
11	75630	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	ND	NP	*T. britovi*	NP
12	75638	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	ND	NP	NP	NP
13	75932	*T. britovi*	NP	*T. britovi*	*T. britovi*	ND	NP	NP	NP
14	75933	*T. britovi*	NP	*T. britovi*	*T. britovi*	ND	NP	NP	NP
15	75996	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	ND	*T. britovi*	*T. britovi*	NS
16	76148	*T. nativa*	*T. nativa*	*T. nativa*	*T. nativa*	ND	NP	*T. nativa*	*T. nativa*
17	76575	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	ND	NP	NP	NP
18	76580	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	ND	NP	NP	NP
19	76643	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	ND	NP	NP	NP
20	76644	*T. britovi*	*T. britovi*	NP	NP	ND	NP	NP	NP
21	76806	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	ND	NP	NP	NP
22	77876	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	ND	NP	NP	NP
23	77885	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	ND	NP	NP	NP
24	77958	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	ND	NP	NP	NP
25	78187	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	ND	ND	ND	ND
26	71102	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	ND	ND	ND	ND
27	71127	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	ND	ND	ND	ND
28	71128	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	ND	ND	ND	ND
29	74449	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	ND	ND	ND	ND
30	74956	*T. britovi*	*T. britovi*	*T. britovi*	*T. britovi*	ND	ND	ND	ND
31	wild boar	ND	*T. britovi*	*T. britovi*	*T. britovi*	ND	ND	ND	ND

Legend to Table 2.
Species identification was performed on pools of 5 larvae (multiplex PCR) and individual *Trichinella* larvae (single larva PCR). PCR on individual non-frozen larvae resulted in product for 93 out of 99 larvae (93.9%). PCR on 72 individual frozen larvae yielded PCR product for only 12 larvae, of which 8 resulted in sequence product. NP: no PCR product was formed. NS: PCR product yielded no sequence results due to poor quality of DNA. ND: not done.

PCR, since banding patterns on gel do overlap (one single band of 127 base pairs (bp) for *T. nativa* and 2 bands of 253 and 127 bp respectively for *T. britovi*).

Single larva PCR

Single larva PCR directed at 5S rDNA on three to four individual non-frozen ML per Latvian fox performed at RIVM (Bilthoven, the Netherlands), confirmed the results of multiplex PCR performed at BIOR, without any mixed *T. britovi* and *T. nativa* infection found. Of in total 96 tested non-frozen *T. britovi* ML, 90 (93.8%) were successfully sequenced, whereas only 6 out of 72 (8.3%) frozen *T. britovi* ML yielded sequences that allowed species determination (Table 2). For the more freeze-resistant *T. nativa*, six out of six non-frozen and two out of six frozen ML were successfully sequenced. The detection limit of the 5S rDNA PCR was 2.5 pg (data not shown).
The single microscopically identified *Trichinella* sp. larva that was recovered from 369 frozen lower forelegs of Dutch foxes appeared severely damaged (Figure 3) and did not result in PCR product after 5S PCR and therefore, no sequence was available for species determination of this isolate.

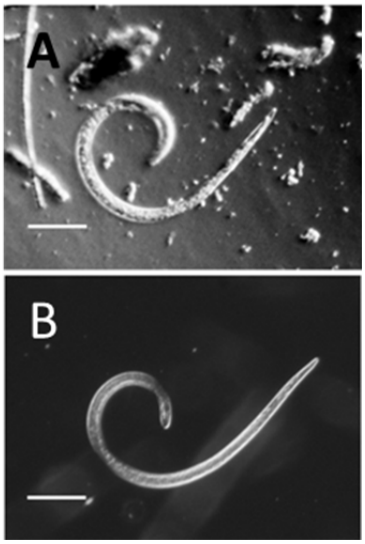

Figure 3 - Single *Trichinella* larva isolated from Dutch red fox
A - One larva was isolated from a fox carcass that had been frozen at -80°C for one week. Note the retracted granular inner structure of the larva. No PCR product could be generated from this specimen.
B - Dead (unfrozen) comma shaped *T. spiralis* larva.
Original magnification 46x, Olympus BH-2 microscope, maximum contrast settings), bars represent 100 µm.

Discussion

A method using sequential sieving (SSM) for the detection of dead *Trichinella* ML from frozen red fox foreleg muscle was validated and was used to analyse trends in time of *Trichinella* in a Dutch red fox population. The SSM is a fast method, since two sedimentation steps of minimally 30 minutes primary sedimentation plus 10 minutes secondary sedimentation (when using the EU-RM) were eliminated and were replaced by a 3-5 minute sieving step in the SSM.

Dead *Trichinella* ML exhibit a lower sedimentation speed than live ML (Dyer and Evje, 1971; Gamble, 1999; Rossi and Pozio, 2008) leading to only 60% recovery of dead *Trichinella* ML from muscle digest using EU-RM, compared to 92% when using SSM, as is shown in this present paper. In comparison, larval counts of frozen fox foreleg muscle obtained with SSM did not differ significantly from larval counts of non-frozen fox foreleg muscle obtained with EU-RM, showing that the SSM was effective to detect dead *Trichinella* larvae. Finding or preparing suitable samples for this type of comparison is a challenge. Henriksen (Henriksen, 1978) used minced and thoroughly mixed experimentally infected rabbit meat to evaluate the effect of freezing on recoverability of *T. spiralis* ML using disposable sieves with mesh size 350 and 20 µm to retain dead larvae. Parasite counts ranged from 82 to 124 ML in that study, irrespective of temperature treatment, despite thorough mixing. In our validation experiment, we found four negative counts after freezing of samples that contained 82-146 larvae when tested before freezing. A plausible biological explanation could be that due to uneven distribution of *Trichinella* larvae in the muscle tissue, these could be missed by chance at second sampling of the same foreleg, near to the primary sampling site. This could also explain the same effect in the other direction, where the post-freezing count value of one sample was 383% of the pre-freezing count. The statistical analysis on the parasite counts in this present study confirmed that parasites follow a contagious distribution in tissues, necessitating GLM methods to accommodate such highly variable counts.

Detection of live and dead *Trichinella* larvae using sequential sieving showed an average sensitivity of 91% (n = 451). Spiked samples with live naked or encapsulated *T. spiralis* ML provide standardized, uniform and quantifiable samples to evaluate test sensitivity of the EU-RM in routine laboratories. This type of samples are generally used by all National Reference Laboratories for *Trichinella* in Europe and elsewhere, with a quantitative sensitivity of 84% (n = 2130, naked larvae) (Riehn et al., 2013) and 81% - 88% (n = 174 - 265, encapsulated larvae) (Vallée et al., 2007) under controlled circumstances. This method however, seems less suitable to validate the SSM presented in this paper, since the test sensitivity dropped from almost 93% (validation of mesh size) to 48% (SSM, n = 60) after freezing of pork samples, that had been spiked with live *T. spiralis* ML, for two weeks (at -80 °C). Test sensitivity of unfrozen spiked control samples that were stored at 4 °C was 80% (n = 30). This low recovery after freezing was confirmed by a study of Nga (Nga, 2008), who analysed pork samples that were spiked with live *T. spiralis* ML (the same strain as was used in this present study) and were subsequently frozen at -20 °C for at least three weeks. Using the EU-RM, the test sensitivity of *Trichinella* detection was

56% (n = 225) after freezing in that study, whereas the test sensitivity was 91% (n = 225) for control samples that had been stored at 4 °C (Nga, 2008). Dead ML were found only occasionally, indicating destruction of *T. spiralis* ML during freezing. In an earlier study, Jackson (Jackson, 1977), demonstrated even 78% loss of *T. spiralis* larvae (compared to non-frozen samples) after freezing at -18 °C. Also in that study, dead larvae were found occasionally.

The use of free larvae without a nurse cell both in the present study and in that of Nga (Nga, 2008) alone, could not explain the large drop in larval recovery after freezing, since Randazzo *et al.* (Randazzo et al., 2011) found no protective effect of the nurse cell capsule against low temperature treatment. An explanation for the lower results with *T. spiralis* spiked frozen samples, could be difference in freeze tolerance between *T. spiralis* and *T. britovi* muscle larvae. Lacour *et al.* (Lacour et al., 2013) indeed found a *T. spiralis* ML inactivation half time of 25 hours at -21°C, whereas 35 hours at -21°C were needed to inactivate half of *T. britovi* ML. However, after one week at -18 to -30 °C, both *T. spiralis* and *T. britovi* that were recovered from either experimentally infected wild boar, rat or mouse muscle tissue, were unable to infect mice (Blaga et al.; Lacour et al., 2013; Malakauskas and Kapel, 2003b; Pozio et al., 1994a). In naturally infected carnivore muscles, the survival time of *T. britovi* at -15 to -20 °C is considerably longer, with 3-6 months, but this trait is lost with the transfer of the parasite to experimental mice (Pozio et al., 1994a). This effect might also have induced the dramatic decline in *T. spiralis* recovery after freezing in our spike experiment and that of Nga (Nga, 2008). The *T. spiralis* strain that Jackson used for his freezing experiment mentioned above, was maintained for almost 40 years (Jackson, 1977). More importantly, these observations underscore our preference for naturally infected fox legs to validate the SSM.

In summary, we validated a fast and effective method to detect dead larvae in meat samples of wildlife. Using this method, we analysed 369 Dutch foxes, of which only one pool of six foxes was positive for *Trichinella.* In this pool, one single larva was isolated and re-tested samples of individual foxes belonging to this pool were all negative, showing a very low infection level.

The *Trichinella* prevalence found in this present study was ten times lower than that described in 1972 by Sluiters *et al.* (Sluiters et al., 1972) and in 1998 by Van der Giessen *et al.* (van der Giessen et al., 1998). Detailed literature concerning historical data regarding *Trichinella* prevalence in red fox from adjacent areas is scarse. However, in the bordering north-western part of Germany (state Hessen), the prevalence of Trichinella in red foxes in the period 1980 - 1983 was 3% (trichinoscopy, six positive, n = 198), whereas in the preceding (1979 - 1980) and following period (1985 - 1987) no positive foxes were found there using artificial digestion (n = 410 and 333 respectively) (Wagner et al., 1988). In Nordrhein-Westfalen, situated in-between Hessen and the Netherlands, *Trichinella* was reported in badger (*Meles meles*, 1985) and in wild boar (1988), however no prevalences were given (Wagner et al., 1988). During the hunting season of 2012, in the eastern part of Belgium (Flanders), one *Trichinella* sp. larva was found in a pool of 20 foxes and also in this occasion, it was not possible to identify an individual positive fox (Claes, 2013),

whereas Geerts *et al.* (Geerts et al., 1995) were not able to demonstrate *Trichinella* in 116 Belgian red fox in 1993.

The decline in *Trichinella* (*britovi*) prevalence in the Netherlands over the past 15 years fits the prevalence patterns of surrounding countries and might be driven by changing feeding habits of the opportunistic red fox in an increasingly densely populated area as the Netherlands. However, not much is known about the natural prevalence fluctuation or infection dynamics of *T. britovi* in red fox. In Slovakia, in contrast to the situation in the Netherlands, the prevalence of *Trichinella* spp. in red fox increased fourfold during the period 2000 - 2007 (Hurnikova and Dubinsky, 2009).

Efforts to identify the species of the single larva found in Dutch foxes by PCR failed, probably due to severe freezing damage, which was clearly visible microscopically. Using validation samples from naturally infected Latvian foxes, we were able to determine a success rate of only 8.3% (n = 72) for molecular speciation of frozen *T. britovi* ML by 5S PCR, against 94% (n = 96) for live larvae prior to freezing. The purpose of testing frozen larvae in our setting was to determine the probability of obtaining positive identification using the 5S PCR on individual larvae that had been submitted to freezing at -80°C for at least one week, since this information was not available in literature up to date.

Several studies report species identification of field samples that were frozen at -20 °C, using single larva multiplex PCR (Beck et al., 2009; Gajadhar and Forbes, 2010; Malakauskas et al., 2007; Reichard et al., 2011; Reichard et al., 2008). None of these studies however, stated the number of single larvae tested per host animal, or the success rate. One study by Pozio *et al*. (Pozio et al., 2001a) on wildlife samples frozen at -20 and -80 °C, used 5 to 10-fold single larva multiplex PCRs to identify the *Trichinella* species, but did not mention how many of these larvae actually were identified. The use of multiple attempts in that study implicates that it was at least anticipated to have a low success rate. Moreover, in a study in coyotes with very low *Trichinella* intensity (0.05-0.6 LPG) (Pozio et al., 2001), *Trichinella* species identification was possible using multiplex PCR in 7 out of 9 animals after freezing of the samples at -20 °C.

The 5S PCR method displayed a test sensitivity of 2.5 pg larval DNA in our laboratory. This level is in range with a sensitivity of 1 pg DNA in a conventional PCR targeted at mitochondrial large subunit RNA of *T. spiralis* as demonstrated by Lin et al. (Lin et al., 2013). Other methods like Q-PCR and multiplex PCR may be more sensitive than the 5S PCR, to identify sheared and otherwise damaged larval DNA after freezing since these PCR methods usually target much smaller DNA fragments. To increase species identification sensitivity, a combination of methods may be considered. Molecular identification of individual *Trichinella* larvae revealed two species in red fox from Latvia: *T. britovi* and *T. nativa*, without any mixed infection in 30 foxes. Malakauskas *et al.* (Malakauskas et al., 2007) demonstrated *Trichinella* spp. prevalence of 29% in foxes in Latvia. In that publication, individual larvae were identified with PCR according to Pozio *et al*. (Pozio et al., 2001a), which showed a distribution of 78% *T. britovi,* 8.5% *T. nativa* and 9.3% mixed infection of the two species in 129 Latvian foxes. Although our sample size of Latvian foxes is much lower and primarily aimed at the validation of our method, we found a

comparable distribution of *T. britovi* and *T. nativa*. The number of isolated *Trichinella* ML from Latvian foxes in this present study might be too low to demonstrate mixed infections.

In conclusion, this study presents a fast and effective sequential sieving method for the detection of dead *Trichinella* larvae in frozen meat. Using this method, we showed that in contrast with a study in the same area fifteen years ago using a comparable method, *Trichinella* prevalence in a Dutch red fox population was significantly lower. Moreover, this study demonstrates that the efficacy of 5S PCR for identification of *Trichinella britovi* single larvae from meat that had been deep-frozen is not more than 8.3%. This is the first time that the effect of deep freezing on *Trichinella* species identification is quantified. To increase species identification sensitivity and at the same time generate DNA sequence information for molecular epidemiology, a combination of methods may be considered.

Competing interests

The authors declare that they do not have competing interests.

Authors' contributions

FF wrote the study design, generated and analyzed parasitological data and wrote the manuscript, GD coordinated the collection of Latvia foxes, generated parasitological data and contributed to the manuscript, ZE generated parasitological data, AH advised with statistical analysis of the results AS, helped with the statistical analysis of the results JvdG wrote the project proposal, coordinated the study and contributed to the manuscript. All authors read and approved the final manuscript.

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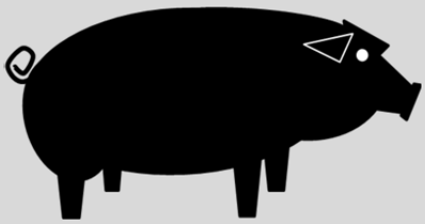
Chapter 4



Quality control of *Trichinella* testing at the slaughterhouse laboratory: evaluation of the use of a 400-um mesh size sieve in the magnetic stirrer method.

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The performance of a 400-µm mesh size sieve ($sieve_{400}$), compared to a sieve with mesh size 180 µm ($sieve_{180}$) is currently unknown. Using spiked pork samples (range 0 - 10 *Trichinella* muscle larvae), this was evaluated using the artificial digestion method to control *Trichinella* in meat producing animals.
We showed that the use of a $sieve_{400}$ results in 12% lower larval counts, 147% more debris and 28% longer counting time, compared to the use of a $sieve_{180}$. On the other hand, no false negative results were obtained, but prolonged counting times may have an impact on performance in a high-throughput environment such as a slaughterhouse laboratory. Based on our results, the $sieve_{180}$ remains the sieve of choice for *Trichinella* meat control at the slaughterhouse labs, according to the EU reference method, laid down in European regulation 2075/2005. Furthermore, the present study may provide a useful contribution to discuss further harmonisation of meat inspection requirements between countries.

Keywords: *Trichinella*, magnetic stirrer method, Meat control; Pork; *Trichinella*

Introduction

Worldwide, around 11 million people are exposed to *Trichinella spiralis*, based on serological evidence (Dupouy-Camet, 2000). However, a far lower number of 65,818 confirmed human cases and 42 deaths have been recorded from outbreak data during the period 1986 – 2009 (Murrell and Pozio, 2011). The majority of outbreaks was reported from Europe (87%) of which 50% was from Romania (Murrell and Pozio, 2011).

Four out of twelve recognised *Trichinella* species circulate in Europe: *Trichinella spiralis, Trichinella nativa, Trichinella britovi* and *Trichinella pseudospiralis* (Pozio, 2005; Pozio et al., 2009b). With respect to food safety, the most important is *T. spiralis*, a cosmopolitan species for which the main reservoirs are domestic swine and wild boar, although also wild carnivores play a role as reservoir (Pozio and Murrell, 2006). The other three *Trichinella* species mainly infect wild boar and other wildlife.

Pork , horse- and wild boar meat that is intended for human consumption is subjected to individual carcass control, although there are exemptions, such as freedom of *Trichinella* testing at slaughter for domestic pigs from controlled housing. The magnetic stirrer method is the recommended test for meat control. This method has been validated for the detection of live *Trichinella* muscle larvae (ML) and critical control points for this method are well described (Forbes and Gajadhar, 1999; Gamble, 1999; Gamble et al., 2000; Rossi and Pozio, 2008). In the European Union, the magnetic stirrer method is mandatory according to European regulation EC 2075/2005 (EU reference method, EU-RM) (European-Commission, 2005). Throughout Europe, national reference laboratories (NRLs) maintain the quality control regarding proper use of the EU-RM in slaughterhouse laboratories, where the analysis on *Trichinella* muscle larvae (ML) is performed routinely.

Despite the recently introduced European legislation to relieve the mandatory individual carcass control for *Trichinella* spp. for pigs that were reared under controlled housing (European-Commission, 2014), mandatory meat control for *Trichinella* will remain in place for pigs not kept under controlled housing conditions, and for horses and wildlife. Moreover, the meat industry may continue testing individual pig carcasses for international trade to third countries.

The EU-RM uses artificial digestion of 100 g meat, consisting of 100 pooled 1 g diaphragm samples of individual pig carcasses. After digestion, two consecutive sedimentation steps are used to isolate *Trichinella* ML that are counted microscopically at 10-20× magnification. Prior to the first sedimentation step, the meat digest is poured through a stainless steel sieve with mesh size 180 µm, to retain crude debris and undigested small meat particles.

The EU-RM is obligatory for the control of meat for trade within the European Union. For export to countries outside the EU (third countries), meat producers may have to comply with regulations according to the food safety authorities of those countries, such as the mandatory use of a 400-µm mesh size sieve ($sieve_{400}$) following artificial digestion, instead of the aforementioned 180-µm mesh size sieve ($sieve_{180}$). To date, the influence of the use of a $sieve_{400}$ on *Trichinella* detection sensitivity in meat remains undocumented.

The aim of the present study was to evaluate the influence of a sieve$_{400}$ on the detection sensitivity of *Trichinella* ML in meat, compared to a sieve$_{180}$. We hypothesised that the use of a sieve$_{400}$ to retain undigested meat particles during the isolation of *Trichinella* ML could result in an increased amount of debris, which may hamper the visibility of *Trichinella* larvae under the microscope and thus may have a negative effect on the test sensitivity. This hypothesis was tested in a comparative study at the Dutch NRL and three routine slaughterhouse laboratories (SLs) in the Netherlands. Dutch SLs perform the EU-RM on a routine basis to test 12,000 – 20,000 pig carcasses daily (120 - 200 pooled samples of 100 animals).

Materials and methods

Trichinella muscle larvae

Trichinella spiralis (ISS 14) larvae for use in spike experiments were obtained from experimentally infected mice by the EU-RM. Isolated larvae were rinsed several times in tap water and stored at 4 °C for further use within two weeks post isolation. The Ethical Committee of the Dutch National Institute for Public Health and the Environment (RIVM) approved this work (DEC permit number 201200223).

Experimental setup

Three rounds of evaluation were conducted. Firstly, an experiment comparing a sieve180 with a sieve400 was conducted at the NRL (Experiment A). Registered parameters were: meat weight, residual meat weight after digestion, volume of collected meat digest after the first sedimentation, *Trichinella* ML count, counting time and amount of debris in the final suspension that was evaluated microscopically. Secondly, the practically realised lower test limit was explored in a comparison of sieve$_{180}$ and sieve$_{400}$, conducted in two laboratories: NRL and a selected SL (SL1), respectively (Experiment B). Due to limited analytic possibilities in SL1, only larva count values were registered for this experiment. Thirdly, both sieves were compared under field conditions, for which pork samples spiked with 3 – 8 Trichinella ML, were analysed in three SL (SL1 – SL3) and in the NRL (Experiment C).

Preparation of spiked meat samples

For Experiment A, 32 samples of 100.02 ± 0.06 gram minced pork from fat free ham were prepared and each sample was spiked with either 4 or 10 *Trichinella* ML, 16 samples for each spike level. For Experiment B, a series of nine 100 g pork samples were spiked with 1, 2, 3 or 4 *Trichinella* ML. A 10th non-spiked 100 g pork sample served as a negative control. Another series of eight 100 g pork samples spiked with 1, 2, and 3 *Trichinella* ML was prepared for the evaluation of the lower test limit at the NRL. For Experiment C, a series of 32 pork samples (100 g each) was spiked with 3 – 8 Trichinella ML. Spike levels were assigned at random to sample series and the selection of sample series for each of

the sieves and labs was randomised. Samples without *Trichinella* ML served as negative control.

To prepare the spiked meat samples, specific numbers of *Trichinella* ML in 10 µl of tap water were carefully transferred to a ditch in the centre of 100 g meatballs using a micropipette. Before and after each transfer of larvae, the pipet tip was checked to ensure that all *Trichinella* ML were transferred to the meatball, and the meatball was closed over the delivered *Trichinella* ML.

Experiment A: comparison of sieves at two spike levels

Stainless steel sieves of mesh size 180 µm and 400 µm (Endecotts Limited, London, UK) were evaluated at the NRL. To evaluate sieve performances, *Trichinella* ML were isolated by eight duplicate digestions at two spike levels (4 larvae and 10 larvae) using a $sieve_{180}$ and eight duplicate digestions using a $sieve_{400}$. Each sample was evaluated microscopically in duplicate by one technician. For each sample, the weight of debris was determined in the microscopical preparation from 16 spiked digestions using $sieve_{180}$ and another series of 16 spiked digestions using $sieve_{400}$. At the end of the isolation procedure, after two sedimentation steps, the meat digest above the bottom 10 ml in a 50 ml glass cylinder was removed and the remaining 10 ml suspension containing debris and *Trichinella* ML was transferred to a Petri dish for microscopical evaluation. Finally, the cylinder was rinsed with tap water and the rinse fluid was added to the Petri dish as well. The amount of debris that is co-isolated with *Trichinella* ML after two consecutive sedimentation steps was determined by filtration of the Petri dish content through a 20-µm mesh size nylon filter. Each filter was weighed using a validated analytical balance with four-digit readout, with debris (after air-drying) and without debris to account for the weight of the clean cellulose filter. The recorded debris weight was corrected for volume of meat digest.

Experiment B: determination of lower test limit

To determine the lower test limit of the $sieve_{400}$, it was decided to test the $sieve_{400}$ in a slaughterhouse lab that showed a long-term performance on the artificial digestion method comparable to the NRL. During the period 2008-2014, the NRL showed a long-term test sensitivity of 87.5% recovered *Trichinella* ML (spike levels 1-19 *Trichinella* ML) upon participation in proficiency tests (PTs) that were organised by the EU reference laboratory (Istituto Superiore di Sanità, Rome, Italy). Over the same period, SL1 exhibited a test sensitivity of 84.0% (spike levels 3-15 *Trichinella* ML) in the PTs organised by the Dutch NRL. The overall test sensitivity realised in SL2 and SL3 were slightly lower, between 70 and 82%.

At SL1, ten samples containing 0, 1, 2, 3, or 4 *Trichinella* ML were processed using the $sieve_{400}$ and analysed microscopically by three technicians, who counted each sample independently. At the NRL, each sample was counted in duplicate by one technician using the $sieve_{180}$. This set up was chosen in view of the fact that any SL can handle only a

limited amount of extra samples during an already fully booked working day, testing 120-200 pooled meat samples daily.

Experiment C: sieve evaluation under field conditions

Two series of four samples containing 0 or 3 – 8 *Trichinella* ML were processed in three SL (SL1 – SL3), using the $sieve_{180}$ or the $sieve_{400}$ for either of two series per SL, and analysed by three technicians per SL, who counted each sample independently. At the NRL, each sample was counted by two technicians independently, using the $sieve_{180}$. Average counting time was estimated from the amount of time needed to evaluate all spiked samples.

Statistical analysis

Statistical analyses were performed using the software package 'R', version 3.0.1 (R-Team, 2008). A linear model approach was used to describe the amount of debris as function of sieve mesh size: $debris \sim Normal(\mu, \sigma),\ \mu = intercept+sieve$. A second linear model was used to describe count time as function of sieve mesh size: $count.time \sim Normal(\mu, \sigma),\ \mu = intercept+sieve$. These models were evaluated by analysis of variance (ANOVA) and subsequent pairwise t-test to identify significant variables. Finally, an F-test was used to evaluate the significance of differing variances of 'debris' with factor 'sieve'. A binomial generalised linear model was chosen to analyse recovery data, since the outcome is a ratio of counts, constrained between zero and one, and we need to take the denominator into account (i.e. 2 out of 4 larvae gives less information than 4 out of 8). The model is defined by $count \sim Binomial(p, spike),\ logit(p) = intercept + (sieve + debris + count.time)^2$. Here 'count' is the larval count, 'spike' the spike level, 'sieve' a factor with levels '$sieve_{400}$' and '$sieve_{180}$', 'debris' the amount of debris in mg, and 'count.time' the counting time in seconds. The square sign indicates that the interactions between all variables are included. Subsequently, the optimal model fit was evaluated by the backward and forward step function in 'R' to select the model with the lowest AIC-value (Akaike's Information Criterion).

Finally, all count data combined were evaluated using a generalised linear model approach to fit the combination of count data and spike level as function of sieve size and technician or lab. In formula: $count \sim Binomial(p, spike),\ logit(p) = intercept + sieve + lab$.

Results and Discussion

Spiked meat samples were digested efficiently

After digestion in Experiment A, the amount of residual meat on the sieves was on average 1.19 ± 0.73 g and did not differ significantly between groups (p = 0,062). In Experiments B and C, amounts of residual meat were estimated 1% or less.

Use of a sieve 400 caused more debris and increased counting time

The volume of collected meat digest after the first sedimentation in experiment A was on average 42.7 ± 1.9 ml (range 40.0 - 48.0 ml). The amount of debris was corrected for volume differences using the formula *corrected debris weight* = *measured weight* × (42.7 / *measured volume*). The amount of debris was on average 1.9 ± 0.7 mg for samples that were processed using the sieve$_{180}$, whereas this was 4.7 ± 2.1 mg for the sieve$_{400}$ (Figure 1).

The time needed for microscopical examination of a whole Petri dish differed between sieves. On average 243 ± 28 sec were needed for samples processed with sieve$_{180}$ against 312 ± 76 sec for samples processed with sieve$_{400}$ (Figure 1). The amount of debris correlated significantly with sieve mesh size ($p = 7.6 \times 10^{-10}$), as did the counting time needed to evaluate a whole Petri dish ($p = 7.7 \times 10^{-6}$).

The best fitting model (displaying the lowest AIC-value) to describe the validation results was $\text{logit}(p) = \text{intercept} + \text{sieve} + \text{debris} + (\text{sieve} + \text{debris})^2$. Categorical variable 'sieve' contributes significantly (p = 0.047, AIC = 139.66) to this model. Moreover, for spike level 10, sieve$_{400}$ did correlate significantly with wider count range (F = 0.2081, df 15, p = 0.00428, F-test to compare two variances). For spike level 4 *Trichinella* ML per sample, the count range was too small to show significance.

The number of recovered *Trichinella* ML is significantly lower using a sieve$_{400}$

No false negative scores were obtained using either sieve$_{180}$ or sieve$_{400}$ sieves at spike levels of 1-4 *Trichinella* ML (Experiment B), but counting times at the SL increased from estimated 8 minutes to 10 minutes respectively per spiked sample. These are unrealistically long counting times, compared to day-to-day testing. A lower SL proficiency level and daily pressures at the slaughterhouse labs, could lead to further decrease of test sensitivity and thus increase the risk of missing positive carcasses when using a sieve$_{400}$, especially at *Trichinella* ML infection levels below 4 larvae / gram).

For all spike levels except 1 *Trichinella* ML, median counts were generally higher with sieve$_{180}$ than with sieve$_{400}$ (Figure 2). Over all (Experiment A – C), significantly more Trichinella ML were recovered using the sieve$_{180}$ (400 out of 444 ML, 90.1%, 96 data points), compared to the sieve$_{400}$ (344 out of 439 ML, 78.4%, 102 data points) ($p = 2 \times 10^{-6}$, Fisher's Exact test). The overall larval recovery using sieve$_{180}$ is slightly higher than test sensitivities obtained in previous studies of the French NRL (81% - 88%, n = 174 - 265) (Vallée et al., 2007) and the German NRL (84%, n = 2130) (Riehn et al., 2013). These results were obtained under highly controlled conditions, suggesting that the EU-RM is prone to loss of larvae during the isolation procedure, mounting to 10-15% of spiked larvae (Riehn et al., 2013). The amount of debris further lowers the test sensitivity when using the sieve$_{400}$. A recently published improvement to the EU-RM, using bottom-up microscopic examination (Makrutzki et al., 2014) might circumvent this disturbing influence of increased debris using the sieve$_{400}$, since debris tends to float above ML and

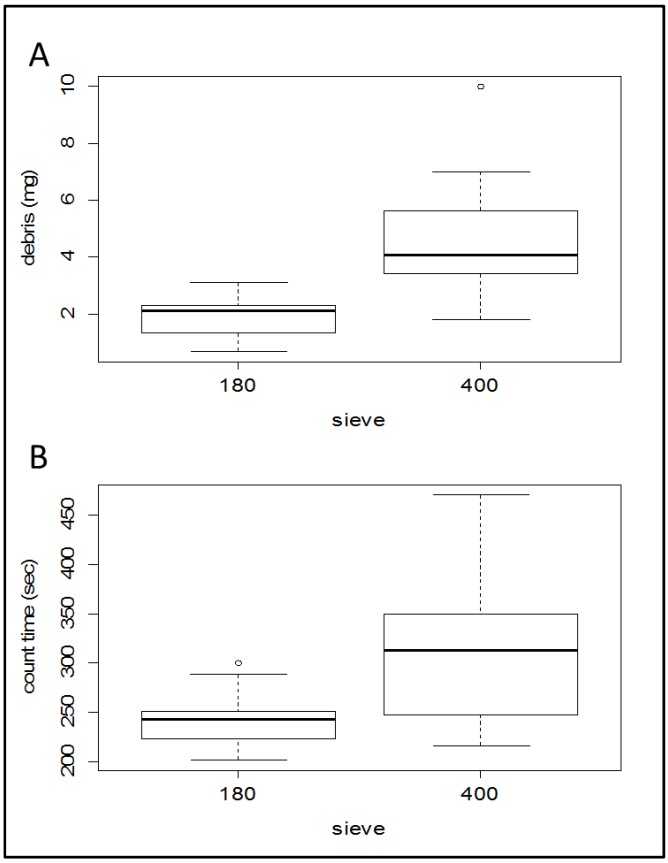

Figure 1. Experiment A: comparison of sieves under expert conditions.
A. A 28% longer counting time correlates significantly with $sieve_{400}$ ($p = 7.7 \times 10^{-6}$).
B. 2.5 times more debris correlates significantly with $sieve_{400}$ ($p = 7.6 \times 10^{-10}$).

thus hides the larvae from being seen by the technician.The best fitting model (displaying the lowest AIC-value, AIC = 326.2) to analyse the count results was $\text{logit}(p) = \text{intecept} + \text{sieve} + \text{lab}$, whereby the factor '$sieve_{400}$' contributed highly significantly ($p = 9.2 \times 10^{-5}$). At four spike levels (2, 4, 5 and 6 *Trichinella* ML), the number of larvae in the sample was overestimated by one technician (overestimation by one larva in all cases). This occurs when curved residual muscle fibres are confused with *Trichinella* ML. These overestimated counts were artificially set to the actual spike level in the meat sample for statistical evaluation. Complete removal of these overestimated counts slightly lowered significance of factor '$sieve_{400}$' ($p = 1.72 \times 10^{-4}$), but the result was still highly significant. According to the model, the probability to find larvae using $sieve_{180}$ is 88.5%, whereas this

is significantly lower using $sieve_{400}$ (78.1%), which fits the actual overall recovery shown above.

The present validation study was conducted with live *Trichinella* ML and a point of concern might be that dead *Trichinella* ML could possibly be retained by a $sieve_{180}$, necessitating the use of a sieve with a larger mesh size. In a previously published study performed at the Dutch NRL (Franssen et al., 2014), live or dead *Trichinella* ML were added to *Trichinella*-free meat digest to evaluate *Trichinella* ML recovery according to either EU 2075/2005 or a newly validated sequential sieving method (SSM). For the latter method, a stack of sieves is used with decreasing mesh size of 300, 63 and 38 µm, for the detection of dead *Trichinella* ML in frozen carcasses (Franssen et al., 2014). In ten replicate tests, on average 9.7 *Trichinella* ML (median 10, range 9 – 10, n = 10) were recovered using a $sieve_{180}$ and 9.3 *Trichinella* ML (median = 9.5, range 8 – 10, n = 10) using a $sieve_{300}$ ($p = 0.223$, t-test), which shows that dead larvae also pass both $sieve_{180}$ and $sieve_{300}$ easily and efficiently. A 400-µm mesh size sieve was not evaluated in that study but it is assumed that this mesh size gives the same results for dead larvae.

According to our results, the 180-µm sieve remains the sieve of choice for the meat inspection at the slaughterhouse labs. With the current study, we provide a useful contribution for decision makers to discuss a further harmonisation of meat inspection requirements between countries.

Acknowledgements

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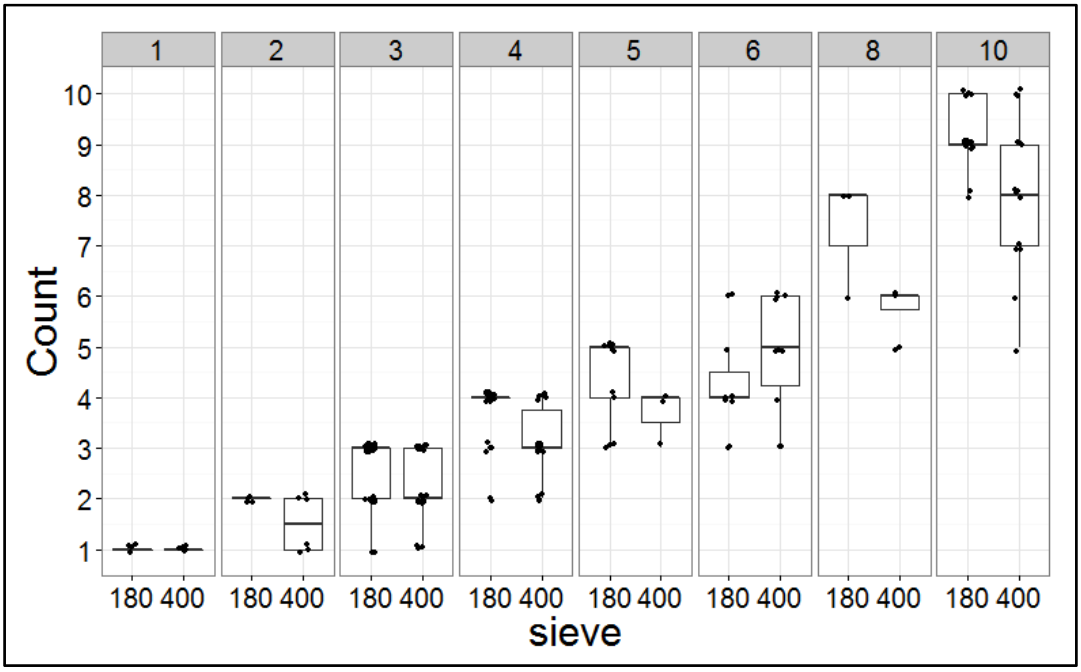

Figure 2. Experiments A – C: realised lower test limit and recoveries of all participating laboratories.

The lower test limit was one larva with both sieves, without time constraints for microscopical examination. Significantly more *Trichinella* ML were recovered with a $sieve_{180}$ (90.1%) than with a $sieve_{400}$ (78.4%, $p = 2 \times 10^{-6}$). Generally, the median count value (horizontal lines in boxplot) was higher with the $sieve_{180}$ and overall, lower *Trichinella* ML recovery correlates significantly with $sieve_{400}$ ($p = 9.2 \times 10^{-5}$). For statistical evaluation, overestimation counts were artificially set to the actual spike level in the meat sample. Analysis without these counts was also highly significant ($p = 1.72 \times 10^{-4}$). Thick horizontal lines in boxplot represent median values and data points are represented 'jittered', to show overlapping data. Spike levels are shown in the grey area above.

Chapter 5

Genetic evidence of interspecies introgression of mitochondrial genomes between *Trichinella spiralis* and *Trichinella britovi* under natural conditions

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Trichinellosis is a zoonotic disease caused by *Trichinella* muscle larvae (ML) through ingestion of raw or undercooked meat. To date, 12 taxa are recognized in this genus, of which four are circulating in Europe (*Trichinella spiralis*, *Trichinella nativa*, *Trichinella britovi* and *Trichinella pseudospiralis*). *T. spiralis* and *T. britovi* circulate in European wildlife and occur simultaneously in the same host species. The possibility of hybrid formation between *T. britovi* and *T. spiralis* has hardly been addressed and so far, results of experimental hybridisation attempts between *T. britovi* and *T. spiralis* are inconclusive. The aim of the present study was to analyse molecular polymorphisms of single *T. spiralis* and *T. britovi* ML from natural infections based on nuclear 5S rDNA intergenic spacer region (5S rDNA-ISR) and mitochondrial cytochrome c oxidase 1 (CO1) gene sequences. Six haplotypes of the 5S rDNA-ISR and 14 of the cytochrome c oxidase 1 CO1 gene were demonstrated in 89 individual *T. britovi* ML from Latvia and Poland. In contrast, only two haplotypes were observed at both 5S rDNA-ISR and CO1 of 57 individual *T. spiralis* ML from Polish wild boar and red foxes. Moreover, this study demonstrates hybridisation in eight individual ML between *T. britovi* and *T. spiralis* under natural conditions in four Polish wild boar and two red foxes, revealed by combining 5S rDNA-ISR and CO1 sequence information of individual *Trichinella* ML. To our knowledge, this is the first report of interspecies hybridisation between *T. spiralis* and *T. britovi* under field conditions.

Keywords: *Trichinella britovi*, *Trichinella spiralis*, haplotypes, natural hybrids, wild boar, red fox, Poland.

Introduction

Nematode worms of the genus *Trichinella* are zoonotic parasites with a high impact on the animal trade and human health (Gottstein et al., 2009). To date, 12 taxa are recognized in this genus, of which four are circulating in Europe (*Trichinella spiralis*, *Trichinella nativa*, *Trichinella britovi* and *Trichinella pseudospiralis*) (Pozio and Zarlenga, 2013). *Trichinella spiralis* is a cosmopolitan species for which the main reservoirs are domestic pig and wild boar and to a lesser extend wild carnivores (Pozio and Murrell, 2006). *T. britovi* predominantly infects canids (e.g. foxes, wolves, raccoon dogs, jackals) and mustelids of the European continent, north-western Africa and south-western Asia, and to a lesser extent domestic pig and wild boar, and humans (Pozio et al., 2009a; Pozio and Zarlenga, 2013). *Trichinella* spp. are widespread in Eastern Europe, with overall prevalence of 0.4% in wild boar (*Sus scrofa*) (EFSA-ECDC, 2014) and 2.7% in red fox (*Vulpes vulpes*) (Chmurzynska et al., 2013) in Poland, and of 2.5% in wild boar and 28.9% in red fox of Latvia (Kirjušina et al., 2015; Malakauskas et al., 2007).

During the period 1995-2014, larvae digested from 82 individual animals were found to be composed of mixtures of *T. britovi* and *T. spiralis*, originating from two Western-European and nine Eastern-European countries and from 9 host species, (http://www.iss.it/site/Trichinella/index.asp). Additionally, several studies documented mixed *T. britovi* and *T. spiralis* (Chmurzynska et al., 2013; Malakauskas et al., 2007; Rodriguez et al., 2008) infections in European wildlife and domestic animals (Liciardi et al., 2009). The genetic diversity within *Trichinella* species have been studied for *T. spiralis* and to a lesser extent for *T. britovi* (La Rosa et al., 2012; Rosenthal et al., 2008; Webb and Rosenthal, 2010), but few researchers have addressed the possibility that hybrids occur between *T. britovi* and *T. spiralis*. One study (Kurdova et al., 2004) describes lack of hybrid formation after experimental cross-infection with *T. britovi* or *T. spiralis* field isolates with *T. spiralis* or *T. britovi* reference larvae obtained from experimentally infected mice. The purpose of that study was to identify the species of *Trichinella* larvae isolated from wildlife, using complementary crossbreeding to corroborate species identification by PCR results, under the apparent assumption that these species do not cross. In contrast, attempts to mate *T. spiralis* from Poland with *T. britovi* from Italy yielded hybrids between female *T. spiralis* and male *T. britovi* , but not in the other direction (Wu et al., 2000).

The aim of the present study was to evaluate molecular polymorphisms of single *T. spiralis* and *T. britovi* muscle larvae (ML) from natural infections based on nuclear 5S rDNA intergenic spacer region (5S rDNA-ISR) and mitochondrial cytochrome c oxidase 1 (CO1) gene sequences. Moreover, we report the occurrence of natural hybrids in field isolates from wild boar and red foxes.

Table 1. Trichinella isolates from Latvia and Poland.

Region of origin (country)	Host	isolate code	# larvae	5S rDNA	CO1	Tb (P)	Ts	Tb/Ts	Ts/Tb	Tb (L)
Lubusz (P)	wild boar	55	4	Ts1	TsA		4			
Lubusz (P)	wild boar	59	2	Ts1	TsA		2			
Kuyavian-Pomeranian (P)	wild boar	61	3	Ts2	TsA		3			
Podlaskie (P)	wild boar	137	1	Tb4	TbA	1				
			1	Tb5	TbE	1				
			2	**Ts1**	**TbA**				**2**	
Lubusz (P)	wild boar	378	1	**Tb1**	**TsA**			**1**		
			1	Ts1	TsA		1			
Kuyavian-Pomeranian (P)	wild boar	143	1	Tb1	TbF	1				
			1	Tb5	TbL	1				
			1	Tb5	TbF	1				
West Pomeranian (P)	wild boar	146	1	Tb1	TbG	1				
			1	Tb6	TbA	1				
			1	Tb6	TbF	1				
Kuyavian-Pomeranian (P)	wild boar	320	2	Ts1	TsA		2			
West Pomeranian (P)	wild boar	322	4	Ts1	TsA		4			
Masovian (P)	wild boar	326	2	Ts1	TsA		2			
West Pomeranian (P)	wild boar	360	4	Ts1	TsA		4			
Kuyavian-Pomeranian (P)	wild boar	425	1	Ts1	TsA		1			
			1	Ts2	TsA		1			
Warmian-Masurian (P)	wild boar	429	1	Tb2	TbB	1				
			1	Tb1	TbI	1				
			1	Tb4	TbI	1				
Greater Poland (P)	wild boar	445	4	Ts2	TsA		4			
Subcarpatian (P)	wild boar	446	2	Tb1	TbM	2				
	wild boar	458	2	Tb4	TbA	2				
			2	Tb5	TbA	2				
Podlaskie (P)	wild boar	474	1	Tb4	TbD	1				
			1	Tb5	TbA	1				
			1	Tb6	TbA	1				
West Pomeranian (P)	wild boar	481	4	Ts1	TsA		4			
Świetokrzyskie (P)	wild boar	485	2	Tb1	TbA	2				
			2	**Ts1**	**TbA**				**2**	
Greater Poland (P)	wild boar	517	1	Tb1	TbA	1				
Warmian-Masurian (P)	wild boar	519	1	Tb1	TbA	1				
			1	Tb1	TbK	1				
West Pomeranian (P)	wild boar	521	1	Ts1	TsA		1			
West Pomeranian (P)	wild boar	539	1	**Tb4**	**TsA**			**1**		
			2	Ts1	TsA		2			
West Pomeranian (P)	wild boar	579	4	Ts1	TsA		4			
West Pomeranian (P)	wild boar	594	4	Ts1	TsA		4			
West Pomeranian (P)	wild boar	597	1	Ts1	TsA		1			
			1	Ts1	TsB		1			
Poland	wild boar	609	1	Ts1	TsA		1			
Masovian (P)	wild boar	610	3	Ts1	TsA		3			
			1	Ts2	TsA		1			

Table 1. Trichinella isolates from Latvia and Poland. (continued)

Region of origin (country)	Host	isolate code	# larvae	5S rDNA	CO1	Tb (P)	Ts	Tb/Ts	Ts/Tb	Tb (L)
Lublin (P)	wild boar	630	3	Tb4	TbA	3				
Opole (P)	wild boar	652	3	Ts1	TsA		3			
Zemgale (L)	wild boar	86149	4	Tb1	TbA					4
			1	Tb1	TbF					1
			1	Tb5	TbN					1
			1	Tb6	TbA					1
Kuyavian-Pomeranian (P)	red fox	FoxA	1	Tb1	TbA	1				
			1	Tb4	TbA	1				
			1	**Ts1**	**TbA**				**1**	
Kuyavian-Pomeranian (P)	red fox	FoxC	3	Tb1	TbA	3				
	red fox	FoxD	1	Ts1	TsA		1			
			3	Ts2	TsA		3			
Pomeranian (P)	red fox	FoxE	1	Tb1	TbC	1				
			1	Tb1	TbF	1				
			1	**Ts1**	**TbA**				**1**	
Pomeranian (P)		FoxF	1	Tb1	TbI	1				
			2	Tb3	TbI	2				
Latgale (L)	red fox	74068	3	Tb1	TbA					3
			1	Tb4	TbA					1
			1	Tb5	TbN					1
			1	Tb6	TbA					1
Kurzeme (L)	red fox	71127	2	Tb1	TbA					2
			1	Tb5	TbA					1
			2	Tb6	TbA					2
Kurzeme (L)	red fox	71128	1	Tb1	TbA					1
			1	Tb1	TbI					1
			1	Tb1	TbN					1
			1	Tb4	TbA					1
Kurzeme (L)	red fox	75475	7	Tb1	TbN					7
Latgale (L)	red fox	75630	1	Tb1	TbA					1
			5	Tb3	TbA					5
			1	Tb4	TbA					1
Vidzeme (L)	red fox	75633	1	Tb1	TbN					1
			1	Tb4	TbA					1
Vidzeme (L)	red fox	75996	4	Tb1	TbA					4
			1	Tb1	TbJ					1
			2	Tb5	TbJ					2
Zemgale (L)	red fox	76643	2	Tb1	TbE					2
			1	Tb4	TbA					1
			1	Tb4	TbE					1
			1	Tb4	TbH					1
			1	Tb6	TbL					1
Totals			154			38	57	2	6	51

Sequence analysis of individual *Trichinella britovi* ML revealed six 5S rDNA-ISR (Tb1-Tb6) haplotypes and 14 CO1 (TbA-TbN) haplotypes, whereas two haplotypes were detected in both 5S rDNA-ISR (Ts1-Ts2) and CO1 (TsA-TsB) of *T spiralis*. Tb (P) *T. britovi* from Poland, Tb (L) *T. britovi* from Latvia.

Materials and methods

Sample collection

Trichinella spp. larvae were collected from 35 wild boar and 6 foxes of Poland and from 1 wild boar and 30 red foxes of Latvia (Franssen et al., 2014), during routine analyses for the detection of *Trichinella* spp. parasites by artificial digestion (European-Commission, 2005). Larvae were stored in 96% ethanol at 4 °C until further use. *Trichinella* T9 (isolate ISS408) reference larvae were obtained from the bio bank of the European Reference Laboratory for Parasites, ISS, Rome, Italy.

Cross breeding experiments

To evaluate the feasibility of hybrid formation, data from an unpublished previously performed experiment from 1996 were used. Muscle larvae of *T. spiralis* (strain ISS003) and *T. britovi* (strain ISS002) were separated into different sexes (Pozio et al., 1999). Subsequently, ten mice (CD1 females of 25 g) were inoculated per os, with pairs of one male and one female muscle larva belonging to different species in both directions (i.e., male of *T. britovi* per female of *T. spiralis* and vice versa) by a stomach tube connected to a 1 ml syringe. Five mice were infected with pairs of single male and female muscle larvae belonging to the same species as a control. Since the enteral niche of laboratory mice does not represent the natural reproductive niche of *T. britovi*, all mice had been immunosuppressed with 4 mg of cyclophosphamide at 0, 4 and 8 days p.i., to improve the probability of producing offspring. Mice were killed 40 days p.i., and the entire skinned and eviscerated carcasses were digested individually and the recovered larvae were counted. To evaluate the fertility of the F1 hybrid muscle larvae produced by these experiments, F1 hybrid larvae were inoculated in bulk *per os* in mice. Mice were processed as reported above. Since these so far unpublished experiments date back to 1998, well before the introduction of the Institute's ethical committee, no approval was required at that time. Moreover, no molecular data concerning these hybrids were available at that time.

DNA isolation and PCR

DNA was extracted from individual ML in one vial each of clearly marked 8-vial PCR strips, using a previously described procedure (Bandi et al., 1993). All PCRs were carried out in 8-vial PCR strips in 50 µl final volume containing 3 µl genomic DNA, 0.5 µl of each forward and reverse primer (50 µM stock) and 25 µl of Qiagen HotstarTaq polymerase master mix (Qiagen NV, Venlo, The Netherlands). The final reaction volume was adjusted to 50 µl with sterile demineralized water. A validated PCR for the identification of Trichinella species directed at the 5S ribosomal DNA (rDNA) intergenic spacer region was performed using forward primer 5'-GCGAATTCTTGGATCGGAGACGGCCTG and reverse primer 5'-GCTCTAGACGAGATGTCGTGCTTTCAACG as described earlier (Liu et al., 1996; Rombout et al., 2001b) resulting in products of approximately 750 base pairs (Rombout et al., 2001a).

Table 2. Overview of haplotypes for *T. britovi* and *T. spiralis*.

A

Haplotype	n	%
***T. britovi* 5S rDNA**		
Tb1	46	51,7%
Tb2	1	1,1%
Tb3	7	7,9%
Tb4	16	18,0%
Tb5	11	12,4%
Tb6	8	9,0%
Total	89	
***T. britovi* CO1**		
TbA	50	56,2%
TbB	1	1,1%
TbC	1	1,1%
TbD	1	1,1%
TbE	4	4,5%
TbF	5	5,6%
TbG	1	1,1%
TbH	1	1,1%
TbI	6	6,7%
TbJ	3	3,4%
TbK	1	1,1%
TbL	2	2,2%
TbM	2	2,2%
TbN	11	12,4%
Total	89	
***T. spiralis* 5S rDNA**		
Ts1	45	78,9%
Ts2	12	21,1%
Total	57	
***T. spiralis* CO1**		
TsA	56	98,2%
TsB	1	1,8%
Total	57	
Total of 146 'pure' larvae		

B

Haplotype	n	%
T. britovi		
Tb1/TbA	23	25,8%
Tb1/TbC	1	1,1%
Tb1/TbE	2	2,2%
Tb1/TbF	3	3,4%
Tb1/TbG	1	1,1%
Tb1/TbI	3	3,4%
Tb1/TbJ	1	1,1%
Tb1/TbK	1	1,1%
Tb1/TbM	2	2,2%
Tb1/TbN	9	10,1%
Tb2/TbB	1	1,1%
Tb3/TbA	5	5,6%
Tb3/TbI	2	2,2%
Tb4/TbA	12	13,5%
Tb4/TbD	1	1,1%
Tb4/TbE	1	1,1%
Tb4/TbH	1	1,1%
Tb4/TbI	1	1,1%
Tb5/TbA	4	4,5%
Tb5/TbE	1	1,1%
Tb5/TbF	1	1,1%
Tb5/TbJ	2	2,2%
Tb5/TbL	1	1,1%
Tb5/TbN	2	2,2%
Tb6/TbA	6	6,7%
Tb6/TbF	1	1,1%
Tb6/TbL	1	1,1%
T. spiralis		
Ts1/TsA	44	77,2%
Ts1/TsB	1	1,8%
Ts2/TsA	12	21,1%
Hybrids		
Tb1/TsA	1	
Tb4/TsA	1	
Ts1/TbA	6	
Total of 154 larvae		

A second PCR was performed to amplify the CO1 gene, using newly developed forward primer 5'-TACCTATACTACTAAGAGGATTCGGA and reverse primer 5'-CTAGTACTCATAGTATGGCTGGTG), resulting in products of approximately 760 base pairs. CO1 PCR conditions were as follows: denaturation at 95 °C for 15 min followed by 40 cycles of 95 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min 30 s, and a final elongation step of 72 °C for 10 min.

DNA sequencing

PCR amplicons were purified using standard procedures (ExoSAP-IT®, Affymetrix, Cleveland, Ohio, USA). Purified PCR products were sequenced in both directions in 20 µl reaction volumes containing 3 µl of amplicon, 7 µl sequence buffer, 1 µl of Big Dye Terminator and 1 µl of forward or reverse PCR primer. Sequence PCR was performed under the following conditions: 95 °C for 1 min, followed by 25 cycles of 96 °C for 10 min, 50 °C for 5 min and finally 60 °C for 4 min. Trace files of the obtained sequences were generated on an automated ABI sequencer at the Institute's sequence facility.

Phylogenetic analysis

DNA sequences were assembled, edited manually and analysed with BioNumerics version 7.1 (Applied Maths NV, Sint-Martens-Latem, Belgium). Sequences with gaps or single strand sequences were excluded from further analysis. Cluster analysis of 5S rDNA-ISR and CO1 sequences was conducted using Neighbour Joining with Jukes-Cantor correction setting of the BioNumerics 7.1 software. Bootstrap values were calculated by the analysis of 10,000 replicates. Reference sequences from other species of *Trichinella* were included for 5S rDNA-ISR (*T. britovi* [GU325734-325737], *T. spiralis* [AY009946], *Trichinella* T8

Table 3. Comparison between *Trichinella britovi* 5S rDNA-ISR haplotypes generated in this study and sequences of other known encapsulated *Trichinella* species and genotypes. The most prevalent *T. britovi* haplotype (Tb1) generated in this study, was used as reference for this comparison. Five different *T. britovi* haplotypes (Tb1-Tb5) were identified based on one to five unique SNPs. Moreover, haplotype Tb6 shares seven additional SNPs (in comparison with Tb1) with most other encapsulated *Trichinella* species. Pink shading represents unique SNPs; grey indicates SNPs that are shared with other Trichinella haplotypes or species.

Table 4. *Trichinella britovi* and *Trichinella spiralis* haplotypes based on CO1 gene sequence. The most prevalent *T. britovi* haplotype (TbA) generated in this study, was used as reference for this comparison. Translation to protein sequence showed that only SNPs indicated in blue resulted in an amino acid change (see Figure 1). Pink shading represents unique SNPs; grey shading indicates SNPs that are shared with other Trichinella haplotypes or species.

Table 3

5S haplotype	28	46	48	68	72	74	89	126	127	154	189	226	247	299	347	379	433	463	472	509	539	548	549	556	582	592	594	597	600
T. britovi Tb1	A	T	T	A	C	T	A	G	A	T	A	T	G	C	A	A	A	A	G	A	G	G	C	C	T	A	T	G	A
T. britovi Tb2	A	T	T	A	C	T	A	G	A	T	A	T	G	C	G	A	A	A	G	A	G	G	C	C	T	A	T	G	A
T. britovi Tb3	A	T	T	A	C	T	A	G	A	T	A	T	G	G	A	A	A	A	G	A	G	G	C	C	T	A	T	G	A
T. britovi Tb4	A	T	T	A	C	T	A	G	A	T	A	T	G	C	A	A	A	A	G	A	G	G	C	C	A	A	T	G	A
T. britovi Tb5	A	C	T	A	C	A	A	G	A	T	A	T	G	C	A	A	A	A	G	A	G	G	C	C	T	A	T	G	A
T. britovi Tb6	T	T	C	A	C	T	T	G	A	C	G	T	C	C	A	A	A	A	C	A	T	T	C	T	T	G	T	G	T
Trichinella T8	A	T	T	A	C	T	A	G	A	C	A	C	C	C	A	C	A	A	G	A	T	T	G	C	T	G	T	G	A
Trichinella T9	A	T	T	G	A	T	A	C	T	C	A	C	C	C	A	A	A	A	G	C	T	T	C	C	T	G	T	A	A
Trichinella murelli	A	T	T	T	A	C	T	A	G	A	C	A	T	C	C	A	A	A	G	A	T	T	C	C	T	G	T	G	A
T. nativa	A	T	T	T	A	C	T	A	G	A	C	A	C	G/C	C	A	A	A	G	A	T	T	G	C	T	G	T	G	T
Trichinella T6	A	T	T	T	A	C	T	A	G	A	C	A	C	C	C	A	A	A	G	A	T	T	G	C	T	C	T	G	T
T. nelsoni	A	T	T	T	A	C	T	A	G	A	C	A	A	T	C	A	A	A	G	A	T	T	G	C	T	G	T	A	T
T. spiralis Ts1	A	T	T	T	G	C	T	A	G	A	C	A	C	T	C	A	A	A	G	A	T	T	G	C	T	C	T	G	T
T. spiralis Ts2	A	T	T	T	G	C	T	A	G	A	C	A	C	T	C	A	A	A	G	A	T	T	G	C	T	C	T	G	T
T. patagoniensis	A	T	T	T	A	C	T	A	G	A	C	A	C	T	C	A	A	A	G	A	T	T	G	T	A	T	T	G	A

Table 4

CO1 haplotype	9	33	52	56	84	90	96	133	156	162	216	292	343	351	381	420	594	609	702
T. britovi TbA	C	C	C	C	T	C	C	C	T	C	G	G	T	C	C	C	T	C	T
T. britovi TbB	C	T	C	C	T	C	C	C	T	C	G	G	T	C	C	C	T	C	T
T. britovi TbC	C	C	C	T	T	C	C	C	T	C	G	G	T	C	C	C	T	C	T
T. britovi TbD	C	C	C	C	T	C	C	C	T	C	G	A	T	C	C	C	T	C	T
T. britovi TbE	C	C	C	C	T	C	C	C	T	C	G	G	T	C	T	C	T	C	T
T. britovi TbF	C	C	C	C	T	C	C	C	T	C	G	G	T	C	C	T	T	C	T
T. britovi TbG	C	C	C	C	T	C	C	C	T	C	G	G	T	C	C	C	T	C	C
T. britovi TbH	C	C	C	C	T	T	C	C	T	C	G	G	T	C	T	C	T	C	T
T. britovi TbI	C	C	C	C	T	C	C	C	T	C	G	G	T	T	C	C	T	C	T
T. britovi TbJ	C	C	C	C	T	C	C	C	T	C	G	G	T	C	C	C	C	T	T
T. britovi TbK	T	C	C	C	C	C	C	C	T	C	G	G	T	C	C	C	C	T	T
T. britovi TbL	C	C	T	C	T	C	C	T	C	C	G	G	C	C	C	C	T	T	T
T. britovi TbM	C	C	T	C	T	C	T	C	C	T	G	G	T	C	C	C	T	T	T
T. britovi TbN	C	C	T	C	T	C	C	C	C	T	G	G	T	C	C	C	T	T	T
T. spiralis TsA	T	T	T	C	T	C	T	C	C	C	A	G	T	C	T	C	T	C	C
T. spiralis TsB	T	T	T	C	T	C	T	C	C	C	G	G	T	C	T	C	T	C	C
Trichinella T9	C	C	C	C	T	C	C	C	C	T	A	G	T	T	T	T	T	C	T

[AY009949], *Trichinella murelli* [AY009947, GU325738, GU325739, GU325740], *Trichinella* T6 [AY009948], *Trichinella nativa* [AY009944], *Trichinella patagoniensis* [JF260993, JF260994, JF260996, JF260999]), *Trichinella nelsoni* [AY009945] and for CO1 (*T. nativa* [AB252966], *Trichinella* T6 [AF129493, DQ007895], *T. murrelli* [DQ007894], *Trichinella* T9 [AB267878, AB267879, AB255885, DQ007898], *Trichinella* T8 [AF129494, DQ007897], *T. spiralis* [AF129486, DQ007890], *T. britovi* [AF129488], *T. nelsoni* [AF129490, DQ007896]). CO1 DNA sequences were trimmed to obtain open reading frames of equal length for all isolates, which were translated to amino acid sequences in BioNumerics 7.1. These were subsequently aligned and analysed using the same software.

Results

DNA sequence analysis

Complete nuclear 5S rDNA-ISR sequences and partial CO1 gene sequences were obtained from 154 *Trichinella* larvae originating from 44 isolates (Table 1). Assignment of individual ML to *T. spiralis* or *T. britovi* was based on identification using the validated 5S rDNA-ISR PCR for the identification of *Trichinella* spp. ML (Rombout et al., 2001b).

Genetic analysis of *Trichinella britovi*

Sequence analysis of *T. britovi* 5S rDNA-ISR intergenic spacer region (609 bp) of 51 ML from 9 Latvian isolates (red foxes) and 38 from 17 Polish isolates (wild boar and red foxes) revealed six haplotypes (coded as Tb1-Tb6, Table 1), whereby Tb1 and Tb4 were the most prevalent (51.7% and 18% respectively) (Table 2A). Haplotypes Tb2-Tb5 differed from Tb1 by one or two single nucleotide polymorphisms (SNPs) (Table 3). Haplotype Tb6 differed by twelve SNPs from Tb1, of which five SNPs were unique compared with all the other encapsulated *Trichinella* species (Table 3). Up to four haplotypes were found simultaneously in the same isolate (Table 1).

Sequence analysis of CO1 (702 bp) from the same 89 larvae reported above, revealed 14 haplotypes (coded as TbA-TbN), based on one to five SNPs, all but one C for T, or T for C substitutions (Table 4). TbA was the most prevalent polymorphism detected in 50 isolates (56.2%); the next most prevalent CO1 haplotype was TbN (12.4%) (Table 2A).

Figure 1. Translated protein sequence of CO1 DNA sequences. All but one *T. spiralis* larvae were homologues and differed two amino acids with *T. britovi* (alanine for threonine at position 3 and threonine for alanine at position 72). *Trichinella britovi* haplotype 3 and 4 showed one amino acid substitution at position 19 and 98 respectively. The reference sequence shown in the table was generated from the aligned amino acid sequences in BioNumerics 7.1.

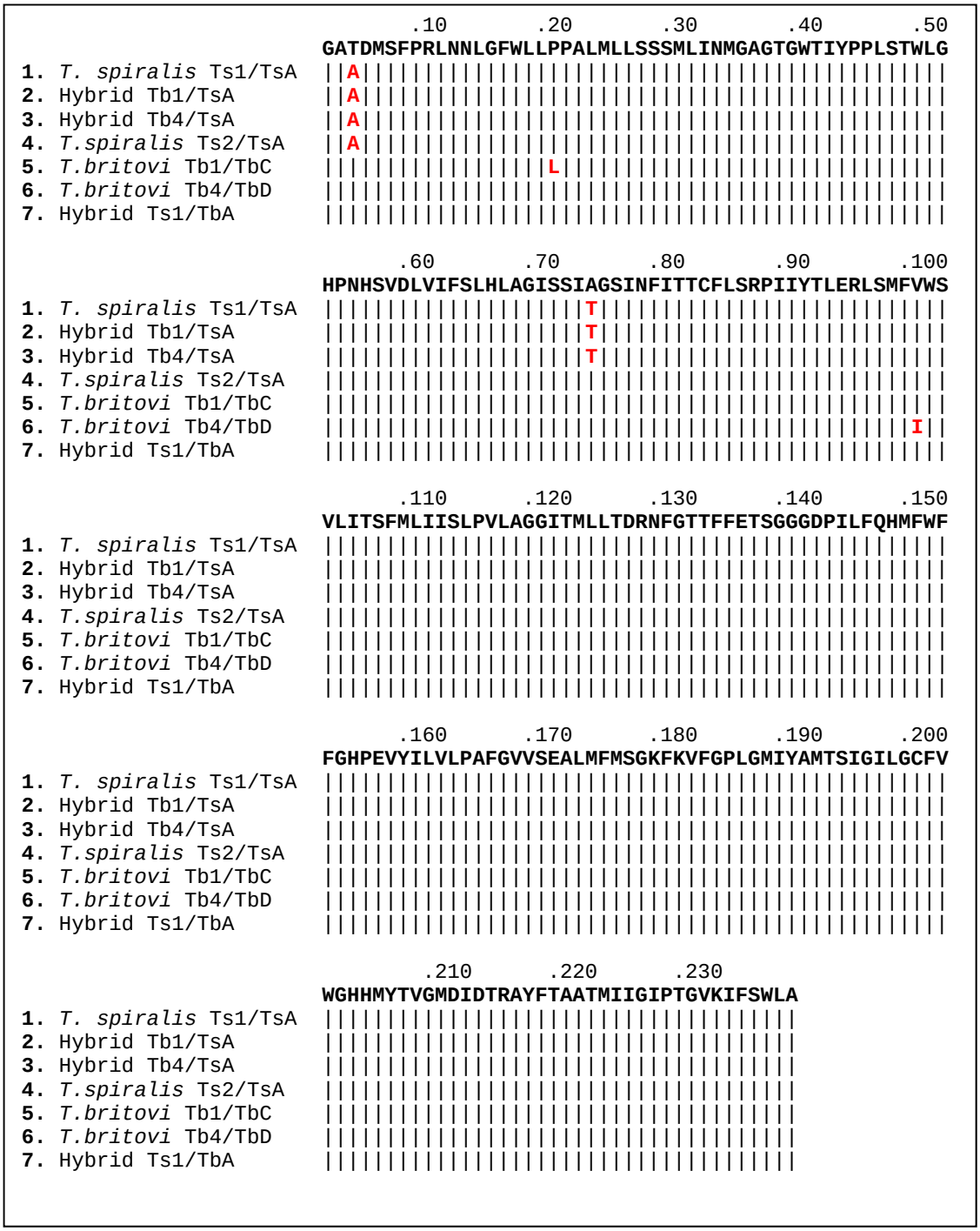
.10 .20 .30 .40 .50
GATDMSFPRLNNLGFWLLPPALMLLSSSMLINMGAGTGWTIYPPLSTWLG
1. T. spiralis Ts1/TsA
2. Hybrid Tb1/TsA
3. Hybrid Tb4/TsA
4. T.spiralis Ts2/TsA
5. T.britovi Tb1/TbC
6. T.britovi Tb4/TbD
7. Hybrid Ts1/TbA
.60 .70 .80 .90 .100
HPNHSVDLVIFSLHLAGISSIAGSINFITTCFLSRPIIYTLERLSMFVWS
.110 .120 .130 .140 .150
VLITSFMLIISLPVLAGGITMLLTDRNFGTTFFETSGGGDPILFQHMFWF
.160 .170 .180 .190 .200
FGHPEVYILVLPAFGVVSEALMFMSGKFKVFGPLGMIYAMTSIGILGCFV
.210 .220 .230
WGHHMYTVGMDIDTRAYFTAATMIIGIPTGVKIFSWLA

Figure 1

Translation of a 714 bp CO1 open reading frame lead to protein sequences of 238 amino acids in length for all isolates. Analysis of the protein sequence alignment revealed that the vast majority of SNP's were silent mutations. However, two *T. britovi* isolates showed one amino acid substitution at position 19 (leucine for proline) and 98 (isoleucine for valine), respectively (Figure 1). The prevalence for all demonstrated *T. britovi* 5S/CO1 haplotype combinations is shown in Table 2B.

Genetic analysis of *Trichinella spiralis*

Among 57 individual *T. spiralis* ML isolated from Polish wild boar and red foxes, two alleles of 5S rDNA-ISR were observed (coded as Ts1 and Ts2, Table 1), whereby the Ts1 allele was the most prevalent (78.9%) (Table 2A). These haplotypes differed by two SNPs (both G for A substitutions, at position 433 and 463, respectively) (Table 3). Both the two 5S rDNA-ISR haplotypes were detected in larvae from two isolates, one from wild boar and the other from red foxes.

Analysis of CO1 sequences of these *T. spiralis* larvae revealed two haplotypes coded as TsA and TsB, of which TsA was the most prevalent (98.2%) (Table 2A). One ML differed from TsA by a single SNP (G for A substitution at nucleotide position 216), which lead to an amino acid substitution in the translated protein (alanine for threonine) at position 72 (Figure 1). The prevalence for all demonstrated *T. spiralis* 5S/CO1 haplotype combinations is shown in Table 2B.

Trichinella species differences

Based on nuclear 5S rDNA-ISR sequences, *T. spiralis* and *T. britovi* differ for 32 SNPs, of which 11 are shown in Table 3. Over all, the CO1 sequences of *T. spiralis* larvae differed from those of *T. britovi* at a total of 64 C/T or T/C single nucleotide substitutions and 14 G/A or A/G substitutions, of which 7 are shown in Table 4. Despite the vast CO1 DNA sequence difference between *T. spiralis* and *T. britovi*, the translated protein of *T. spiralis* differed from that of *T. britovi* only at position 3 (alanine for threonine) and 72 (threonine for alanine) (Figure 1). Combination of 5S rDNA-ISR and CO1 sequence information revealed sexual recombination between *T. britovi* and *T. spiralis*, as illustrated in Figure 2.

Natural hybrids

Simultaneous infections with *T. spiralis* and *T. britovi* larvae were detected in four wild boars and two red foxes from Poland based on nuclear 5S rDNA-ISR sequences (isolates 137, 378, 485, 539, Fox A and Fox C). Jointly considering 5S rDNA-ISR and CO1 sequences revealed apparent crossbreeding between the two *Trichinella* species: in eight out of 19 individual larvae, the mitochondrial CO1 haplotype corresponded to haplotypes of the alternative *Trichinella* species. Six larval hybrid offspring of male *T. spiralis* x female *T. britovi* were identified in two wild boars and two foxes, while two larval hybrid offspring of male *T. britovi* x female *T. spiralis* were isolated from two wild boars (Table 1, Table 2B, Figure 3). The *Trichinella* hybrids demonstrated in our study are a combination of the

most prevalent haplotypes of each species: Tb1 (52%) and Tb4 (18%) of *T. britovi* with TsA (98%) of *T. spiralis* at one hand, and Ts1 (79%) of *T. spiralis* with TbA (56%) of *T. britovi* on the other hand (Table 2A). Events leading to hybrids that were demonstrated in this study are visualized in the upper part of Figure 3. Hybrid larvae carrying *T. spiralis* and *T. britovi* 5S rDNA-ISR alleles simultaneously, were not seen in the same host animal. Only clear and distinctive single peaks were found in the trace files of 5S rDNA-ISR sequences of both strands at all sites where *T. britovi* and *T. spiralis* differ.

In contrast, some ambiguity in base calls at one of both strands was seen at variable positions in a minority of *T. britovi* isolates. In those few cases, the other (unambiguous) strand was used to corroborate the predominance of overlapping peaks. Both 'pure' parental strains were not demonstrated simultaneously in the same host. *Trichinella spiralis* larvae were not detected in Latvian isolates.

The probability that these results could be the consequence of technical errors is highly unlikely, since all individual vials of each 8-vial PCR strip containing *Trichinella* ML were clearly marked. Moreover, each vial was checked microscopically to verify the presence of only one single larva per vial. Inverting the orientation of the 8-vial PCR strips during laboratory analysis was ruled out theoretically, which was confirmed by repeated CO1 PCR and sequencing of the larval DNA extracts from all 8-vial strips containing hybrid larva.

Phylogenetic analysis

A Neighbour Joining tree inferred from the 5S rDNA-ISR sequences (including all described SNPs) of this study and *Trichinella* spp. sequences present in Genbank, shows four clades in the lower part of the tree (*Trichinella* T9, *T. britovi* Tb6, *T. murrelli* and *T. britovi* Tb1-5) that cluster two-by-two, supported by a high bootstrap value (94%, Figure 4). One cluster harbours *T. britovi* Tb6/TbA, Tb6/TbF and Tb6/TbL (submitted to Genbank: KP900340, KP900341 and KP900342, respectively) and *Trichinella* T9 (ISS408, reference larvae TrichT9a, TrichT9d and TrichT9h, submitted to Genbank: KP9003343, KP900344 and KP900345, respectively). The second cluster harbours T. murrelli and the *T. britovi* Tb1-5 haplotypes, the latter of which are identical to Genbank entries GU325734 - GU325737 (Figure 4). The NJ tree inferred from CO1 720 bp sequences of these larvae revealed that *T. britovi* Tb6 (submitted to Genbank: KP900334 - KP900336) was associated with three different *T. britovi* CO1 matrilineages (Table 2B), but not with the *Trichinella* T9 CO1 sequence (submitted to Genbank: KP900337 - KP900339) (Figure 5). The latter of which is identical to Genbank entries AB255878, AB255879, A255885 and DQ007898 (Figure 5), indicating homology of both alleles.

Cross-breeding experiments

In experimental infection of laboratory mice, *T. spiralis* and *T. britovi* larvae succeeded in producing fertile F1 larvae (Table 5). The reproductive capacity obtained by cross breeding was far lower than the reproductive capacity of 'pure' *T. britovi* (213 ML per mouse) and *T. spiralis* (627 ML per mouse), with respectively 35 Tb/Ts hybrids in one out

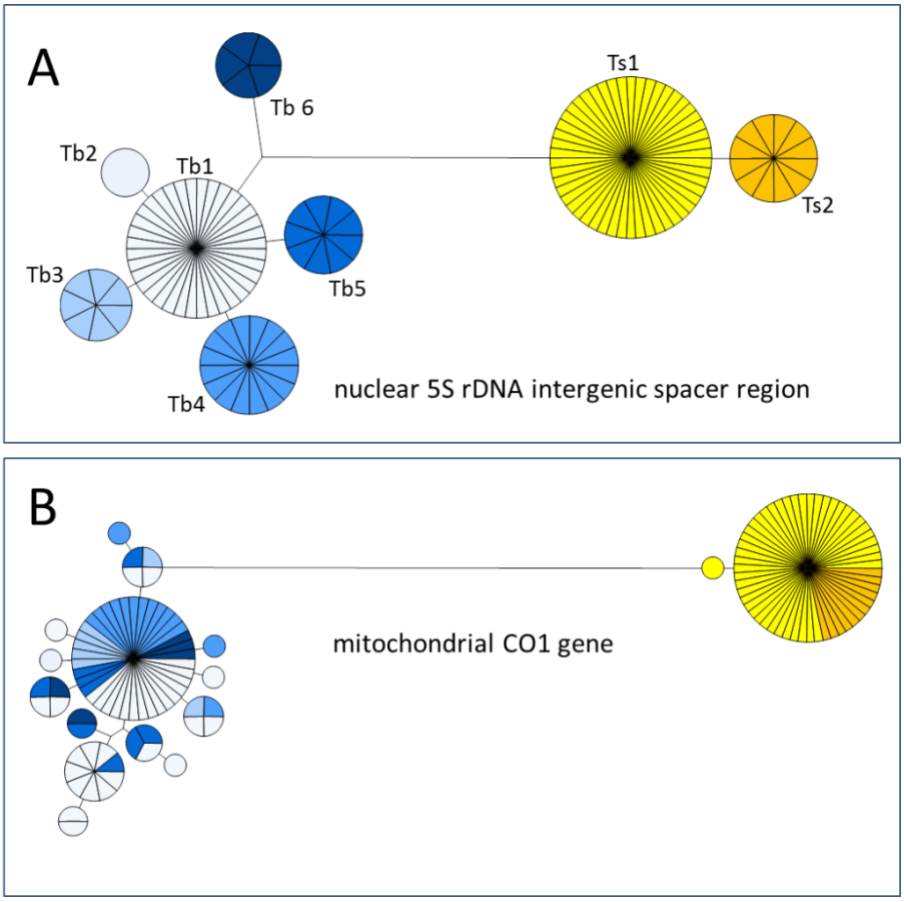

Figure 2. Nuclear 5S rDNA-ISR alleles are associated with different matrilineages.

A. A Maximum Parsimony network based on 5S rDNA-ISR (609 bp) from individual larvae revealed six *T. britovi* (blue shades) and two *T. spiralis* haplotypes (yellow / orange shades). Each wedge represents one individual *Trichinella* larva.

B. A Maximum Parsimony network based on CO1 sequences from individual larvae revealed 14 matrilineages for *T. britovi* and 2 for *T. spiralis*, where colour coding in blue (*T. britovi*) and yellow (*T. spiralis*) represents nuclear 5S rDNA-ISR haplotype. *T. britovi* 5S rDNA-ISR did not segregate with any specific mitochondrial haplotype. The *T. britovi* Tb1 5S nuclear allele was associated with eleven matrilineages, Tb2 with two matrilineages, Tb4 with three, Tb5 with five and Tb6 with three. All but one *T. spiralis* larvae shared an identical mitochondrial haplotype and the sole exception differed by only one substitution.

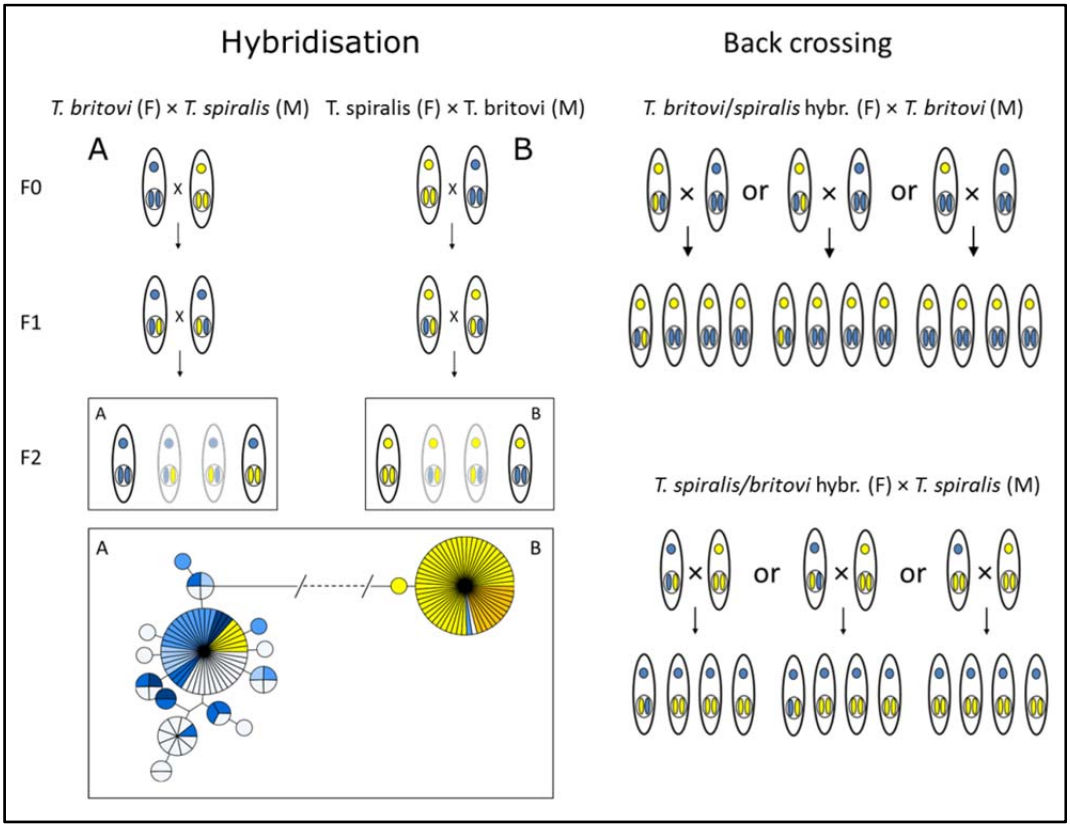

Figure 3. Scenario explaining observed hybridisation between *T. spiralis* and *T. britovi* in the most predominant matrilineage of both species. Recombination events leading to possible outcomes of hybridisation between *T. britovi* and *T. spiralis* and subsequent backcrossing events with parental species are shown.

Hybridisation - Scenario **A** shows hybridisation between female *T. britovi* and male *T. spiralis* resulting in F1 individuals heterogeneous for 5S rDNA-ISR, with matrilineal (*T. britovi*) CO1 gene. Scenario **B** shows a hybridisation event between a female *T. spiralis* and male *T. britovi* resulting in F1 individuals heterogeneous for 5S rDNA-ISR, with matrilineal (*T. spiralis*) CO1 gene. Small (upper) filled circles represent mitochondria, large (lower) filled circles represent nucleus with two 5S rDNA-ISR alleles.

Uptake of these F1 individuals by a subsequent mammalian host would result in F2 progeny depicted in scenarios A and B and indeed, two out of three possible outcomes were demonstrated in wild boar and red fox. Heterogeneous hybrid larvae possessing both *T. britovi* and *T. spiralis* 5S alleles (light grey individuals in boxes A and B) were not observed.

Lower box: A Maximum Parsimony network based on CO1 sequences from individual larvae as shown in Figure 2B, but here including the observed hybrids, where colour coding in blue (*T. britovi*) and yellow (*T. spiralis*) represents nuclear 5S rDNA-ISR haplotypes.

Legend to Figure 3 (continued)
Lower box (continued): **A:** Female *T. britovi* Ts1 x male *T. spiralis* (yellow individuals amidst blue ones) were observed in two wild boars and two red foxes. **B.** Female *T. spiralis* x male *T. britovi* Tb1 (bright blue individuals amidst yellow) and female *T. spiralis* x male *T. britovi* Tb4 (blue individuals amidst yellow) were observed in two wild boars.
Backcrossing - Theoretical back crossing of female F2 hybrid *Trichinella* individuals with parental species male individual results in 2heterogeneous 5S rDNA-ISR haplotypes and 10 homogeneous 5S-rDNA-ISR haplotypes, all bearing mitochondria of the alternative species. Backcrossing of male hybrid individuals with female parental individuals, results in restauration of a parental species-specific mitochondrion, but theoretical maintenance of the heterogeneous 5S rDNA-ISR haplotype in a 2 to 10 ratio (not shown).

of ten mice and 8 Ts/Tb hybrids in one out of ten mice (Table 5). Retrieved F1 larvae that were inoculated into new mice produced 49 and 17 *Trichinella* ML per mouse respectively. The progeny of the cross breeding experiments was not characterised by molecular analyses.

Discussion

The aim of the present study was to evaluate molecular polymorphisms of single *T. spiralis* and *T. britovi* ML from natural infections in wildlife originating from Latvian red foxes and in Polish wild boar and red foxes, based on nuclear 5S rDNA-ISR and mitochondrial CO1 gene sequences.
Analysis of DNA sequences derived from *T. britovi* single larvae revealed 6 and 14 haplotypes in the 5S rDNA-ISR and CO1 gene sequences respectively. Only two haplotypes for both the 5S rDNA-ISR and CO1 gene sequences have been detected in *T. spiralis*, suggesting a high degree of homology within this species recent common ancestry. This can be explained by the relatively recent introduction of *T. spiralis* in Europe, estimated at 1000 years before the present (BP) (La Rosa et al., 2012; Rosenthal et al., 2008), in contrast to the expansion of *T. britovi* into Europe, which was estimated to be 15 - 20 million years BP (Pozio et al., 2009a; Pozio and Zarlenga, 2013; Zarlenga et al., 2006). In a tRNA-Ile sequence, six SNPs were demonstrated in mitochondrial protein coding sequences and one SNP by comparison of deep sequencing data of two geographically and temporally distinct *T. spiralis* isolates (Webb and Rosenthal, 2010). Additionally, much greater haplotype variation was demonstrated in mitochondrial DNA and in microsatellites of *T. spiralis* isolates from Asia than in *T. spiralis* isolates from Europe and South-America (La Rosa et al., 2012; Rosenthal et al., 2008) and with the same markers, more variation was seen in European *T. britovi* than in European *T. spiralis* (Rosenthal et al., 2008). Out of 154 tested individual *Trichinella* ML, eight larvae were hybrids between *T. britovi* and *T. spiralis*, which were isolated from four wild boar and two red foxes from

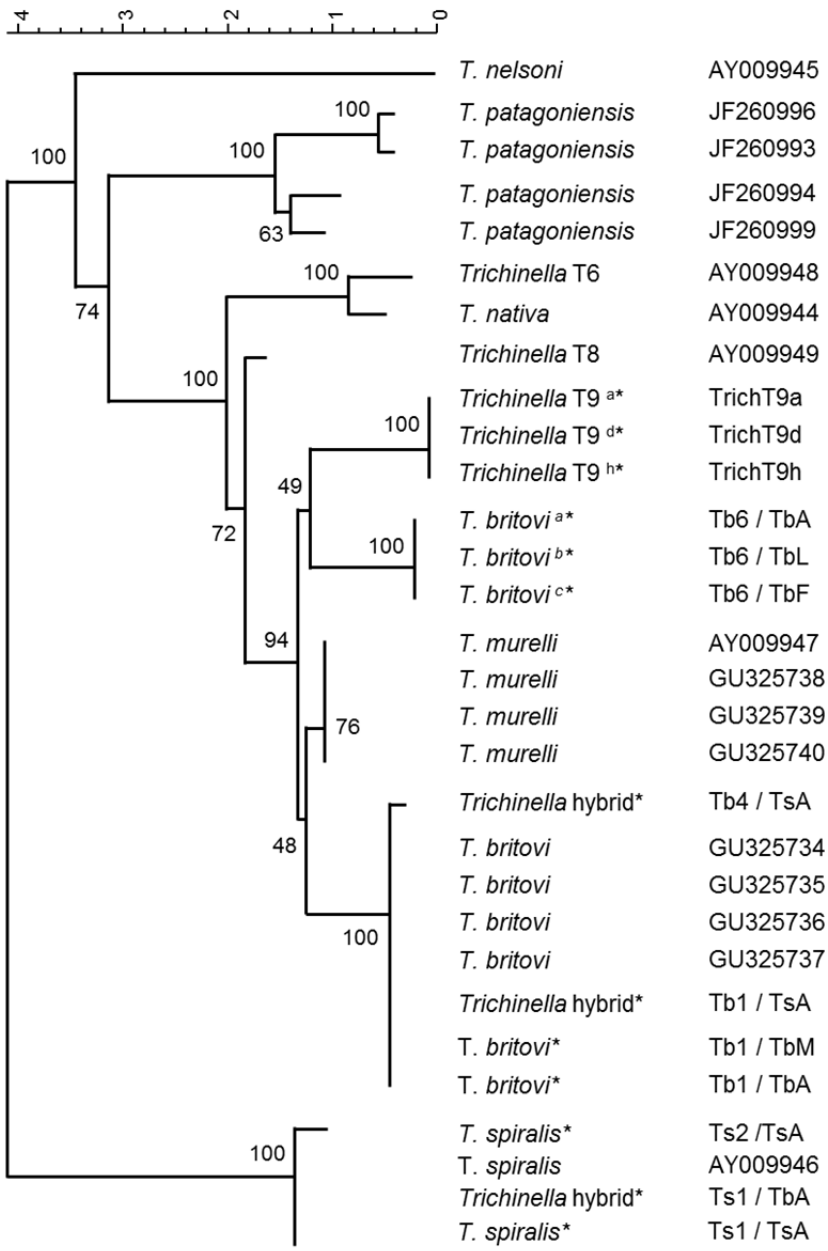

Figure 4. Neighbour Joining tree inferred from 5S rDNA-ISR sequences of *Trichinella* species. * 5S rDNA-ISR sequences generated in this study. *Trichinella* T9 [a]*: submitted to Genbank, KP900343. *Trichinella* T9 [d]*: KP900344. *Trichinella* T9 [h]*: KP900345. *T. britovi* [a]*: KP900340. *T. britovi* [b]*: KP900341. *T. britovi* [c]*: KP900342. Bootstrap values were calculated from 10,000 simulations.

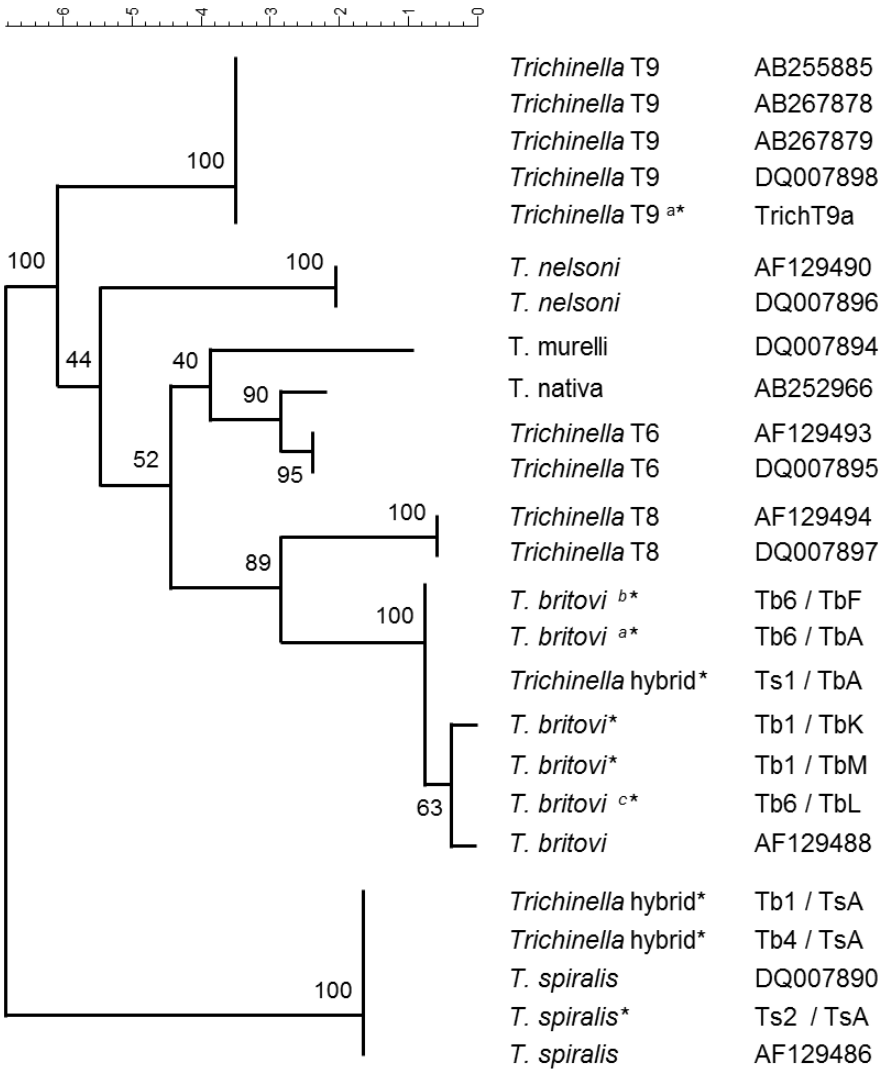

Figure 5. NJ tree inferred from CO1 nucleotide sequences of *Trichinella* species. Due to difference in sequence length between Genbank entries and sequences generated in this study (720 bp), this neighbour joining tree is based on 288 overlapping base pairs, which does not allow display of sequence differences between *T. britovi* CO1 haplotypes depicted in the NJ tree. * CO1 sequences generated in this study. *Trichinella* T9 [a]*: submitted to Genbank, KP900337. *T. britovi* [a]*: KP900334. *T. britovi* [b]*: KP900335. *T. britovi* [c]*: KP900336. Bootstrap values of 10,000 simulations.

Poland. All hybrids were combinations of the most prevalent haplotypes, which implies that possibly more hybrid haplotype combinations could exist, for which analysis of a far greater sample size is needed to demonstrate those combinations. Infection experiments with pairs of single male and female larvae of either *Trichinella* species in the present study showed that hybridisation comes with the apparent cost of reduced offspring in F1, in comparison to the parent *Trichinella* species. This cost appears higher for male *T. spiralis* with female *T. britovi* hybridisation than for the other direction (female *T. spiralis* with male *T. britovi*) ($p < 0.0001$, Fisher's Exact test). Cross-experiments between *T. spiralis* from Poland and *T. britovi* from Italy only yielded hybrids between female *T. spiralis* and male *T. britovi*, but not in the other direction (Wu et al., 2000), which corroborates our finding. Preference of hybridisation direction is also seen with other *Trichinella* species. Cross-breeding experiments between male *T. nativa* and female *Trichinella* T6 revealed fertile F1, which were shown to produce F2 offspring, whereas crosses of *T. nativa* females and *Trichinella* T6 males were not fertile (La Rosa et al., 2003b).

The apparent cost of hybridisation as seen in our cross breeding experiments could drive the F2 and later progeny towards segregation of rDNA alleles, resulting in the transmission of one matrilineal haplotype to two distinct genomic backgrounds. Our observation that no hybrid 5S rDNA-ISR was seen in individual hybrid larvae suggests that the actual hybridisation events took place in the past in all observed cases, after which frequent subsequent backcrossing occurred, resulting in larvae carrying a 5S rDNA-ISR haplotype of one species and a matrilineal CO1 haplotype of the alternative species. The fact that all hybrids displayed a homozygous 5S rDNA-ISR sequence, may be explained by too small a number of hybrids detected in our study to reveal heterozygosity on just one nuclear marker used. The theoretically limited occurrence of heterozygous hybrids by introgression may support our observations, since *Trichinella* individuals carrying homologous 5S rDNA-ISR would outnumber individuals carrying heterogeneous 5S rDNA-ISR in a 10 to 2 ratio following backcrossing of all possible F2 outcome depicted in this study (Figure 3). In Canadian wolverines, introgression or back-crossing between closely related *T. nativa* and *Trichinella* T6 was demonstrated in pooled larval DNA extracts and in single larvae. In that study, *Trichinella* T6 larvae (based on nuclear microsatellite

Table 5. Cross-breeding results between single male and female larvae of *Trichinella britovi* and *Trichinella spiralis* in mice.

Code	1 male	1 female	No. of mice	No. offspring larvae (infected mice)
A	*T. britovi*	*T. spiralis*	10	35 (1)
B	*T. spiralis*	*T. britovi*	10	8 (1)
C	*T. britovi*	*T. britovi*	5	average larvae/mouse 213 (5)
D	*T. spiralis*	*T. spiralis*	5	average larvae/mouse 627 (5)
F1A	15 larvae, mixed sexes		2	average F2 larvae/mouse 49 (2)
F1B	8 larvae, mixed sexes		1	F2 larvae/mouse 17

markers) displayed a *T. nativa* matrilineage (Dunams-Morel et al., 2012). It is tempting to contemplate on the practical implications of *Trichinella* hybrids, but it is at the same time highly speculative to draw any conclusion with respect to phenotypic traits such as freeze resistance or pathogenic characteristics. While the practical implications of the existence of *Trichinella* hybrids are unclear, interrogression/hybridisation events between digenean schistosomes infecting humans (*S. haematobium*, *S. mansoni*), bovines (*S. bovis*) and sheep, goats and bovines (*S. curassoni*) in Senegal have resulted in novel (combinations of) traits, which among others, result in expansion of host range and decreased success of drug treatment in cattle (Huyse et al., 2013; Webster et al., 2013).

In the present study, the topology of the phylogenetic tree inferred from 5S rDNA-ISR sequences resembles the hypothesized phylogeny for the genus *Trichinella* as published recently (Pozio et al., 2009a), and is identical to a NJ tree inferred from multilocus analysis of 11 isoenzymes (La Rosa et al., 2003a). The *T. britovi* Tb6 haplotype that was identified in the present study, shows a 5S rDNA-ISR sequence that is deviant from *T. britovi* Tb1 (12 SNPs). Of the observed SNPs, five were shared between *T. britovi* Tb6 and all other encapsulated species, except *T. britovi* Tb1-5. At the same time, five other SNPs were unique to *T. britovi* Tb6 5S rDNA-ISR. As a result, *T. britovi* Tb6 forms a clade in the 5S rDNA-ISR phylogenetic tree and clusters with *Trichinella* T9, well away from a second cluster harbouring *T. murelli* and the clade consisting of *T. britovi* haplotypes Tb1-Tb5 and the two *T. britovi* / *T. spiralis* hybrids. At the mitochondrial level however, *T. britovi* Tb6 displays three *T. britovi* matrilineages, which are distant from *T. murelli* and *Trichinella* T9. However, more research is needed to survey many more genes to evaluate the composition of the hybrid genome and the *T. britovi* Tb6 genome.

For routine analysis, *Trichinella* species determination relies on banding pattern using gel electrophoresis after PCR of the mitochondrial CO1 gene alone (Nagano et al., 1999) or in combination with nuclear markers when using multiplex PCR (Zarlenga et al., 1999). However, using these techniques, underlying DNA sequence information or more precisely variation thereof remains unseen and interspecies hybrids are missed. Moreover, use of CO1 PCR banding patterns solely may lead to misidentification of the *Trichinella* species in the case of hybrid larvae.

In conclusion, we demonstrate by combination of 5S rDNA-ISR and CO1 sequence information of individual *Trichinella* ML from field isolates, that interspecies recombination between *Trichinella spiralis* and *Trichinella britovi* occurs under natural conditions.

Competing interests

The authors declare that they do not have competing interests.

Author's contributions

FF wrote the study design, generated and analysed molecular data and wrote the manuscript. EB wrote the study design, generated and analysed molecular data, contributed to the manuscript. GD coordinated Latvian hunters to collect *Trichinella* spp. positive foxes, generated parasitological data and contributed to the manuscript. HS advised with regard to molecular analysis and contributed to the manuscript. EP conducted the cross-breeding experiments and contributed to the manuscript. BR helped with the interpretation of molecular results and contributed to the manuscript. MR wrote the project proposal and coordinated the study in Poland, and contributed to the manuscript. JvdG wrote the project proposal, coordinated the study in the Netherlands and contributed to the manuscript.

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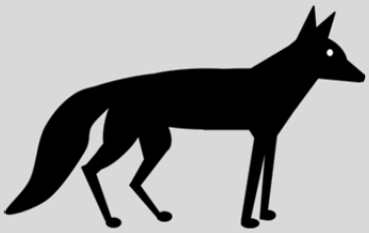
Chapter 6



Increase in number of helminth species from Dutch red foxes over a 35-year period

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The red fox (*Vulpes vulpes*) is host to a community of zoonotic and other helminth species. Tracking their community structure and dynamics over decades is one way to monitor the long term risk of parasitic infectious diseases relevant to public and veterinary health.

We identified 17 helminth species from 136 foxes by mucosal scraping, centrifugal sedimentation / flotation and the washing and sieving technique. We applied rarefaction analysis to our samples and compared the resulting curve to the helminth community reported in literature 35 years ago.

Fox helminth species significantly increased in number in the last 35 years (p-value <0.025). *Toxascaris leonina*, *Mesocestoides litteratus, Trichuris vulpis* and *Angiostrongylus vasorum* are four new veterinary-relevant species. The zoonotic fox tapeworm (*E. multilocularis*) was found outside the previously described endemic regions in the Netherlands.

In conclusion: helminth fauna in Dutch red foxes increased in biodiversity over the last three decades.

Keywords: Helminth fauna, Red fox, Biodiversity, Molecular analysis, *Echinococcus*, *Toxocara*, *Taenia*, *Alaria*

Introduction

Long-term studies on parasite communities of marine and terrestrial wildlife hosts were instrumental to evaluating the influence of natural and anthropogenic factors on environmental changes, especially when sampling series span more than ten years (Dzikowski et al., 2003; Haukisalmi and Henttonen, 1990; Spratt, 1987).

For larger mammals, like the red fox, many cross-sectional studies report on the parasitic helminth fauna (Borgsteede, 1984; Bruzinskaite-Schmidhalter et al., 2012; Eira, 2006; Gortazar et al., 1998; Lucius et al., 1988; Magi et al., 2009; Nickel et al., 1980; Rajkovic-Janje et al., 2002; Richards et al., 1995; Saeed et al., 2006) or focus on limited parasite species (Barabási, 2010; Criado-Fornelio et al., 2000; Letkova et al., 2006; Rajkovic-Janje et al., 2002; Richards et al., 1995; Suchentrunk and Sattmann, 1994; Vervaeke, 2005; Wolfe, 2001), but long-term studies are rare (Nickel et al., 1980).

In the eighties of the last century, Borgsteede (Borgsteede, 1984) studied the helminth fauna in foxes from the border region in the eastern part of The Netherlands, collected between February 1978 and May 1979. For ensuing decades, this study has been the sole large scale surveillance of helminth fauna in red foxes in the Netherlands.

A series of additional large scale surveillance in red foxes became reality since the initial detection of *Echinococcus multilocularis* in the Netherlands in 1996 (van der Giessen et al., 1999). *E. multilocularis* tends to increase in the fox population over the last decades in Europe (Hegglin and Deplazes, 2013) and therefore, the European Food Safety Authority (EFSA) recommends monitoring this parasite in foxes, especially at the borders of its distribution area in Europe (EFSA, 2010). Following the initial detection in the Netherlands, *E. multilocularis* in foxes was found to disperse in southern Limburg, but not in the central and western part of the Netherlands (van der Giessen et al., 1999). Since the Netherlands are a densely populated country with an average human population density of 497/km^2 (CBS, 2013) and a pet population of around 1.5 million dogs (Cijfers, 2011), a high density of red foxes (0.5 to 4.0 per square kilometre) might potentially lead to exposure of humans and dogs to zoonotic parasites, like *E. multilocularis* (Vervaeke, 2005).

Here, we compared our recent large-scale surveillance of helminth fauna in the population of red foxes from the border region in the eastern part of The Netherlands with the historic studies more than 35 years ago. We evaluated trends in parasite richness by applying the rarefaction analysis (Colwell et al., 2012; Gotelli and Colwell, 2001) . In addition, we discuss the relevance of our findings for public health.

Materials and methods

Animals

From October 2010 until April 2012, routinely shot foxes were collected by hunters and sent to the National Institute for Public Health and Environment (RIVM, Bilthoven, The

Netherlands). The chosen fox sample size (288) originated from a strip with a width of 15 km and a length of 266 km at the border with Germany, between Groningen and Limburg (4000 km^2), excluding the formerly found positive districts (Figure 1). Upon arrival, fox carcasses were stored at -80˚C to inactivate the eggs of *E. multilocularis* (Veit et al., 1995), according to WHO guidelines (WHO, 1984). After a minimum period at -80˚C of one week, carcasses were thawed and dissected. Data on weight, measurements, age and gender were collected after thawing. From weight and body size, condition was estimated as the ratio of body weight in gram over body length (nose - anus) in millimetres (body weight / length, BWL index).

The age of the foxes was evaluated by examining tooth wear, especially the wear of the lower incisors and the upper and lower molars and by cutting the root of one or two canines into several 0.15 mm thin slices, which were examined microscopically (magnification 20-40 times) under horizontal cross light (Grue and Jensen, 1979). Foxes without signs of wear were classified as first year animals (Mulder et al., 2004) .

During dissection, the jejunum and faecal material (if present) from the distal colon/rectum of each fox were sampled. The whole small intestines of 262 foxes were evaluated by microscopic examination of mucosal scrapings and macroscopic examination of the opened small intestine. Moreover, distal colon content was used for PCR (see *E. multilocularis*-specific PCR identification); 158 foxes had sufficient faecal content in the colon to be used for additional microscopic analysis after centrifugal sedimentation / flotation.

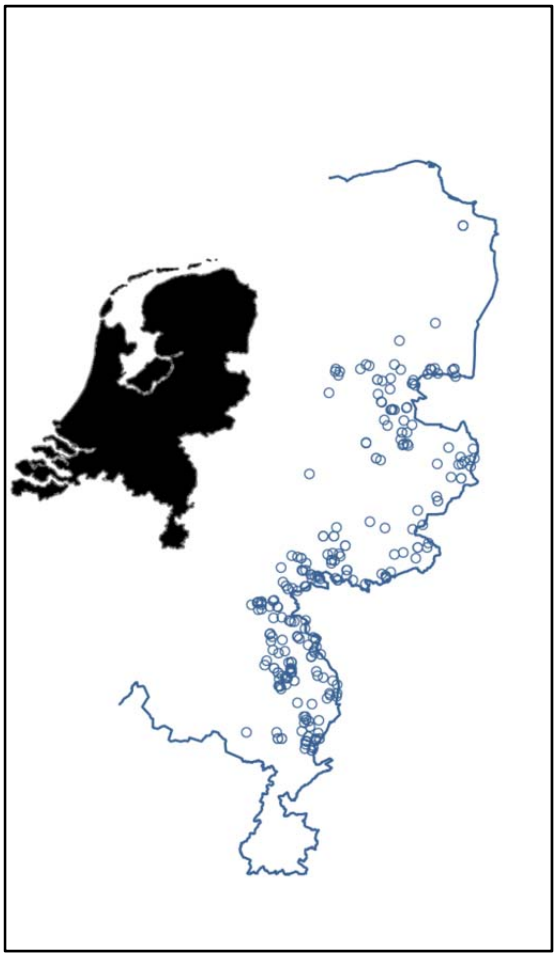

Figure 1 - Geographical origin of individual foxes. This figure shows the study area along the eastern border of the Netherlands in blue, with a representation of the whole country in black. Circles show the geographical origin of the foxes collected for this study.

Microscopical examination of parasites

Small intestine mucosal scraping

The small intestine of each fox was separated and opened. Macroscopically visible helminths were scored and noted. Subsequently, mucosal scrapings were made to screen the mucosal content for small helminths microscopically (Deplazes and Eckert, 1996; Eckert et al., 2001). The presence of intestinal helminths was scored semi-quantitatively: '+' 1-2 individuals, '++' 3-10, '+++' 11-50, '++++' 51-100 and '+++++' >100. Parasites were identified morphometrically and in cases where difficult to identify young adult stages were found, or the freezing/thawing process had damaged the morphology of cestode species, morphological identification was confirmed by PCR (see Molecular identification of parasites). For this purpose, parasite specimens were collected and stored in 70% ethanol until further use.

Sedimentation / flotation on gut content

When available, about 3 grams of distal colon content were suspended in 50 ml tap water, an 11 ml centrifuge tube was filled with this suspension and the product of centrifugal sedimentation / flotation was examined microscopically. A sucrose solution of 1.28 - 1.3 g/cm^3 was used as flotation medium for the faecal examination of eggs and larvae. The centrifugal step for flotation was performed with the cover slip on top of the tube and one slide was examined per sample. The results were scored semi quantitatively using '+' for 1-10 eggs per slide; higher numbers were scored as '++' for one to five per microscopic field at 100x (10x10) magnification and '+++' for more than five per microscopic field at the same magnification.

Since fox carcasses were frozen to inactivate zoonotic parasites, the Baermann method could not be used to isolate first stage larvae of *Crenosoma vulpis* and *Angiostrongylus vasorum*. Larvae that were found by CSF, which were not too damaged by the freezing and thawing process were identified morphologically according to McGarry and Morgan (McGarry and Morgan, 2009).

Screening for cardio-pulmonary helminths

The lungs and hearts of 97 foxes were examined for helminths by opening the right heart and pulmonary arteries up to the level of small branches in the lungs (Bourque AC, 2008). The bronchi were opened, examined and washed with water, which was sieved through a 150 µm mesh size sieve. The same procedure was used for heart and vessels. Adult and juvenile worms were removed from the sieve and identified morphologically up to species level (Morgan et al., 2008; Rosen et al., 1970).

Screening for helminths in the urinary bladder

In addition, four urinary bladders were opened to look for adult worms of *Pearsonema plica*.

Helminth species number

To evaluate a possible change in helminth species richness, we applied rarefaction analysis (Colwell et al., 2012; Gotelli and Colwell, 2001) to the number of distinct helminth species that we identified in 136 foxes. We calculated the rarefaction curve with the software package EstimateS 9.0 (Colwell, 2013; Colwell et al., 2012; Gotelli and Colwell, 2001) with default settings. Based on the rarefaction curve, we compared our findings with those of historical studies (Borgsteede, 1984; Bruzinskaite-Schmidhalter et al., 2012; Lucius et al., 1988; Nickel et al., 1980; Saeed et al., 2006)

Foxes, for which biological parameters or geographical data were missing, were excluded from analysis. This limited the available dataset for multifactorial analysis to 136 foxes. For each parasite species, prevalence was calculated and significance of prevalence difference was analyzed with Fisher's Exact test. Correlations between body condition, age, gender and parasite prevalence were determined by ANOVA (analysis of variance). Fisher's exact test and ANOVA were performed and the resulting P-values were calculated using Quickcalc (GraphPad Software, Inc. La Jolla, California, USA) and the data analysis module of Microsoft Excel 2007.

E. multilocularis-specific PCR identification

To analyse the presence of *E. multilocularis* at sub-microscopical level, three grams of colon contents were tested in a single tube nested 12S ribosomal DNA PCR as described previously (van der Giessen et al., 1999). PCR products were specified by southern blot hybridization, using *E. multilocularis*- specific probes as described previously (van der Giessen et al., 2004).

Molecular identification of parasites

DNA isolation and PCR

Parasites were transferred from 70% ethanol and soaked in demineralized water. DNA was isolated using the Qiagen Blood and Tissue Kit (Qiagen NV, Venlo, The Netherlands), according to the manufacturer's instructions. To confirm the identification of cestode species, a fragment of the mitochondrial cytochrome oxidase 1 (CO1) gene was amplified as described by Bowles et al. (Bowles et al., 1992). All PCRs were carried out in 50 µl final volume containing 3 µl genomic DNA, 0.5 µl of each forward and reverse primer (50 µM stock) and 25 µl of Qiagen HotstarTaq polymerase master mix (Qiagen NV, Venlo, The Netherlands). The final reaction volume was adjusted to 50 µl with sterile demineralized water. PCR amplification of the partial CO1 gene was performed using the following conditions: denaturation at 95 °C for 15 min, followed by 35 cycles of 1 min denaturation at 95 °C, 1 min annealing at 45 °C and 1:15 min elongation at 72 °C, followed by a final extension step of 7 min at 72 °C.

DNA sequencing of amplicons

PCR amplicons were purified using standard procedures (ExoSAP-IT®, Affymetrix, Cleveland, Ohio, USA). All DNA sequence PCR reactions were carried out on both DNA strands in 20 µl final volume containing 3 µl of amplicate, 7 µl sequence buffer, 1 µl of Big Dye Terminator and 1 µl of each PCR primer. Sequence PCR was performed under the following conditions: 95 °C for 1min, followed by 25 cycles of 96 °C for 10 min, 50 °C for 5 min and finally 60 °C for 4 min. Trace files of the obtained sequences were generated on an automated ABI sequencer at the Institute's DNA sequence facility.

DNA and phylogenetic analysis

DNA sequences were assembled, edited, and analysed with BioNumerics version 6.6 (Applied Maths NV, Sint-Martens-Latem, Belgium). Obtained CO1 gene sequences were compared to reference sequences present in Genbank after subtraction of the primer sequences. Cluster analysis of the sequences was conducted using the unweighted neighbour-joining algorithm of the BioNumerics program. Bootstrap proportions were calculated by the analysis of 2500 replicates for neighbour-joining trees. Available CO1 sequences of cestodes and trematodes from Genbank were included in the alignment. Sequence homology ≥99% and homology of morphological criteria were considered as proof of identity between isolated and Genbank species. Unequivocally identified *Alaria alata* isolates from foxes from this study served as out-group in phylogenetic analysis.

Results

Animal age, gender and body weight

In total, 262 foxes were collected. Seventy per cent of the foxes were 7 - 12 months old at the time of sampling and seven foxes were older than 5 years. This age distribution of shot foxes indicates high hunting pressure as found in previous studies (Mulder, 2005; Mulder et al., 2004).

Overall, 55% of the sampled foxes were males and 45% were females, which were evenly distributed over the study area (Figure 1). Males were heavier than females; average body weight / length (BWL) index of males and females differed significantly (ANOVA, P-value < 0.0001). Correlation between BWL index and infection classes was absent for both male (P-value = 0.626) and female foxes (P-value = 0.232).

Analysis of helminth species number

Seventeen helminth species were identified from our reference data set of 136 foxes. The 95% confidence interval was 14.39 – 19.61 parasite species. The number of parasite species in 137 foxes that were sampled 35 years ago (Borgsteede, 1984) was twelve species, which is a significantly lower species richness (P-value < 0.025) (Figure 2).

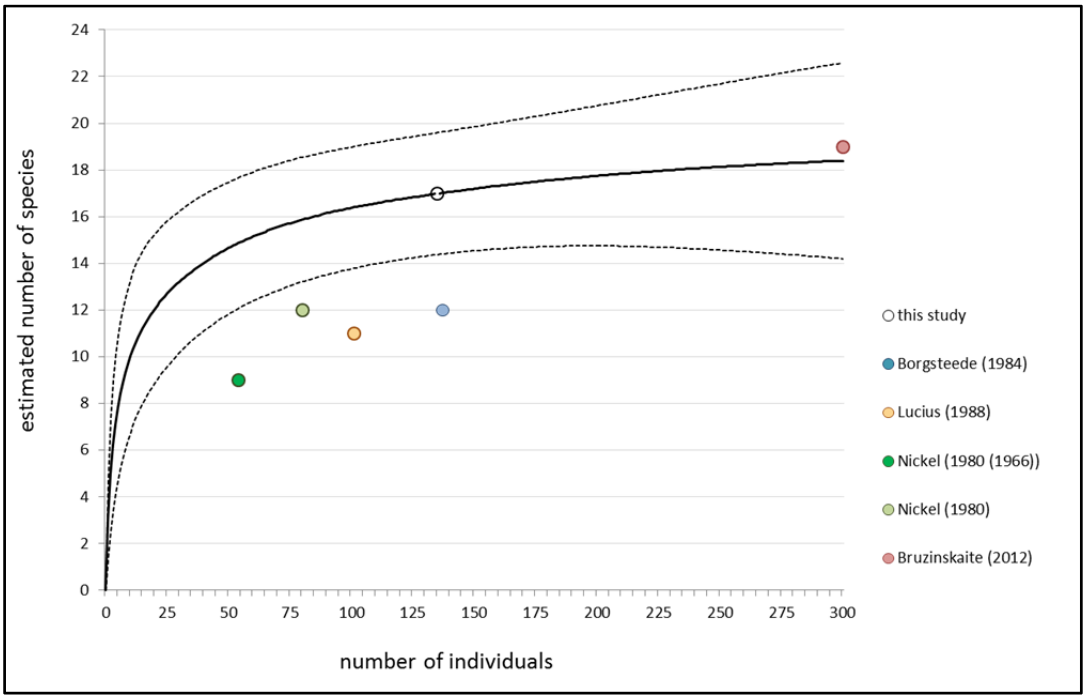

Figure 2 - Analysis of fox parasite species by rarefaction method
Open circle: the number of distinct parasite species identified from 136 Dutch foxes in this study. Solid circle: the number of distinct parasite species identified from the foxes described in a cited study. Solid line: expected number of distinct parasite species estimated by the rarefaction method based on our data set (i.e. open circle). Dotted line: 95% confidence interval. Nickel *et al.* reported two independent fox populations from different regions, sampled in 1966 (green solid circle) and in 1980 (light green solid circle) respectively.

Multiple infections per fox

On average 97.1% of the foxes were infected with one or more out of 17 helminth species, with maximum co-infection levels of eight different species. Foxes younger than 10 months were more frequently infected (35 - 37%) with 2 to 3 parasite species than foxes older than 10 months (10-27%) (Figure 3).

Prevalence per helminth species and comparison with other studies

Parasite prevalence was higher in male foxes for the majority of the parasite species (Table 1), although this was only significant for *Toxocara canis* (Fisher's Exact test, P=0.013). *T. canis* and *U. stenocephala* were the most prevalent intestinal fox parasites in our study, like in other Western European countries (Criado-Fornelio et al., 2000; Eira, 2006; Lucius et al., 1988; Saeed et al., 2006; Vervaeke, 2005; Wolfe, 2001). The prevalence of *T. canis* and *Taenia* spp. were significantly lower in this study compared to

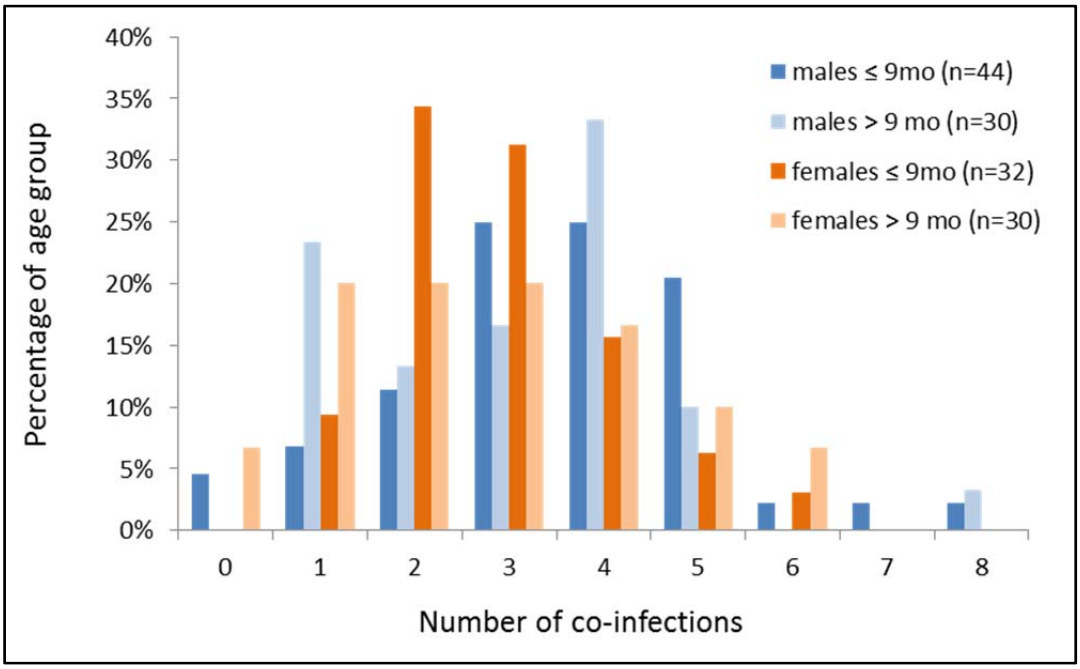

Figure 3 - Number of co-infections per age group and per gender.
Male foxes peak at three to four co-infections, females nine months of age and younger peak at two to three co-infections. Male foxes exhibit the highest numbers of co-infection (8). Zero co-infections mean no infection at all. Total number of foxes is 136.

the earlier study of Borgsteede (Borgsteede, 1984) (Table 2). The combined prevalence of *Toxocara canis* and *Toxascaris leonina* reported in Belgian foxes in 2005 (Vervaeke, 2005) was not different (Fisher's Exact test, P=0.315) from the prevalence in our study. The prevalence of *T. canis* in Danish foxes in 2006 was 59.4%, which is almost identical to the level found in this present study, as was the case for *Taenia* species. In contrast, the prevalence of *Uncinaria stenocephala* was significantly higher in Denmark (Saeed et al., 2006), compared to either our data (Fisher's Exact test, P=0.0018), historical data from northern Germany (Lucius et al., 1988) (Fisher's Exact test, P=0.002), or historical data from the Netherlands (Borgsteede, 1984) (Fisher's Exact test, P=0.054).
The prevalence of *Strongyloides* sp., *Eucoleus aerophilus* and *Crenosoma vulpis* was significantly higher than reported in 1984 by Borgsteede (Table 2). *Trichuris vulpis*, *Angiostrongylus vasorum*, *Mesocestoides litteratus* and *Echinococcus multilocularis* were new species in de studied area. The trematode *Apophallus donicus*, of which one individual was found by Borgsteede (Borgsteede, 1984) was not identified in the present study. This was also the case for *Hymenolepis* spp., for which rodents are definitive hosts. Adult *Hymenolepids* are regarded as passing species from prey, as is *Molineus patens*, and these were thus excluded from analysis of helminth species parasitic to red fox.

Table 1 - Overview of parasitic helminths found in Dutch red fox.

Helminth species	males (n=73)		females (n=63)		overall (n=136)		Means of infection	Method
Intestinal nematodes	%	n	%	n	%	n		
1. *Toxocara canis*[1]	71.2	52	49.2	31	61	83	worm eggs, paratenic hosts	D, CSF
2. *Toxascaris leonina*	1.4	1	3.2	2	2.2	3	worm eggs, paratenic hosts	CSF
3. *Trichuris vulpis*[2]	20.5	15	12.7	8	16.9	23	worm eggs	CSF
4. *Uncinaria stenocephala*	60.3	44	47.6	30	54.4	74	free larvae, paratenic hots	CSF, MS
5. *Strongyloides* sp.	9.6	7	20.6	13	14.7	20	free larvae	CSF, MS
Other nematodes								
6. *Eucoleus aerophilus* (n=96)	71.4	35	63.8	30	67.7	65	earthworms, worm eggs	WS
7. *Pearsonema plica*	(2/2)		(2/2)		(4/4)*		worm eggs	D. WS
8. *Capillaria* spp.[3]	52.1	38	47.6	30	50	68	worm eggs	CSF
9. *Angiostrongylus vasorum* (n=96)	6.1	3	2.1	1	4.2	4	terrestrial gastropods, frogs	WS, CSF
10. *Crenosoma vulpis* (n=96)	24.5	12	8.5	4	16.7	16	terrestrial gastropods	WS, CSF
Intestinal cestodes								
11. *Taenia crassiceps* / 12. *Taenia polyacantha*	21.9	16	22.2	14	22.1	30	rodents, lagomorpha	D, MS, PCR
13. *Mesocestoides litteratus*	6.8	5	4.8	3	5.9	8	frogs, intermediate hosts	D, MS, PCR
14. *Echinococcus multilocularis*	1.4	1	0	0	0.7	1	rodents, lagomorpha	PCR
Intestinal trematodes								
15. *Cryptocotyle lingua*	4.1	3	3.2	2	3.7	5	fish	MS
16. *Isthmiophora melis*	1.4	1	0	0	0.7	1	tadpoles	MS
17. *Alaria alata*	17.8	13	15.9	10	16.9	23	tadpoles, frogs	MS, PCR

[1] The observed prevalence in *T. canis* between male and female foxes is significantly different (Fisher's Exact test, P=0.013). [2]This diagnosis was not confirmed by demonstrating adult worms in the colon. [3]*Capillaria* spp. eggs were not identified to species level due to morphological changes as a result of freezing and thawing. Methods used for detection and speciation. D: dissection, CSF: centrifugal sedimentation / flotation, MS: mucosal scraping, WS: washing and sieving. Species number 6, 9 and 10 were obtained from heart and lung washings for which 96 foxes were available. *: Four out of four urine bladders were found positive for this species, but prevalence was not extrapolated from this limited number of analyses.

Table 2 - Parasite prevalence in red fox compared to 35 years ago.

	Zoonotic species	Netherlands Borgsteede (1984)	Netherlands This study	Fisher Exact P (2-sided)
Intestinal nematodes		**% (n=137)**	**% (n=136)**	
Toxocara canis	Yes	73.7	61.0	0.028
Toxascaris leonina	No	0	2.2	0.122
Trichuris sp.	No	0	16.9	<0.0001
Uncinaria stenocephala	Yes	59.9	54.4	0.393
Strongyloides sp.	Yes[1]	0.7	14.7	<0.0001
other nematodes				
Eucoleus aerophilus	No	46.8	67.7	0.285
Pearsonema plica	No	23.5	(4/4)[2]	-
Capillaria spp.			50.0	-
Angiostrongylus vasorum adults/larvae	No	(0)[3]	4.2	0.028
Crenosoma vulpis adults/larvae	No	4.5	16.7	0.008
Cestodes				
Taenia spp.[4]	Yes[5]	53.3	22.1	<0.0001
Mesocestoides sp.	No	0	5.9	0.003
Echinococcus multilocularis	Yes	0	0.7	0.498
Trematodes				
Cryptocotyle lingua	No	3.6	3.7	1
Eupariphium melis	No	1.5	0.7	1
Alaria alata	No	10.9	16.9	0.166
Opistorchis felineus	Yes	0	0	-
Apophallus donicus	No	0.7	0	0.498
noninfected (over-all)		2.9	2.9	

Differences between this study and the Borgsteede study (1984) are indicated (Fisher's exact test). **[1]** *Strongyloides* species are non-zoonotic, whereas *S. stercoralis* is infectious to humans and is a species of warm geographical zones, although found in a dog kennel in Finland (Dillard et al., 2007). **[2]** This species was present in four analysed urinary bladders, therefore prevalence difference was not analysed. **[3]** The first documented cases of autochthonous French heartworm were seen in 2009.

Legend to Table 2 (continued)
[4] Data on *Taenia* species were combined to facilitate comparison with other studies. [5] In our study, *T. crassiceps* and *T. polyacantha* were found, the former of which is zoonotic.

E. Multilocularis-specific PCR identification

All foxes were negative for this species by microscopical examination of mucosal scrapings, but one out of 262 investigated foxes was positive for *E. multilocularis* (prevalence 0.7%; 95%CI 0.02-2.1%), using the 12S single tube nested PCR and subsequent southern blot analysis on faecal content. This positive result was confirmed after repeated testing of the fecal content. Up to this study, no positive foxes were identified in the presently studied area.

Molecular characterisation of intestinal parasites

PCR products of *Taenia polyacantha*, *Taenia crassiceps* and *Alaria alata* were all 403 bp in length. These DNA sequences were submitted to Genbank [accession numbers KF751222 - KF751223 (*T. crassiceps*, isolates V1382 and V1336), KF751225 - KF751226 (*T. polyacantha*, V1361 and V1269) and KF751233 - KF751234 (*A. alata*, V1338 and V1359)].
Microscopic identification of cestodes was confirmed by cluster analysis of the partial CO1 gene sequences. The inferred Neighbour Joining tree shows very high homology between obtained CO1 sequences and Genbank entries for *T. crassiceps* from Russia and Norway (EU544549), *T. polyacantha* from Denmark and Finland (EU544583, EU544584, EU544585 and EU544586) and for the trematode *A. alata* from Lithuania and Germany (HM022221, HN022222 and HM022224), the latter of which served as outgroup (Figure 4).

Discussion

This study shows an increased diversity in the helminth parasite community of Dutch red foxes compared to a study conducted in the same region 35 years ago (Borgsteede, 1984). We report four new records of veterinary importance: *Toxascaris leonina*, *Mesocestoides litteratus, Trichuris vulpis* and *Angiostrongylus vasorum*. The finding of a fifth (zoonotic) species –*Echinococcus multilocularis*– has been described earlier for the Netherlands (van der Giessen et al., 1999), but not in this same geographical area.
We used a combination of microscopic and molecular techniques to evaluate the helminth fauna of red fox as described above, whereas Borgsteede (1984) and Lucius et al. (1988) used microscopy following the washing and sieving technique. Use of the more sensitive PCR technique in this present study might have biased the observed biodiversity to some extent, since it was not available in the period of the study of Borgsteede, but this does not explain the observed biodiversity increase compared to older studies. Confirmation of the identity of cestode species that had been found microscopically by

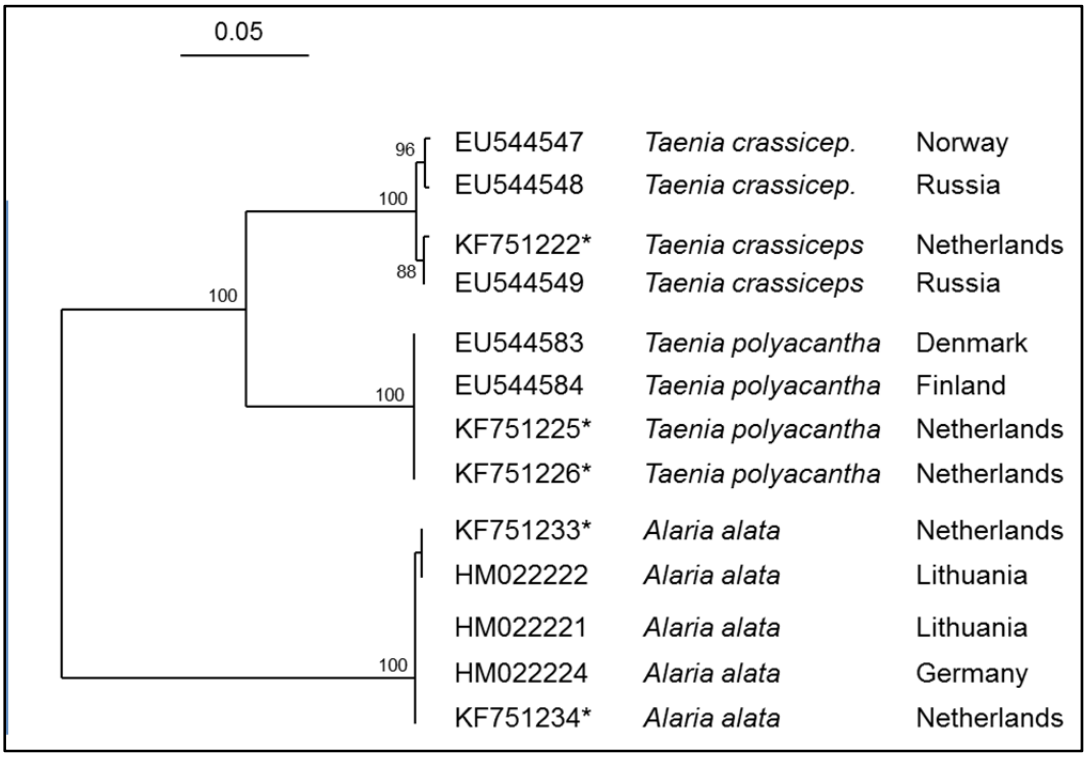

Figure 4 - CO1 Neighbour Joining Tree of European fox cestode isolates.
Taenia species found in red fox (* this study) show high homology with other European isolates found in Genbank (bootstrap values of 2500 simulations). Alaria alata is used as outgroup and here too, the Dutch isolates show high homology with other European isolates from Genbank. Bar indicates base substitutions per site.

PCR in this present study, did not lead to more cestode species compared to historic data. Moreover, even without *E. multilocularis*, which was demonstrated only by PCR, significantly more helminth species were found in this present study, compared to historical data (result not shown). The introduction of *E. multilocularis* and *A. vasorum* into the Netherlands is documented (van der Giessen et al., 2004; van der Giessen et al., 1999; van Doorn et al., 2009); these independent studies support the increased biodiversity of helminth fauna in the population of red foxes in the Netherlands. The study of van der Giessen et al. (van der Giessen et al., 1999), for which a combination of mucosal scraping and PCR was used, demonstrated presence of *E. multilocularis* in the eastern border region, both north and south to the present study area, but not in the latter, which was included in that study as well. This finding confirmed the observation of Borgsteede (Borgsteede, 1984) at that time. Parasites indicated as *Capillaria* spp. might include more fox specific species, like *Eucoleus boehmi*, which is endemic to the

Netherlands (H. Cremers, unpublished data), and other species passing through the gut after predation; however these were not further identified to species level.
Rarefaction and extrapolation of parasite richness and abundance data (this study), revealed a significant increase of species richness compared to 12 different fox parasite species determined by Borgsteede (Borgsteede, 1984), 11 species found by Lucius et al. (Lucius et al., 1988) and 9-12 species found in two regions of the former German Democratic Republic respectively in 1966 and in 1980 (Nickel et al., 1980). Recent studies in the Northern European hemisphere (Bruzinskaite-Schmidhalter et al., 2012; Saeed et al., 2006) show species richness that fits the asymptotic maximum of the estimated species richness calculated from our data. This increase might be driven by a combination of natural developments and or anthropogenic causes (global warming, climatic fluctuations). It is however, beyond the scope of this paper to identify the drivers for the observed increase in the parasite biodiversity.
Parasites of veterinary importance may be introduced into the environment through pet travel or translocation of wildlife hosts. *Angiostrongylus vasorum* only recently became endemic to the Netherlands (van Doorn et al., 2009) and is known for its endemic foci in Dutch dogs (van Doorn et al., 2009). In the present study, we found *A. vasorum*-positive foxes in the southern half of the study area, outside and distant from the published endemic foci, which demonstrates a wider endemic area sustained by the red fox.
In this study, *E. multilocularis* parasite DNA was identified by PCR in the intestinal content of one red fox in the northern part of the Dutch-German border area. The identification based solely on molecular techniques suggests a very low intestinal abundance in the infected fox, well below the detection level of microscopy. Previous studies showed PCR to be more sensitive, compared to the mucosal scraping method, especially at low endemicity (Takumi et al., 2008; van der Giessen et al., 1999).
The observed *T. canis* prevalence decline in foxes (-17%) is also recognised in the human population, since data from a Dutch cohort study show a moderate but significant decrease of *T. canis* exposure between 1998 and 2004 (Pinelli et al., 2011). However, this is not recognised in prevalence of patent infections in dogs (le Nobel WE et al., 2004; Overgaauw, 1997; Overgaauw et al., 2009; Rep, 1980).
The prevalence of *Taenia* spp. showed the sharpest decline (-59%), followed by *T. canis* (-17%), compared to the study by Borgsteede (Borgsteede, 1984). Among fox prey are rodents, which are obligate intermediate hosts in the lifecycle of cestode parasites like *E. multilocularis* and *Taenia* spp., and facultative intermediate hosts of nematodes like *T. canis.* Small mammals, especially voles (*Microtus arvalis* and *Arvicola terrestris*), comprise almost 50 % of the fox's prey during autumn and winter (Klanszki et al., 1999; Mulder et al., 2004; Voigt, 1999). The decreasing prevalence of *Taenia* spp. and *T. canis* in foxes might be correlated with the decreasing abundance of rodents (Apeldoorn, 2005; Broekhuizen, 1992), which is also indicated by decline of raptor species exclusively preying on rodents (Bijlsma, 2011; Herremans, 2011). We were able to identify *Taenia crassiceps* and *T. polyacantha* from frozen material, using morphological data in

combination with molecular techniques. A combination of detection techniques as presented in this study might be useful to increase sensitivity and specificity and to differentiate host-specific parasites from parasite eggs and/or larvae passing after ingestion of prey. CO1 gene sequences of *A. alata*, *T. crassiceps* and *T. polyacantha* from Dutch fox (this study) were homologous with isolates from European countries at the North or East of the Netherlands (Germany, Denmark, Lithuania, Finland and Russia). Previously, spatial prevalence analysis across borders demonstrated radiation of *E. multilocularis,* from the adjacent Belgian fox population to the southern Dutch fox population (van der Giessen et al., 2004; Vervaeke et al., 2006).

In conclusion, we infer a significant increase in parasitic helminths diversity in the fox population at the eastern border of the Netherlands over a period of 35 years. In the same period, the prevalence of two zoonotic helminths species belonging to different genera declined. In addition, four veterinary-important species were identified for the first time in this present study, and three additional species showed higher prevalence over that period. We identified the fox tapeworm *E. multilocularis* for the first time outside the previously described endemic spots in the Netherlands. Due to the very low prevalence and abundance, the infection risk for humans in the studied area is considered limited. It remains important, however, to follow the spread of *E. multilocularis* in this area in the future.

Competing interests

The authors declare that they do not have competing interests.

Authors' contributions

FF generated and analysed parasitological data, performed molecular lab work and sequence analysis, and wrote the manuscript, RN generated parasitological data and wrote the manuscript, JM generated biological data concerning the collected foxes, HC generated parasitological data concerning non-intestinal helminths, CD did the molecular lab work concerning *E. multilocularis*, KT wrote the study design and manuscript, and helped with statistical analysis, JvdG wrote the study design, conceived and wrote the project proposal, coordinated the study, generated parasitological data and contributed to the manuscript. All authors read and approved the final manuscript.

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Chapter 7

Antibody response against *Trichinella spiralis* in experimentally infected rats is dose dependent

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Domestic pigs are the main representatives of the domestic cycle of *Trichinella spiralis* that play a role in transmission to humans. In Europe, back yard pigs of small household farms are the most important risks for humans to obtain trichinellosis. Rats might play a role in the transmission of *Trichinella spiralis* from domestic to sylvatic animals and *vice versa*. In order to be able to investigate the role of wild rats in the epidemiology of *T. spiralis* in the Netherlands, we studied the dynamics of antibody response after *T. spiralis* infections in experimental rats, using infection doses ranging from very low (10 muscle larvae, ML, per rat) to very high (16,000 ML per rat). To evaluate the feasibility of rats surviving high infection doses with *T. spiralis*, clinical and pathological parameters were quantified. Serological tools for detecting *T. spiralis* in rats were developed to quantitatively study the correlation between parasite load and immunological response. Results showed that an infection dose dependent antibody response was developed in rats after infection with as low as 10 ML up to a level of 10,000 ML. A positive correlation was found between the number of recovered ML and serum antibody levels, although specific measured antibody levels correspond to a wide range of LPG values. Serum antibodies of rats that were infected even with 10 or 25 ML could readily be detected by use of the *T. spiralis* western blot 2 weeks post infection. We conclude that based on these low infection doses, serologic tests are a useful tool to survey *T. spiralis* in wild rats.

Keywords: *Trichinella spiralis*, rats, dose dependent response, experimental infection

Introduction

Trichinella spiralis is the only known *Trichinella* species out of 12 recognized species or genotypes (Pozio et al., 2009a) that is transmitted and maintained in both a domestic and sylvatic cycle. The *T. spiralis* sylvatic cycle involves omnivores like wild boar, carnivores like wolf and fox, but also scavenger wild rodents (Pozio, 2000; Pozio et al., 2009b). *T. spiralis* is distributed worldwide and maintained in pigs as one of the most important representatives of the domestic cycle. In Europe, free ranging pigs of small household farms are the most important risk for public health (Pozio et al., 2009b).

Rats play a role in the transmission of *T. spiralis* from domestic to sylvatic animals and *vice versa*. It has been shown that pigs exposed to rats were infected more often, whereas pigs that were physically separated from rats remained free of *Trichinella* (Schad et al., 1987). Rats in the vicinity of pig farms were infected only when *T. spiralis* occurred in pigs on those farms under low sanitation level (Leiby et al., 1990; Stojcevic et al., 2004). However, it has been shown that even in the absence of a known source of infection on farm level, *T. spiralis* is able to persist in rats (Leiby et al., 1990).

In the geographical spread and maintenance of *T. spiralis* in nature, humans play a major role. Disposal of infected carcasses of pigs or hunted wild boar, wolves and foxes in nature or on waste disposal sites might be a driving force in spreading *T. spiralis* infections in wild rat populations (Mikkonen et al., 2005; Neghina, 2010). Circumstantial evidence indicated that an outbreak of *T. spiralis* in outdoor farmed wild boar could be attributed to an invasion of rats from an improperly closed down landfill in the vicinity of the farm (Jovic et al., 2001; Oivanen et al., 2002). Jovic *et al*. have shown by bioassay using rats, that *T. spiralis* larvae in artificially infected pork meat that had been buried in the ground at a depth of 30-100 cm, remain infective for rats for more than 91 days [10].

Rats were shown to be a potential reservoir host species for *Trichinella* using mathematical models, provided that cannibalism occurs (Takumi et al., 2010). It was argued in that study that rats should be included in the minimal set of wildlife species that maintain the cycle of *T. spiralis*. Even if rats do not represent an important route of *Trichinella* distribution, but are merely sentinel species, it might be useful to monitor rats for *Trichinella* in a wildlife monitoring programme. Wildlife monitoring is one of the tools indicated by the EU regulation 2075/2005EU to control Trichinella (European-Commission, 2005). The results of a rodent monitoring might give additional information about Trichinella dynamics in wildlife and might also be useful in a more generic wildlife monitoring programme.

In this study, we developed serological tools to quantitatively study the correlation between parasite load and immunological response of artificially *T. spiralis* infected rats at different infection levels. To augment the dynamics of *T. spiralis* in infected rats using different infection doses, and to evaluate the probability of rats surviving high infection doses with *T.spiralis*, clinical and pathological parameters are quantitatively described as well.

Material and methods

Experimental infection

Male Wistar Unilever rats weighing 230-280 g were infected with *T. spiralis* muscle larvae (strain ISS 14), which had been isolated by pepsin-HCl digestion from previously infected mice or rats. To assess low dose infection, thirty-six rats were divided into six groups of six animals per group and individual rats were infected with 10, 25, 50, 100, 200 and 400 ML per group. To assess high dose infection, additional thirty rats were divided into ten groups of three animals per group and individual rats were infected with 200, 400, 2,000, 4,000, 6,000, 8,000, 10,000, 12,000, 14,000 and 16,000 ML per group. On the first day of the experiments, all rats received ML which were delivered directly into the stomach through a gastric tube. Doses of 10 to 400 ML were prepared by counting individual larvae. Doses between 2,000 and 16,000 ML were prepared by a series of dilutions. At each dilution step, the suspensions were mixed continuously by magnetic stirring to avoid possible sedimentation of the muscle larvae. Blood from rats of both the low and high dose experiments was collected weekly via orbital puncture and sera were stored at -20°C until further use. Rats in the groups of 10,000 ML or higher showed signs of severe clinical illness and their diets were changed to soft food to prevent premature termination. Forty-two days post infection (dpi) the rats were euthanized. Body weights and clinical scores were determined daily. Clinical scores were defined as follows: 0 - no signs, 1 - rough coat and light diarrhea, 2 - rough coat, animal inactive and severe diarrhea, 3 - rough coat, inactivity, severe diarrhea and weight loss, 4 - rough coat, inactivity, severe diarrhea, more than 20% weight loss and decrease in body temperature, 5 - death. The experimental protocol was approved by the RIVM Animal Experiments Committee.

Pathology

At terminal sacrifice at 42 days p.i., samples of front leg muscle (1 animal per group, highest level of recovered muscle larvae), duodenum, heart, liver, thymus, spleen and mandibular lymph node were collected and fixed in 4% buffered formaldehyde. Tissues were processed to paraffin blocks and 4µm sections were cut and stained with H&E. All organs were examined in the lowest and highest infection dose groups, and affected organs (spleen, thymus, lymph node and muscle) also from intermediate groups.
At day 8 p.i., an autopsy was performed on the animals of the highest dose group (16,000 ML), of which the organs were examined as described above . In addition, lungs and three muscles (diaphragm, musculus masseter and quadriceps femoris muscles) and the intestines were examined histologically (small intestine 4 locations, large intestine 3 locations). Selected organs were stained with PAS additional to H&E.

Isolation of muscle larvae

From each rat, the muscle tissue was separated and *T. spiralis* ML were isolated by artificial digestion according to EU regulation 2075/2005 (European-Commission, 2005). Briefly, after weighing the carcass, all muscle tissue from each rat was isolated, its weight

established and digested using pepsin-HCl for 30 minutes at 45 ± 1°C. After two successive sedimentation steps, the resulting suspension containing ML was transferred to Petri dishes and the number of isolated larvae was determined by two technicians. These counts were used to calculate average values and the number of larvae per gram (LPG) muscle tissue per rat.

Production of *Trichinella* ES antigen

T. spiralis excretory/secretory (E/S) antigen was produced as described previously by Gamble (Gamble, 1985). Briefly, *Trichinella* ML (ISS 14) were isolated from mouse muscle tissue by artificial digestion and washed three times in PBS containing penicillin (12 mg per 100 ml) and streptomycin (20 mg). Washed ML were incubated at a concentration of 10,000 larvae per ml in a CO_2 incubator for 18 hours at 37 °C in 250 cm^2 culture flasks containing 70 ml RPMI 1640 culture medium (supplemented with 1% glutamine and 1% penicillin/streptomycin, Gibco 10378, Invitrogen, Bleiswijk, The Netherlands). The culture medium was separated from the ML by centrifugation and the supernatant was concentrated in a dialysis membrane (Spectra/Por molecularporous membrane MWCO 6-8,000, Spectrum Laboratories, Inc., Rancho Dominguez, USA) by dehydration using polyethylene glycol 20,000 (PEG, Fluka 81300, Sigma-Aldrich Chemie GMBH, Steinheim, Germany). The concentrated culture medium was dialyzed two times over-night against PBS at 4 °C and the dialyzed and concentrated solution containing *T. spiralis* ES proteins was further concentrated using 5 kD molecular weight cut-off filters (Amicon Ultra Centrifugal Filter Devices, Millipore, Carrigtwohill, Ireland). The protein concentration was determined by BCA protein assay (Pierce, Rockford, IL).

In-house ELISA

Antibody responses of the infected rats were determined by an in-house ELISA. Flat-bottom ELISA plates (Nunc 66904) were coated with 0.125 µg of *T. spiralis* ES antigen per well for one hour at 37 °C. After washing (PBS/0.05% Tween 20 (PBS-T)) and saturation (1% BSA w/v in PBS-T at 4 °C overnight), sera were applied (100 µl per well, 1/100 dilution in PBS-T) for one hour at 37 °C. After washing, each well was incubated for one hour at 37 °C with 100 µl Goat anti-Rat IgG conjugated to Horse Radish Peroxidase (1/8000 in 1% BSA/PBS-T). After washing, antigen-antibody interaction was visualized by adding 100 µl Sure Blue™ TMB substrate solution (KPL, Gaithersburg, USA) per well and incubation for ten minutes at room temperature. The reaction was stopped with 100 µl 2M H_2SO_4 and optical density (OD) values were determined with an ELISA reader (Bio-Tek EL808 Ultra Microplate Reader, Bio-Tek Instruments, Inc.) at 450 nm.

Calculation of normalised OD and cut-off

Sera were tested in duplicate and normalized OD minus blank (OD_n) values were calculated using the formula $OD_n = (OD-b)/a$, where OD is the average OD minus blank value of the duplicates, a is the x-variable and b the intercept from the linear regression analysis tool in Microsoft Excel.Positive and negative rat control sera were used to

determine variables *a* and *b* for each ELISA plate to correct for inter-plate and day-to-day variation. The OD_n values of pre-immune (day 0) sera of all experimental rats was used to define a cut-off level as average OD_n plus 2 times standard deviation.

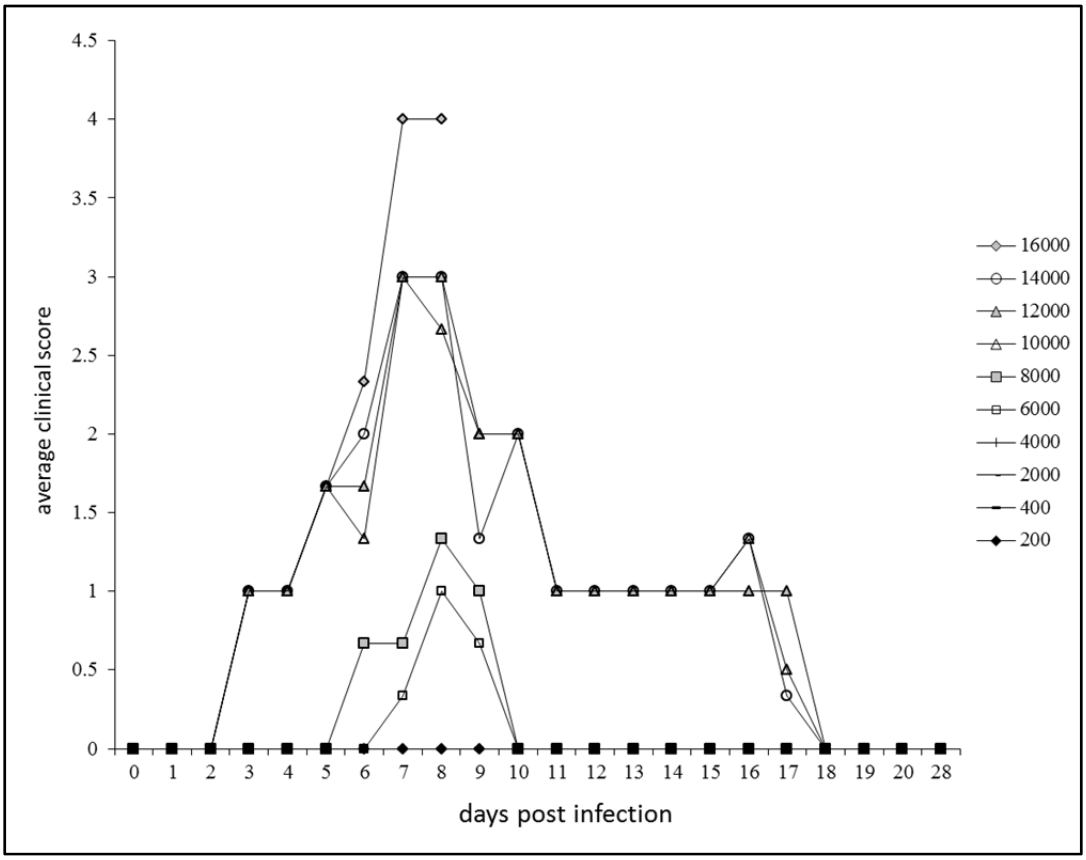


Figure 1. Average clinical scores per dose group (n=3). Clinical signs develop in animals that were infected with 6000 ML or more, mainly during the intestinal phase of *Trichinella*. In animals that were infected with 10000-16000 ML, clinical symptoms set on earlier and lasted longer as compared to rats that received lower doses.

Western blot

T.spiralis ES antigen (1 µg total protein per lane) was separated by SDS-Page and transferred to nitrocellulose membrane (Trans-Blot ® Transfer Medium, Bio-Rad Laboratories, Hercules, CA). After saturation of the membrane with 1% BSA in PBS-T (BSA/PBS-T), serum dilutions (1:50 or 1:300 (positive control) in BSA/PBS-T) were applied and immunoreactions were visualized with Goat anti Rat IgG conjugated to Horse Radish Peroxidase (Sigma-Aldrich, St. Louis, USA, 1:5000 in BSA/PBS-T) and ECL Detection Reagent (GE Healthcare Ltd, Little Chalfont, Buckinghamshire, UK), followed by detection

on a Luminescent Image Analyzer (LAS-3000, Fuji Photo Film Co., Ltd, Tokyo, Japan). Rats infected with 10, 25 or 50 ML with OD_n values roughly equal to the average OD_n in the *T.spiralis* ES ELISA were selected for testing in the western blot

Statistical analysis

The correlation between measured OD_n values and ^{10}log transformed values of recovered larvae per gram (LPG) were determined using Microsoft Excel statistical section. From the 2D normal distribution for OD_n and LPG, low estimate (0.05 percentile) and high estimate (0.95 percentile) of the conditional distribution of LPG given the OD_n value was determined.

For analysis of the dose effect on OD_n per time point, the Generalized Linear Model (GLM) Fit of Mathematica 8.0.1.0 (Wolfram, Inc, Champaing IL, USA) was used. The combined effect of time and dose was calculated in the GLM using the formula $OD_n = a+b\cdot\text{dose}\cdot\text{day}$. Probability levels less than 0.05 were considered significant.

Results

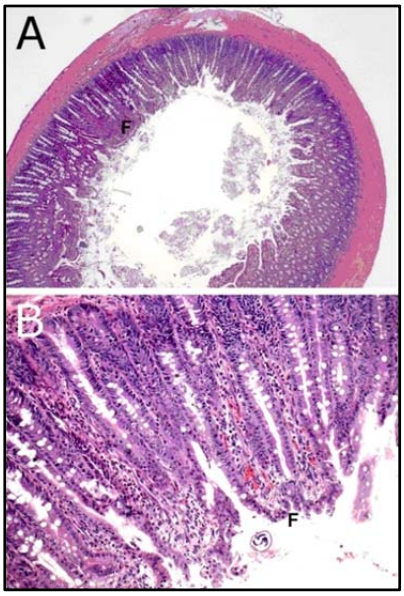
Clinical scores and body weight

No clinical signs were observed in animals that were infected with less than 6,000 ML. The rats that were infected with 6,000 or 8,000 ML showed mild clinical symptoms (maximum average clinical score 1-1.25 on day 8 p.i.) from day 5 to day 10 p.i.. In animals that were infected with 10,000-14,000 ML, clinical symptoms started earlier (day 2 p.i.), lasted longer (day 18 p.i.) and the maximum average clinical score reached level 3 in these animals (Figure1). The animals in the highest dose group (16,000 ML) were severely affected at day 8 p.i. (clinical score level 4, body weight loss 21 $\pm$ 2.6%, Fig. 1) and it was decided to euthanize these animals at day 8 p.i.. The average bodyweight of rats infected with 200-2,000 ML showed no decrease after infection with *T. spiralis* ML. The animals in the higher infection dose groups lost bodyweight in a dose dependent manner (Takumi et al., 2010). Briefly, between day 5 and day 8 p.i., the average bodyweight of the rats infected with 4,000 ML decreased with 7.2 $\pm$ 5.5%. Rats infected with 6,000-14,000 ML lost 15.8% $\pm$ 4.3% bodyweight. From day 10 onwards, the rats of the 4,000-14,000 ML infection groups regained bodyweight (129 g $\pm$ 24 g from 8 days p.i. to 42 days p.i.) at the same rate as the animals in the 200-2,000 ML groups (123 g $\pm$ 4 g).

Pathology

The rats that received 16,000 ML were euthanized at day 8 p.i. and were necropsied immediately after euthanasia. All three animals showed hypermotility of the pale intestine. The duodenum up to ascending colon were distended with clear, slightly viscous fluid containing minute flakes. Microscopically, shed villus tips were visible in these contents. The stomach contained only bile. Peyers patches and mesenteric lymph nodes were markedly enlarged. The spleen was small and the thymus very small. No body

fat was present.Histopathologically, intestinal lesions were most severe in the proximal small intestine and caecum. *Trichinella* females were most numerous in the proximal small intestine but present up to the descending colon. The mucosa of duodenum and jejunum was covered with a thick layer of PAS-positive mucus containing extruded epithelial cells. Erosion was minimal due to obvious efficient fusion of the remaining short and distorted villi (Fig. 2). Immaturity of superficial epithelium was generally observed. Inflammatory infiltration in the *lamina propria* consisted mainly of eosinophilic leucocytes, many of which were degranulated. In the caecum the submucosa was infiltrated and oedematous. Plasmacytosis of the medullary cords was responsible for the enlargement of the mesenteric lymph nodes. Other lymph nodes showed lymphoid atrophy (Fig 3A). Splenic peri-arteriolar lymphocyte sheath (PALS) and marginal zones were atrophic (Fig 3C). Follicle centres in spleen and lymph nodes showed slight necrosis. In the thymus cortex, there was complete loss of lymphocytes (Fig 3E). No abnormalities were found in liver, heart and lung. In muscles, notably in the diaphragm, acute degeneration of myocytes was seen, together with some newborn larvae. At terminal sacrifice of the other animals on day 42 p.i., no abnormalities in heart, liver and duodenum were found in any of the infection groups. The cellularity in the lamina propria of the duodenum was prominent but no difference between the 200 and the14,000 ML dose groups was observed. Mandibular lymph nodes showed an activated appearance as seen by abundant secondary follicles, paracortex and medullary cords (Fig. 3B). The spleen was often congested with prominent marginal zones (Fig. 3D). The thymus was well developed and showed no signs of regression (Fig. 3F). Only a single muscle sample was taken per treatment group. The most prominent changes were seen in the highest dose groups. Individual myofibers were swollen and degenerated and contained pale amorphous material harboring larvae (nurse cells). Muscle fibers in the vicinity were often degenerated. Inflammatory cells (predominantly macrophages) were abundantly surrounding affected fibers, but typically not directly in or on the nurse cells (the larvae or capsules) (Fig 4).



No difference was found in lymphoid organs from the intermediate dose groups. Numbers of muscle larvae increased with infection dose as evident after counting, but these data are of limited quantitative value, as only 1 muscle sample per treatment group was taken, and significant variation per muscle sample is to be expected.

Figure 2. A. Histopathology of duodenum at 8 days p.i. (16,000 ML, H&E). A. Villi are fused (F) and contain small hemorrhages as seen at magnification 20x. **B.** detail from A at magnification 100x

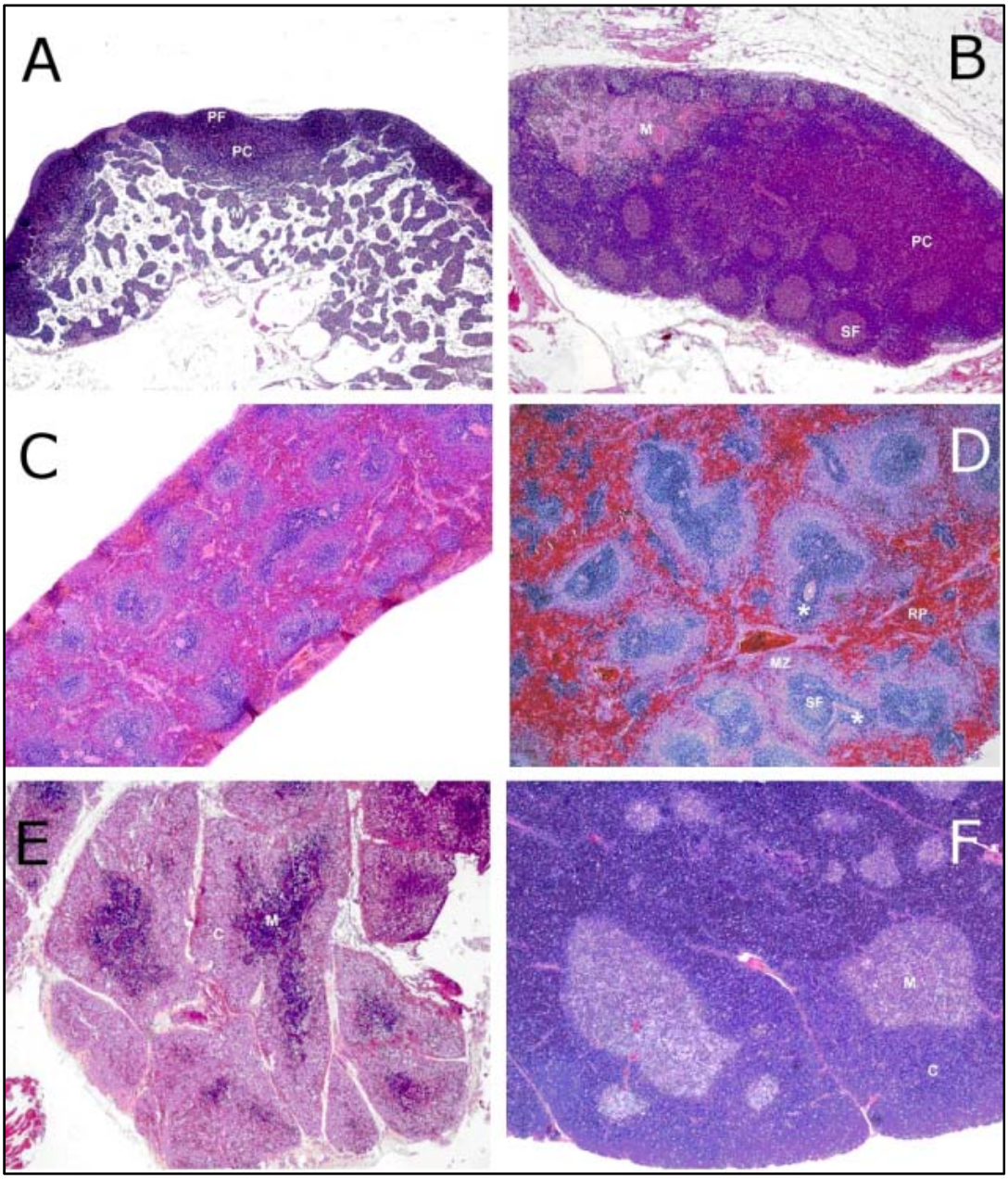


Figure 3. Histopathology of rat lymphoid organs on day 8 p.i. (16,000 ML, left panel) and day 42 p.i. (14,000 ML, right panel), H&E, magnification 40x. A. Mandibular lymph node 8 dpi; note small size, inactive (primary) follicles (PF) and paracortex (PC). **B.** Mandibular lymph node

medullary chords (M) **C.** Spleen 8 dpi, small and inactive appearance compared to **D.** Spleen 42 dpi, activated appearance: prominent secundary follicles (SF) and marginal zone (MZ) **E.** Thymus 8 dpi, smaller than F. with disappearance of cortex producing a reverse corticomedullary contrast compared to **F.** Thymus 42 dpi, prominent size and appearance with clear contrast between cortex (C) and medulla (M)

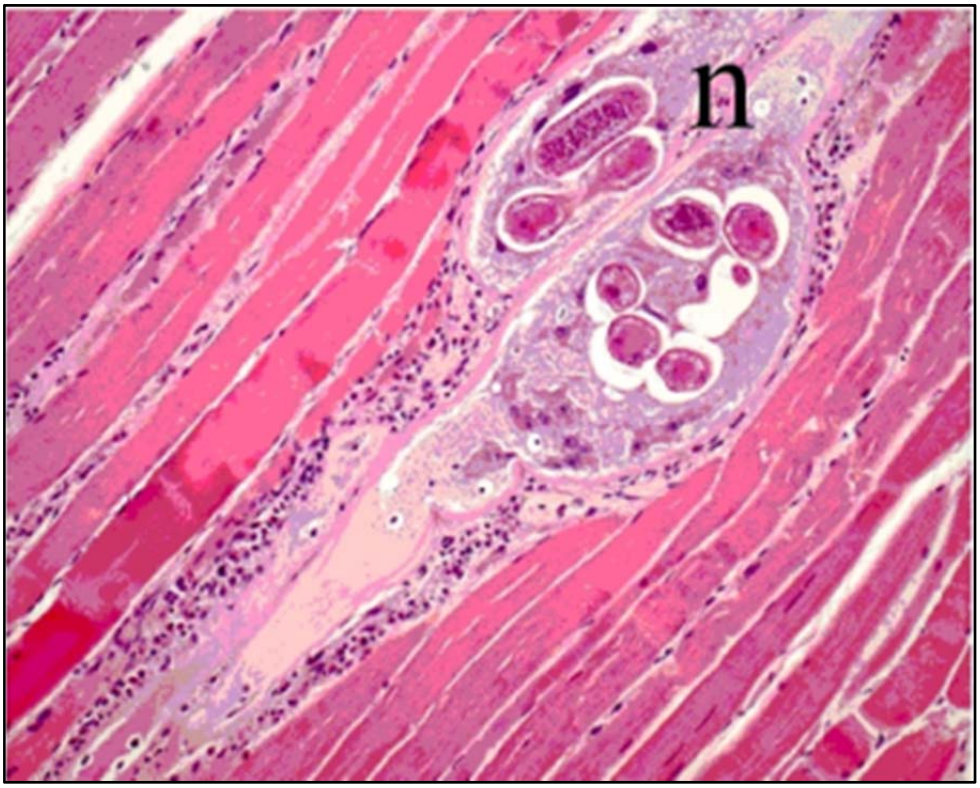


Figure 4. Histopathology of muscle tissue at 42 dpi (14,000 ML, H&E, magnification 200x). Swollen transformed muscle fibres contain live muscle larvae (Nurse cells, n) surrounded by inflammatory cells (mainly macrophages).

Recovery of muscle larvae

Administration of 10 *T. spiralis* ML resulted in active infections in all animals from which on average 6.96 $\pm$ 4.42 larvae per gram (LPG) could be recovered. Increasing infection doses resulted in increasing numbers of recovered larvae, in a non-linear manner as was demonstrated in our laboratory (Takumi et al., 2010). Infection doses above 10,000 ML

resulted in decreasing numbers of recovered muscle larvae at day 42 p.i.. No ML could be recovered on day 8 p.i. from muscle tissue of animals infected with 16,000 ML.

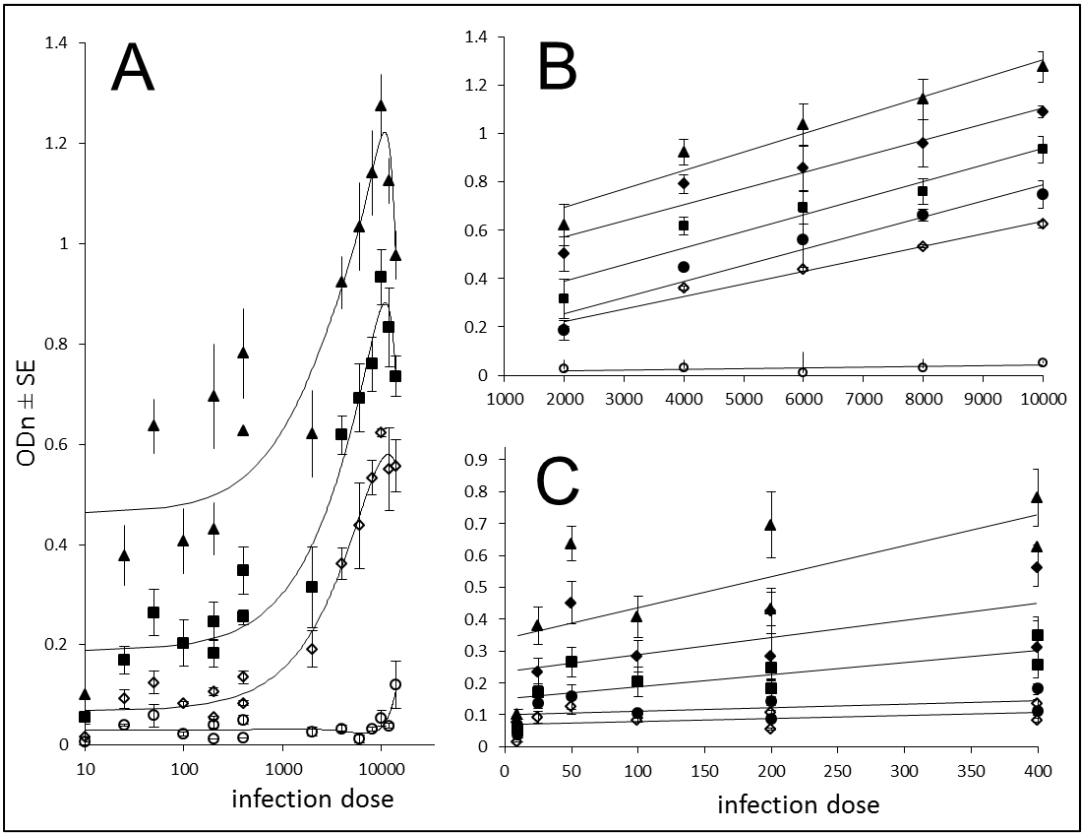


Figure 5. Measured ODn values plotted against infection dose. Immunological response on day 7 (open circle), day 14 (open diamont), day 21 (closed circle), day 28 (closed square),day 35 (closed diamond) and day 42 (closed triangle) post infection. Infection doses 200 and 400 ML of both low and high dose experiments are displayed. **A.** Average $OD_n \pm SE$ values show a positive correlation with infection, both in time and infection dose, although less pronounced at low infection doses (10-400 ML). At the highest doses (12,000-14,000 ML), OD_n values decline between 14 and 42 dpi as compared to the 10,000 ML infection group. Intermediate time points are not shown for the sake of clarity.
B. Average OD_n values of infection groups 2,000-10,000 ML correlate significantly ($P<0.05$) with infection dose on all time points **C.** Average OD_n values of infection groups 10-400 ML correlate significantly ($P<0.05$) with infection dose on all time points, except 7 dpi.

Correlation between OD and LPG

All experimentally infected rats seroconverted between 7 and 14 days p.i. with OD levels well above cut-off, except for the animals of the 10 ML infection group, which seroconverted between 14 and 21 days p.i.. In general, there is a positive correlation between OD_n and infection dose, although there is a remarkable difference between low (10-400 ML) and high (2,000-10,000 ML) infection dose ranges (Fig. 5A). OD_n values of rats that were infected with 12,000 and 14,000 ML declined as compared to the 10,000 ML infection group, reflecting the decline in LPG yield at 42 days p.i. in these infection groups (data not shown). There is a significant positive correlation between OD_n values and infection dose in the range 2,000-10,000 ML at all time points ($P<0.05$, Fig. 5B). In the lower dose groups there is a significant positive correlation at all time points, except 7 days p.i. ($P<0.05$, Fig. 5C). The increase of OD_n values over time is significant for all infection groups ($P<0.05$).

OD_n values of individual animals measured at 42 days p.i. plotted against ^{10}log recovered LPG yielded a positive correlation ($R^2=0.668$, Figure 6). However, a wide range of LPG values corresponds to a specific OD_n value (Table 1).

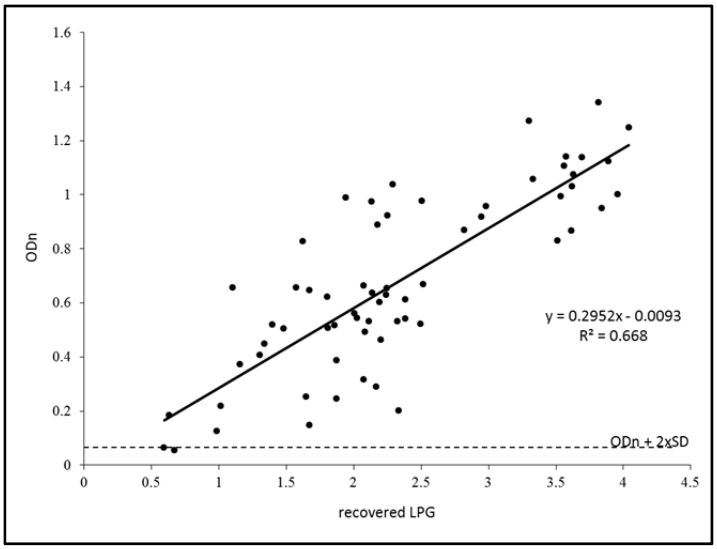


Figure 6. Relation between antibody level and recovered LPG. Individual OD_n values were plotted against ^{10}log transformed LPG, calculated from the number of larvae per rat that was isolated by artificial digestion. Data points represent individual animals of both the low- and high dose infection experiments. All but one points are above the cut-off level (OD_n 0.061).

Western blot

Infection with as little as 10 ML is sufficient to generate an IgG response on 14 days p.i. in the *T. spiralis* ES western blot. Although the signal was fairly weak with a serum dilution of 1:50, bands were clearly visible (Figure 7). Moreover, by use of Western blot, seroconversion is detected at 14 dpi, which is one week earlier for this low infection level, as compared to ELISA. The seroconversion between day 14 and 21 p.i. as measured with ELISA is confirmed by an increase in signal with Western blot. With increasing infection dose, the number and intensity of recognized bands is enhanced.

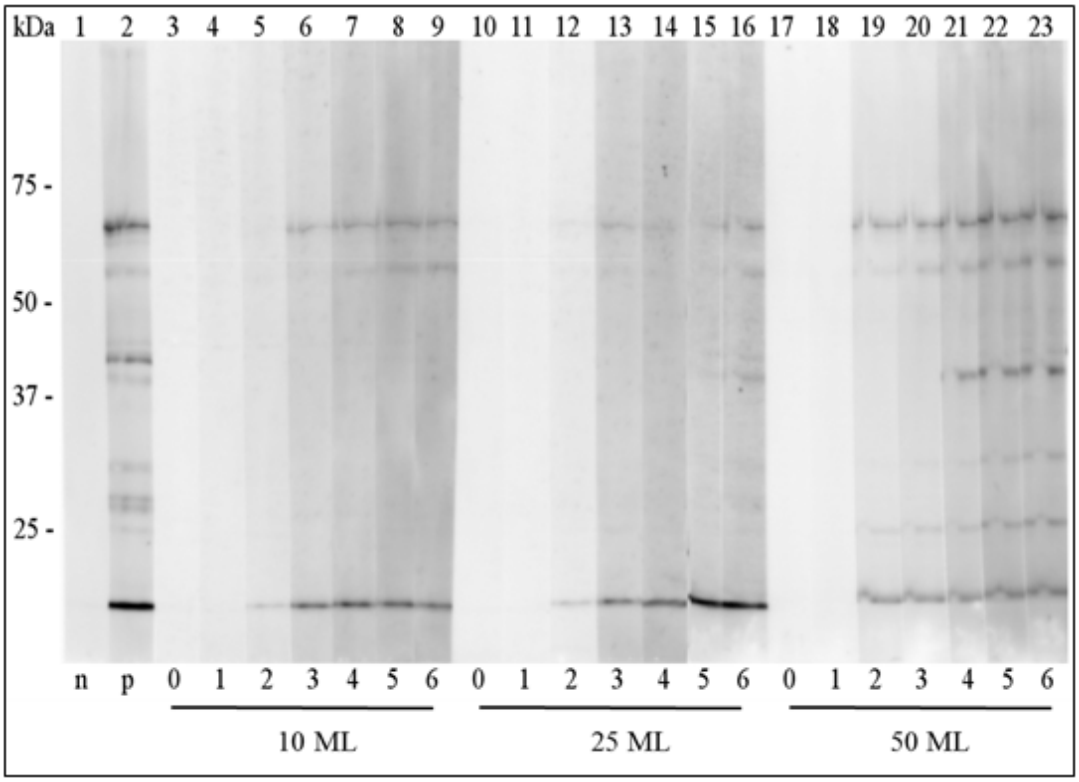


Figure 7. Immune response of experimentally *T. spiralis* infected rats as determined in Western blot. Lane 1: negative control (pooled pre-immune rat sera), lane 2: positive control (day 42 serum of a rat infected with 200 *T.spiralis* ML), lanes 3 - 9: experimental rat serum 0-6 weeks p.i. with 10 ML infection dose, lanes 10 – 16: experimental rat serum 0-6 weeks p.i. with 25 ML infection dose, lanes 17-23: experimental rat serum 0-6 weeks p.i. with 50 ML infection dose.

Discussion

The present study confirms that *T. spiralis* is highly infective at very low doses in Wistar rats and can easily be detected by serology or pathogen detection using digestion. We describe the dynamics of *T. spiralis* infection in rats using doses ranging from very low (10 ML per rat) to very high (16,000 ML), with the aim to study the usefulness of rats as sentinel animals in a wildlife monitoring program for *Trichinella*. Rats are sentinels for several human pathogens and have several advantages for epidemiological studies as mentioned by Psaroulaki *et al*. (Psaroulaki et al., 2010). This is the first report describing

the clinical, pathological, immunological and parasitological findings using this wide a dose range of

Table 1. Low estimate (0.05 percentile) and high estimate (0.95 percentile) of the conditional distribution of expected LPG given the OD value.

OD measured	Low estimate LPG	High estimate LPG
0	3	19
0,1	5	31
0,2	7	51
0,3	12	84
0,4	19	138
0,5	31	228
0,6	51	377
0,7	84	623
0,8	138	1029
0,9	228	1700
1	376	2809
1,1	621	4642
1,2	1025	7672
1,3	1694	12678
1,4	2799	20953

T. spiralis in experimental infection in rats. Infection with 10-2,000 ML caused no clinical symptoms in experimentally infected rats. Body weights of rats infected with 4,000 ML or more, declined in a dose-dependent manner until day 8-9 p.i. This is probably caused by diarrhea and a lesser food intake during the intestinal phase of *T. spiralis* infection as observed before by Aulí and Fernández (Auli and Fernandez, 2005). Although the probability of survival might have been biased by administering soft food, all animals, except the rats in the highest dose group (16,000 ML) were able to overcome illness, regained weight and survived until the end of the experiment. This is corroborated by the differences in post mortem pathological findings between rats euthanized and examined at day 8 p.i. (16,000 ML infection group) and rats euthanized at day 42 (200-14,000 ML). During the intestinal phase of *T. spiralis* (day 8 p.i., 16,000 ML), rats showed increased mucus production and intestinal hypermotility. This is in accordance with the model of Khan and Collins (Khan and Collins, 2004), which describes enteritis induced Th2 immune response resulting in intestinal muscle hyperactivity and increased mucus production by goblet cells.

Histological changes were most prominent in the duodenum at day 8 p.i., while on day 42 the duodenum was not altered in any of the other infection groups. Also the thymus showed severe atrophy at day 8 p.i., but showed no pathological alterations after 42 days p.i. in the other infection groups. Both features indicate successful regeneration of these organs after regression and inactivity indicative of severe biological stress. Findings in the

spleen and (mandibular) lymph nodes seen on day 42 p.i. were considered indicative of systemic immune stimulation. The finding of wild caught *Rattus norvegicus* with very high LPG, ranging from 5,720 to 7,692 (Leiby et al., 1990; Mikkonen et al., 2005), indicates that also in nature wild rats are able to survive these high infection levels. Infection with high doses of *T. spiralis* ML is characterized by a large variability in the numbers of muscle larvae that developed in individual rats. The maximum number of recovered muscle larvae was found in rats infected with 10,000 ML. Rats infected with 12,000 ML developed fewer muscle larvae and infection with 14,000 ML produced fewest muscle larvae. This is probably due to severe competition for space (in intestinal epithelium as well as in muscle fibers) and nutrients, in combination with expulsion of *T. spiralis* intestinal stages by the host. No ML could be recovered on day 8 p.i. from muscle tissue of animals infected with 16,000 ML, although some ML could be identified in diaphragm after histology. This might indicate that the intramuscular larvae might have been destroyed after digestion at this early developmental stage, since the protective nurse cell formation process starts at 8 days p.i. and is completed only on day 26 p.i. (Despommier, 1998).

One of our goals was to determine whether the ELISA was a suitable tool for detecting *T. spiralis* in rats. Therefore, it would be necessary to determine (1) the correlation between OD and infection dose in time and (2) the correlation between measured OD and exact numbers of *T. spiralis* ML in the rat. This is important to be able to evaluate ELISA results of future serological surveys in wild rats.

There was a clear antibody response in all infection groups. Seroconversion in rats infected with 25 ML or more, took place between days 7 and 14 p.i., but already at day 7 p.i., OD_n levels of rats infected with 14,000-16,000 ML were above cut-off. Rats infected with 10 ML showed a gradual linear increase in OD above cut-off level from 14 to 42 days p.i. We found a positive correlation between OD_n values and infection dose, although less pronounced in the 10-400 ML infection groups. Some studies, also performed in rats, focused mainly on immune response with a limited number of infective doses. Salinas-Tobon *et al*. (Salinas-Tobon et al., 2007) performed experiments in rats with infection doses of 700, 2,000, 4,000 and 8,000 ML. Specific antibodies did not increase proportionally with the different ML doses tested, but the seroconversion period that occurred between 10 and 19 days p.i., varied according to the infection dose.

We found a positive correlation between the number of recovered ML and serum antibody levels, although the predictive value of measured OD to estimate infection levels is limited, reflecting host variation in the immune response against a *Trichinella* infection. The kinetics of anti-*T. spiralis* newborne larvae (NBL) immunity and its dose effects were studied *in vivo* by Wang (Wang, 1998). In that study, rats were infected with 500, 2,000, 5,000 and 6,000 ML and immune response was measured as reduction in numbers of recoverable NBL after intravenous challenge with 10,000-100,000 NBL on day 16 p.i.. One of the results of that study was that infection with 2,000 ML *per os* induced the strongest immunity and that high dose immunization might induce a suppressive effect on host

immunity. Our results demonstrated the exhausting effect on rats for doses above 8,000 ML and the more moderate clinical effects for doses of 6,000 and 8,000 ML. The lower immune effects with 5,000 and 6,000 ML observed by Wang (Wang, 1998) could reflect this strong impact on rat health rather than a real suppressive effect on the immune system. Serum antibodies were not measured in that study.

In our study, infection dose and OD correlated linearly at all time points after infection with 2,000-10,000 *T. spiralis* ML. Infection with lower doses resulted in a positive correlation with OD, but only after 14 days p.i. or more, and at a lower, though increasing level with time. Infection with higher doses (12,000 and 14,000 ML) resulted in lower numbers of recovered larvae as compared to the 10,000 ML infection group (70% and 60% respectively), which explains the observed comparable decline in OD_n between 28 and 42 days p.i. However, at day 7 p.i. the OD_n values in the 12,000-16,000 ML infection groups are considerably higher than those of the 10,000 ML group.

Rats that were infected with 10, 25 or 50 ML showed a serological response in the *T.spiralis* western blot 2 weeks post infection. Other rodents like mice have been experimentally infected with low doses of 5, 10, and 50 ML of *Trichinella spiralis* per animal (Dvoroznakova et al., 2011; Reiterova et al., 2009). Seroconversion was observed in these experiments at 30 days p.i. with an ES-ELISA and specific antibodies increased until 80 days p.i.. Measured splenic T-lymphocyte activity increased from day 10 to day 15 p.i., even with 10 ML as infective dose (Dvoroznakova et al., 2011). This implies that for epidemiological studies, low infection levels can be detected by serology, both in rats and in mice and we confirm that low infection doses of *T. spiralis* larvae induce the production of specific antibodies at detectable levels in rats. These low doses reflect the infection level that we can find in the sylvatic cycle. Hurnikova and Dublinsky (Hurnikova and Dubinsky, 2009) underlined that *Trichinella* (*britovi, pseudospiralis* and *spiralis*) infection in wild foxes is usually below 20 LPG and far less in wild boar. In The Netherlands, *Trichinella britovi* infection in wild foxes is even lower with LPG ranging from 0.04 to 0.71 (van der Giessen et al., 1998). For confirmation of rat sera from animals with low *Trichinella* infection levels or higher infection levels early in the time course of infection, that exhibit OD values around the cut-off, we showed that western blots are a suitable instrument.

The results of our study confirmed previously conducted studies that were performed with very limited infection dose ranges in other host species like cattle (Smith et al., 1990), sheep (Pajersky et al., 1996; Tomasovicova et al., 1991), goat (Korinkova et al., 2006; Reina et al., 1996), horse (Pozio et al., 2002; Smith and Snowdon, 1987; Soule et al., 1993), wild boar, pig and fox (Korinkova et al., 2008; Moller et al., 2005; Nockler et al., 2005). In these studies, animals were experimentally infected with *T. spiralis* or other *Trichinella* species. As in our study, in most of these experiments the time point of seroconversion and specific antibodies titer were also dose dependent.

In summary, we showed that rats, even infected with a low dose of 10 *Trichinella* ML, develop an immune response, which can be detected by use of serological assays and this

immunological response is dose dependent up to an infection level of 10,000 ML. This indicates that the *Trichinella* ES-ELISA and Western blot are useful instruments to detect the presence of *T. spiralis* in sentinel populations like wild rats, which easily cross sylvatic-domestic borders. We also showed that antibody levels can not be used to calculate exact LPG values in rats due to high variation in infection rates.

Ethical issues

Experimental infections in rats were conducted according to the Dutch laws applicable. The Central Animal Laboratory of the National Institute for Public Health and the Environment, the Netherlands, possesses a license under the Dutch 'Animal Experiments Act'. In accordance with Section 14 of this act, an officer has been appointed to supervise the welfare of laboratory animals.

Competing interests

The authors declare that they have no competing interests

Author's contributions

FFJF generated and analyzed parasitological and serological data, and wrote the manuscript first draft. MF and AG contributed to the generation of parasitological data. KT carried out the statistical analysis of the data. MAK and PWW carried out the pathological and histochemical analysis, and data interpretation. IV and PB contributed to the design and analysis of the studies. JWBG conceived and designed the experiments, coordinated the study and contributed to the manuscript first draft. All authors read, approved and contributed to the final manuscript.

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Chapter 8

Zoonotic helminths in black rats (*Rattus rattus*) and brown rats (*Rattus norvegicus*) from different environments in the Netherlands

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Rattus norvegicus (brown rat) and *Rattus rattus* (black rat) are known carriers of bacteria, viruses and parasites of zoonotic and veterinary importance. Moreover, rats may play a role in the transmission of muscle larvae of the zoonotic nematode *Trichinella spiralis* to farm animals. We aimed to study the intestinal and intramuscular helminths in wild rats from three different environments to assess the relevance of rats as carrier of zoonotic parasites for public health.

Wild brown rats (117 individuals) and black rats (44 individuals) were captured at farms, in urban and in rural environments in the Netherlands. Intestinal helminths were isolated and identified morphologically. Artificial digestion was used to isolate muscle larvae.

Morphological analysis of rat intestinal contents yielded six nematode species (*Syphacia muris, Heterakis spumosa, Aonchotheca murissylvatici, Trichuris muris, Nippostrongylus brasiliensis* and *Strongyloides* sp.), three cestode species (*Hymenolepis diminuta*, *H. nana* and *Hymenolepis* (=*Rodentolepis*) *fraterna*) and four trematode species (*Plagiorchis muris, Plagiorchis proximus, Echinostoma chloropodis* and *Notocotylus imbricatus*).

Black rats at farms displayed the lowest intestinal helminth species variation (6 species) and carried overall on average 0.93 species simultaneously. In comparison, brown rats at farms carried 7 helminth species and 1.91 species simultaneously. Brown rats from suburban environments displayed the highest species variation (11 species) at 1.82 simultaneous helminth species. Absence of trematodes from rats at farms may suggest limited exchange of rats between farms and surrounding wet rural environments. We report four species of veterinary (*Syphacia muris*) or zoonotic relevance (*Hymenolepis diminuta*, *Hymenolepis nana* and *Plagiorchis muris*). We did not find *Trichinella* muscle larvae, consistent with long-term prevalence in Dutch wild rats.

Key words: brown rat, *Rattus norvegicus*, black rat, *Rattus rattus*, intestinal helminths, Hymenolepis, Syphacia, Trichinella

Introduction

Rattus norvegicus (brown rat) and *Rattus rattus* (black rat) are known carriers of bacteria, viruses and parasites of zoonotic and veterinary importance (Meerburg et al., 2009; Reperant et al., 2009). In the Netherlands, wild rats from livestock farms have been studied to elucidate their role in spreading of *Coxiella burnetti* (Reusken et al., 2011) and methicillin-resistant *Staphylococcus aureus* (van de Giessen et al., 2009). Some decades earlier, *Trichinella* prevalence was monitored in Dutch wild rats, mice, voles, badgers, martens and muskrats (Franchimont et al., 1993; Kampelmacher et al., 1966; van Knapen et al., 1993). However, a comprehensive recent parasitological survey in wild rats has not been published before in the Netherlands. We were particularly interested in zoonotic parasites to assess the relevance of rats for veterinary public health.

Wild brown rats (117 individuals) and black rats (44 individuals) were captured at different farm types, in urban environments and in rural environments in the Netherlands. Of each rat, intestinal helminths and presence of muscle larvae (*Trichinella* spp.) were analysed. Parasitological data were analysed statistically to evaluate helminth species variation and simultaneous helminth infections in black rats and brown rats from different environments

The aim of the present paper was to study the intestinal and intramuscular helminths in wild rats and to evaluate parasite species distribution in three different environments, to assess the relevance of rats carrying zoonotic parasites for public health.

Materials and methods

Wild rats

The highest concentrations of animal husbandry in the Netherlands is found in the two southern provinces Noord-Brabant (Brabant for short) and Limburg; therefore, most rats were captured in 43 six-digit postal code areas of this part of the country. Brown rats were captured at twelve farms and black rats at fourteen farms. All animals were captured based on convenience sampling by a pest control agency using live traps (Killgerm Inc., Turnhout, Belgium) baited with food.

Location type

Six-digit postal code areas were checked using Google Maps in the Internet with satellite view, and used in combination with other database entries, to divide trapping locations into three categories: farms (pigs, cattle, goats, and poultry), rural environments

(locations outside town boundaries, including ditch banks, agricultural use) and suburban environments (outskirts of rural towns and small cities, including ditch banks).

Age

The age of brown rats was estimated using weight categories adapted from Davis (Davis, 1949). Characteristic indicators for sexual maturity of rats are scrotal testis for males and vaginal perforation and pregnancy for females. We defined weight categories for brown rat males: juvenile (< 100 g), young adult (101 – 200 g) and adult (> 200 g), and for females: juvenile (< 100 g), young adult (101 – 175 g) and adult (> 175 g).
For black rat, the age categories were defined as follows; for males: juvenile (< 100 g), young adult (100 – 150 g), adult (> 150 g), and for females: juvenile (< 90 g), young adult (90 – 130 g) and adult (> 130 g).

Season

We used meteorological boundaries for season: spring (March – May), summer (June – August), autumn (September – November) and winter (December – February).

Samples for analysis

After direct transportation to the National Institute for Public Health and the Environment (RIVM, Bilthoven, the Netherlands), rat species, sex and weight, location and location type of capture were recorded. The rats were anaesthetized with isoflurane and were euthanized by cardiac puncture.
Upon death, the intestinal tract including the stomach was taken from each rat for parasitological examination. Diaphragm and muscles of both hind legs of 132 wild rats were separated for *Trichinella* analysis by artificial digestion. All biological materials were stored either at 4 °C for immediate evaluation or at -20 °C until further use. The Institute's Animal Ethical Committee has approved all experiments using wild rodents prior to the study (RIVM/DEC permits 200900164 and 201200208).

Isolation of muscle helminths

The diaphragm and muscle tissue of both hind legs were separated from each rat and analysed by artificial digestion according to EU regulation 2075/2005 (European-Commission, 2005). Since the diaphragm is a predilection site for *Trichinella* in most mammals, *Trichinella* ML can be expected in this muscle, even at low levels of infection. For analysis, 3.6-11.8 g muscle tissue per rat (depending on age and size of the animal) was pooled up to a maximum of 115 g in total. Subsequently, the pooled muscle tissue was minced and digested in aqueous pepsin-HCl for 30-60 minutes at 46 °C. After two successive sedimentation steps, the resulting suspension was transferred to petri dishes

and examined microscopically for isolated larvae. Muscle samples of 20 animals that had been frozen before, were analysed using a validated sequential sieving method to detect *Trichinella* larvae (Franssen et al., 2014).

Isolation of intestinal helminths

Intestines of the brown rats and black rats were separated individually into small (duodenum up to ileum) and large intestine (including caecum and colon) and their contents were firmly squeezed into 40 ml of PBS using forceps, and subsequently were homogenized by vigorous shaking. The resulting suspension was poured over a 63 µm mesh size sieve and the retentate was extensively washed with tap water to remove debris. The washed retentate was flushed into a 14 cm diameter Petri dish and the suspension was meticulously examined to collect all intestinal parasites under a dissection microscope. We were confident that the squeezing technique was efficient, judging the large amount of villus epithelium in the isolates. The stomach was opened to screen for parasites macroscopically and microscopically. Isolated parasites were counted, sexed and identified morphologically. Subsequently, the parasites were stored in labelled, screw-capped glass tubes containing 70% ethanol until further use.

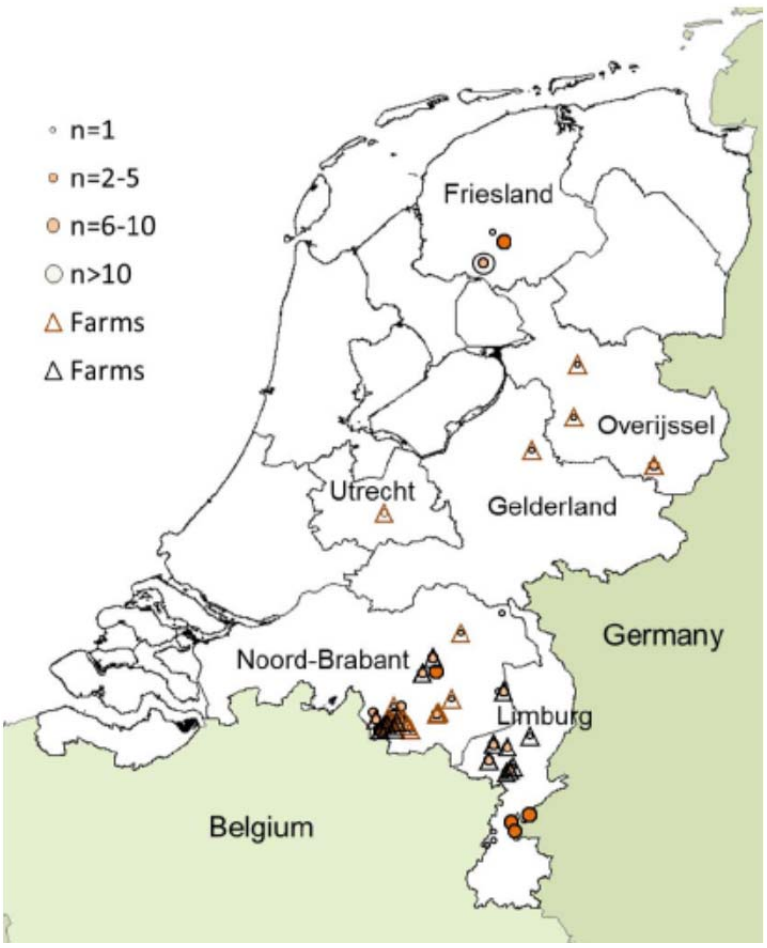
Generalised Linear Model (GLM) analysis of helminth data

Prevalence was determined for all isolated helminth species, stratified by rat host species and location type where rats were captured. Numbers of simultaneously occurring helminth species infesting each individual rat, were analysed in relation to the categorical predictors: 'host species (brown / black)', 'host sex (male / female)', 'host age (juvenile / young adult / adult)', 'season (spring / summer / autumn / winter)', 'year', 'province (Brabant / Limburg / Friesland / Others)', 'location type (farm / rural / suburban)'. Additionally, the square root of the cumulative helminth abundance was included as numerical, as indicator for exposure. In formula: $nHelminth\ species \sim host\ species + host\ sex + host\ age + season + (year + province + location\ type)^2 + \sqrt{(cumulative\ abundance)}$. The square sign indicates interaction between the factors year, province and location type. The square root transformation of variable cumulative abundance is based on visual inspection of the scatterplot for helminth species against cumulative abundance (Figure 5B). Since the number of species is a non-negative count ($n \geq 0$), a Poisson regression with logarithmic link function was applied. Subsequently, the optimal model fit was evaluated by backward and forward model selection in 'R' to select the model with the lowest AIC-value (Akaike's Information Criterion).

Results

Wild rats

From December 2009 until December 2010, 108 wild brown rats and black rats were captured in the provinces Limburg and Noord-Brabant at farms, in rural and in suburban environments. Five brown rats were captured in February 2010 on a goat farm in the province of Overijssel, two brown rats were captured in March and April the province of Gelderland (of which one from a goat farm). One brown rat was captured in April on a goat farm in the province of Utrecht. In November and December 2011, an additional 25 brown rats were captured in urban and rural environments in the province of Limburg. Finally, 23 brown rats were captured in rural environments in Friesland between May 2012 and August 2013 (Figure 1). In the two southern provinces of Brabant and Limburg, 133 rats were captured, divided over 43 six-digit postal code areas. Table I provides further geographical and temporal details.



Rat species

In total, 117 brown rats and 44 black rats were captured; black rats were captured only in Noord-Brabant and Limburg, which is the prime distribution area for this species in the Netherlands (Figure 1). In the provinces of Brabant and Limburg, the majority of black

Figure 1. Geographical origin of captured wild rats. Dots indicate number of rats that were captured. Triangles indicate farms where brown (brown triangle) and black rats (black triangle) were captured.

rats (93.2%) were caught on farms, whereas only 25.8% of brown rats were captured at farms, consistent with habitat preferences of the two species. Proportionally, most black rats were captured in summer and least in winter (Figure 2A); the majority of black rats was captured in 2010 (Figure 2B). Overall, slightly more males (50.9%) than females (46%) were captured and the majority of rats were adults (54.0%) or young adults (25.5%); brown rat males (244 ± 95 g) weighed more than females (206 ± 78 g), whereas black rat females (132 ± 60 g) were heavier than males (119 ± 68 g) (Table 1).

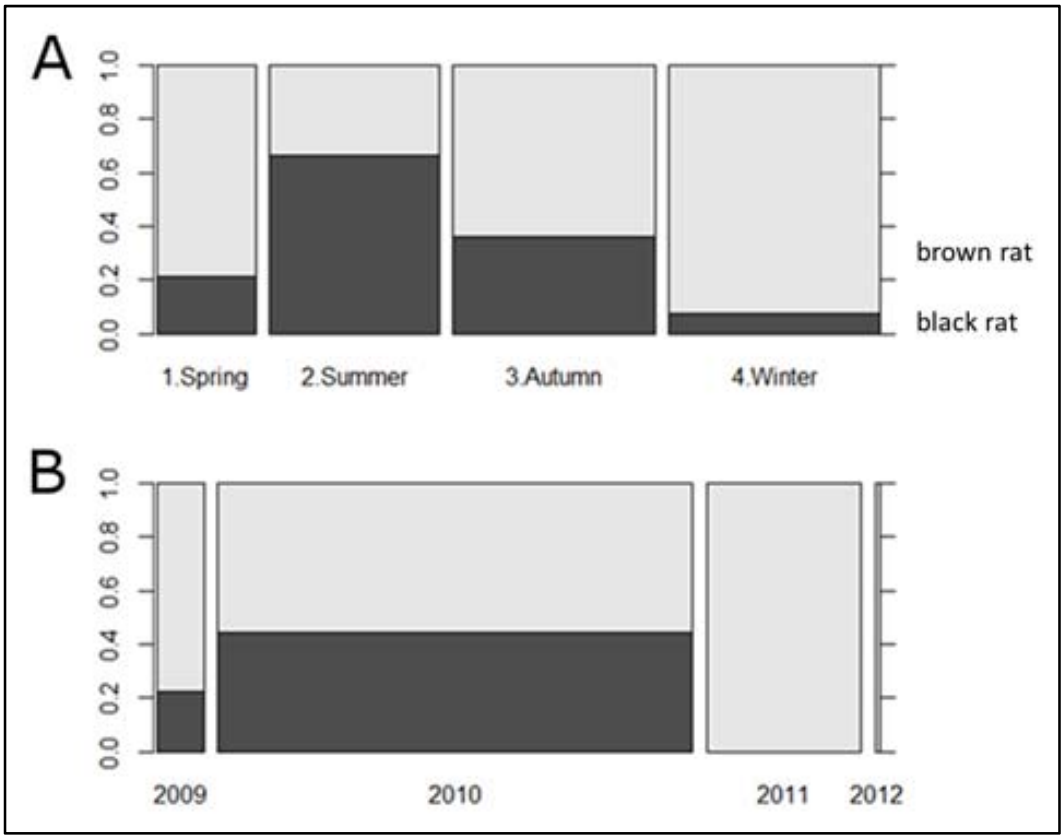


Figure 2. Distribution of rat species from the southern provinces per season (A) and per year (B).
The width of the blocks is proportional to the number of rats per factor indicated underneath. The percentage of rat species is indicated at right vertical axis.

Table I. Geographical, temporal and biological data of captured rats. Counts are given per variable, with prevalence (%) between brackets.

Location of capture		Time of capture			Brown rat	Black rat	Overall
Province	**n = 161**	**Year**	**n = 161**	**Sex**	**n = 117**	**n = 44**	**n = 161**
Friesland	23 (14.3)	2009	9 (5.6)	Female	54 (46.2)	20 (45.5)	74 (46.0)
Overijssel	4 (2.5)	2010	98 (60.9)	Male	59 (50.4)	23 (52.3)	82 (50.9)
Gelderland	2 (1.2)	2011	30 (18.6)	unknown	4 (3.4)	1 (2.3)	5 (3.1)
Utrecht	1 (0.6)	2012	6 (3.7)	**Age**			
Noord-Brabant	82 (50.9)	2013	18 (11.2)	Adult	70 (59.8)	17 (38.6)	87 (54.0)
Limburg	49 (30.4)			Young adult	34 (29.1)	7 (15.9)	41 (25.5)
		Season		Juvenile	8 (6.8)	16 (36.4)	24 (14.9)
Location type		spring	25 (15.5)	unknown	5 (4.3)	4 (9.1)	9 (5.6)
Farm	71 (44.1)	summer	51 (31.7)	**Weight**			
Rural	50 (31.1)	autumn	39 (24.2)	Female	206 ± 78 g	132 ± 60 g	-
Suburban	40 (24.8)	winter	46 (28.6)	Male	244 ± 95 g	119 ± 68 g	-

Helminth species

Morphological analysis of intestinal contents of the 117 brown rats and 44 black rats, yielded six nematode species (*Syphacia muris, Heterakis spumosa, Aonchotheca murissylvatici, Trichuris muris, Nippostrongylus brasiliensis* and *Strongyloides* sp.), which were identified morphologically using Anderson et al**.** (Anderson et al., 2009).

Three cestode species (*Hymenolepis diminuta*, *H. nana* and *Hymenolepis* (=*Rodentolepis*) *fraterna*) were identified morphologically and confirmed by molecular analyses (data not shown.).

Finally, four trematode species (*Plagiorchis muris* Figure 3A)*, Plagiorchis proximus* (Figure 3B)*, Echinostoma* sp. and *Notocotylus imbricatus* (Figure 3C) (Table II - IV) were identified using morphological examination. Seven specimen of juvenile *Echinostoma* sp. were isolated, displaying a conspicuous collar bearing 47 spines around the oral sucker. The arrangement of the collar spines was consistent with that of a group of seven *Echinostoma* spp. (*E. chloropodis, E. corvi*, *E. hystricosum*, *E. necopinum*, *E. rousseloti*, *E. sarcinum* and *E. travassosi*) (Figure 3.9 in (Kanev et al., 2009)).

Notocotylus spp. were morphologically identified using relevant literature (Cribb, 1991; Dönges, 1962; Pike, 1969). *N. imbricatus* is clearly distinct from other *Notocotylus* species parasitizing rodents in Europe (Schuster, 1986; Simon-Vincente et al., 1985).

Artificial digestion of the diaphragm and both hind legs of each wild rat revealed no *Trichinella* (or any other) muscle larvae, and thus the best estimate for the prevalence is zero (binomial 95% CI: 0% – 0.03%).

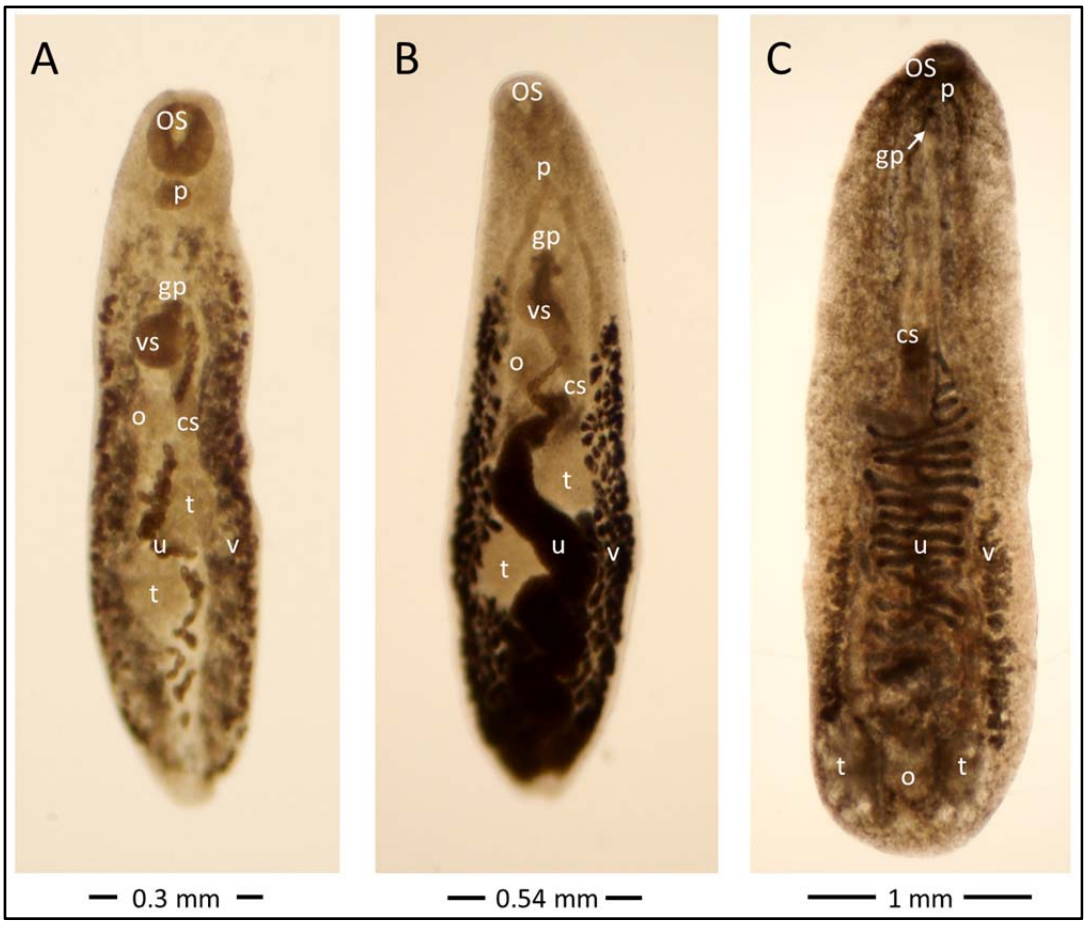


Figure 3. ***Plagiorchis muris*** **(A),** ***Plagiorchis proximus*** **(B) and** ***Notocotylus imbricatus*** **(C) isolated from wild rats.**
Unstained trematodes (A and B ventral view, C dorsal view) as seen with enhanced contrast microscopy. **cs** cirrus sac, **gp** genital pore, **o** ovarium, **os** oral sucker, **p** pharynx, **t** testis, **u** uterus, **vs** ventral sucker, **v** vitellaria.

Helminth variation per rat species

Overall, six helminth species were isolated from black rats, which occurred almost exclusively on farms, whereas in total 13 different species were isolated from brown rats originating from all three environments, and without exception, maximum species abundance was much higher in brown rats than in black rats (Table II). However, these data differed between provinces. Overall, brown rats from farms carried eight helminth species, rats from rural environment carried nine species and rats from suburban

Table II. Prevalence of helminth species in rats, differentiated to location type and host (brown rat and black rat).
[L]: only in rats captured in Limburg and in one rat captured in Friesland, [F]: only in rats captured in Friesland, [L]: only in rats captured in Limburg. Max*: highest recorded abundance. ** Total number of rats carrying hymenolepidid or trematode species.

	Helminth species	Farm (n = 71)						Rural environment (n = 50)						Suburban environment (n = 40)					
		brown (n = 30)			black (n = 41)			brown (n = 49)			black			brown (n = 38)			black		
		%	n	max*	%	n	max*	%	n	max*	n = 1		max*	%	n	max*	n = 2		max*
1	Aonchotheca murissylvatici	13.3%	4	160	9.8%	4	10	16.3%	8	48	1		4	34.2%	13	74	1		4
2	Syphacia muris	36.7%	11	1700	56.1%	23	134	28.6%	14	159	0		-	21.1%	8	200	0		0
3	Heterakis spumosa	53.3%	16	173	4.9%	2	1	28.6%	14	70	0		-	50.0%	19	7	0		0
4	Strongyloides sp.	13.3%	4	198	0.0%	0	0	6.1%	3	2	0		-	5.3%	2	0	0		0
5	Nippostrongylus brasiliensis	0.0%	0	0	0.0%	0	0	14.3%	7[L,F]	63	0		-	36.8%	14[L]	0	0		0
6	Trichuris muris	3.3%	1	0	2.4%	1	1	0.0%	0	0	0		-	2.6%	1	0	0		0
7	Hymenolepis diminuta,	50.0%	15	31	2.4%	1	1	10.2%	5	3	0		-	10.5%	4	7	0		0
8	Rodentolepis fraterna	10.0%	3	1	19.5%	8	37	12.2%	6	58	0		-	5.3%	2	0	0		0
9	Hymenolepis nana	3.3%	1	1	0.0%	0	0	4.1%	2	1	0		-	0.0%	0	0	0		0
	Prevalence hymenolepidid species**	63.3%	19	-	22.0%	9	-	26.5%	13	-	0		-	15.8%	6	-	0		-
10	Echinostoma sp.	0.0%	0	0	0.0%	0	0	0.0%	0	0	0		-	5.3%	2[L]	0	0		0
11	Notocotylus imbricatus	0.0%	0	0	0.0%	0	0	0.0%	0	0	0		-	10.5%	4[L]	0	0		0
12	Plagiorchis proximus	0.0%	0	0	0.0%	0	0	0.0%	0	0	0		-	7.9%	3[L]	0	0		0
13	Plagiorchis muris	0.0%	0	0	0.0%	0	0	4.1%	2[F]	1	0		-	0.0%	0	0	0		0
	Prevalence trematode species**	0.0%	0	-	0.0%	0	-	0.0%	0	-	0		-	23.7%	9	-	0		0
	Total number of species		8			6			9		1				11		1		
	average #helminths species/rat		1.91			0.93			1.00		1				1.82			0.50	

environment carried eleven different helminth species, to which rats from the province of Limburg contributed substantially (due to three species of trematodes and *N. brasiliensis*). Black rats from farms carried six helminth species (Tables II - IV).

Helminth prevalence in relation to location type

N. brasiliensis was absent from both black and brown rats at farms, whereas it was prevalent in brown rats from rural and suburban environment in Limburg (Table II), and it was demonstrated in one rat captured in rural environment in Friesland (Table II).

Syphacia muris prevalence was high (> 21%) in rats from all environments, at comparably high abundance (300 – 500), with an exceptional peak of 1700 *S. muris* in one brown rat from a farm. *H. spumosa* was highly prevalent in brown rats from all environments (28.8 – 53.3%), but far less in black rats (4.9%).

Overall, *Hymenolepis* spp. prevalence in rats from farms (39.4%, n = 71) was significantly higher than in rats from other environments combined (21.1%, n = 90) (p = 0.0001, Fisher's exact test). *Hymenolepis* species prevalence in brown rats captured at farms was 63.3%, which is significantly higher than in both rural (26.5%, p = 0.0088,

Fisher's exact test) and urban environments (15.8%, p < 0.0001, Fisher's exact test). In the province of Brabant, *Hymenolepis* spp. prevalence in rats from farms (52.6%, n = 55) was higher than in other environments combined (15.6%, n = 32), although not significant (P = 0.1987). In the province of Limburg, *Hymenolepis* spp. prevalence in rats from farms (69.2%, n = 13) was significantly higher than in other environments combined (12.1%, n = 33) (p = 0.0003, Fisher's exact test). Data for the other provinces were too few to draw conclusions.

Three different trematode species, which depend on aquatic intermediate hosts to complete their lifecycle, were mostly demonstrated in rats from suburban environments, which were captured at or nearby banks of small streams in Limburg. Additionally, a fourth trematode species was demonstrated in one out of four rats captured in Friesland. Trematode species were absent from both black and brown rats at farms.

Helminth profile per six-digit postal code area

The helminth species that were retrieved from forty-three

Table III. Prevalence of helminth species captured in the province of Brabant, differentiated to location type and host (brown rat and black rat).

Max*: highest recorded abundance. ** Total number of rats carrying hymenolepidid or trematode species.

Brabant		Farm (n = 55)						Rural environment (n = 15)						Suburban environment (n = 17)					
		brown (n = 19)			black (n = 36)			brown (n = 15)			black			brown (n = 15)			black		
	Helminth species	%	n	max*	%	n	max*	%	n	max*	n = 0		max*	%	n	max*	n = 2		max*
1	*Aonchotheca murissylvatici*	15.8%	3	160	5.6%	2	4	16.7%	3	48	0			26.7%	4	74	1		4
2	*Syphacia muris*	52.6%	10	1700	55.6%	20	134	22.2%	4	159	0		-	33.3%	5	200	0		0
3	*Heterakis spumosa*	52.6%	10	173	5.6%	2	1	22.2%	4	1	0		-	13.3%	2	7	0		0
4	*Strongyloides sp.*	21.1%	4	198	0.0%	0	0	0.0%	0	0	0		-	0.0%	0	0	0		0
5	*Nippostrongylus brasiliensis*	0.0%	0	0	0.0%	0	0	0.0%	0	0	0		-	0.0%	0	0	0		0
6	*Trichuris muris*	0.0%	0	0	0.0%	0	0	0.0%	0	0	0		-	0.0%	0	0	0		0
7	*Hymenolepis diminuta,*	36.8%	7	4	0.0%	0	0	11.1%	2	1	0		-	20.0%	3	7	0		0
8	*Rodentolepis fraterna*	15.8%	3	1	16.7%	6	37	0.0%	0	0	0		-	0.0%	0	0	0		0
9	*Hymenolepis nana*	0.0%	0	0	0.0%	0	0	0.0%	0	0	0		-	0.0%	0	0	0		0
	Prevalence hymenolepidid species**	52.6%	10	-	16.7%	6	-	11.1%	2	-	0		-	20.0%	3	-	0		-
10	*Echinostoma sp.*	0.0%	0	0	0.0%	0	0	0.0%	0	0	0		-	0.0%	0	0	0		0
11	*Notocotylus imbricatus*	0.0%	0	0	0.0%	0	0	0.0%	0	0	0		-	0.0%	0	0	0		0
12	*Plagiorchis proximus*	0.0%	0	0	0.0%	0	0	0.0%	0	0	0		-	0.0%	0	0	0		0
13	*Plagiorchis muris*	0.0%	0	0	0.0%	0	0	0.0%	0	0	0		-	0.0%	0	0	0		0
	Prevalence trematode species**	0.0%	0	-	0.0%	0	-	0.0%	0	-	0		-	60.0%	9	-	0		0
	Total number of species		6			4			4		0				4		1		
	average #helminths species/rat		1.92			0.83			0.93		-				0.87			0.50	

Table IV. Prevalence of helminth species captured in the province of Limburg, differentiated to location type and host (brown rat and black rat).

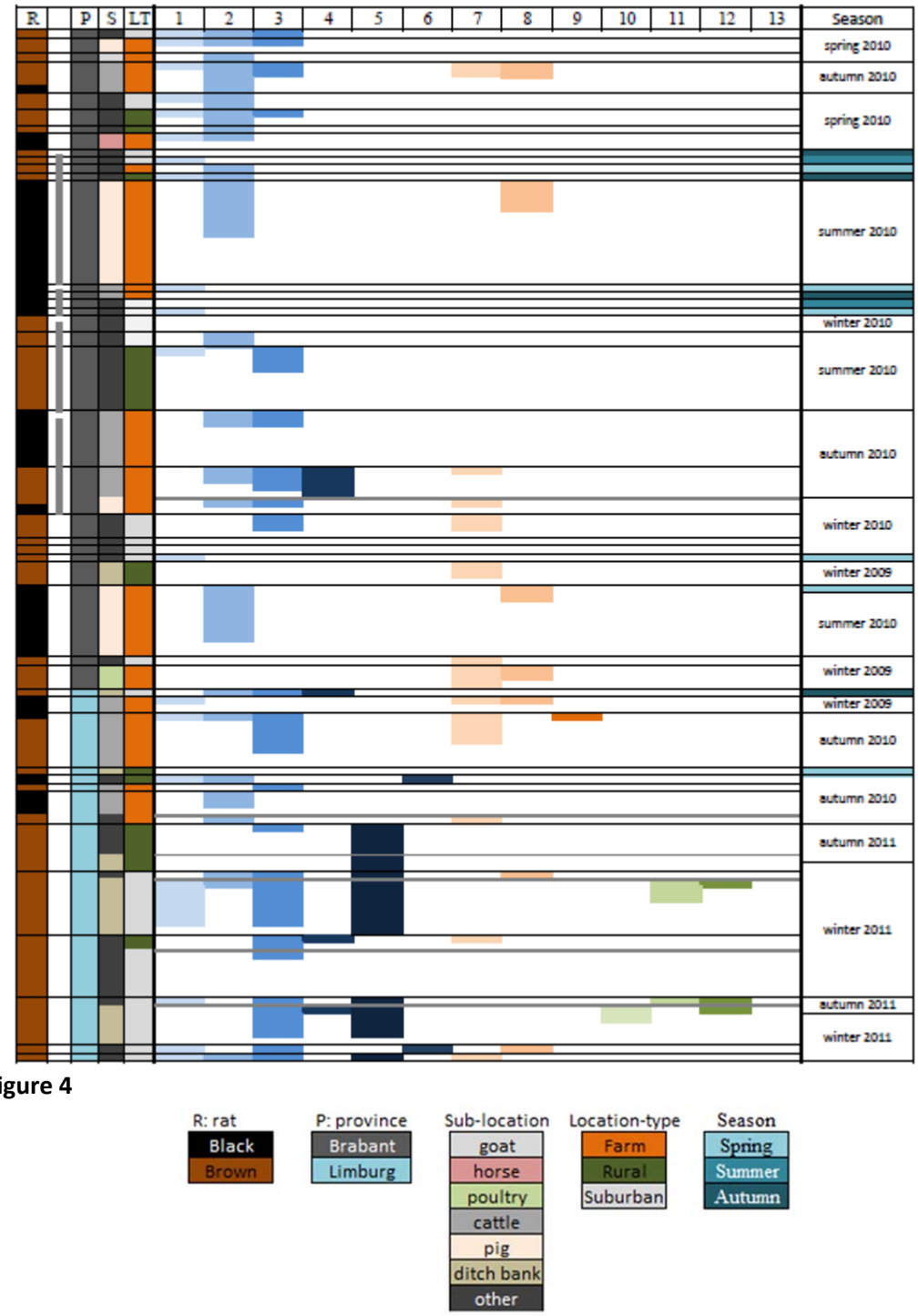
Max*: highest recorded abundance. ** Total number of rats carrying hymenolepidid or trematode species.

Limburg		Farm (n = 13)						Rural environment (n = 9)						Suburban environment (n = 24)					
		brown (n = 7)			black (n = 6)			brown (n = 9)			black			brown (n = 24)			black		
	Helminth species	%	n	max*	%	n	max*	%	n	max*	n = 0		max*	%	n	max*	n = 0		max*
1	*Aonchotheca murissylvatici*	14.3%	1	1	33.3%	2	10	11.1%	1	6	0		0	37.5%	9	76	0		0
2	*Syphacia muris*	14.3%	1	1	50.0%	3	47	0.0%	0	0	0		0	12.5%	3	40	0		0
3	*Heterakis spumosa*	71.4%	5	33	0.0%	0	0	33.3%	3	70	0		0	70.8%	17	116	0		0
4	*Strongyloides* sp.	0.0%	0	0	0.0%	0	0	11.1%	1	1	0		0	8.3%	2	63	0		0
5	*Nippostrongylus brasiliensis*	0.0%	0	0	0.0%	0	0	66.7%	6	63	0		0	58.3%	14	289	0		0
6	*Trichuris muris*	14.3%	1	1	16.7%	1	1	0.0%	0	0	0		0	4.2%	1	2	0		0
7	*Hymenolepis diminuta,*	71.4%	5	31	16.7%	1	1	11.1%	1	1	0		0	4.2%	1	6	0		0
8	*Rodentolepis fraterna*	0.0%	0	0	33.3%	2	2	0.0%	0	0	0		0	8.3%	2	1	0		0
9	*Hymenolepis nana*	14.3%	1	1	0.0%	0	0	0.0%	0	0	0		0	0.0%	0	0	0		0
	Prevalence hymenolepidid species**	85.7%	6	-	50.0%	3	-	11.1%	1	-	0		-	12.5%	3	-	0		-
10	*Echinostoma* sp.	0.0%	0	0	0.0%	0	0	0.0%	0	0	0		0	8.3%	2	4	0		0
11	*Notocotylus imbricatus*	0.0%	0	0	0.0%	0	0	0.0%	0	0	0		0	16.7%	4	8	0		0
12	*Plagiorchis proximus*	0.0%	0	0	0.0%	0	0	0.0%	0	0	0		0	12.5%	3	5	0		0
13	*Plagiorchis muris*	0.0%	0	0	0.0%	0	0	0.0%	0	0	0		0	0.0%	0	0	0		0
	Prevalence trematode species**	0.0%	0	-	0.0%	0	-	0.0%	0	-	0		0	37.5%	9	-	0		0
	Total number of species		6			5			5			0			11			0	
	average #helminths species/rat		2.12			1.00			1.33			-			2.42			0.50	

individual, sometimes-neighbouring, six-digit postal codes. Figure 4 shows that the absence / presence pattern of species is structured for most codes, especially the ones where more than one rat was captured. Season may influence species variation, e.g. most of pig farms were sampled in summer (except 3 rats captured in April / May and 2 rats captured in December), whereas cattle farms were sampled in autumn (September – October, except 2 rats captured in December). Postal codes exhibiting the highest helminth variation as a result of trematode prevalence, were all sampled in November – December in Limburg.

GLM analysis of simultaneous helminth infections

Since most brown rats and all black rats were captured in the two southern provinces of Brabant and Limburg, we excluded the other provinces from the final GLM analysis, although the same results were obtained with and without rats from the other provinces. Factors 'season' and 'location' correlated strongly (Table V), as did 'location type' and 'province', and therefore could not be tested separately in the model.


R
P
S
LT
1
2
3
4
5
6
7
8
9
10
11
12
13
Season
spring 2010
autumn 2010
spring 2010
summer 2010
winter 2010
summer 2010
autumn 2010
winter 2010
winter 2009
summer 2010
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winter 2009
autumn 2010
autumn 2010
autumn 2011
winter 2011
autumn 2011
winter 2011
R: rat
Black
Brown
P: province
Brabant
Limburg
Sub-location
goat
horse
poultry
cattle
pig
ditch bank
other
Location-type
Farm
Rural
Suburban
Season
Spring
Summer
Autumn

Figure 4

Legend to Figure 4. Helminth profile per six-digit postal code (anonymised) from the provinces of Brabant and Limburg, where both brown and black rats were captured. Forty-three sometimes-neighbouring (vertical grey bars), six-digit postal codes were sampled and arranged top down in increasing order. Each row represents one postal code and row height indicates the number of rats captured. R: rat species, P: province of capture, S: sub-location type, LT: location type. See legend for colour coding. Helminth codes: blue shades: nematodes, pink shades: cestodes, green shades: trematodes. Horizontal numbers refer to species given in Table II.

Table V. Numbers of captured rats per season, stratified for location type and year.

	Location type			Year			
Season	**Farm**	**Rural**	**Suburban**	**2009**	**2010**	**2011**	**2012**
Autumn	25	6	4	0	28	7	0
Spring	7	4	4	0	14	0	1
Summer	21	7	3	0	31	0	0
Winter	7	6	28	8	10	23	0

We chose 'location' rather than 'season', in view of ecological expectations. The best model fit, using the GLM approach, was obtained with the function *nHelminth species* $\sim \sqrt{(cumulative\ abundance)} + province$ (AIC = 321.17). The baseline (intercept) was rats in Brabant. In comparison with this baseline, factors '√(cumulative abundance)' ($p = 7.16\times10^{-11}$) and 'Limburg' ($p = 2.54\times10^{-3}$) contributed significantly to the model. The model predicts on average 1.6 times more helminth species in rats in Limburg than in Brabant, at equal cumulative abundance. The model fit was excellent ($P(Chi)^2 = 0.9678$) (Figure 5A) and the normal Q-Q plot (depicting residuals between observed and predicted values) only deviated at both extremities (results not shown). According to the model, √(cumulative abundance) predicts 1.09 times increase of simultaneously carried helminth species, which means that e.g. a four-fold increase in cumulative abundance, results in $\sqrt{4}$ (=2) times 1.09 (= 2.18) more helminth species (Figure 5B). Due to non-randomized sampling, factors 'province' and 'location type' correlated significantly ($p = 1.33\times10^{-4}$, Fisher's exact test). Although factor 'province' is maintained in the best model, the determining factor might actually be location type.

Factors 'year' ($p > 0.4720$), 'location type' ($p > 0.1633$) 'host' ($p = 0.1709$), 'age' ($p > 0.6351$), 'sex' ($p = 0.9587$) and 'season' ($p > 0.4926$), did not contribute significantly.

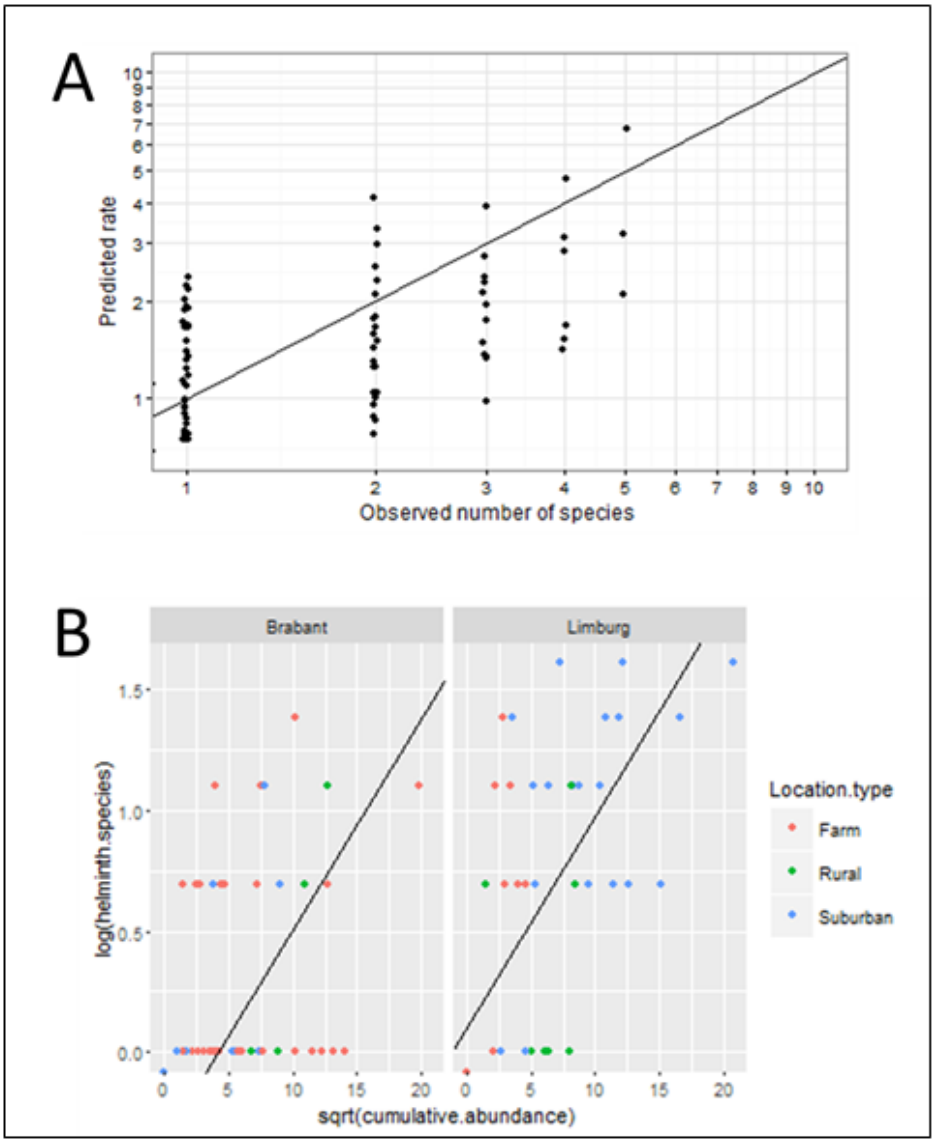

Figure 5. The number of simultaneous helminth species correlates with cumulative helminth abundance. A. The numbers of helminth species predicted by the generalised linear model (Predicted rate) correlate with observed number of helminth species ($P(Chi)^2 = 0.9678$). Note that the model predicts fractions, whereas helminths are counted in discrete numbers. Data points are presented jittered, to minimise overlap. **B.** Simultaneous helminth infections correlated positively with cumulative abundance. The dots show observed data; the line shows model fit to the data, which differs between provinces. Note that in the province of Limburg, data are segregated by location type. Since the model compares $\sqrt{}$(*cumulative abundance*) with log transformed number of helminths , count value 0 is placed below the horizontal axis.

Discussion

The aim of the present paper was to study the intestinal and intramuscular helminths in wild rats and to evaluate parasite species distribution in three different environments, to assess the relevance of rats carrying zoonotic parasites for public health.

Rats were captured as a convenience sample, which caused that neither the effect of season and location type, nor the effect of province and host could be separated in our Generalised Linear Model analysis. E.g. most black rats (38 out of 44) were captured in Brabant, most of which (36 out of 44) at farms. Moreover, Factors 'province' and 'location type' correlated significantly ($p = 1.33\times10^{-4}$, Fisher's exact test). Although random sampling would have been a preferable method and probably could have prevented these complications, it is far more difficult and expensive and therefore less realistic to perform.

The vast majority of black rats was captured only at farms in the southern provinces Brabant and Limburg, most in summer (22) and autumn (14) and least in winter (3) and spring (5), following typical farm population dynamic, where rat numbers start to increase in spring and start decreasing in autumn to reach the lowest number in winter(Davis, 1948; McGuire et al., 2006).

Heterakis spumosa, a typically soil-transmitted helminth species, was demonstrated in only 4.9% of black rats, whereas the prevalence in brown rats at farms was more than ten times higher (52.6%). The extent to which black and brown rats have soil contact, may explain differences in the number of helminth species seen in in our study. In a study on black rats in an old-growth forest in California, only 5% of the nests of black rats were underground burrows (Whisson et al., 2007). In contrast, brown rats may use buildings during winter, but breed in fields and moist environments during summer (Macdonald et al., 1999), sometimes occupying two burrows, e.g. one in a ditch bank and a second 875 metres away in a barn (Macdonald and Fenn, 1995), or use underground burrows at the premises of the farm (Akande, 2008).

In the present study, *N. brasiliensis* was prevalent in brown rats from rural and suburban ditch banks in Limburg. Also trematode species, which depend on aquatic intermediate hosts to complete their lifecycle, were demonstrated in rats captured along ditch banks in Limburg. The probability of exchange of these specific rats carrying trematodes and *N. brasiliensis* between rural environments and farms was considered minute, since these rats were captured some 20 kilometres separated from the nearest farm included in this study. Interestingly, despite presence of ditches and occasionally small streams in rural environments surrounding farms, neither *N. brasiliensis* nor trematode species were seen in farm rats. This may suggest that rats from farms in our study do not forage in

surrounding rural areas, which may explain absence of *N. brasiliensis* from rats at farms. Alternatively, intermediate hosts relevant for trematodes recorded in our study (lymnaeid snails) may be absent from ditches in the direct surroundings of farms, rendering rats at farms not prone to trematode infections, but that does not explain absence of *N. brasiliensis* from farm rats. In the Netherlands, six-digit postal codes (four numbers plus two letters) refer to one street, which may be up to 1500 metres in length in rural areas, or sections of one street between junctions, which may be 50 – 100 metres in length in suburban areas, where additionally even- and odd numbered sides have different letter-codes. The differing helminth profiles observed in (neighbouring) six-digit postal code areas may suggest that postal codes coincided with separate rat territories, although this hypothesis should be put to the test by conducting more research, controlling temporal and geographic variation. However, literature seems to provide supportive data for this hypothesis. The maximum distance that brown rats travers (primarily within their territories) within farm boundaries and margins of fields surrounding a farm, was 26 – 131 m in two studies using radio tracking of rats (Gómez Villafañe et al., 2008; Landreth, 1972). Also black rats display comparable limited motility, with maximum distances of 97 – 179 m in their natural woodland environment (Hooker and Innes, 1995; Whisson et al., 2007). At farms, with a constant supply of food, motility of black rats (<56 m) is even less than that of brown rats (Gómez Villafañe et al., 2008).

We found two representatives of the genus *Plagiorchis* in the intestines of brown rats. *Plagiorchis muris*, which was previously recorded in wood mouse (*Apodemus sylvaticus*) (Rogan et al., 2007) and has been described as zoonotic species on a few occasions in Asia, although without any clinical signs (Asada et al., 1962; Hong et al., 1996); final hosts are mice (*Mus musculus* and *Microtus* spp.), brown rat (*R. norvegicus*), pigeon (*Columba livia*) and humans (Blankespoor, 1970). The second was *Plagiorchis proximus*, which was previously recorded from the duodenum of the American muskrat (*Fiber zibethicus*) (Barker, 1915).

Notocotylus imbricatus, which is a cosmopolitan digenean species, was found in five individual Brown rats; this trematode usually parasitizes ducks (*Anatidae* and *Spatula clypeata*) (Dönges, 1962; Pike, 1969), although it was also found in an Australian water rat (*Hydromys chrysogaster*) (Cribb, 1991). *N. imbricatus* is clearly distinct from other *Notocotylus* species parasitizing rodents in Europe (Schuster, 1986; Simon-Vincente et al., 1985). To our best knowledge, none of these trematodes were previously recorded in rats from the Netherlands.

Syphacia muris, which is primarily a rat-to-rat transmitted species, was most prevalent in black rats at farms, although not significantly different from brown rats at farms (p =

0.1494, Fisher's exact test), but significantly higher compared to brown rats from rural (p = 0.0103, Fisher's exact test) and urban environment (p = 0.0025, Fisher's exact test).

S.muris gravid females actively leave their host's intestine and deposit the eggs around their host's anus, after which (auto)infection and transmission between rats takes place through grooming and social behaviour. *S. muris* is of veterinary importance in relation to experimental rodent colonies (Easterbrook et al., 2008). In a survey in 2006, respondents from 35 US research institutions reported *S. muris* in 17% of mouse colonies and 42% of rat colonies (Carty, 2008). Although *S. muris* is considered non-pathogenic, *S. muris* infections have been shown to exert significant negative effect on digestibility of nutrients and consequently growth of laboratory rats, which could interfere with experiment outcomes (Plachy et al., 2016).

In our study, *Hymenolepis* spp. prevalence in rats from farms (39.4%, n = 71) was significantly higher than in rats from other environments combined (21%, n = 90, p = 0.0001, Fisher's exact test). *Hymenolepis* species prevalence in brown rats captured at farms was 63.3%, which is significantly higher than in both rural (26.5%, p = 0.0020, Fisher's exact test) and suburban environments (15.8%, p < 0.0001, Fisher's exact test). In a study in Brown rats captured at nine farms in the UK, the prevalence of *H. diminuta* was significantly lower (21.8%, n=243) than in brown rats at twelve farms in the present study (63.3%, n = 30, p = 0.0002, Fisher's exact test). The prevalence of *H. nana* in that study was 11% (n=243), which is higher than the prevalence in the present study (3.3%) (Webster and Macdonald, 1995). *R. fraterna*, the third *Hymenolepis* species recorded in our study (prevalence 10%), was not demonstrated in British rats (Webster and Macdonald, 1995). Both *H. nana* and *H. diminuta* are zoonotic cestode helminths, although the latter is infrequently seen in humans. *H. diminuta* is transmitted to humans by ingestion of *Tribolium confusum* (flour beetle, intermediate
host) with infested cereals, or by the faecal-oral route. *H. nana* is transmitted through faecal-oral contact (eggs), or by accidental ingestion of intermediate hosts harbouring cysticercoids. *Hymenolepis* spp. infections in humans are mostly asymptomatic, although weakness, headache, abdominal pain and diarrhoea may occur (CDC, 2016).

Artificial digestion of the diaphragm and both hind legs of each wild rat revealed no *Trichinella* muscle larvae (binomial 95% CI: 0% – 0.03%). This does not differ significantly from historic *Trichinella* spp. prevalence reported in the years 1970, (2.4%, $p = 8.5 \times 10^{-2}$, Fisher's Exact test) and 1972 – 1992, (0 – 1.6% , p > 0.20, Fisher's Exact test) (van Knapen et al., 1993). Only in the year 1971, a significantly higher *Trichinella* prevalence was recorded (6.6%, $p = 4.3 \times 10^{-3}$, Fisher's Exact test) (Figure 6).

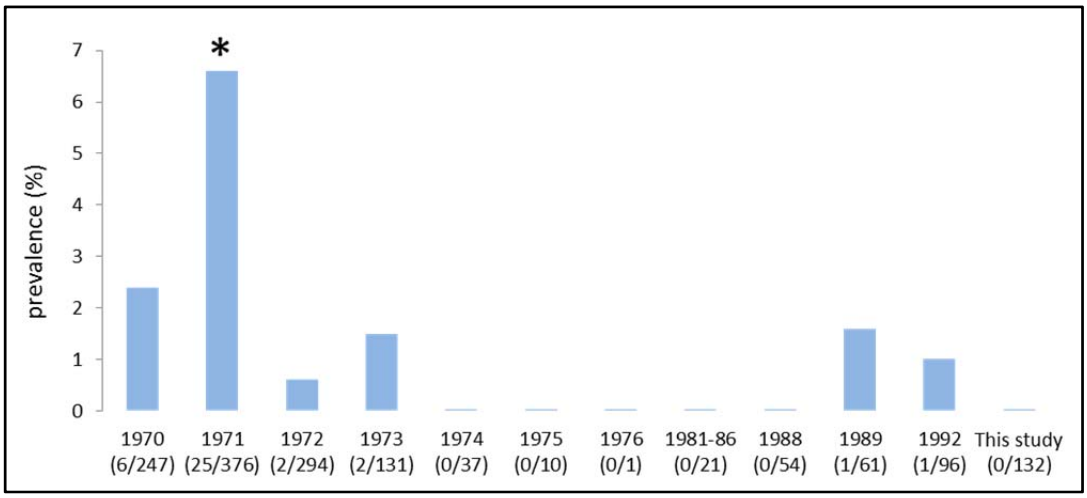

Figure 6. Historic *Trichinella* prevalence in wild rats in The Netherlands during the period 1970 – 1992 (adapted from (van Knapen et al., 1993) and (Franchimont et al., 1993)) and results from the present study . The observed prevalence did not differ significantly from year to year, except for the year 1971 (Fisher's exact test). * indicates significance.

In conclusion, we demonstrate considerable differences in helminth species variation between environments. The lowest intestinal helminth species variation (6 species) and simultaneous parasite infections (0.93) were recorded in black rats. In comparison, brown rats at farms displayed the lowest species variation (8 helminth species), but at the same time the highest number of simultaneous infections (1.91), compared to brown rats from other environments. Absence of trematodes from rats at farms may suggest limited exchange of rats between farms and the surrounding rural environments. We report four species of veterinary (*Syphacia muris*) or zoonotic relevance (*Hymenolepis diminuta*, *Hymenolepis nana* and *Plagiorchis muris*). We did not find *Trichinella* muscle larvae, consistent with long-term prevalence in Dutch wild rats.

Conflict of interest

The authors declare that they do not have conflicts of interest.

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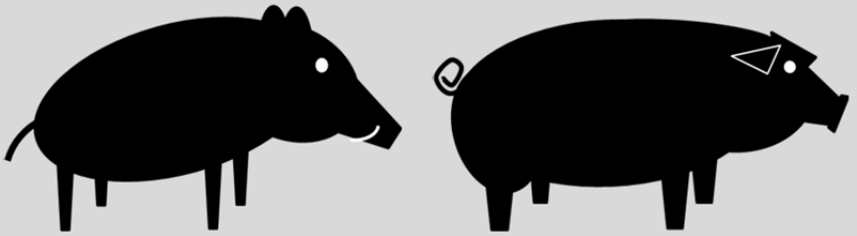
Chapter 9



Parasite to Patient: a Quantitative Risk Model for *Trichinella* spp. in pork and wild boar meat

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Consumption of raw or inadequately cooked pork meat may result in trichinellosis, a human disease due to nematodes of the genus *Trichinella*. In many countries worldwide, individual control of pig carcasses at meat inspection is mandatory but incurs high costs in relation to absence of positive carcasses from pigs reared under controlled housing. EU regulation 2015/1375 implements an alternative risk-based approach, in view of absence of positive findings in pigs under controlled housing conditions. Moreover, Codex Alimentarius guidelines for the control of *Trichinella* spp. in meat of suidae have been published (CAC, 2015) and used in conjunction with the OIE terrestrial Animal health code, to provide guidance to governments and industry on risk based control measures to prevent human exposure to *Trichinella* spp. and to facilitate international pork trade.

To further support such a risk-based approach, we model the risk of human trichinellosis due to consumption of meat from infected pigs, raised under non-controlled housing, and wild boar, using Quantitative Microbial Risk Assessment (QMRA) methods. Our model quantifies the distribution of *Trichinella* ML in swine, test sensitivity at carcass control, partitioning of edible pork parts, *Trichinella* ML distribution in edible muscle types, heat inactivation by cooking and portion sizes. The resulting exposure estimate is combined with a dose response model for *Trichinella species* to estimate the incidence of human illness after consumption of infected meat. Paramater estimation is based on experimental and observational datasets.

In Poland, which served as example, we estimated an average incidence of 1.56 (95%CI: 0 – 5.52) trichinellosis cases per million persons per year (Mpy) due to consumption of pork from pigs that were reared under non-controlled housing, and 1.97 (95%CI: 0.82 – 4.00) cases per Mpy due to consumption of wild boar.

The total estimated incidence of human trichinellosis attributed to pigs from non-controlled housing and wild boar in Poland, is similar to the incidence of human trichinellosis in that country reported by EFSA. Overall, in Europe, we estimated an upper incidence limit of 5.3×10^{-5} cases per Mpy, or less than one predicted case of trichinellosis in the European Union every 39.4 years, due to consumption of pork from controlled housing. Therefore, *Trichinella* testing of pigs under controlled housing is not adding any value to protect human health. We suggest applying our farm-to-fork QMRA model to further support decision making on the global scale.

Keywords: *Trichinella*, QMRA, model, meat inspection, inactivation, domestic pig, wild boar

Introduction

Trichinellosis is a meat borne zoonotic disease in humans caused by nematodes of the genus *Trichinella*. Within this genus, twelve taxa are recognized (nine encapsulated and three non-encapsulated species) which infect a wide range of carnivores and omnivores (Pozio et al., 2009a; Pozio and Murrell, 2006; Pozio and Zarlenga, 2013). Domestic pigs, wild boar, and horses are the main animal species through which humans acquire the infection by consuming contaminated meat.

Infection by *Trichinella* muscle larvae (ML) of humans and other mammalian hosts is followed by maturation of the ingested larvae into adult worms, mating and subsequent release of new born larvae in the small intestine. At least one male and one female larva are required for reproduction, implying that a serving of meat containing one single larva or two larvae of the same sex cannot lead to infection. Newborn larvae penetrate the gut wall and migrate to striated muscle tissues. Clinical disease follows the developmental sequence of *Trichinella*, with varying symptoms depending on the ingested dose and *Trichinella* species (Pozio et al., 2003).

To prevent the disease in humans, domestic pigs and horses, but also wildlife intended for human consumption (e.g. wild boar) are tested for *Trichinella* at slaughter. The parasite preferentially nestles in diaphragm, tongue and masseter in domestic pigs, tongue and masseter in horses and tongue and diaphragm in wild boar (Forbes and Gajadhar, 1999) (Kapel, 2000; Kapel and Gamble, 2000; Kapel et al., 2005). Samples from either of these locations may be taken for testing at slaughter, with preference for diaphragm in domestic pigs, since this is not commercially relevant and is easy to digest, in contrast with tongue. (European-Commission, 2005).

According to Codex Alimentarius, risk management should include a primary production to consumption approach, in order to identify all steps in the food chain where control measures are required (CAC, 2015). Domestic pigs reared in non-controlled housing may become infected under poor hygienic conditions and improper management, involving feeding of non-cooked scraps, offal from slaughter, wildlife remains or ingestion of infected rodents (Oivanen et al., 2002; Pozio, 2001; Pozio et al., 2001a; Schad et al., 1987; Stojcevic et al., 2004). Mandatory requirements for controlled housing in the EU explicitly strive to exclude these risks by maintenance of an efficient rodent control programme, acquiring feed from certified producers and storage of feed in closed rodent-proof containers (European-Commission, 2015).

Moreover, the adapted and recently approved EU Regulation 2015/1375 prescribes the method for detecting *Trichinella* ML in muscle tissue (European-Commission, 2015) when controlled housing for pigs is not in place and for all other susceptible animals intended for human consumption. In this method, 100 samples of 1 gram diaphragm from domestic pigs or 20 samples of 5 gram from wild boar or horse are pooled. Hence, a theoretical test sensitivity of 1 larva per gram is expected for domestic pigs and 1 larva per 20 g for wild boar and horse. However, *Trichinella* spp. ML are not evenly distributed over and within the different muscle tissues of their host (Franssen et al., 2014; Kapel et

al., 2005). For this reason, *Trichinella* ML may be missed by random effects at sample collection. To complicate the matter, *Trichinella* counts in diaphragm or other predilection sites differ from each other, and from other muscle tissues, which are used for consumption, such as ham and loin (Kapel et al., 2005).

Trichinella ML that escape detection at meat inspection may be inactivated during household cooking or freezing, but this is prone to failure. Heat inactivation is subject to culinary customs and traditions and undercooked or raw pork or wildlife meat, or raw meat products are frequently consumed. In Eastern Europe, homemade raw salami type sausages made of wild boar meat are traditionally eaten fresh, prior to the two weeks period needed to inactivate *Trichinella* ML larvae in such products (Neghina, 2010; Smith et al., 1989). Meat that is cooked 'rare' or 'medium done', may contain live *Trichinella* ML in its raw core. Some studies have been published concerning heat inactivation of *Trichinella spiralis* larvae through cooking of experimentally *Trichinella* infected pork (Carlin et al., 1969; Kotula et al., 1983) or temperature treatment of encapsulated or naked *T. spiralis* larvae in water (Randazzo et al., 2011).

Risk-based monitoring in the EU has been implemented since 2006 (European-Commission, 2005), which allowed risk-based *Trichinella* testing of domestic pigs in Member States, with derogation from testing for pigs raised in (holdings of) farms under negligible risk, or countries with proven negligible risk. However, since the World Organisation for Animal Health (OIE) no longer recognised a negligible risk status for a country or region, such recognition is linked to compartments of one or more holdings applying specific controlled housing conditions. The negligible risk country status, which was only applied to Denmark and Belgium, was replaced in 2014 by derogation of testing, depending on approval of the controlled housing level of farms or compartments. In parallel, guidelines for risk based control measures of pig meat for global trade have been prepared at the FAO/WHO Codex Alimentarius Committee on Food Hygiene (CCFH)(CAC, 2015). At present, risk-based *Trichinella* monitoring is not supported by a quantitative model to determine the role and impact of measures that restrict the presence of *Trichinella* spp. in pork. Recently, CCFH developed a model to evaluate residual risks of infection with *Trichinella* spp. from *Trichinella*-tested domestic pigs under different hypothetical scenarios (FAO-WHO, 2014b). The model in its present form does not include distribution of *Trichinella* numbers over different muscle types and only provides exposure assessment. A dose-response relationship for *Trichinella* infections in humans to translate exposure to human health endpoints is a critical component of quantitative microbial risk assessment (Haas et al., 1999). Other factors relevant to testing for *Trichinella* at meat inspection, such as clustering at animal level, probability of detection or the effect of pooling test samples, are missing as well in the presently available model.

The aim of the present study was to develop a farm-to-fork quantitative microbial risk assessment (QMRA) model that simulates occurrence of the parasite in wildlife, its transmission dynamics through the food chain from meat inspection to consumption of

pork or wild boar meat, and consequent human trichinellosis risks. We focus on meat from shoulder, belly and loin, since these meat cuts are purchased raw and cooked by consumers at home. Using the model, we estimate the number of human trichinellosis cases from consuming pork reared in different husbandry systems and from consuming wild boar meat. For this purpose, we evaluated the meat production system in a *Trichinella* endemic country in Europe (Poland), identified critical points at which *Trichinella* ML may escape detection or inactivation, collected and critically appraised relevant datasets to estimate model parameters and developed a stochastic model representing variability due to systematic and random effects. Reported incidence rates of human trichinellosis over a period of six years in Poland are used to evaluate the outcomes of our model. We did not evaluate input uncertainty; however, we did include uncertainty in the dose response model and we evaluate uncertainty of model outcome for different cooking scenarios.

Model

The conceptual model for the *Trichinella* QMRA is shown in Figure 1, addressing the chain of events between *Trichinella* infection in pigs or wild boar and illness in humans. The next sections describe its modules, for which Table II shows the model equations, Table III provides the data input and parameters that were used to build the model. All distributions reflect variability. We model numbers of *Trichinella* ML in 100 gram of pork originating from fattening pigs from non-controlled housing and wild boar meat, at each step of the food chain to human consumption.

The output of the model is the expected incidence of human trichinellosis in our model country, Poland. We perform many simulations, with each simulation representing a year; therefore, variability over simulations can be interpreted as variability over years. Within each simulation, we model *Trichinella* ML numbers in all portions from 5000 randomly generated carcasses (Figure 2).

Modules

Trichinella larvae distribution in swine

Data from Polish *Trichinella* control at slaughter in the period 2007 – 2012 were used to estimate the average *Trichinella* prevalence for wild boar and domestic pigs from non-controlled housing. A negative binomial distribution empirically describes the number of larvae in an animal's diaphragm, also accommodating observed zero larvae for uninfected animals. For parameter estimation, we applied the maximum likelihood method to data sets from Polish surveillance data. These were (1) *Trichinella* ML abundance in 50 g diaphragm samples from thirty-four wild boar (larval loads of 0.3 – 211 larvae per gram, a number *z* of *Trichinella* ML is present in the 50 g diaphragm with probability p(z | m, k) (Equation I), where the parameter *m* is the mean number of *Trichinella* ML in 50 g of median 4.92, n = 34, Table I), and (2) the above-mentioned prevalence of *Trichinella* in

Polish wild boar diaphragm, averaged over all tested wild boar, and the parameter *k* describes the clustering of *Trichinella* ML among individual wild boar. When the value of *k* is much less than one, the majority of the total larvae in the population of all tested wild boar are present in relatively few animals, while when *k* approaches infinity, the number of larvae per sample follows a Poisson distribution (no heterogeneity between animals). Each of the *n* wild boar tested in the surveillance program was labelled by index *j*. The likelihood of parasite number z_j is calculated according to Equation 2. For those wild boar where only absence/presence data was available we used *p(0,m,k)* and *1-p(0,m,k)* for the probabilities of parasite absence or presence, respectively. Likelihood of *x* absent and *y* present test-outcomes follows a binomial distribution (Equation 3). We numerically maximized the log-likelihood to calculate the maximum likelihood estimates for the parameters *m* and *k*, using the software Mathematica version 10 (Wolfram Research, Champaign, IL). Now, two 50 g diaphragms make up the total 100 g portion size. The sum of two negative binomial random variables is again a negative binomial distribution with parameters *2k* and *2m*.

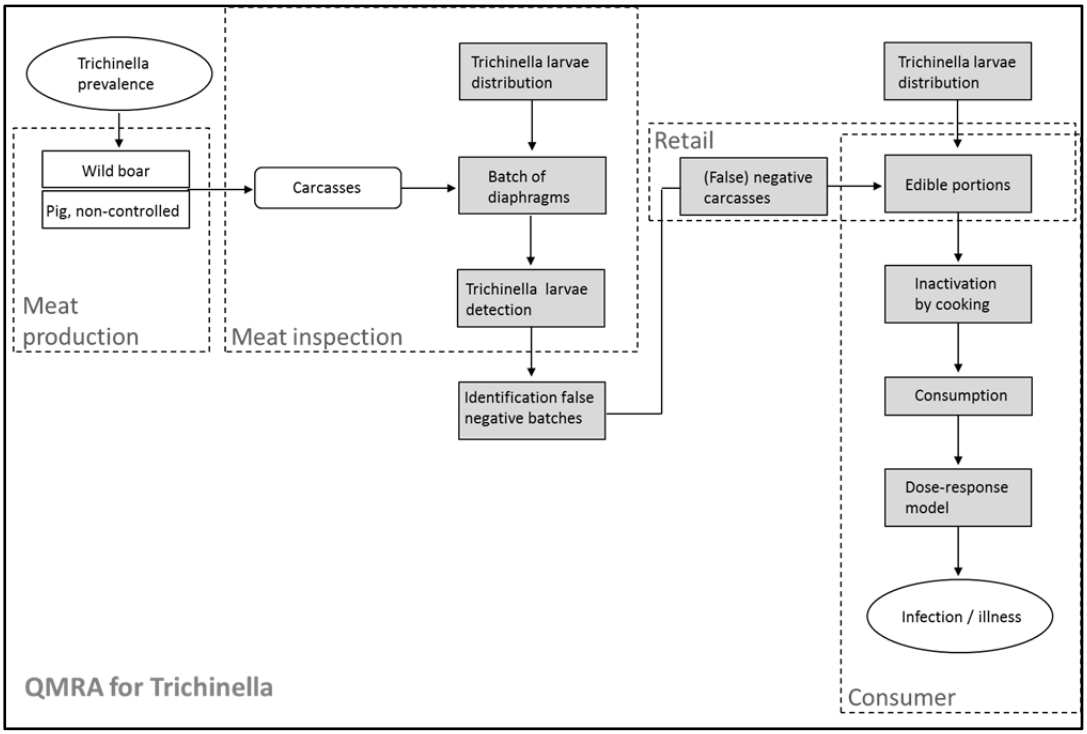


Figure 1. Conceptual model structure for the quantitative microbial risk analysis for *Trichinella* in the food chain. Shaded boxes represent modules of the QMRA model.

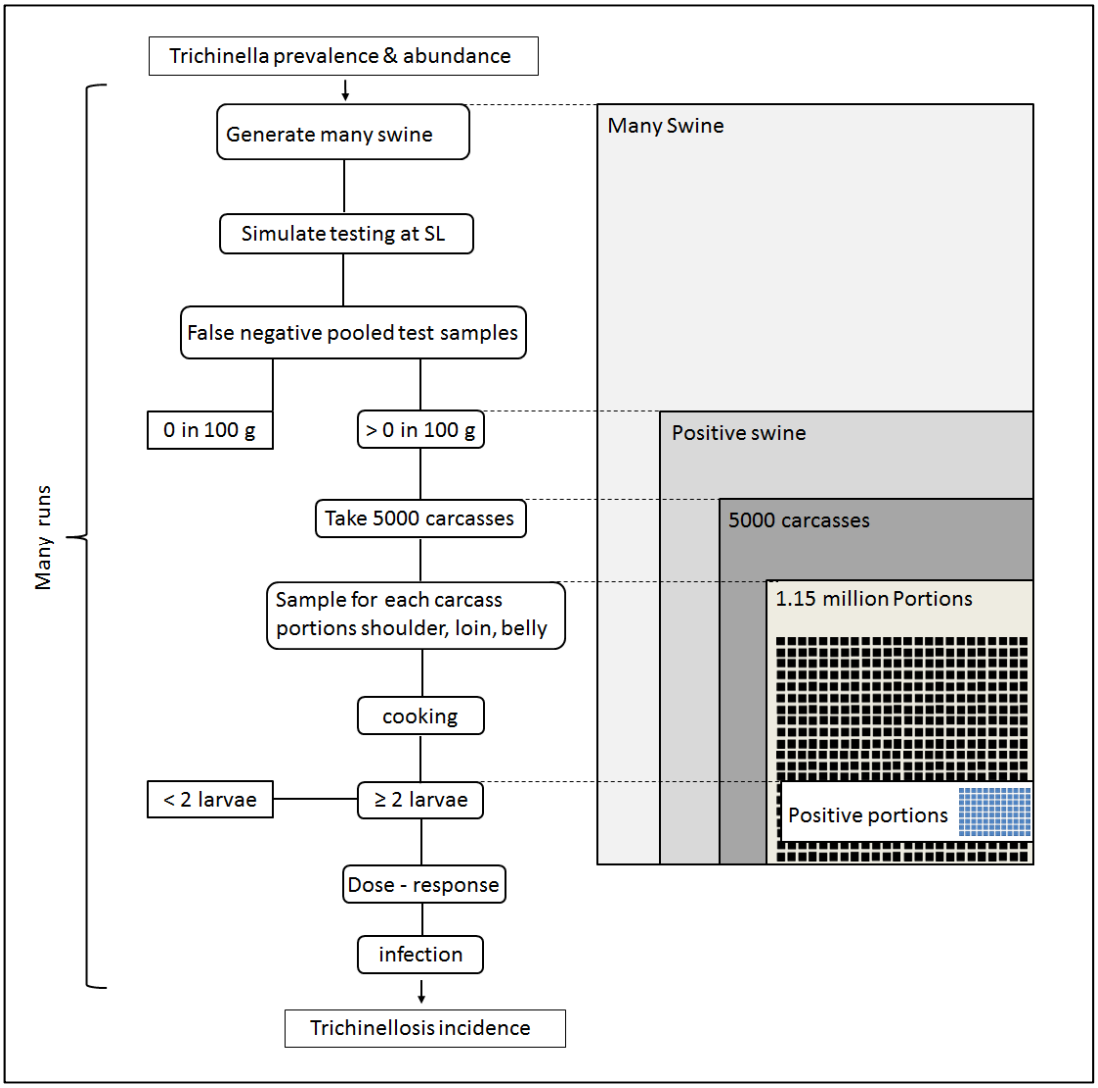


Figure 2. Model layout

We also calculated parameters *m* and *k* for domestic pigs from non-controlled housing. We substituted for larva-present animals the larval loads determined in wild boar, since count data were not available for domestic pigs from non-controlled housing, and it was anticipated that the distribution of *Trichinella* is comparable in pigs and wild boar, since both are related animal species and potentially exposed to the same environment.

Table I. Trichinella larval burden in 34 individual Polish wild boar. ID: identification of individual wild boar; LPG: larvae per gram.

ID	province	species	LPG	ID	province	species	LPG
20	warmińsko - mazurskie	*T. britovi*	7.6	467	mazowiecie	*T. spiralis*	0.22
55	lubuskie	*T. spiralis*	4.02	474	podlaskie	*T. britovi*	89
59	zachodnio - pomorskie	*T. britovi*	0.3	481	zachodnio - pomorskie	*T. spiralis*	19.52
61	kujawsko - pomorskie	*T. spiralis*	42.8	485	świętokrzyskie	*T. britovi*	47
137	podlaskie	*T. britovi*	33.1	513	pomorskie	*T. britovi*	2.4
143	warmińsko - mazurskie	*T. britovi*	12	519	warmińsko - mazurskie	*T. britovi*	3
146	zachodnio - pomorskie	*T.britovi*	13	521	zachodnio - pomorskie	*T. spiralis*	4.1
203	warmińsko - mazurskie	*T. spiralis*	0.3	539	zachodnio - pomorskie	*T. spiralis*	69
320	kujawsko - pomorskie	*T. spiralis*	41	579	lubelskie	*T. spiralis*	4.92
322	zachodnio - pomorskie	*T. spiralis*	1.9	594	zachodnio - pomorskie	*T. spiralis*	56
326	mazowieckie	*T. spiralis*	4	597	kujawsko - pomorskie	*T. spiralis*	211
378	lubuskie	*T. spiralis*	10	609	zachodnio - pomorskie	*Trichinella sp*	0.8
415	pomorskie	*T. spiralis*	2.4	610	mazowiecie	*T. spiralis*	0.9
429	warmińsko - mazurskie	*T. britovi*	1.4	622	wielkopolskie	*T. spiralis*	94
432	warmińsko o mazurskie	*T. britovi*	6	624	wielkopolskie	*T. spiralis*	0.56
445	wielkopolskie	*T. spiralis*	63	630	lubelskie	*T. britovi*	2.12
458	zachodnio - pomorskie	*T. britovi*	4.92	652	opolskie	*T. spiralis*	1.5

Batch of diaphragm samples for meat inspection

According to EU legislation, for wild boar, the *Trichinella* artificial digestion test procedure at meat inspection utilises 5 g of diaphragm muscle of 20 wild boar each, in a pool of in total 100 g muscle. For pigs, 1g of diaphragm muscle is used, and samples from 100 pigs are pooled. In this study, we simulated a situation in which 600,000 wild boar or 114×10^6 pigs from non-controlled housing were tested during meat inspection at slaughter in a simulated year. These numbers represent the average slaughter volumes in Poland during the period 2007-2012.

We label each swine with an index *j* and sample for each animal according to the negative binomial distribution with parameters *2m* and *2k* a number Y_j of *Trichinella* ML in 100 g of diaphragm (Equation 4).

Testing 5 g diaphragm sample of wild boar or 1 g diaphragm sample for pig, involves determining a subset of *Trichinella* ML that are present in the diaphragm of each animal. We chose to model sampling from 100 g of diaphragm, which is the standardised portion size throughout the model, instead of modelling sampling from 200 g of diaphragm (estimated total weight) and subsequent downscaling. Sampling is a binomial process with Y_j trials and probability of $p = 0.05$ = 5 g / 100 g for wild boar and $p = 0.01$ = 1 gram / 100 gram for pigs for each larva to be present in a fraction of the diaphragm weighing 5 g and 1 g, respectively. A random draw y_q from the binomial distribution with parameters *p*

Table II. Model equations (including pages 159 – 162)

Model step	Symbol	Equation	Eq.
Trichinella larvae distribution in swine	*p*: probability density function *z*: number of *Trichinella* muscle larvae (ML) *m*: mean number of *Trichinella* ML in 50 g *k*: a measure of unevenness in *Trichinella* ML counts among wild boars	$p(z\|m,k) = \frac{(k+z-1)!}{z!\,(k-1)!}\left(1+\frac{m}{k}\right)^{-k-z}\left(\frac{m}{k}\right)^{z}$	1
	L: likelihood *n*: number of swine observed *j*: index of individual swine z_j: number of *Trichinella* ML in individual swine	$L(m,k\|z_j) = \prod_{j=1}^{n} p(z_j,m,k)$	2
	B: likelihood *x*: number of swine with no *Trichinella* ML present *y*: number of swine with *Trichinella* ML present	$B(x,y\|m,k) = \binom{x+y}{y}\left(1-p(0,m,k)\right)^{y} p(0,m,k)^{x}$	3
Batch of diaphragm samples for meat inspection	*p*, *z*, *m*, *k* as in 2.1.1	$p(z\|2m,2k) = \frac{(2k+z-1)!}{z!\,(2k-1)!}\left(1+\frac{m}{k}\right)^{-2k-z}\left(\frac{m}{k}\right)^{z}$	4
	Q: number of animals in a pool y_q: number of *Trichinella* ML in a diaphragm sample from an individual animal *R*: number of *Trichinella* ML in a pool	$R = \sum_{q=1}^{Q} y_q$	5
Trichinella larvae detection	*g*: probability density function *U*: number of *Trichinella* ML per test sample *u*: *Trichinella* ML recovered by technician	$g(U,u) = \binom{U}{u}\frac{\text{Beta}(u+a,U-u+b)}{\text{Beta}(a,b)}$	6
	Beta: Euler beta function *a*: a shape parameter of beta distribution *b*: parameters of the beta function	$\text{Beta(a,b)} = \int_0^1 t^{a-1}(1-t)^{b-1}\,dt$	7
Number of edible portions and *Trichinella* ML distribution	n_E: number of edible pork portions of muscle group *m* *w*: weight of muscle group *m* in kg m_{fract}: lean mass fraction of muscle	$n_E = (w \times m_{fract}) / 0.100$	8

	group *m*		
	C: vector of *Trichinella* ML counts in muscle parts *M*: number of distinct muscle parts p_i: see Table III line 2.1.5 Y_j: observed number of *Trichinella* ML in muscle part *M* + 1 (diaphragm)	$P(C = c) = \binom{Y_j + M - 1}{M}\binom{M}{c_1 \cdots c_M}\prod_{i=1}^{M} p_i^{c_i} p_{M+1}^{Y_j}$ $C = (C_1, \ldots, C_M)$ $c = (c_1, \ldots, c_M)$ l_i is a random draw from C	9
	f: probability density function l_i: number of *Trichinella* ML to distribute for muscle part i *v*: vector of *Trichinella* ML counts over portions	$f(v, l) = \frac{l_i!}{\prod_{k=1}^{n_E} x_k!} 2^{-\sum_{k=1}^{n_E} x_k}$ *h_i is a random draw from f*	10
Inactivation by cooking	I_1: remaining number of *Trichinella* ML after inactivation at time point t_1 h_i: starting number of *Trichinella* ML after inactivation at time point t_0 α^+: maximum inactivation possible *K*: steepness at point T^* T^*: point of maximal slope T_0: lower temperature T_1: upper temperature t_1: time point at which T_1 is reached	$\log(\mathrm{I}_1) = \log(h_i) + \frac{\alpha^+ \mathrm{t}_1}{k(\mathrm{T}_0 - T_1)} \log\left(\frac{1 + e^{k(\mathrm{T}_1 - \mathrm{T}^*)}}{1 + e^{k(\mathrm{T}_0 - \mathrm{T}^*)}}\right)$ Note that this equation is applied several times, for different phases in the cooking process. We do not repeat the formula for each step for clarity.	11
Consumption patterns	A_{Wild}: available number of consumable portions per year N_{wild}: total number of wild boar $\sum p$: total number of portions shoulder, loin and belly per carcass PL_C: total population in Poland (period 2007 – 2012)	$A_{Wild} = N_{wild} \times \sum p \,/\, PL_c$	12
	N_{con}: number of consumed portions of pork per year in Poland P_{nc}: proportion of non-controlled housing in Poland C_{Pork}: average number of consumed portions of pork (100g) for Poland per year PL_A: average population in Poland (period 2007 – 2012)	$N_{con} = P_{nc} \times C_{Pork} \times PL_A$	13
Dose response modelling	P_{ill}: probability of illness I_1: number of *Trichinella* ML after cooking P_m: *Trichinella* ML survival probability	$P_{\mathrm{ill}}(I_1 \mid P_m, r) = 1 + e^{-I_1 p_m} - e^{-I_1 p_m (1-r)} - e^{-I_1 p_m r}$ $P_m \sim Beta(\alpha, \beta)$	14

	r: sex ratio (e.g. proportion of females)		
Risk characterisation	$P_{ill,Wild}$: total predicted human cases per year from consumption of wild boar meat A_{Wild}: available number of consumable portions per year (see 2.1.7) $N_{ill\ Wild}$: average illness per million consumable portions (see 2.1.7)	$P_{Wild} = A_{Wild} \times N_{ill,Wild}$	15
	N_{con}: number of consumed portions of pork per year (see 2.1.7) P_{nc}: proportion of non-controlled housing in Poland (see 2.1.7) C_{Pork}: average number of consumed portions of pork (100g) for Poland per year (see 2.1.7) $N_{ill,pork}$: average number of illness per million portions of pork from non-controlled housing $P_{ill,pork}$: total predicted human cases per year from consumption of pork from non-controlled housing	$P_{ill,pork} = N_{con} \times N_{ill,pork}$	16
Extrapolation to pigs from controlled housing	g: probability density function n: number of swine for testing u: possible number of positive swine A: number of positive swine in past tests B: number of swine tested in past tests	$g(n,u) = \binom{n}{u} \frac{\text{Beta}(u+A+1, n-u+B+1)}{\text{Beta}(A+1, B+1)}$	17
	U_p: upper prevalence limit T_{EU}: number of annually tested pigs from controlled housing T_p: testing period	$U_P = (T_{EU} \times T_p)^{-1}$	18
Extrapolation to pigs from controlled housing	$P_{ill,extra}$: maximum extrapolated number of human trichinellosis cases per year from consumption of pork from controlled housing U_P: upper limit in prevalence for controlled housing p = prevalence in pigs from non-controlled housing $P_{ill,pork}$: total predicted human cases per year from consumption of pork	$P_{ill,extra} = U_P \times p^{-1} \times P_{ill,pork}$	19

	from non-controlled housing (see 2.2)		
	$P_{ill,extra}$: maximum extrapolated number of human trichinellosis cases per year from consumption of pork from controlled housing F_{ill}: occurrence of $P_{ill,extra}$ *in years*	$F_{ill} = (P_{ill,extra} \times 508)^{-1}$	20

and Y_j determines the number of larvae in 5 or 1 g diaphragm for each wild boar or domestic pig, respectively. Add up each realisation per animal in the pool, to arrive at the total number R of larva in the 100 g pool (Equation 5). We keep track of which animal contributes to which pool, to trace individual animals in a false negative batch later on.

Trichinella muscle larvae detection

We model *Trichinella* ML detection at meat inspection by quantifying the probability of detecting one single larva in the pool. For this, we used 280 proficiency test records of the Dutch National Reference Laboratory for Parasites (RIVM, Bilthoven, the Netherlands), in which a known number of *Trichinella* ML were added to 100 g of muscle tissue and 14 technicians from four different routine *Trichinella* laboratories counted the recovered *Trichinella* ML (Table IV). Recovery of each larva is a random process, with the probability of recovery for each larva unlikely to be the same for all larvae, because different technicians and laboratories are involved in such analyses. We describe the variability in the recovery probability by a Beta binomial distribution *g(U,u)* (Equation 6), which is a mixture of binomial and beta distributions.

In a testing meat sample to which U larvae were added, a technician recovered a number of u larvae following a Beta distribution with parameters a and b, which is written in terms of the Euler beta function (Equation 7).

Using the software Mathematica (Wolfram Research, Champaign, IL) the log-likelihood was numerically maximized to calculate the maximum likelihood estimates for the parameters a and b (Table III).

Identification of false-negative batches

All carcasses that test positive for *Trichinella* ML at meat inspection are withdrawn from the food chain and therefore do not constitute a risk to consumers. However, a batch containing $R > 0$ larvae may pass the meat inspection, i.e. *Trichinella* ML are not detected, with a probability *g(R,0)*. If this happens, we mark the batch as a false-negative. After identification of the false-negative batches, we trace back the individual infected swine(s) in this batch. For each of these batches, we list the number Y_j of *Trichinella* ML present in the 100 g diaphragm(s) of the individual animal(s).

Table III. Data inputs and parameters in the model.

Model part	Symbol	Unit	Wild boar	Pig non-controlled	Reference
Trichinella larvae distribution in swine	m: mean number of *Trichinella* ML in 50 g	Larvae / 50 g	5.24	0.00159125	This study
	k: larvae distribution between wild boars	-	4.47×10^{-4}	1.3523×10^{-7}	This study
	n: number of swine tested	Swine	685,595	114,395,817	EFSA-ECDC 2010-2014
	z_i: number of *Trichinella* ML in individual swine	*Trichinella* ML/animal	0.3 - 211	0.3 - 211	Observed (Table I)
	x: number of *Trichinella* ML -absent swine	Swine	682,763	114,395,672	EFSA-ECDC 2010-2014
	y: number of *Trichinella* ML -present swine	Swine	2,832	145	EFSA-ECDC 2010-2014
Batch of diaphragm samples for meat inspection	p: probability for a *Trichinella* ML to be present in G grams of diaphragm	-	0.05	0.01	This study
	Q: number of animals per pool	Swine	20	100	EU 2075/2005
Trichinella larvae detection	U: number of *Trichinella* ML per test sample	*Trichinella* ML / 100 g	0 – 9 (median = 5)	0 – 9 (median = 5)	Experimental data
	u: *Trichinella* ML recovered by technician	*Trichinella* ML	0 – 10 (median = 4)	0 – 10 (median = 4)	NRL-P (NL)
	a: shape parameter	-	1.54	1.54	This study
	b: shape parameter	-	0.29	0.29	This study
Identification of false-negative batches	G: weight of diaphragm sample	g	5 g	1 g	EU 2075/2005
	Q: number of animals per pool	Swine	20	100	EU 2075/2005
Number of edible portions and *Trichinella* ML distribution	n_E: number of edible pork portions	Portions	367	395	Estimated (Table VI)
	w: weight of muscle group m in kg	kg	8.00 – 21.03 kg	7.86 – 29.28 kg	Estimated (Table VI)
	m_{fmct}: lean mass fraction of muscle group m*	percent	0.57%	44 – 62%	Marcoux et al. 2007; Monziols et al., 2006; Skewes et al., 2008
	n_p: number of portions calculated from weight (shoulder, belly [3], loin)	Portions	50, 76, 103	35, 54, 144	This study (Table VI)
	P_i: probability of allocation to muscle part (shoulder, belly[2], loin)	-	0.103, 0.278, 0.101	0.059, 0.162, 0.116	This study (Table VII)

Table III. Data inputs and parameters in the model. (continued)

Model part	Symbol	Unit	Wild boar	Pig non-controlled	Reference
Inactivation by cooking	T^*: point of maximal slope	°C	59.3	59.3	Swart & Franssen, in preparation
	K: steepness at point T^*	-	0.17	0.17	
	α^*: maximum inactivation possible	-	0.63	0.63	
Consumption patterns	A_{wild}: available number of 100 g portions of wild boar meat	Portion/person/year	0.68		This study
	N_{wild}: total number of tested wild boar (period 2007 – 2012)	Animals	685595	-	EFSA-ECDC 2010, 2013, 2014
	$\sum p$: total number of portions shoulder, loin and belly per carcass	Portions	229	223	Estimated (Table VI)
	PL_C: total population in Poland (period 2007 – 2012)	Humans	230,020,458	230020458	EFSA-ECDC 2010, 2013, 2014
	$Ncon$: number of consumed portions of pork per year in Poland	Portions	2.71×10^{7}	1.54×10^{10}	This study
	P_{nc}: proportion of non-controlled housing in Poland	Proportion	-	0.784	(Pozio, 2014)
	C_{Pork}: average number of consumed portions (100g) for Poland per year	Portions	0.68	514	This study
	PL_A: average population in Poland (period 2007 – 2012)	Persons	3.83×10^{7}	3.83×10^{7}	This study
Dose response modelling	r: sex ratio (e.g. proportion of females)	Proportion	0.7	0.7	Teunis, 2012
	α, β: dose response parameters		sampled from uncertainty distribution	sampled from uncertainty distribution	Supplied by Teunis
Extrapolation to pigs from controlled housing	A: number of positive swine in past tests	-	0	0	EFSA-ECDC 2010-2014
	B: number of swine tested in past tests	Pigs	-	139,729,393	EFSA-ECDC 2010-2014

* Only average value without variation given for wild boar in Skewes et al., 2008

Table IV. Results of proficiency tests organised by the Dutch NRL-P during the period 2012-2014.

S	R	S	R	S	R	S	R	S	R	S	R	S	R
5	5	5	4	3	3	3	3	5	5	4	4	8	6
5	5	0	0	3	3	0	0	0	0	8	6	0	0
7	7	5	3	8	7	3	1	5	4	0	0	4	4
0	0	7	6	0	0	8	3	9	6	6	6	8	7
9	4	9	9	8	6	8	6	9	6	8	8	6	5
5	5	5	4	3	4	3	3	9	7	4	5	6	6
5	4	0	0	3	3	0	0	5	4	8	8	8	8
7	7	5	3	8	7	3	1	0	0	0	0	0	0
0	0	7	4	0	0	8	3	9	9	6	6	8	8
9	4	9	8	8	8	8	6	5	4	8	8	4	4
5	5	7	5	3	3	3	3	9	6	4	2	6	5
5	4	9	8	3	3	0	0	5	5	8	5	8	6
7	7	5	4	8	7	3	1	0	0	0	0	0	0
0	0	0	0	0	0	8	3	9	8	6	6	8	7
9	6	5	3	8	7	8	5	5	6	8	7	4	4
5	4	7	5	3	3	3	3	9	7	4	3	6	4
5	4	9	6	3	3	0	0	5	4	8	6	8	6
7	7	5	4	8	7	3	1	0	0	0	0	0	0
0	0	0	0	0	0	8	3	9	9	6	6	8	6
9	4	5	4	8	8	8	6	5	4	8	8	4	4
9	8	7	4	3	3	3	3	5	5	4	4	8	6
5	5	9	8	0	0	0	0	0	0	8	6	0	0
0	0	5	3	3	2	3	1	9	8	0	0	4	4
5	5	0	0	8	6	8	3	5	3	6	6	6	5
7	6	5	2	8	5	8	6	9	8	8	8	8	4
9	7	3	3	3	2	5	5	5	6	8	7	8	5
5	6	3	2	0	0	0	0	0	0	0	0	0	0
0	0	8	7	3	2	5	4	9	8	4	3	4	3
5	4	0	0	8	6	9	6	5	3	8	8	6	3
7	7	8	6	8	5	9	7	9	8	6	6	8	6
9	8	3	3	3	3	5	4	5	5	8	5	8	6
5	5	3	3	0	0	0	0	0	0	0	0	0	0
0	0	8	7	3	2	5	3	9	7	4	4	4	2
5	4	0	0	8	7	9	8	5	3	8	8	6	3
7	6	8	6	8	5	9	7	9	8	6	6	8	5
5	4	3	3	3	3	5	5	5	5	8	5	8	7
0	0	3	2	0	0	0	0	0	0	0	0	0	0
5	3	8	7	3	1	5	3	9	10	4	4	4	3
7	5	0	0	8	3	9	6	5	3	8	7	6	4
9	9	8	6	8	5	9	7	9	8	6	6	8	3

Data represent *Trichinella* muscle larvae that were spiked in 100 g fat-free minced pork balls, which were recovered by different technicians at slaughterhouse labs after artificial digestion according to EU regulation 2075/2005. Rec: recovered. S: spike, R: recovered.

Table V. Muscle weight distribution of pork cuts. (adapted from Monziols *et al*., 2006 and Skewes *et al*., 2008).

Domestic Pig	Weight (kg)	% Muscle	Muscle (Kg)	# Portions	Lean fraction
Live weight	115				
Carcass	81,84	0,50	40,59	406	
Shoulder[1]	20,66	0,45	9,30	93	11,4%
Shoulder[2]	7,86	0,45	3,54	35	4,3%
Foreleg[3]	8,82	0,45	3,97	40	4,9%
Belly[3]	12,36	0,44	5,43	54	6,6%
Loin[4]	29,28	0,49	14,44	144	17,6%
Ham	19,56	0,62	12,13	121	14,8%
Total			39,49	395	48,3%

Domestic pigs.
Average of 24 pigs, 6 each of four different breeds. [1] Shoulder in Canadian cuts includes shoulder, neck and foreleg of a pig's carcass. [2] % of carcass weight (Marcoux et al., 2007) was used to separate shoulder and foreleg (European cut) from Schoulder[1] (Canadian cut). [3] Belly includes intercostal and abdominal muscles. [4] neck loin and loin are considered jointly, since they will probably be prepared similarly by consumers.

Table V. Muscle weight distribution of pork cuts (continued)

Wild Boar	Weight (kg)	% Muscle	Muscle (kg)	# Portions	Lean fraction
Carcass[1]	81.84	0.57	46.65	466	
Shoulder[2]	20.01	0.57	11.41	114	24.5%
Shoulder[3]	8.84	0.57	5.04	50	10.8%
Foreleg[3]	3.17	0.57	1.81	18	3.9%
Belly[4]	13.32	0.57	7.59	76	16.3%
Neck loin[3]	8	0.57	4.56	46	9.80%
Loin	9.98	0.57	5.69	57	12.20%
Loin[5]	17.98	0.57	10.25	103	22.0%
Ham	21.03	0.57	11.99	120	25.7%
Total:			36.67	367	78.7%

Wild boar.
[1] Carcass weight given in the paper was 47.2 kg for farmed wild boar of 39 weeks old; this weight was standardised to the average weight for domestic pigs, which would represent the weight of an average adult wild boar (live weight 75 – 160 kg, depending on age and gender). [2] Chilean pork cut is identical to Canadian cut. [3] these separate parts were given in the paper by Skewes et al. [4] Pork cut 'Belly' is called 'Spare rib' in Chilean cut. [5] neck loin and loin are considered jointly, since they will probably be prepared similarly by consumers.

Table VI. Number of *Trichinella* ML detected in different muscle types of experimentally infected pigs, and estimated distribution of ML over 100 g portions of edible tissue from 1 pig.

Year	2005	2006	2007	2008	2009	2010	2012	2013							
Infection dose	37000	40000	40000	20000	40000	40000	40000	40000		Wild boar			Domestic pig		
Incubation time (weeks)	23	19	19	26	16	10	13	16	Row sums	Portions	Total ML	Fraction	Portions	Total ML	Fraction
Diaphragm pillar	460	469	645	288	1148	239	1332	821	5402	2[a]	10804	0.0134	2[a]	10804	0.0110
Shoulder	94	232	194	102	297	107.5	396	230	1652.5	50	82625	0.1028	35	57837	0.0590
Belly	303	214	272	146	658	155.5	725	463	2936.5	76	223174	0.2776	54	158571	0.1618
Loin	72	79	59	44	132	40.5	206	156	788.5	103	81215	0.1010	144	113544	0.1159
Ham	80	93	110	76	217	53	309	161	1099	120	131880	0.1640	121	132979	0.1357
Other[b]	1225	1112	1322	752	1895	559	2199	1480	10544	26	274144	0.3410	48	506112	0.5165
Column sums	2234	2199	2602	1408	4347	1154.5	5167	3311	22422.5		803842	1		979847	1

[a] the weight of diaphragm was estimated at 200 g;

[b] 'other' represents tongue, masseter and foreleg; the weights of tongue and masseter were estimated at 400 g each, the weight of foreleg is shown in Table IV. Data adapted from (Mayer-Scholl et al., 2012; Mayer-Scholl et al., 2009, 2010; Mayer-Scholl et al., 2011; Nöckler and Reckinger, 2005)

Number of edible portions and *Trichinella* ML distribution

The output of the previous paragraph is the number of *Trichinella* ML per unit of 100 g diaphragm in an infected carcass that was tested negative during meat inspection and the prevalence of such samples. This output needs three further steps to identify the number of *Trichinella* ML for each consumable portion of pork or wild boar meat.

Step 1 is to determine the number of portions that are available for consumption from relevant muscle parts. The average weight of commercially relevant swine muscle parts was determined using published experimental data of several pig breeds and of farmed wild boar (Marcoux et al., 2007; Monziols et al., 2006; Skewes et al., 2008) (Table V). Commercial pork cuts are shoulder, foreleg, loin, rib, belly and ham, but the most relevant pork parts were considered to be loin, shoulder and belly (including intercostal and abdominal muscles), since the consumer purchases these parts raw and food safety relies on proper home cooking procedures. Foreleg is generally eaten well cooked and thus we excluded this type

of meat. For each selected pork part, we calculated the number of edible 100 g portions (Equation 8).
In step 2, we estimated the number of *Trichinella* ML in edible muscle parts from the number of *Trichinella* ML in 100 g of diaphragm. Experimental infection data obtained over eight years, using one domestic pig per year, revealed the distribution of *Trichinella* ML over 100 g of diaphragm, tongue, masseter, shoulder, foreleg, abdominal and intercostal muscles (= belly), loin and ham of each animal (Table VI).
Given the numbers present, the distribution of the larvae over the muscle groups is assumed multinomial, but unlike the common situation where one knows the total, and is interested in counts in categories, we now know counts in a single category (diaphragm) and we wish to estimate the counts in the other categories. For this purpose the negative multinomial distribution is suitable ((Lange, 2010) page 139), where C is a vector of larvae counts, and the probability distribution describes the probability of finding $C_1=c_1, \ldots, C_M=c_M$ larvae (Equation 9). The number of muscle parts is $M+1$, with category $M+1$ being the diaphragm for which the number of larvae Y_j is known in the model. The probabilities p_i are estimated in the usual way, by dividing the number of larvae in category i by the total (see columns 'Fraction' Table VI).
In step 3, the number of *Trichinella* ML in each muscle part was assigned to the portions by using a multinomial distribution with equal probability for each portion (Equation 10). This output entered the next part of the model.

Inactivation by cooking

The risk of contracting trichinellosis is related to the number of eating occasions and portion size, the number of false negative pools that escape detection at meat inspection, the prevalence of positive carcasses in those pools, the number of larvae per gram of edible meat, Trichinella ML that escape inactivation by cooking and the sex distribution of *Trichinella* ML.
To evaluate heat-inactivation of *Trichinella* ML in meat, we extracted data from the limited available literature on *Trichinella* ML inactivation by oven cooking of pork from experimentally infected pigs (Carlin et al., 1969) and cooking of naked larvae in test tubes containing water (Randazzo et al., 2011). We modelled *Trichinella* ML inactivation as a function of temperature and time. The model for the probability of *Trichinella* ML inactivation as a function of temperature at a given cooking time, is approximated by an ascending sigmoidal curve, which has a transition temperature at which the initial horizontal component (0% inactivation) changes into a linear ascending part, until a second transition temperature is reached, above which inactivation is 100%. In the linear ascending part of the model, inactivation depends on temperature to which a portion of meat is heated and the time during which heating takes place. T^* is the point where the temperature inactivation curve reaches its maximal slope. Equation 11 describes residual numbers of larvae (I_1) after inactivation of a portion of meat containing I_0 larvae at time

point t_0 = 0 and temperature T_0, following heating to temperature T_1 at time point t_1. (Swart and Franssen, manuscript in preparation).

Finding a realistic cooking scenario in literature is difficult and published experiments seldom fit ones purpose exactly. For the model, we used a set of cooking scenarios, which included an experimental temperature-time profile for the preparation of pan-fried patties weighing 115 g (dimensions: 2,5 cm thick, 8.5 cm in diameter) (Swart et al., 2015). Furthermore, we used USDA temperature recommendations for consumer pork cooking (USDA, 2015), and results of a small scale questionnaire, which revealed that 15% of responders cooked pork chops medium done (scenario 'Medium Swart') and 85% cooked pork chops well done (scenario 'Chef') (http:// donetemperature.com/) or 'Traditional' (well done plus extra cooking time, Table VII).

Cooking preferences are personal and diverse, often passed on between generations and difficult to capture. Consumer preference data from UK (Food & you, 2014) and Dutch reports (Swart et al., 2015) showed that 8 – 12% of the population eats meat 'that has pink or red juices', indicating medium or rare cooked meats, including pork and sausages. The frequency at which this happened was reported as 'some times' to 'regularly'. For the model, we assumed that 10% of the general population eats risky meat, defined as pork that has been cooked to a core temperature of no more than 63 °C (scenario 'Medium, USDA') and 90% of the population cooks pork to 'Well done Chef'. All scenarios include scenario 'Rare, Swart' as first stage, after which each scenario continues with its own profile (Figure 3).

Table VII. Different cooking scenarios for pork and wild boar meat.

Scenario	T_0 (°C)	T_1 (°C)	t_1 (min)	Extra (min)
Rare, Swart	20	54	2.5	-
Medium Swart	54	63	4.2	-
Well done Swart	54	68	5.3	2.0[a]
Medium, USDA	54	63	4	3.0[b]
Well done, USDA	54	71	9	-
Well done, Chef	54	76.7	10	-
Well done, Traditional	54	76.7	10	10.0[c]

The first stage of every scenario is 'Rare, Swart', e.g. scenario Medium Swart utilises 2.5 minutes to reach 54 °C, followed by 1.7 minutes continued cooking to reach a final temperature of 63 °C, and a final cooking time of 4.2 minutes. See Figure 4 for an overview of cooking scenario profiles.

[a] additional cooking time at 68 °C; [b] resting time at 63 °C; [c] additional cooking time at 76.7 °C.

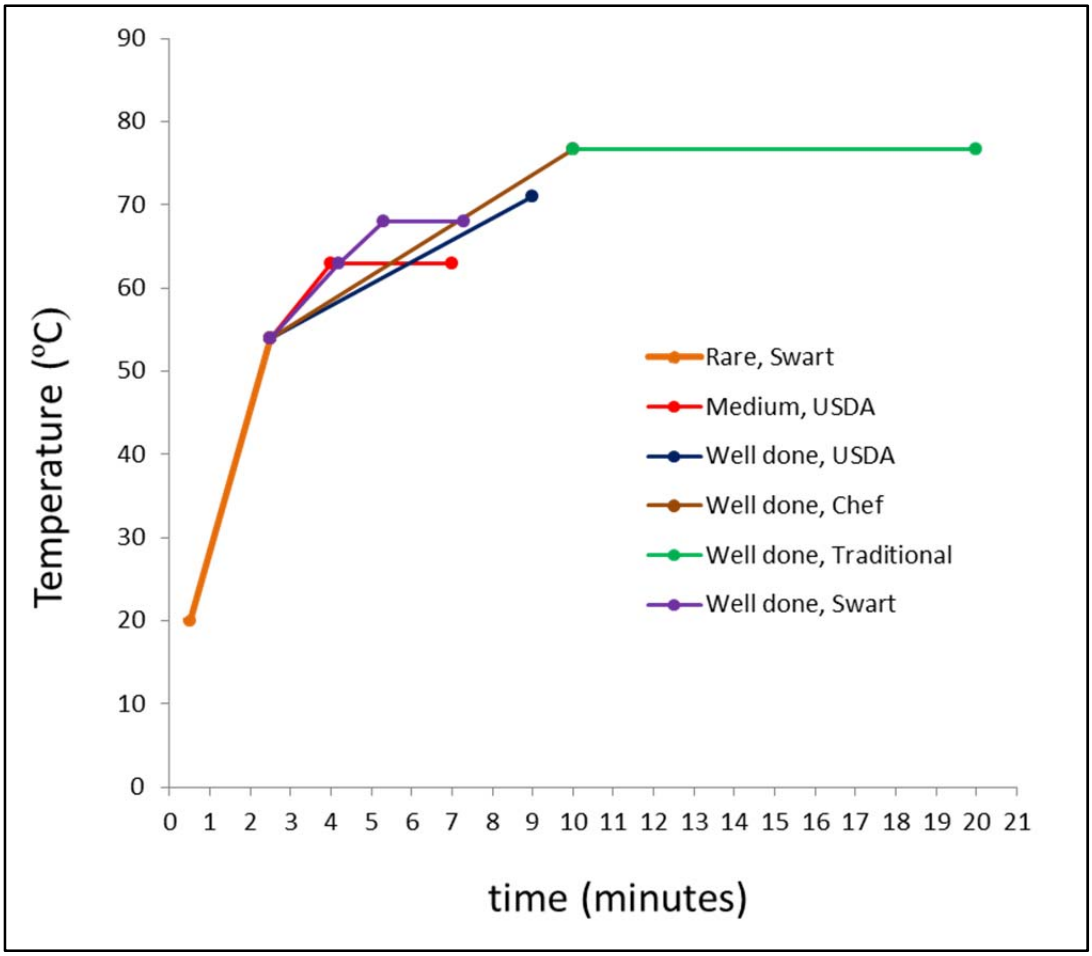


Figure 3. Cooking scenario profiles.
Overview of cooking scenarios built on basic scenario 'Rare', which runs from 20 ºC until 53 ºC. All other scenarios continue from there, except scenario 'Well done, Traditional', which continues from the final temperature of scenario 'Well done, Chef'.

Consumption patterns

Since consumption data for wild boar meat in Poland are lacking, we calculated the average number of consumable portions per person per year in that country as the total number of consumable portions of wild boar meat for the period 2007 – 2012, divided by the total population for Poland over the same period (Equation 12). The total number of consumable portions was calculated by multiplying the total number of tested wild boar over the period 2007 – 2012 by the total number of portions shoulder, loin and belly from a single wild boar (Table V). To calculate the total number of consumed portions of pork, first, the average number of consumed portions of pork weighing 100 g was calculated

from consumption data during the period 2008 – 2013 (AHDB, 2015). Second, the annual consumption in Poland was calculated using Equation 13, taking into account that 78.4% of Polish pig farms keep their animals under non-controlled housing (Pozio, 2014)

Dose response modelling

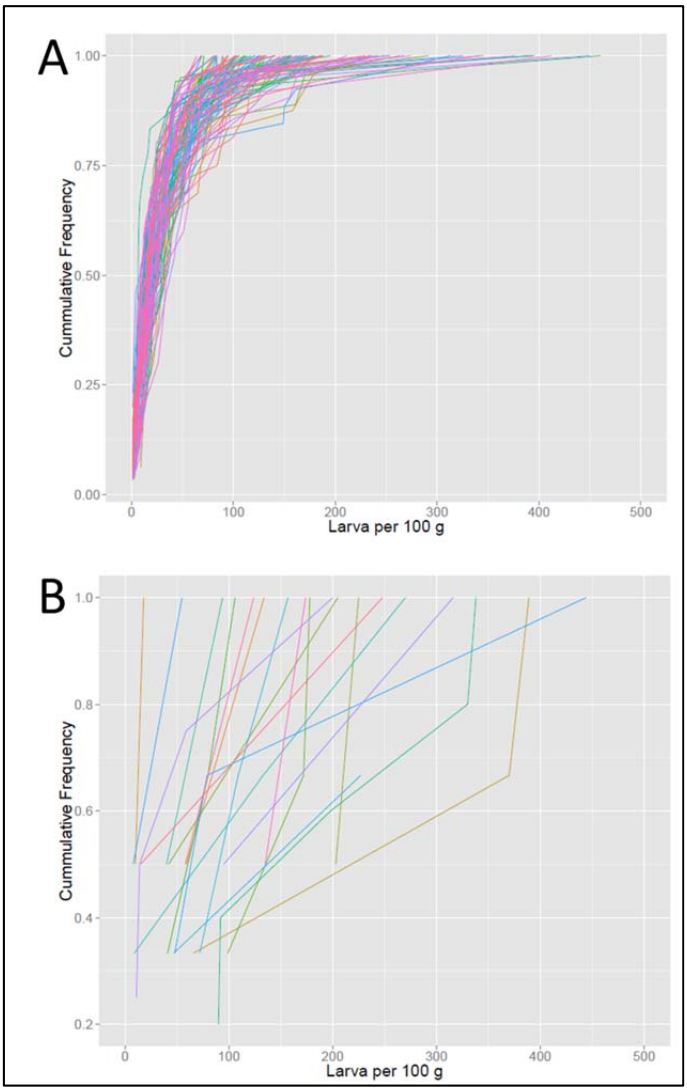
Finally, to model disease risk from exposure to *Trichinella* ML, we used a previously published dose response model that includes different Trichinella species (Teunis et al., 2012) (Equation 14). This model takes into account the number of ingested larvae, but also the distribution of male and female worms that produce the next generation (new borne larvae), which cause disease. The output of the dose response model is the probability of illness, given exposure to a single portion of undercooked meat, given presence of a known number of *Trichinella* ML in that meat.

Risk characterisation

The output of this module is distributions of human illness cases, simulated over year, with each year a new realisation of the animal infection distribution (Equation 1). For each year, we simulate all portions from 5000 randomly generated carcasses. We modelled the average number of portions of undercooked pork or wild boar meat from false negative batches that cause illness, per million portions per year. Using this number and the consumption data for wild boar meat and pork as described above, the incidence rate due to consumption of pork or wild boar meat was calculated using Equation 15 (wild boar) and Equation 16 (pork from non-controlled housing), resulting in an estimate of the number of human trichinellosis cases per year. Finally, the incidence was divided by the population number (in millions) to generate incidence rate estimates, expressed as cases of human trichinellosis per million persons per year (Mpy).

Extrapolation to pigs from controlled housing

Seventy-eight percent of pigs produced in Poland are from non-controlled housing and data from controlled housing are unavailable. Hence, we resorted to data from the Netherlands, where the vast majority of pigs is kept under controlled housing. In the Netherlands, 139,729,393 pigs were tested in the period 2003 – 2013 and not a single pig was tested positive during that period. It is not feasible to use these data for calculations in the present model because it is impossible to determine a measurable prevalence of *Trichinella* ML in these pigs, to feed the model. However, even if in the past this many pigs were all tested negative, a future test may turn out positive. Therefore, we calculated the probability of finding one or more *Trichinella* positive pigs in 1 million (10% of the hypothetical slaughter volume) to 10 million pigs tested in the following year.



Taking into account all the observed negative outcomes in the past, the beta distribution is the posterior probability distribution of a positive outcome for an additional test in the following year. For additional n tests in the following year, the number of positive outcomes is the beta binomial distribution (Equation 17). Assuming a uniform beta prior and taking into account 139,729,393 pigs tested in the past, we set two parameters of the beta binomial distribution at A = 0 (zero positives) and B = 139,729,393 to model possible positive outcomes from testing an additional number of n pigs raised under controlled housing, with posterior Beta (A+1, B-A+1).

Figure 4. Cumulative frequency of larvae in the diaphragm of positive animals that are missed at the meat inspection. A. Frequency distribution for 114,266 wild boar. **B.** Frequency distribution for 22,879,163 domestic pigs from non-controlled housing. Note the lower cumulative frequencies in comparison with wild boar, which is due to a lower overall prevalence of positive animals in the population.

Results

Data from Polish *Trichinella* control at slaughter in the period 2007 – 2012 revealed an average *Trichinella* prevalence for wild boar of 0.0041 (n = 685,595, y = 2,832, 95%CI 0.004 – 0.0043, Table III) while for domestic pigs from non-controlled housing, the

prevalence was 1.27×10^{-6} (n = 114×10^{6}, y = 145, 95%CI 1.06×10^{-6} – 1.48×10^{-6}, Table III). The mean number of *Trichinella* ML in 50 g of diaphragm was 5.24 and 0.0016 for wild boar and pigs from non-controlled housing, respectively. *Trichinella* ML distribution among wild boars was 4.47×10^{-4}, whereas this was 1.3523×10^{-7} in pigs from non-controlled housing (Table III).

Trichinella testing at the meat inspection

The probability of recovering at least one *Trichinella* ML was estimated at 0.841 for one larva present in the test sample, 0.927 for two larvae, 0.956 for three larvae, rapidly increasing to 0.970 – 0.991 for 4 – 10 larvae that were present in the pooled test sample.
The estimated prevalence of false negatives in all pooled samples was 3.73×10^{-3} (95%CI 2.17×10^{-3} – 4.9×10^{-3}) for wild boar and 6.03×10^{-6} (95%CI 0.00 - 1.65×10^{-5}) for domestic pigs from non-controlled housing (Table VIII (A)). Only a minority of individual animals in these false negative pools was truly positive: 5.32×10^{-2} for wild boar (95%CI 5.00×10^{-2} – 5.93×10^{-2}) and 0.010 for domestic pigs from non-controlled housing (95%CI 0.010 – 0.010) (Table VIII (B)). Figure 4 shows the cumulative frequency distribution of identifying larvae in 100 g of diaphragm of positive animals in a false negative test pool. Table VIII (C) shows the prevalence of *Trichinella* ML infected portions for each of the modelled muscle types before cooking, over all portions of positive carcasses. The consumers cooked these infected portions.

Inactivation by cooking

Cooking meat to scenario 'Rare' hardly inactivates any *Trichinella* ML. Cooking scenario 'Well done, Traditional' is the only scenario that totally inactivates *Trichinella* ML in meat. However, we expect that this scenario applies to a minority of consumers. Finally, we chose a combination scenario of 10% of portions cooked to 'Medium, USDA' and 90% of portions cooked to 'Well done Chef' to include consumer preference in the inactivation-by-cooking model. Results for this combination of cooking preferences for each of the muscle types are shown in Table VIII (D). The probability of illness for each of the portions from positive carcasses (from a false-negative batch) after cooking is given in Table VIII (E).

Risk characterisation

An individual person on average consumed 0.68 portions of wild boar and 403 portions of pork each year.

Wild boar

The estimated annual prevalence of portions of wild boar potentially causing human illness was 2.89 (95%CI 1.21 – 5.88) per million cooked and consumed portions of shoulder, belly, or loin combined (Table VIII (G)). This resulted in 75.3 (95%CI 31.6 – 153) cases of human trichinellosis per year in Poland, or 1.97 (95%CI 0.82 – 4.00) human cases per million persons per year Table VIII (K and L, respectively).

Table VIII. Model outcomes calculated for three swine production systems: hunted wild boar, pigs from non-controlled housing and pigs from controlled housing.

	Wild boar		Pig, non-controlled housing	
Carcass testing	***mean***	***95% CI***	***mean***	***95% CI***
A. False negative pools	3.63×10^{-3}	$2.17\times10^{-3} – 4.9\times10^{-3}$	6.03×10^{-6}	$0.00 – 1.65\times10^{-5}$
B. Positive carcasses in those pools	5.32×10^{-2}	$5.00\times10^{-2} – 5.93\times10^{-2}$	0.010	0.010 – 0.010
C. *Trichinella* infected portions				
Shoulder	0.912	0.821 - 0.986	0.739	0.000 - 1.000
Belly	0.716	0.576 - 0.869	0.731	0.000 - 1.000
Loin	0.751	0.615 - 0.890	0.697	0.000 - 1.000
D. Residual infected cooked portions				
Shoulder	0.273	0.155 - 0.408	0.536	0.000 - 1.000
Belly	0.080	0.048 - 0.148	0.411	0.000 - 1.000
Loin	0.090	0.053 - 0.156	0.240	0.000 - 0.990
E. P illness from cooked portions				
Shoulder	0.029	0.016 - 0.050	0.081	0.000 - 0.248
Belly	0.007	0.003 - 0.017	0.046	0.000 - 0.162
Loin	0.009	0.004 - 0.020	0.021	0.000 - 0.084
F. N illness per million portions				
Shoulder	5.55	2.440 – 10.0	0.006	0.000 - 0.021
Belly	1.41	0.541 – 3.41	0.003	0.000 - 0.013
Loin	1.71	0.655 – 3.95	0.001	0.000 - 0.006
G. Average N illness per million portions	2.89	1.210 – 5.880	0.004	0.000 - 0.013
H. N consumptions of 100 g pp/year	-	6.80×10^{-1}	-	403[a]
I. Average population size 2007 - 2012	-	3.83×10^{7}	-	3.83×10^{7}
J. Total consumed portions	-	2.61×10^{7}	-	1.54×10^{10}
K. Total predicted human cases / year	75.3	31.6 - 153	59.0	0 - 212
L. Predicted human cases / million / year	1.97	0.82 – 4.00	1.56	0.00 – 5.52

A. Probability that a swine is from a false negative batch. **B.** Prevalence of positive carcasses from false negative batches; **C.** Prevalence of positive portions before cooking, over all portions of positive carcasses; **D** Prevalence of positive portions after cooking, over all portions of positive carcasses; **E** Probability of illness, for each of the portions from positive carcasses; **F** Number of illnesses per million portions of 100g. **G.** Average number of illnesses per million portions of any muscle type.

[a] The average pork consumption per year from non-controlled housing is estimated at 78.4% (Pozio, 2014) of 514 portions.

Domestic pigs from non-controlled housing

The estimated annual prevalence of portions potentially causing human trichinellosis per million portions of pork from pigs from non-controlled housing was 0.004 (95%CI: 0 – 0.013) per million cooked and consumed portions of shoulder, belly, and loin combined (Table VIII (G)). This was equivalent to 59 (95%CI 0 -212) cases of human trichinellosis for the Polish situation, or 1.56 (95%CI 0 – 5.52) cases of trichinellosis per million persons per year Table VIII (K and L, respectively).

Table IX. Analysis of trichinellosis incidence following different cooking scenarios.

Wild boar	**Trichinellosis cases / year**			**Cases / million persons / year**		
Scenario	**Average**	**2.5 perc**	**97.5 perc**	**Average**	**2.5 perc**	**97.5 perc**
Uncooked	747	430	1120	19.5	11.2	29.2
Rare, Swart	713	405	1090	18.6	10.6	28.3
Medium Swart	511	268	810	13.3	6.98	21.1
Well done Swart	220	96.7	574	5.74	2.52	10.3
Medium, USDA	190	85.2	34.2	4.95	2.22	8.91
Well done, USDA	72.8	23.5	158	1.90	0.61	4.13
Well done, Chef	25.5	4.14	74.2	0.66	0.11	1.94
Well done, Traditional	0	0	0	0	0	0

Pigs from NCH*	**Trichinellosis cases / year**			**Cases / million persons / year**		
Scenario	**Average**	**2.5 perc**	**97.5 perc**	**Average**	**2.5 perc**	**97.5 perc**
Uncooked	318	0	878	8.30	0	22.9
Rare, Swart	308	0	853	8.05	0	22.2
Medium Swart	255	0	736	6.65	0	19.2
Well done Swart	153	0	478	3.99	0	12.5
Medium, USDA	140	0	445	3.65	0	11.6
Well done, USDA	77.3	0	271	2.02	0	7.06
Well done, Chef	38.7	0	163	1.01	0	4.25
Well done, Traditional	0	0	0	0	0	0

NCH*: non-controlled housing

Risk analysis for different cooking scenarios

All cooking scenarios were evaluated against scenario 'Rare, Swart' using estimated annual human trichinellosis as a measure (Table VII, Figure 3). In each scenario, the same cooking methodology applies to all edible portions. Resulting incidence estimates for each of the modelled cooking scenarios for both wild boar and pigs from non-controlled housing are shown in Table IX. These results allow quantification of the effect of cooking to prevent human trichinellosis. Without cooking, the hypothetical number of cases of

trichinellosis per year from consumption of wild boar meat, is estimated 747 (95%CI 430 - 1120), and 318 (95%CI 0 – 878) for consumption of pork from non-controlled housing.

Comparison with epidemiologic estimates

Incidence rate estimates from our model are 1.97 per Mpy for wild boar and 1.56 per Mpy for pigs from non-controlled housing (Table VIII (L)), which results in a total incidence rate estimate of 3.53 cases of human trichinellosis per million inhabitants per year. The observed incidence rate for Poland for the period 2007 – 2012 is 1.15 (EFSA-ECDC, 2010, 2013, 2014). Our estimate is therefore not inconsistent with best-available independent evidence, although direct comparison between the epidemiological evidence and our model is not straightforward (see the Discussion)

Extrapolation to pigs from controlled housing

In view of the huge numbers of test-negative pigs from controlled housing in the Netherlands, the probability of finding *Trichinella* ML in future tests is minute. At a theoretical slaughter volume of 10 million pigs in the next year, the probability of finding one positive pig out of 1 million tested is 0.0007. It takes testing of 7.3 million pigs to achieve a probability of 0.05 of finding one or more positive pigs, but even considering all 10 million hypothetical pigs, the probability of finding one or more positive pig is still only 0.067 (Table X).

Table X. Probability of finding positive pigs when no positives were found out of 140 million tested in the preceding ten years, when testing 0.1 million, 1 million or 10 million pigs the following year.

Larva/ pig	0.1 million	1 million	7.3 million	10 million
0	0.9993	0.9929	0.9503	0.9332
1	0.0007	0.0070	0.0472	0.0623
2	5.11×10^{-7}	5.01×10^{-5}	0.0023	0.0041
3	3.65×10^{-10}	3.56×10^{-7}	0.0001	2.70×10^{-4}
4	2.61×10^{-13}	2.53×10^{-9}	5.77×10^{-6}	1.85×10^{-5}
5	1.87×10^{-16}	1.79×10^{-11}	2.86×10^{-7}	1.24×10^{-6}

Alternatively, EU data enabled us to better estimate the upper prevalence limit, since far more pigs from controlled housing have been tested in the past. Roughly 120 million pigs from controlled housing are slaughtered and tested annually in the European Union (Pozio, 2014), without any *Trichinella* ML findings in the last two decades. As a result, the observed EU-wide prevalence is less than one per 2,400 million pigs from controlled housing. From this data, an upper limit of prevalence for pigs from controlled housing is estimated using Equation 18. From the upper prevalence limit, we estimated that the risk from eating pork from controlled housing is less than 5.3×10^{-5} human cases per million per year (Equation 19). At a total EU population of 508 million, this boils down to less

than one citizen of the European Union with trichinellosis due to eating pork from controlled housing in 39.4 years (Equation 20).

Discussion

This paper presents a quantitative microbial risk assessment (QMRA) model for *Trichinella*, based on experimental and literature data. We modelled human trichinellosis incidence from consumption of both wild boar and domestic pig meat from non-controlled housing, using Poland as an example. We implemented in our model the distribution of *Trichinella* spp. numbers in meat, inactivation of *Trichinella* ML by cooking and dose-response relationships for *Trichinella* spp. infections in humans.

Infected swine that escape carcass control pose a risk for human trichinellosis. In the present study, we assumed the lower limit for *Trichinella* ML detection at meat inspection to be one larva per gram, representing one positive animal in a pooled sample, the lowest theoretically achievable detection level using the magnetic stirrer test, which is the standard reference method (European-Commission, 2015). Our estimates on the probability of finding one larva in a 100 g pooled sample are consistent with a previous study using experimentally infected pigs, which reported a test sensitivity of 73% for pooled digestion of 100 samples of 1 g at an infection level of 1.0 – 1.4 larvae per gram (Forbes and Gajadhar, 1999). Sensitivity in that study when testing a lower infection level (0.001 – 0.9 larvae per gram) dropped to 40%, which is understandable in view of high probability of missing larvae at this level. It is precisely this situation where we expect our model to perform well. Indeed, our model predicts a range between 1 and 500 *Trichinella* muscle larvae per 100 g of diaphragm (on average 0.01 – 5 larvae per gram) of positive animals that were missed at meat inspection and allows subsequent estimation of resulting cases of human trichinellosis.

Our estimates are based on data regarding the hazard in Poland, and the reported human trichinellosis incidence rate in Poland should be the most compatible, alternative measure of risk. Comparable magnitude in our QMRA estimates and in the reported incidence rate is a fair support for our modelling approach.

Our QMRA model shows an estimated annual incidence rate in Poland of 75.3 human trichinellosis cases from consumption of wild boar (95%CI 31.6 – 153 cases). For consumption of pork from pigs reared under non-controlled housing, the estimated incidence rate was 59.0 trichinellosis cases per year (95%CI 0 – 212 cases). The reported total number of human trichinellosis cases in Poland over the period 2007 – 2012 (EFSA-ECDC, 2010, 2013, 2014), ranged between 1 and 216 confirmed cases per year for the whole population. The reported numbers appear congruent to the sum of our estimates. Note however, that our model predicts the number of sporadic cases, while the reported number includes clustered outbreaks of trichinellosis. However, as the number of infected animals will be low, also the disease cases modelled here will be clustered, even though this is not explicit in our model.

Our QMRA model includes all stages in the chain of events from *Trichinella* prevalence and distribution in animals to exposure, infection and illness in humans. This allows quantification of risk when one or more parts in the chain change due to a locally-specific factor, such as country-specific *Trichinella* prevalence and abundance, varying test sensitivity at meat inspection, different proportions of controlled housing in a country, and consumption data. For the sake of clarity, we did not include heterogeneity into all aspects of the QMRA model. One example of heterogeneity that is not included in the presented model is consumption pattern in a country, where a group of non-pork consumers may be present. A recently published consumer survey, conducted in 2008 in five European countries (Belgium, Denmark, Germany, Greece and Poland), showed that 12% of respondents (mostly women living alone) never eat pork. The survey reported in addition: 18% rarely eat pork (predominantly single women), 51% eat one serving of fresh or processed pork per day (families and other non-single households), and 19% eat several servings per day of both fresh and processed pork (predominantly less educated overweight males) (Verbeke et al., 2010). In our approach, all persons in the population are assumed to consume slightly more than one portion of pork per day. The current model would need further development to evaluate the effect of a combination of different consumption patterns.

Another example of heterogeneity that is not included in the presented model is consumable portions weighing more than 100 grams. The per capita pork consumption in Western Europe ranged from 24.1 kg (UK) to 57.6 kg (Austria) in the years 2008-2013, indicating a pork consumption of on average 66-158 g per capita per day. The highest average pork consumption was recorded in Austria, Germany, Poland and Spain (50.2 - 57.6 kg) (AHDB, 2015). The average pork consumption does not discriminate between groups within a society and the actual portion size may be higher: the young and the elderly generally eat less meat, and men generally eat more than women do. Perhaps the actual portion size per occasion might well be 100 – 300 g (Opsteegh, 2012) (Teunis et al., 2011). Consumer data concerning wild boar meat is limitedly available from papers describing trichinellosis outbreaks, which mention portion sizes of 58 – 396 gram (reviewed in (Teunis et al., 2011)).

We did include heterogeneity in consumer preferences to prepare a meal in an accustomed way. We chose a combination of 10% of portions cooked to scenario 'Medium, USDA' and 90% of portions cooked to 'Well done Chef' to model inactivation by cooking. In a published study using experimental pan frying of different types of meat, pork chops cooked for 6.5 minutes to an internal temperature of 70 ˚C, were considered well done (Lahou et al., 2015). This approaches our cooking scenario 'Well done, USDA' and may be an alternative to 'Well done Chef' in our setting. However, Lahou and co-workers state that in three replicate experiments, their scenario failed to achieve an internal temperature of 70 ˚C for two minutes for pork chops, which they considered necessary to render the meat microbiologically safe. This implies that 6.5 minutes cooking time may be too short, which supports the longer cooking time in both scenario 'Well

done, USDA' and 'Well done Chef' in our setting. Inclusion of a third scenario into our QMRA may be needed to achieve a more accurate approximation of consumer preference, since we used scenario 'Medium, Swart' to include consumption of meat that has pink or red juices into the model, the latter of which may implicate lower doneness than achieved with scenario 'Medium, Swart'.

Table XI. Published *Trichinella* outbreaks 2007-2014.

***Trichinella* species**	**Country**	**Cases**	**Source**	**Confirmation**	**Reference**
T. spiralis	Spain, Sweden	21	Home-made sausage of wild boar meat from Spain	Yes (trichinoscopy)	(Gallardo et al., 2007)
T. spiralis	Poland, Ireland, Germany	219	Raw sausage of pork from West-Pomerania, Poland (one producer)	Not stated	(Golab et al., 2007b))
T. spiralis	Germany	3	Cured sausage of home slaughtered pig from Romania	Not stated	(Nockler et al., 2007)
T. spiralis	Slovac Republic	23	Home-slaughtered pork and/or smoked pork product	No	(Reiterova et al., 2007)
Not stated	Denmark	7	Sausage of raw pork meat from West-Pomerania, Poland	Not stated	(Stensvold et al., 2007)
T. britovi	Turkey	1098	Illegal mixed pork into beef	Not stated	(Akkoc et al., 2009)
Not stated	Italy	5	Pork products of non-tested pork from Romania	No	(Angheben et al., 2008)
T. spiralis	Spain	4	Wild boar meat	No	(Arévalo et al., 2009)
Not stated	Lithuania	107	Sausage of wild boar meat	No	(Bartuliene et al., 2009)
T. spiralis	Romania	15	Home-slaughtered pork	No	(Neghina et al., 2010a)
T. spiralis	Argentina	64	Home-slaughtered pork	No	(Calcagno et al., 2014)[b]
T. spiralis	Iowa, USA	6	Wild boar ham roast	No	(Holzbauer et al., 2014)[c]
T. spiralis	Germany	101	Wild boar meat	Yes[a]	(Faber et al., 2015)
T. britovi	Italy	38	Home-made raw sausages of wild boar meat	No	(Fichi et al., 2015)[b]

[a] The identity of a wild boar that was found positive at testing was switched accidentally with a negative wild boar. [b] The regions were these outbreaks occurred were wrongly considered to be non-endemic for *Trichinella* spp.. [c] The wild boar was hunted and eviscerated on a game farm; infection of a father and his son may have taken place during processing the wild boar.

In our QMRA model, we did not include some factors that might influence the results, such as consumption of raw meat products. The term 'raw meat products' refers to an inhomogeneous group of different product types, varying from dried or smoked ham to complicated, combined products, such as sausages, each with its own preparation method (Savic, 1985). Industrially produced raw sausages are prepared from *Trichinella* tested and certified pork, following strict regulations, surrounded by QA measures, to guarantee efficient inactivation of *Trichinella* muscle larva and other potential pathogens that might be present in the raw meat (Essien, 2003; Porto-Fett et al., 2010; Smith et al., 1989). In contrast, fresh home-made raw sausages, one of the main causes for trichinellosis in Europe (Table XI), are often prepared from non-tested wild boar meat or non-tested pork from back-yard pigs (Sadkowska-Todys and Golab, 2013). Typically, these sausages are shared with family and friends, and often cause small and clustered outbreaks, not only in Poland, but also in other *Trichinella*-endemic countries (Neghina, 2010). Eight outbreaks due to consumption of *Trichinella* infested raw wild boar sausages have been described in seven European countries (including Poland) between 2007 and 2015, causing 7 – 219 confirmed human cases per outbreak (Table XI). Although raw products were not explicitly included in our QMRA model, the Polish outbreak and other human trichinellosis cases reported for Poland for the period 2007 – 2013 (EFSA-ECDC, 2010, 2013, 2014), fit within incidence limits estimated in our model.

We did not include *Trichinella* inactivation through home freezing by consumers, who will probably freeze a proportion of purchased fresh pork or wild boar meat, to prolong its shelf life. The extent to which this takes place is difficult to establish. In a British survey, 80% of households own a consumer freezer, of which 51% would freeze fresh meat (not specifically pork) (Maxey and Oliver, 2010). In a Scottish high-income consumer survey, 60% of responders would freeze wild game meat (FSAS, 2012). The efficacy of freezing to kill *Trichinella* ML depends on freezing temperature, frozen storage time, *Trichinella* species and host species, but one week freezing at -18 °C or -21 °C effectively kills *T. spiralis* and *T. britovi* in infected meat (Hill et al., 2009; Lacour et al., 2013; Malakauskas and Kapel, 2003a). If a proportion of consumers would freeze fresh meat, this could proportionally lower human exposure to *Trichinella* ML and the resulting incidence of human trichinellosis estimated in our model. More research is needed to establish to which extent consumers actually do freeze pork or wild boar meat, and whether these home-freezing conditions fully inactivate *Trichinella* ML.

We included variability, in our model, except for the dose response module, where we sampled parameters from an uncertainty distribution. More work is needed to evaluate uncertainty in the other parts of our model. E.g. for weight of diaphragm samples, we used the required weight according to EU Regulation 2015/1375, whereas the actual weight may vary and depends on correct functioning of sampling devices. Cooking, which is a highly subjective factor that may harbour considerable uncertainty, is another example, since most people will use a rough combination of time, estimated cooking heat and meat weight, to reach a certain doneness of the cooked meat. As a result, the actual

temperature inactivation of *Trichinella* ML that may be achieved is highly variable, as illustrated in Table IX.

In the present paper, we estimated that the risk from eating pork from controlled housing is less than 5.3×10^{-5} human cases per million per year or less than one citizen of the European Union with trichinellosis due to eating pork from controlled housing in 39.4 years. This corroborates derogation from *Trichinella* testing for pigs that are reared under controlled housing and justifies alternative surveillance strategies for animals from this type of husbandry. EU Regulation 2015/1375 (European-Commission, 2015) (Article 2a) stipulates that under controlled housing, at least 10% of the annually slaughtered pigs should be tested, among which all sows and boars, which have a longer life and therefore a higher theoretical risk of infection with *Trichinella*. However, in view of complete absence of positive findings at meat inspection, testing of 10% of animals from controlled housing to demonstrate the absence of *Trichinella* is questionable. In the Dutch example, described in this paper, at a volume of 140 million *Trichinella*-negative pigs at meat inspection in ten preceding years, the probability of finding a positive pig is not more than 0.0007 if testing 10% of pigs from controlled housing, including sows and boars. In the Dutch example, testing of about 7.3 million pigs (50 – 60% of the annual slaughter volume) is needed to obtain a 5% probability of finding a positive pig. Under approved controlled housing as shown here for the Dutch situation, *Trichinella* testing is not adding any value to protect human health, although for the time being, 100% of Dutch slaughter pigs will remain tested for *Trichinella*, due to export requirements.

In conclusion, our QMRA model provides estimates for human trichinellosis incidence from different meat production systems. Comparable magnitude in our QMRA estimates and reported incidence rate, supports the validity of our modelling approach. Our model may prove useful to evaluate alternative scenarios for the quantification of regional consumer-related variables, such as meat consumption, meat portion size and cooking habits, raw sausage consumption and *Trichinella* inactivation through home-freezing of pork, as well as production system-related variables. Finally, our QMRA model may be used to support development of meaningful risk-based monitoring programmes to control *Trichinella* in pigs from different housing systems.

Conflict of interest

The authors declare no conflict of interests.

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Chapter 10

Discussion

General discussion

Four out of twelve recognised species and genotypes of *Trichinella* (*T. spiralis*, *T. britovi*, *T. nativa* and *T. pseudospiralis*) circulate in European carnivores, such as red foxes, wolves, lynxes and bears, and in omnivores such as wild boar and raccoon dogs (Pozio et al., 2009a). Domestic pigs can become infected with *Trichinella spp.* through outdoor access (Boutsini et al., 2014), improper farm management and poor hygienic standards (Neghina, 2010; Stojcevic et al., 2004) or illegal practices (Akkoc et al., 2009), (Kapel, 2001). The most important sources for trichinellosis outbreaks in humans in Europe are ingestion of *Trichinella* muscle larvae (ML) in improperly cooked meat from wild boar and pigs not kept under controlled housing conditions and not tested for Trichinella (Angheben et al., 2008; Arévalo et al., 2009; Bartuliene et al., 2009; Gallardo et al., 2007; Golab et al., 2007a; Neghina et al., 2010a; Nockler et al., 2007; Reiterova et al., 2007; Stensvold et al., 2007). Also illegal practices, such as mixing untested meat from *Trichinella* susceptible animal species may cause large outbreaks as seen in Turkey, where *Trichinella* infected meat was mixed with beef, which affected 1098 people (Akkoc et al., 2009).

The overall aim of this thesis was to study the presence and prevalence of *Trichinella* spp. in wildlife host species, that play a role in the ecology of the parasite and its presence in the food chain, which could be used to develop a quantitative microbial risk analysis (QMRA) model for Trichinella. The presence and prevalence of *Trichinella* spp. in wildlife host species was studied to generate data over longer periods of time, which may provide insight in the natural fluctuations of *Trichinella* spp. in wildlife populations. Wildlife data, meat inspection data and experimental data, were used to develop a quantitative microbial risk analysis (QMRA) model for *Trichinella*. Such a QMRA model may be used to identify hazards in the chain of events, from *Trichinella* presence in domestic livestock or wildlife, to *Trichinella* infection of humans. Additionally, such a QMRA model could be used to quantify attribution of factors such as husbandry system (e.g. controlled housing, free ranging), types of meat and meat products (e.g. different pork cuts, fresh meat), or carcass control (e.g. introduction of alternative equipment), to evaluate risk based control measures.

To develop the QMRA model, we used *Trichinella* prevalence and abundance data from Polish wild boar and prevalence data from domestic pigs reared under non-controlled housing in Poland, and we incorporated experimental data from the literature and practical data obtained from proficiency tests of Dutch slaughterhouse labs. Finally, we used human *Trichinella* incidence data, based on confirmed cases (EFSA-ECDC, 2010, 2013, 2014) to validate the QMRA model and subsequently, the validated QMRA model

was used to quantify human infection risk following different scenarios. Finally, these results can be used to evaluate risk based control measures and to inform about risk based surveillance.

Detection of *Trichinella* spp. in wildlife and pigs

The artificial digestion method according to EU 2015/1375 (European-Commission, 2015), (updating the previous Regulation EU 2075/2005 (European-Commission, 2005) with its amendments EU 216/2014 (European-Commission, 2014)) is used at slaughterhouse laboratories to detect *Trichinella* ML at meat inspection of pigs, horses, wild boar and other susceptible meat producing animals intended for human consumption. Coiled live *Trichinella* ML in fresh meat samples, would efficiently pass a 180-µm mesh size sieve at meat inspection. However, concerns were raised by an international pork trade partner that a 400-µm mesh size sieve should be used instead, to prevent loss of larvae, as has been reported in 1999 (Gamble, 1999). That paper stated that both motile and dead larvae would not pass a 180-µm mesh size sieve as efficiently as live, coiled larvae do. In contrast, we show that the use of a 400-µm mesh size sieve to isolate live larvae, leads to significantly lower larval counts, due to more debris and longer counting time, compared to the use of a 180-µm mesh size sieve (Chapter 4). It seems that the main factor to explain the discrepancy between the two papers could be the amount of residual meat after digestion that was retained at the sieves in the paper of Gamble et al (2.372 ± 0.803 g dry weight), which was considerably higher than recorded in our experiments (1.19 ± 0.73 g wet weight). Such an amount of residual meat could easily trap especially dead larvae in the debris on the sieve. Moreover, the experiments carried out by Gamble were performed with infected pork cubes, which could explain differences between experiments, since *Trichinella* ML are not evenly distributed among and within muscles, whereas we used individually counted *Trichinella* ML to spike meatballs. Finally, both the EU reference method and the OIE Terrestrial Manual (OIE-Manual, 2012) recommend the use of a 180-µm mesh size sieve for the detection of *Trichinella* ML for meat inspection at slaughter.

Red fox, wild boar and wild rats were studied to get more insight in sylvatic circulation of *Trichinella* spp. in the Netherlands. As a biosafety measure, all collected red foxes are to be frozen at -80 ˚C, to prevent the risk of *Echinococcus multilocularis* infection, since the Netherlands is endemic for the fox tapeworm. However, freezing at -80 ˚C, will also kill *Trichinella* ML, which influences their sedimentation characteristics and thus test

sensitivity using the digestion method. We validated an alternative method to isolate dead *Trichinella* ML from artificial digest of frozen fox muscle (Chapter 3).

The prevalence of *Trichinella* in red foxes in the Netherlands was very low at only one *Trichinella* ML found in a pool of six foxes. No *Trichinella* ML were found in 160 wild rats collected during the years 2009 – 2013, using the artificial digestion method on diaphragm and hind legs of the rats (Chapter 8). These data suggest that *Trichinella* spp. circulate at very low prevalence in the Netherlands, or that the levels in infected animals are below the detection limit of 1 larva / 100 g in pooled test samples. In addition, no *Trichinella* ML were detected in pigs, wild boar and horses at meat inspection in Dutch slaughterhouses, during the period 2007-2015. Since serological methods are estimated 30-100 times more sensitive than direct detection methods used at meat inspection, we evaluated the serological response to *Trichinella* infection, to study *Trichinella* infections in the sylvatic cycle at levels below 1 LPG. The in house ELISA for Dutch wild boar was validated and serological results were compared with artificial digestion results using 45 gram of meat sample per wild boar over the period 2004 – 2005. Subsequently, we used the in house ELISA to monitor *Trichinella* in wild boar over the period 2007 – 2015 and confirmed the positive findings with Western blot (Chapter 2). Three animals out of 3,944 wild boar tested, were confirmed positive and another wild boar was found positive by ELISA only, but no *Trichinella* ML were demonstrated in that animal. Moreover, in the study of 2004 – 2005, another – ELISA negative – wild boar carried muscle larvae of *T. pseudospiralis*, as demonstrated by artificial digestion using 45 gram of meat. This resulted in an overall *Trichinella* spp. seroprevalence of 0.076% for Dutch wild boar, which indeed is considerably lower compared to seroprevalence seen in *Trichinella* endemic European regions of 0.68 – 0.74% in Poland (Bien, 2006, 2007), 2.01% on Corsica, France (2006 – 2008) (Richomme et al., 2010) and 2.2% in Italy (2007 – 2008) (Gomez-Morales et al., 2014) ($p<0.0001$). Note that only in the latter study, seroprevalence was confirmed by Western blot and therefore, only this result can be directly compared to ours.

The very low seroprevalence in Dutch wild boar also suggests low infection pressure towards organic and free-ranging pig holdings, in contrast to endemic countries. Indeed, in a study conducted in 2006 in the Netherlands, involving forty organic pig farms, eleven free-ranging farms and 24 intensive farms, only one out of 402 animals from an organic farm tested positive for *Trichinella* spp. in the in-house ELISA (van der Giessen et al., 2007).

We demonstrated that the in house ELISA is applicable for monitoring *Trichinella* infections in laboratory rats and showed that obtained antibody titres were dependent on

infection dose (Chapter 7). However, to study serological responses to *Trichinella* in wild rats we needed to validate the in-house ELISA first, to define seropositivity. The validation of the ELISA was hampered by the lack of well-defined field sera, which otherwise might allow identification of positive and negative sub-populations and determination of a cut-off level (e.g. by binary mixture modelling). Since all tested diaphragms and hind legs of wild rats were negative for *Trichinella* ML in our study, it was not possible to evaluate serological data.

Trichinella spp. characterisation

Trichinella species muscle larvae are virtually indistinguishable morphologically, and therefore molecular techniques are widely used to type all twelve species and genotypes. This may be accomplished by different methods such as multiplex PCR (Zarlenga et al., 1999), analysis of banding pattern using gel electrophoresis in combination with PCR of the mitochondrial CO1 gene (Nagano et al., 1999), or a combination of PCR of the partial ITS-2 gene and restriction fragment length polymorphism (Mayer-Scholl et al., 2014). Additionally, *Trichinella* spp. may be identified using PCR of the 5S intergenic spacer in combination with reverse line blotting (Rombout et al., 2001) or sequencing (De Bruyne et al., 2005), or finally, pyrosequencing of a PCR amplicon of the mitochondrial large subunit ribosomal RNA gene (Sadaow et al., 2015). The multiplex PCR (Zarlenga et al., 1999), is regarded the reference method by the European Reference Laboratory (Istituto Superiore di Sanitá, Rome , Italy). This method utilises the PCR product of the nuclear expansion segment V (ESV) gene to provide a baseline on gel that varies in base pair length between species. *Trichinella* species that exhibit the same baseline are further differentiated simultaneously by a second PCR product of either internal transcribed spacer 1 (ITS1) or 2 (ITS2). Since this method is based on banding pattern analysis on gel, no differences in DNA sequence will be identified.

We used PCR and sequencing of two markers: the 5S intergenic spacer region (Liu et al., 1996; Rombout et al., 2001b) and the mitochondrial CO1 gene, to identify *Trichinella* ML from wild boar and red fox, originating from twelve regions (voivodships) in Poland and from four regions in Latvia. With this approach, we demonstrated genetic evidence for the first time for inter-species hybridisation between *T. spiralis* and *T. britovi*, in Polish regions where both species co-occur (Chapter 5). More specifically, we found interspecies introgression of mitochondrial genomes between *T. spiralis* and *T. britovi*, which may have consequences for species-typing based on either nuclear or mitochondrial markers

alone. The extent to which hybridisation between *T. spiralis* and *T. britovi* has influenced phenotypic traits remains unknown and needs further research.

Trichinella infection dynamics and monitoring in wildlife

No *Trichinella* ML were seen in 18,088 wild boar tested at meat inspection in the Netherlands over the period 2007 – 2015, using artificial digestion according to EU 2015/1375. *Trichinella* ML were found in one Dutch wild boar out of in total 166 tested in 2004, using nine times the usual sample test weight, and using serological testing (ELISA), indicating that *Trichinella* occurs at both very low endemicity (prevalence 0.079%) and very low individual infection level (< 1 LPG) (Chapter 2).

The prevalence of *Trichinella* spp. in Dutch red foxes (0.27%) as shown in the Dutch border region with Germany in 2010, was roughly ten times lower than reported for the same area in 1972 (2.8%) (Sluiters et al., 1972) and in 1998 (3.9%) (van der Giessen et al., 1998), which fits observations in adjacent parts of Germany (Wagner et al., 1988) and Belgium (Claes, 2013). We analysed other parasitic infections, additional to *Trichinella* spp. in Dutch red fox, to study whether the decline in *Trichinella* infection reflects a general decline in parasite infections in red fox. In contrast, we demonstrated a significant increase in parasitic helminth diversity in the fox population at the eastern border of the Netherlands in comparison to 35 years ago, but at the same time, a decline in the prevalence of another two zoonotic helminths species (*Toxocara canis* and *Taenia* spp.), additional to *Trichinella* spp. (Chapter 6).

The finding of *T. spiralis* (van der Giessen et al., 2001) and *T. pseudospiralis* (Chapter 2) in wild boar and the finding of *T. britovi* (van der Giessen et al., 1998) in red foxes, reflects the general host preference of these species as seen elsewhere in Europe (Pozio, 2005; Pozio et al., 2009b). In Romania, an endemic region for *Trichinella* spp., red foxes were shown to be reservoir species for *T. britovi*, which is considered less effectively transmitted to pigs (Kapel and Gamble, 2000), but not for *T. spiralis*, although previously, red fox was considered the indicator species to determine *Trichinella* prevalence in wildlife. In 40% of hunting grounds, *Trichinella* prevalence in red fox was 21.5%; 96% of isolates was *T. britovi* and 4% *T. spiralis* (Imre et al., 2015). Consequently, wild boar and outdoor raised pigs may be the better indicator for surveillance of human infection risks. However, as wild boar all tested negative, using artificial digestion at meat inspection in the Netherlands, serological monitoring of wild boar might be a more sensitive surveillance system than artificial digestion. Results of serosurveillance in Dutch wild boar

showed that the prevalence is very low since 2007, confirming the overall findings of low *Trichinella* prevalence in the sylvatic cycle in the Netherlands (Chapter 2).

Transmission risks

In order to study the role of rats as a reservoir and risk of transmission to pigs, we studied wild rats from different environments. No *Trichinella* ML were identified using artificial digestion in 132 wild rats captured at farms, in suburban environments and in rural environments (Chapter 8). During the period 1974 – 1992, a single positive rat was identified both in 1989 (n = 61) and in 1992 (n = 97)(Franchimont et al., 1993; van Knapen et al., 1993). Additionally, we have demonstrated significant differences in helminth profiles and numbers of (simultaneous) intestinal helminth infections between location types, suggesting limited exchange of rats between farms and surrounding wet rural environments. Different helminth profiles were observed in (neighbouring) six-digit postal code areas, which may suggest that postal codes coincided with separate rat territories, with little, if any, exchange of hosts or parasites, although more research is needed to confirm this hypothesis.

Recent studies in Germany have shown that the raccoon dog (*Nyctereutes procyonoides*) is also a good indicator host for *Trichinella* species. Raccoon dogs have been seen in the North-eastern part of the Netherlands since 2001 (Mulder, 2013b) and both in 2012 and 2013, reproduction of wild raccoon dogs was recorded in the Dutch province of Drenthe (Meijer and Klop, 2014; Mulder, 2013a). Large part of the Netherlands are suitable habitat for raccoon dogs and it is expected that this species will spread to most of the country within 15-30 years (Meijer and Klop, 2014). Recently, a heavily *T. spiralis* infected road killed raccoon dog (89 *T. spiralis* larvae / g) was found in the eastern part of the Netherlands (Maas et al., manuscript in preparation), which shows that this invasive species may play a role in (re)introduction of *T. spiralis* in the Netherlands. In the eastern part of Germany (Federal State of Mecklenburg-Western Pomerania), the number of hunted raccoon dogs increased from 541 in 1993 – 1996, to 78,311 during the period 2005 – 2008. This increase coincided with an increase of *Trichinella* prevalence in wild boar, compared to the rest of Germany (Pannwitz et al., 2010). The prevalence of *Trichinella* spp. in raccoon dogs in that study ranged from 4 – 6.5%, the majority of which were *T. spiralis*, the rest were *T. pseudospiralis*.

In the Netherlands, as in other European countries, wild boar populations increased over the period 1990 – 2014 in the Veluwe region (Groot Bruinderink et al., 1999; Spek, 2015) and in the southeast of the Netherlands (Guldemond et al., 2015). Despite the low

Trichinella prevalence in Dutch natural habitats at present, an increasing wild boar population, together with a spread of racoon dogs could lead to an increasing *Trichinella* biomass in the sylvatic cycle. Recent measures, such as leaving wild boar in woodlands to be eaten by scavengers, could further contribute to an unwanted and efficient spread of zoonotic parasites, such as *T. spiralis*, alongside the advantageous effects on biodiversity in woodlands. Previously, improper hunting practice, such as leaving skinned carcasses and viscera openly in the field, has been shown to have a major impact on the prevalence of *Trichinella* spp. prevalence in wildlife in endemic countries (Pozio et al., 2001a). Surveillance of wild boar and raccoon dogs is therefore important to assess the risk of *Trichinella*, especially to free ranging pigs in future.

Quantitative Microbial Risk Assessment (QMRA) of human infection

Human trichinellosis is caused by ingestion of raw or improperly cooked pork or meat from wildlife, such as wild boar, bear and walrus (Pozio, 2005). The present work focused on *Trichinella* species for which the most relevant hosts are domestic pig and wild boar. European *Trichinella* outbreaks mainly involved non-tested wild boar and non-tested home-slaughtered back-yard pigs. At official slaughter control, *Trichinella* ML were only found in domestic pigs from non-controlled housing and in hunted wild boar, throughout the European member states, but not in pigs from controlled housing, while an estimated 2.4 billion of pigs have been tested during the last two decades in the European Union (Pozio, 2014). Consequently, large sums are spent on the control of a population of animals that is virtually free of *Trichinella*, which warrants a risk-based approach of *Trichinella* testing (Alban et al., 2008; Alban et al., 2011; Pozio, 2014). For global pork trade, the FAO-WHO Codex Alimentarius Committee on Food Hygiene, preparing guidelines for food safety on a global level, recently adopted guidelines for a risk based control of *Trichinella* in meat of suidae (CAC, 2015) and developed a spreadsheet model to evaluate residual risks of infection with *Trichinella* spp. from *Trichinella*-tested domestic pigs from controlled housing (FAO-WHO, 2014b). However, this model did not include *Trichinella* ML distribution within and among different types of edible meat and dose-response relationships for *Trichinella* spp. infections in humans.

We built a QMRA model, describing the whole chain of events from *Trichinella* infection in domestic pigs or wild boar, to testing at meat inspection, inactivation by cooking and finally infection and illness in humans. We used prevalence data for wild boar and pigs from non-controlled housing in Poland as an example to build the QMRA model, and incidence data for human trichinellosis in Poland to validate the model. We used

consumer survey data, to identify a proportion of consumers that eat undercooked meat. The actual consumer risk of infection and disease was modelled, following consumption of different cuts of pork or wild boar meat that had been cooked to different degrees. We were thus able to model (residual) risk of infection and illness from meat produced under different holding systems to corroborate risk-based surveillance.

Our model allows evaluation of several factors that are included in the model, such as cooking style. The most effective means to inactivate *Trichinella* ML is thorough cooking. When comparing cooking recommendations published online by the USDA to inform US consumers (USDA, 2015) with scenario 'Traditional' cooking (Chapter 9) in our model, the latter was shown to eliminate infection risk with *Trichinella* in our endemic example country Poland. In contrast, cooking all pork or wild boar meat according to the new USDA recommendation would result in a residual risk of 4.95 (95%CI 2.22 – 8.91) cases of human trichinellosis per million persons per year for wild boar and 3.65 (95%CI 0 – 11.60) for pork from non-controlled housing for Poland. In comparison, cooking all pork or wild boar meat according to scenario 'Well done, Chef' would result in 0.66 (95%CI 0.11 – 1.94) cases of human trichinellosis per million persons per year for wild boar and 1.01 (95%CI 0 – 4.25) cases for pigs from non-controlled housing. Note, that these estimates are generated from animals that were missed at meat inspection (false negatives) with Poland as an example. However, the USDA recommendation was issued given virtual elimination of the *Trichinella* risk from pork in the USA over the last decades (Davies, 2011; USDA, 2015), which is corroborated by the virtual absence of *Trichinella* from pigs from controlled housing in Europe. Nevertheless, wide adaptation to these new recommendations, which are intended to improve the structure and taste of *Trichinella*-free pork from controlled housing, may lead to more human cases of trichinellosis, when applied in *Trichinella* endemic countries, or in cases where meat inspection fails. The latter happened in December 2014 in Belgium, where imported wild boar meat from Spain caused clinical trichinellosis and hospitalisation of at least nine people who ate wild boar meat in a small number of restaurants (Claes et al., 2015).

We calculated the actual risk of human trichinellosis according to a model scenario by which 10% of consumers eat medium done meat (scenario Medium, USDA, Chapter 9) and 90% eat well done pork (scenario Well done, Chef, Chapter 9). Using this model scenario, the number of predicted human trichinellosis cases per million of Polish inhabitants was 1.97 (95% CI 1.21 – 5.88) for wild boar and 1.56 (95% CI 0 – 5.52) for domestic pigs from non-controlled housing, thus in total 3.53 predicted cases per million per year. The observed incidence over the period 2007 – 2012 in Poland was 1.15 per

million per year (EFSA-ECDC, 2010, 2013, 2014). Our estimate is therefore consistent with reported human cases.

In the FAO-WHO study (FAO-WHO, 2014b), the residual risk after testing 100 million pigs from controlled housing and no positive findings was estimated at 16.7 – 0.0017 infective portions of pork per million servings anticipating that 10% of portions would not be rendered safe after cooking and that infected portions actually cause illness. We anticipated that loin, belly and shoulder are the most relevant pig parts, since consumers purchase these raw. Our model predicts a residual risk of 0.032, 0.024 and 0.014 infected cooked portions per million servings for loin, belly and shoulder respectively, after testing 114 million pigs from non-controlled housing at a *Trichinella* prevalence of 1.267×10^{-6}. Incorporation of a dose response module into our QMRA shows that the modelled actual residual risk from pigs from non-controlled housing is on average 0.004 cases of human trichinellosis per million portions of cooked pork, leading to 1.56 trichinellosis cases per million persons per year (in the Polish situation, Chapter 9). Extrapolation of the outcome of our model for pork of pigs from non-controlled housing to pigs from controlled housing resulted in an estimate of less than 5.3×10^{-5} human trichinellosis cases per million persons per year, or less than 1 European citizen every 39.4 years (Chapter 9). This confirms the effectiveness of controlled housing to reduce human health risks and justifies a risk based surveillance system using alternative strategies for animals from this type of husbandry.

Main conclusions from this thesis

The studies presented in this thesis show that the *Trichinella* prevalence in Dutch wildlife is very low, both in prevalence and in larval burden, and has undergone a steep decline, compared with historic data. However, exotic wildlife species, such as the raccoon dog may (re)introduce *T. spiralis* into the Dutch wildlife. There is no evidence that *Trichinella* presence in Dutch wildlife at the current low levels affects the domestic pigs in the Netherlands, which are almost exclusively kept under controlled housing. However, free ranging or outdoor access of pigs might present a risk and surveillance of wildlife is therefore important.

The developed QMRA model for *Trichinella* allows extrapolation of residual risk of infection from consumption of undercooked pork, which shows that the residual infection risk from pigs under controlled housing is virtually absent. The model also shows that in a *Trichinella* endemic country, modern cooking styles that deliberately undercook pork to improve structure and taste of the meat (Medium, USDA, 8.6 cases/million/year),

increases the incidence of human trichinellosis by five times, compared to the modern version of well done meat (Well done Chef, 1.67 cases/million/year), due to *Trichinella* positive pigs from non-controlled housing and wild boar that were missed at meat inspection. It also shows that traditional cooking, eliminates the risk of trichinellosis. Needless to say, the consumption of raw meat (raw sausages, carpaccio of wild boar) is an even more risky practice.

It is impossible to model illegal or improper practice, such as home-slaughter of non-*Trichinella* tested back yard pigs or wild boar. It is however possible to estimate the relative risk from pork produced under different housing systems. Our QMRA for *Trichinella* shows that in the Polish example, consumption of *Trichinella*-tested wild boar meat is responsible for 55% of the modelled cases of human trichinellosis, while the number of tested wild boar is 167.6 times less than the number of tested pigs from non-controlled housing. At the same time, *Trichinella* prevalence in wild boar is 4,100 times higher than in pigs from non-controlled housing in the Polish example.

EU Regulation 853/2004 , laying down specific hygiene rules for foodstuffs, stipulates that 'this regulation does not apply in relation to domestic preparation of food for private domestic consumption' and to 'hunters who supply small quantities of wild game or wild game meat directly to the final consumer or local retail establishments directly supplying the final consumer'((European-Commission, 2004), Chapter I, Article 3b and 3e). However, the same Regulation states that the Member States should adopt measures to prevent that *Trichinella*-infested wild boar meat reaches the final consumer ((European-Commission, 2004) Chapter I, Article 4). Compulsory meat inspection of all *Trichinella* susceptible game meat intended for human consumption (e.g. wild boar), is a logical step to further improve food safety regarding *Trichinella*, whether game meat is intended for own consumption or not. Especially in *Trichinella* endemic regions where homemade raw products are traditionally produced and enjoyed, compulsory game meat inspection may prevent outbreaks of trichinellosis that are seen in endemic countries, but also across their borders (Chapter 9).

At a volume of 140 million *Trichinella*-negative pigs at meat inspection in ten preceding years, the probability of finding a positive pig is not more than 0.0007 when testing 10% of pigs from controlled housing, including sows and boars, as shown in this thesis. In the Dutch example, testing of about 7.3 million pigs (50 – 60% of the annual slaughter volume) is needed to obtain a 5% probability of finding a positive pig. Under approved controlled housing as shown here for the Dutch situation, *Trichinella* testing is not adding any value to protect human health, although for the time being, 100% of Dutch slaughter pigs will remain tested for *Trichinella*, due to export requirements. There is no evidence

that *Trichinella* presence in Dutch wildlife at the current low levels affects the domestic pigs in the Netherlands, which are almost exclusively kept under controlled housing, but free ranging or outdoor access of pigs might cause a risk and surveillance of wildlife is therefore important.

In industrialised countries, social movements propagate more 'natural' pig production systems, with ample outdoor access and more attention for animal welfare (essentially back to traditional small scale mixed farming), or utterly oppose to pig holding under controlled housing (Davies, 2011). It should be clear however, that such decisions can only be made when policy makers are scientifically informed about the risks.

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Summary

Summary

Nematodes of the genus *Trichinella* are parasites of many different species of mammals, birds and reptiles. Six encapsulated *Trichinella* species (*T. spiralis, T. britovi, T. nativa, T. murelli, T. patagoniensis* and *T. nelsoni*) and three encapsulated genotypes (*Trichinella* T6, T8 and T9) have been recognized, which infect carnivorous and omnivorous mammals (Pozio, 2005; Pozio et al., 2009b). Three non-encapsulated *Trichinella* species infect birds (*T. pseudospiralis*) and crocodiles (*T. zimbabwensis* and *T. papuae*). No *Trichinella* infections have been demonstrated in amphibians or fish (Pozio, 2005). *T. spiralis* and *T. pseudospiralis* have a cosmopolitan distribution, due to dispersion by humans and birds respectively, whereas all other species have a more restricted geographical distribution (Pozio et al., 2009a)(Chapter 1, Table 1).

The overall aim of this thesis was to study the presence and prevalence of *Trichinella* spp. in wildlife host species that play a role in the ecology of the parasite and its presence in the food chain, which could be used to develop a quantitative microbial risk analysis (QMRA) model for *Trichinella*. Such a QMRA model may be used to identify hazards in the chain of events from the presence of *Trichinella* parasites in domestic life stock or wildlife, to *Trichinella* infection of humans. Additionally, a QMRA model could be used to quantify attributions of factors such as husbandry system (e.g. controlled housing, free ranging), types of meat and meat products (e.g. different pork cuts, fresh meat), or carcass control (e.g. introduction of alternative equipment).

In **Chapter 2**, we evaluated the *Trichinella* prevalence in Dutch wild boar. Meat originating from wild boar and pigs under non-controlled housing condition is the most important source for trichinellosis outbreaks in humans. Artificial digestion, which is used for routine meat inspection at the slaughterhouse to identify *Trichinella* positive animals, is a less suitable instrument to monitor *Trichinella* spp. at population level; it is labour intensive and less sensitive than serological techniques. We included previously unpublished serological data of wild boar during the period 1999 – 2005, and we tested 3778 wild boar sera collected during the period 2007 – 2015, in a *Trichinella* in-house ELISA. Since validation of serological assays for wildlife surveillance is often hampered by a lack of well-defined field sera, we included wild boar sera originating from Poland and Romania, which are regarded endemic for *Trichinella* spp., to determine an appropriate cut-off value. The aim of the present study was to evaluate serological response of Dutch wild boar to *Trichinella* spp. during the last fifteen years. Over the years 2007 – 2015, six wild boar sera tested positive by ELISA, of which three were confirmed by Western blot, resulting in an overall seroprevalence of 0.079%, which was low compared to 2% seroprevalence in 2005 ($p = 0.0473$). The overall seroprevalence in the preceding period (1999 – 2004) was 5.7%.

We developed an alternative method for detection of Trichinella ML in frozen carcasses of red fox (**Chapter 3**). Freezing of fox carcasses to minimize professional hazard of infection with *Echinococcus multilocularis* is recommended in endemic areas, but this could influence the detection sensitivity of *Trichinella* larvae in the same host species. A

method based on artificial digestion of frozen fox muscle in combination with larva isolation by a sequential sieving method (SSM), was validated using naturally infected foxes from Latvia. The validated SSM was used to detect dead Trichinella muscle larvae (ML) in frozen muscle samples of 369 red foxes from the Netherlands, of which one fox was positive (0.067 larvae per gram, LPG). This result was compared with historical *Trichinella* findings in Dutch red foxes of more than 15 years ago. Molecular analysis showed that both *T. britovi* and *T. nativa* were present in the Latvian foxes; no mixed infection was found. Of 96 non-frozen *T. britovi* ML, 94% was successfully sequenced, whereas this was the case for only 8% of 72 frozen T. britovi ML. The single *Trichinella* sp. larva that was recovered from a frozen foreleg of a Dutch fox did not yield PCR product, probably due to severe freeze-damage. In conclusion, the SSM presented in this thesis is a fast and effective method to detect dead Trichinella larvae in frozen meat. We showed that the *Trichinella* prevalence in Dutch red fox was 0.27% (95% CI 0.065-1.5%), in contrast to 3.9% demonstrated in the same study area fifteen years ago.

Besides freezing of meat, equipment may influence test sensitivity at meat inspection. EU Regulation 2015/1375 stipulates a standard artificial digestion method for detection of *Trichinella* ML by artificial digestion (EU reference method), for which chemicals and equipment involved, is described in detail. In **Chapter 4**, the performance of an alternative sieve for *Trichinella* control at meat inspection, as required by a trade partner, was evaluated. The standard digestion method uses a 180 µm sieve to retain undigested meat particles; a trade partner required use of a 400 µm sieve instead. Using spiked pork samples (range 0 - 10 *Trichinella* muscle larvae), the performance of a 400 µm mesh size sieve ($sieve_{400}$), compared to a sieve with mesh size 180 µm ($sieve_{180}$), was evaluated using the EU reference method for artificial digestion. We showed that the use of a $sieve_{400}$ results in 12% lower larval counts, 147% more debris and 28% longer counting time, compared to the use of a $sieve_{180}$. On the other hand, no false negative results were obtained, but prolonged counting times may have an impact on performance in a high-throughput environment such as a slaughterhouse laboratory. Based on our results, the $sieve_{180}$ remains the sieve of choice for *Trichinella* meat control at the slaughterhouse labs, according to the EU reference method, laid down in European regulation 2075/2005.

In **Capter 5** various *Trichinella* isolates from individual *T. spiralis* and *T. britovi* larvae isolated from wild boar and red fox from Poland and Latvia were analysed. Molecular identification of *Trichinella* ML is important for risk evaluation, since different *Trichinella* spp. have different infection characteristics in domestic pigs or wild boar and ultimately in humans. To date, 12 taxa are recognized in the genus *Trichinella*, of which four are circulating in Europe (*Trichinella spiralis*, *Trichinella nativa*, *Trichinella britovi* and *Trichinella pseudospiralis*). *T. spiralis* and *T. britovi* circulate in European wildlife and occur simultaneously in the same host species. We studied molecular polymorphisms of single *T. spiralis* and *T. britovi* muscle larvae from natural infections, using PCR and

sequencing of the nuclear 5S rDNA intergenic spacer region (5S rDNA-ISR) and the mitochondrial cytochrome c oxidase 1 (CO1) gene. We demonstrated six haplotypes of the 5S rDNA-ISR and 14 of the CO1 gene in 89 individual *T. britovi* ML from Latvia and Poland. In contrast, only two haplotypes were observed at both 5S rDNA-ISR and CO1 of 57 individual *T. spiralis* ML from Polish wild boar and red foxes. Moreover, this study demonstrates hybridisation in eight individual ML between *T. britovi* and *T. spiralis* under natural conditions in four Polish wild boar and two red foxes, revealed by combining 5S rDNA-ISR and CO1 sequence information of individual Trichinella ML.

Trichinella prevalence declined in the Netherlands as shown in previous chapters of this thesis. To investigate whether this was unique for *Trichinella* spp., we studied long-term data of parasitic infections in red fox (**Chapter 6**). The red fox is host to a community of zoonotic and other helminth species and tracking their community structure and dynamics over decades is one way to monitor the long term risk of parasitic infectious diseases relevant to public and veterinary health. We identified 17 helminth species from 136 foxes by mucosal scraping, centrifugal sedimentation / flotation and by the washing and sieving technique. We applied rarefaction analysis to our samples and compared the resulting curve to the helminth community reported in literature 35 years ago. In comparison, fox helminth species significantly increased in number in the last 35 years (p-value <0.025). *Toxascaris leonina*, *Mesocestoides litteratus, Trichuris vulpis* and *Angiostrongylus vasorum* are four new veterinary-relevant species that were demonstrated in this study. The zoonotic fox tapeworm (*E. multilocularis*) was found outside its previously described endemic regions in the Netherlands. In conclusion, helminth fauna in Dutch red foxes increased in biodiversity over the last three decades. It is uncertain what mechanism induced decline of *Trichinella* in red fox in the Netherlands.

Small mammals, such as rats may play a role as transport host between the sylvatic and the domestic cycle of *Trichinella*. To study *Trichinella* seroprevalence in rats, we studied dose response to a wide range of *Trichinella* infection doses in experimentally infected laboratory rats (**Chapter 7**). We studied the dynamics of anti-Trichinella IgG antibody response after *T. spiralis* infections in experimentally infected rats, using infection doses ranging from very low (10 *Trichinella* ML, per rat) to very high (16,000 *Trichinella* ML per rat). To evaluate the feasibility of rats surviving high infection doses with *T. spiralis*, clinical and pathological parameters were quantified. Serological tools for detecting *T. spiralis* in rats were developed to quantitatively study the correlation between parasite load and immunological response. An infection dose dependent antibody response developed in rats after infection with as low as 10 ML up to a level of 10,000 ML. Additionally a positive correlation was found between the number of recovered ML and serum antibody levels, although specific measured antibody levels correspond to a wide range of LPG values. Serum antibodies of rats that were infected even with 10 or 25 ML could be detected by use of the *T.spiralis* western blot, 2 weeks post infection. We concluded that based on these low infection doses, serologic tests are a useful tool to survey *T. spiralis* in wild rats, which survive even high doses of *T. spiralis.*

In **Chapter 8** we studied parasitic infections including *Trichinella* in 161 wild rats that were caught in different environments, such as farms, urban environments and suburban environments, for which intestinal helminths were isolated and identified morphologically and artificial digestion (EU reference method) was used to isolate muscle larvae. Morphological analysis of rat intestinal contents yielded six nematode species (*Syphacia muris, Heterakis spumosa, Aonchotheca murissylvatici, Trichuris muris, Nippostrongylus brasiliensis* and *Strongyloides* sp.), three cestode species (*Hymenolepis diminuta*, *H. nana* and *Hymenolepis* (=*Rodentolepis*) *fraterna*) and four trematode species (*Plagiorchis muris, Plagiorchis proximus, Echinostoma chloropodis* and *Notocotylus imbricatus*). Black rats at farms displayed the lowest intestinal helminth species variation (6 species) and carried overall on average 0.93 species simultaneously. In comparison, brown rats at farms carried 7 helminth species and 1.91 species simultaneously. Brown rats from suburban environments displayed the highest species variation (11 species) at 1.82 simultaneous helminth species. Absence of trematodes from rats at farms may suggest limited exchange of rats between farms and surrounding wet rural environments. We report four species of veterinary (*Syphacia muris*) or zoonotic relevance (*Hymenolepis diminuta*, *Hymenolepis nana* and *Plagiorchis muris*). We did not find *Trichinella* muscle larvae, consistent with long-term low prevalence in Dutch wild rats.

Finally, we developed a quantitative microbial risk assessment (QMRA) model for *Trichinella* in **Chapter 9**, to assess the actual consumer risk of infection and disease, following consumption of pork or wild boar meat. We included the whole chain of events, from distribution of *Trichinella* larvae in and between pigs and wild boar, test characteristics using artificial digestion, parasite inactivation by cooking of meat, consumer preference data concerning eating undercooked meat and the dose response relation of *Trichinella* infections in humans. In many countries worldwide, individual *Trichinella* control of pig carcasses at meat inspection is mandatory but incurs high costs in relation to the number of positive carcasses found, especially regarding pigs reared under controlled housing. EU regulation 2015/1375 implements an alternative risk-based approach, in view of absence of positive findings in pigs under controlled housing conditions. Moreover, Codex Alimentarius guidelines for the control of *Trichinella* spp. in meat of suidae have been published and used in conjunction with the OIE terrestrial Animal health code, to provide guidance to governments and industry on risk based control measures to prevent human exposure to *Trichinella* spp. and to facilitate international pork trade. To further support such a risk-based approach, we modelled the risk of human trichinellosis due to consumption of meat from infected pigs, raised under non-controlled housing, and wild boar, using Quantitative Microbial Risk Assessment (QMRA) methods.

Our QMRA model quantifies the distribution of *Trichinella* ML in and among swine, test sensitivity at carcass control, partitioning of edible pork parts, *Trichinella* ML distribution in edible muscle types, heat inactivation by cooking and portion size. The resulting exposure estimates were combined with a dose response model for *Trichinella species* to

estimate the incidence of human illness after consumption of infected meat. Paramater estimation was based on experimental and observational datasets. In Poland, which served as example, we estimated an average incidence of 1.56 (95%CI: 0 – 5.52) trichinellosis cases per million persons per year (Mpy) due to consumption of pork from pigs that were reared under non-controlled housing, and 1.97 (95%CI: 0.83 – 4.00) cases per Mpy due to consumption of wild boar. The total estimated incidence of human trichinellosis attributed to pigs from non-controlled housing and wild boar in Poland, is similar to the incidence of human trichinellosis in that country reported by EFSA. Overall, in the European Union, we estimated an upper incidence limit of 5.3×10^{-5} cases per Mpy, or less than one predicted case of trichinellosis in the European Union every 39.4 years, due to consumption of pork from controlled housing. Therefore, *Trichinella* testing of pigs under controlled housing is not adding any value to protect human health.

In the General Discussion of this thesis, detection of *Trichinella* spp. in wildlife and domestic pigs, *Trichinella* spp. characterisation, infection dynamics and monitoring, as well as transmission risks and the development of a QMRA for *Trichinella* are discussed in a broader perspective. The last part of the discussion provides implementations of the work presented in this thesis. It is argued that at present, *Trichinella* prevalence in Dutch wildlife is very low and there is no evidence that *Trichinella* presence in Dutch wildlife at the current low levels affects domestic pigs in the Netherlands, which are almost exclusively kept under controlled housing. However, free ranging or outdoor access of pigs might present a risk and surveillance of wildlife is therefore important.

The developed QMRA model for *Trichinella* showed that residual risk of infection from consumption of undercooked pork from pigs under controlled housing is virtually absent. This warrants modern cooking styles that deliberately undercook pork to improve structure and taste of the meat. However, introduction of such cooking styles in endemic countries, as shown for Poland in this thesis, increases the incidence of human trichinellosis by five times, compared to well done meat, due to *Trichinella* positive pigs from non-controlled housing and wild boar that were missed at meat inspection. Needless to say, the consumption of raw meat (raw sausages, carpaccio of wild boar) is an even more risky practice. Our QMRA for *Trichinella* shows that in the Polish example, consumption of *Trichinella*-tested wild boar meat is responsible for 55% of the modelled cases of human trichinellosis, while the number of tested wild boar is 167.6 times less than the number of tested pigs from non-controlled housing. At the same time, *Trichinella* prevalence in wild boar is 4,100 times higher than in pigs from non-controlled housing in the Polish example. EU Regulation 2015/1375 permits game meat that is 'directly supplied to the final consumer or to local retail establishments directly supplying the final consumer' not to be tested for *Trichinella.* Especially in *Trichinella* endemic regions where homemade raw products are traditionally produced and enjoyed, compulsory game meat inspection may prevent outbreaks of trichinellosis that are seen in endemic countries, but also across their borders. There is no evidence that *Trichinella* presence in Dutch wildlife at the current low levels affects the domestic pigs in the

Netherlands, which are kept almost exclusively under controlled housing but free ranging, or outdoor access of pigs might present a risk and surveillance of wildlife is therefore important.

At a volume of 140 million *Trichinella*-negative pigs at meat inspection in ten preceding years in the Netherlands, the probability of finding a positive pig is not more than 0.0007 if testing 10% of pigs from controlled housing, including sows and boars, as shown in this thesis. In the Dutch example, testing of about 7.3 million pigs (50 – 60% of the annual slaughter volume) is needed to obtain a 5% probability of finding a positive pig. Under approved controlled housing as shown here for the Dutch situation, *Trichinella* testing is not adding any value to protect human health, although for the time being, 100% of Dutch slaughter pigs will remain tested for *Trichinella*, due to export requirements. In industrialised countries, social movements propagate more 'natural' pig production systems, with ample outdoor access and more attention for animal wellfare (essentially back to traditional small scale mixed farming), or utterly oppose to pig holding under controlled housing (Davies, 2011). It should be clear however, that such decisions can only be made when policy makers are scientifically informed about the risks.

Samen

vatting

Rondwormen van het geslacht *Trichinella* komen voor in de spieren van wild en varkens. Mensen kunnen ziek worden van *Trichinella* door het eten van besmet vlees; de ernst van de ziekte hangt af van het opgenomen aantal en de soort *Trichinella*.

Doel van de studie was onderzoek naar het voor komen van *Trichinella* in Nederlands wild en analyse van infectierisico's voor de mens. Een kwantitatieve microbiële risico analyse (QMRA) kan de mate van risico bepalen van verschillende onderdelen van de vleesproductieketen en daarmee een risico-gestuurde aanpak ondersteunen voor *Trichinella* controle.

We hebben vastgesteld dat *Trichinella* in Nederlandse vossen en wilde zwijnen nu significant minder voor komt in vergelijking met historische gegevens. In ratten is *Trichinella* niet gevonden. In Polen komt *Trichinella* nog steeds veelvuldig voor in wild en in varkens van kleinschalige, niet gecontroleerde houderij. *Trichinella* gegevens uit Polen werd gebruikt om het nieuw ontwikkelde QMRA model voor *Trichinella* te testen. Met dit model kunnen alle stappen in de keten van productie (*Trichinella*-larven bij dieren, karkascontrole, *Trichinella* in vlees porties, afdoden van *Trichinella* door koken) berekend worden. Vervolgens werd een eerder in ons lab ontwikkeld dosis-responsmodel voor *Trichinella* opgenomen in de QMRA, om te schatten hoeveel mensen ziek werden na consumptie van *Trichinella*-besmet vlees (incidentie).

De resultaten van ons nieuwe QMRA model voor Trichinella komen goed overeen met de gemeten incidentie van trichinellosis in een endemisch gebied. Ons model laat zien dat het individueel testen van varkens uit gecontroleerde houderij niet verder bijdraagt aan de bescherming van de volksgezondheid.

Samenvatting

Rondwormen van het geslacht *Trichinella* parasiteren veel verschillende soorten zoogdieren, vogels en reptielen. Ze leven in spiercellen van hun gastheer en overdacht gebeurt via consumptie van besmet vlees. Zes *Trichinella* soorten (*T. spiralis, T. britovi, T. nativa, T. murelli, T. patagoniensis* en *T. nelsoni*) en drie *Trichinella* genotypen (*Trichinella* T6, T8 and T9) zijn omgeven door een kapsel in de spiercel. Deze Trichinella soorten infecteren carnivore (vleesetende) en omnivore (allesetende) zoogdieren (Pozio, 2005; Pozio et al., 2009b). Drie andere *Trichinella* soorten vormen geen kapsel in de spieren van hun gastheer en zijn infectieus voor roofvogels (*T. pseudospiralis*) en krokodillen (*T. zimbabwensis* and *T. papuae*). Er zijn tot nog toe geen *Trichinella* infecties aangetroffen in amfibieën en vissen. *T. spiralis* and *T. pseudospiralis* komen wereldwijd voor, waarbij de mens en vogels een rol spelen. Alle andere *Trichinella* soorten hebben een beperkte geografische verspreiding (Pozio et al., 2009a)(Hoofdstuk 1, Tabel 1).

Het doel van de studie die leidde tot dit proefschrift, was onderzoek naar het voor komen van *Trichinella* in gastheersoorten die een rol spelen in de ecologie van de parasiet en in de voedselketen. Gegevens die voortkomen uit deze studie, kunnen wellicht gebruikt worden om een kwantitatief microbiologische risico analyse (*quantitative microbial risk analysis* (QMRA)) model te ontwikkelen voor *Trichinella*. Een dergelijk QMRA model kan gebruikt worden om bijdragen te kwantificeren van factoren zoals het type varkenshouderij (bijvoorbeeld gecontroleerde houderij, vrije uitloop varkens), type vlees of vleesproduct (bijvoorbeeld verschillende delen van het varken, vers vlees) of karkascontrole (bijvoorbeeld introductie van afwijkend instrumentarium).

In **Hoofdstuk 2** hebben we gekeken naar de Trichinella prevalentie (percentage positieve dieren) van *Trichinella* soorten in wilde zwijnen. Vlees van wilde zwijnen en van varkens die gehouden worden in open stallen (*non-controlled housing*), vormen de belangrijkste bron voor *Trichinella* uitbraken in de mens. Kunstmatige digestie, die gebruikt wordt om *Trichinella* infecties aan te tonen bij de routine vleesinspectie, is een minder geschikte methode om *Trichinella* soorten te monitoren op populatie niveau. Het is een arbeidsintensieve methode, die minder gevoelig is dan serologische technieken. We hebben niet eerder gepubliceerde serologische gegevens gebruikt van wilde zwijnen uit de periode 1999 – 2005 en we hebben 3778 wilde zwijnensera getest in de periode 2007 – 2015, waarvoor we een in-huis-ELISA hebben gebruikt. Aangezien validatie van serologische methoden voor wild wordt bemoeilijkt door het ontbreken van goed gedefinieerde veldsera, hebben we wilde zwijnensera uit Polen en Roemenië getest in onze ELISA. Polen en Roemenië worden beschouwd als *Trichinella* endemische gebieden; wilde zwijnensera uit die gebieden zouden dus voor een deel *Trichinella*-positief kunnen zijn, en daardoor geschikt om een bruikbare afkapwaarde te bepalen. Het doel van het onderzoek in Hoofdstuk 2 was de serologische respons van Nederlandse wilde zwijnen te bepalen over een periode van vijftien jaar. Over de periode 2007 – 2015 werden zes wilde zwijnen positief bevonden voor *Trichinella* in de ELISA, waarvan er drie bevestigd werden met een tweede techniek, de Western blot. Dit resulteerde in een seroprevalentie van

0.079%, hetgeen significant lager is dan 2% seroprevalentie in 2005 (p = 0.0473). In de voorgaande periode 1999 – 2004 was de seroprevalentie 5.7%.
We ontwikkelden een alternatieve methode voor detectie van *Trichinella* spierlarven in diepgevroren karkassen van vossen (**Hoofdstuk 3**). Diepvriezen van vossenkarkassen dient om het risico van besmetting met de vossenlintworm (*Echinococcus multilocularis*) te beperken voor de onderzoekers, maar die behandeling kan ook de detectiegevoeligheid van *Trichinella* spierlarven beïnvloeden in die vossen. We hebben een methode gevalideerd, die gebaseerd was op de kunstmatige digestiemethode, gevolgd door een *sequential sieving* methode (SSM) om eventueel aanwezige *Trichinella* spierlarven te isoleren. De SSM werd gevalideerd met natuurlijk geïnfecteerde vossen uit Letland en de gevalideerde SSM werd gebruikt om dode *Trichinella* spierlarven te detecteren in spiermonsters van 369 Nederlandse vossen, waarvan één dier positief werd bevonden. Dit resultaat werd vergeleken met historische gegevens van *Trichinella* bevindingen in Nederlandse vossen, van meer dan vijftien jaar geleden. DNA-analyse toonde aan dat zowel *T. britovi* als *T. nativa* voor kwamen in de vossen uit Letland, maar er werden geen gemengde infecties van beide *Trichinella* soorten waargenomen. Van 96 niet diepgevroren *T. britovi* spierlarven kon DNA worden geïsoleerd, wat in 94% van de gevallen leidde tot een succesvolle identificatie (DNA sequentie), terwijl dit maar in 8% van 72 diepgevroren *T. britovi* larven het geval was. De enige *Trichinella* spierlarve die werd gevonden in de Nederlandse vos, was te beschadigd om DNA uit te verkrijgen dat resulteerde in PCR product. Concluderend is de SSM een snelle en effectieve methode om dode *Trichinella* spierlarven te detecteren in vlees dat is diepgevroren. We hebben aangetoond dat de *Trichinella* prevalentie in Nederlandse vossen 0.27% was (95% betrouwbaarheidsinterval 0.065 – 1.5%), terwijl dat 3.9% was in een voorgaande studie in hetzelfde gebied, vijftien jaar geleden.
Naast het diepvriezen van vlees, kunnen gebruikte materialen van invloed zijn op de gevoeligheid van *Trichinella* detectie bij de vleeskeuring. Volgens EU Verordening 2015/1375 moet detectie van *Trichinella* spierlarven plaats vinden met de standaard kunstmatige digestiemethode (EU referentiemethode), met in detail beschreven chemicaliën en materialen. In **Hoofdstuk 4** hebben we een andere zeef dan voorgeschreven in de EU referentiemethode geëvalueerd, die door een van de handelspartners met Nederland wordt vereist. De EU Referentiemethode maakt gebruik van een 180 µm zeef om niet verteerde vleesdeeltjes te blokkeren; de handelspartner verlangt gebruik van een 400 µm zeef voor dat doel. Met gebruik van 100 g varkensvleesmonsters, waaraan 0 – 10 *Trichinella* spierlarven werden toegevoegd, werd de werking van de 400 µm zeef ($zeef_{400}$) vergeleken met resultaten verkregen met een 180 µm zeef ($zeef_{180}$, EU Referentiemethode). We laten zien dat het gebruik van de $zeef_{400}$ leidt tot 12% lagere larventellingen, 147% meer onverteerde spierdeeltjes en 28% langere tijd die nodig is om aanwezige *Trichinella* spierlarven te tellen, in vergelijking met de $zeef_{180}$. Aan de andere kant werden geen fout negatieve uitslagen verkregen met de $zeef_{400}$, maar er moet rekening worden gehouden met het effect van langere

beoordelingstijden in een omgeving met hoge doorloopsnelheden van monsters, zoals dat het geval is in slachthuislaboratoria. Gebaseerd op onze resultaten, moet worden geconcludeerd dat de zeef$_{180}$ de eerste keuze blijft voor *Trichinella* controles in het slachthuis volgens EU Verordening 2015/1375.

Moleculaire identificatie van *Trichinella* spierlarven is van belang voor de risicoschatting, aangezien verschillende *Trichinella* soorten verschillen in eigenschappen van infecties in varkens, wilde zwijnen en in mensen. Analyse van verschillende *Trichinella*-isolaten van individuele *T. spiralis* en *T. britovi* spierlarven uit vossen en wilde zwijnen uit Polen en Letland wordt beschreven in **Hoofdstuk 5**. Tot nu toe worden 12 verschillende soorten onderkend in het geslacht Trichinella, waarvan er vier circuleren in Europa (*T. spiralis*, *T. nativa*, *T. britovi* en *T. pseudospiralis*). *T. spiralis* en *T. britovi* circuleren in Europees wild en kunnen tegelijk voorkomen in hetzelfde dier. We hebben moleculaire polymorfismen bestudeerd van individuele *T. spiralis* en *T. britovi* spierlarven van natuurlijke infecties, gebruik makend van PCR en sequensen van de nucleaire 5S *intergenic spacer* regio (5S rDNA-ISR) en het mitochondriaal cytochroom c oxidase 1 (CO1) gen. We hebben zes haplotypes van 5S rDNA-ISR en 14 van CO1 aangetoond in 89 individuele *T. britovi* spierlarven uit Letland en Polen. Echter, in 57 individuele *T. spiralis* spierlarven uit Poolse vossen en wilde zwijnen, werden slechts twee haplotypes aangetoond in zowel 5S rDNA-ISR als CO1. Bovendien werden in deze studie niet eerder beschreven kruisingen aangetoond van *T. spiralis* en *T. britovi*, in acht individuele spierlarven uit vier Poolse wilde zwijnen en twee vossen, door 5S rDNA-ISR en CO1 gegevens van individuele larven te combineren.

Zoals aangetoond in voorgaande hoofdstukken van dit proefschrift, is de *Trichinella* prevalentie gedaald in Nederlands wild. Om te bestuderen of deze afname uniek is voor *Trichinella* soorten, hebben we lange termijn gegevens van parasitaire infecties in de vos bestudeerd (**Hoofdstuk 6**). De vos is gastheer voor een breed scala aan zoönotische en andere parasieten, en studie van de parasieten samenstelling en veranderingen daarin over langere tijd, is een manier om het risico op parasitaire infecties voor mens en dier te volgen. We hebben 17 verschillende worminfecties aangetoond in 136 vossen uit de oostelijke grensstreek, met de *mucosal scraping* techniek, sedimentatie /flotatie techniek en met de was en zeeftechniek. Met behulp van *rarefaction analysis*, een modelleermethode die, rekening houdend met prevalentie en aantallen parasieten per soort per individueel gastheerdier, een schatting maakt van het aantal te verwachten parasietensoorten gebaseerd op observaties, met de mogelijkheid om te schatten wat het aantal parasietensoorten zou zijn in maximaal 2,5 keer het aantal geobserveerde gastheerdieren. Vergelijking van de berekende *rarefaction*-curve met parasitologische gegevens van 35 jaar geleden van vossen uit dezelfde regio die de huidige studie bestrijkt, toont een significantie toename aan in het aantal parasieten ($p < 0.025$). Vier veterinair relevante parasietensoorten werden voor het eerst aangetoond in deze studie (*Toxascaris leonina*, *Mesocestoides litteratus, Trichuris vulpis* en *Angiostrongylus vasorum*). De voor de mens infectieuze vossenlintworm (*E. multilocularis*) werd voor het eerst buiten de

bekende endemische gebieden in Nederland gevonden. Concluderend kan vastgesteld worden dat de parasitaire diversiteit in de vos is toegenomen in Nederland, over de laatste 35 jaar. Welk mechanisme verantwoordelijk is voor de afname van *Trichinella* prevalentie in de Nederlandse vossen is echter onzeker.

Kleine zoogdieren, zoals ratten, kunnen mogelijk een rol spelen bij de overgang van *Trichinella* van de wildcyclus naar de domestische cyclus. Om *Trichinella* seroprevalentie in ratten te kunnen vast stellen, werd de antilichaamrespons van ratten tegen een brede reeks aan experimentele *Trichinella* infectie doses bestudeerd (**Hoofdstuk 7**). We analyseerden de dynamiek van de anti-*Trichinella* IgG-antilichaam respons, van zeer lage dosis (10 *Trichinella* spierlarven per rat) tot zeer hoog (16.000 spierlarven per rat). Om de waarschijnlijkheid te evalueren dat ratten dergelijke hoge infectiedoses zouden overleven, werden klinische en pathologische parameters gekwantificeerd. De anti-*Trichinella* IgG-antilichaamrespons nam gradueel toe met de infectiedosis, van 10 tot en met 10.000 larven per rat. Daarnaast werd er een positieve correlatie gevonden tussen het aantal spierlarven dat werd geïsoleerd uit een rat en de immuunrespons van die rat, hoewel omgekeerd het niveau van die antilichaamrespons niet correspondeerde met een exact bepaald aantal spierlarven in die rat. Anti-*Trichinella* antilichamen konden worden aangetoond vanaf twee weken na infectie met behulp van de Western blot, vanaf een infectiedosis van 10 – 25 Trichinella spierlarven per rat. Deze resultaten tonen aan dat serologische technieken gebruikt kunnen worden om *Trichinella* infecties te volgen in (wilde) ratten, die zelfs infecties met hoge aantallen *Trichinella* larven overleven.

Hoofdstuk 8 beschrijft de studie van parasitaire infecties, inclusief *Trichinella* soorten, in 161 wilde ratten, die werden gevangen in drie verschillende biotopen, zoals boerderijen, platteland en buitenwijken van plattelandsgemeenten en kleinere steden. Hiervoor werden maag- en darmparasieten geïsoleerd en morfologisch geïdentificeerd; kunstmatige digestie volgens de EU Referentiemethode werd gebruikt om spierlarven te isoleren. Er werden zes nematoden (rondworm) soorten geïdentificeerd (*Syphacia muris, Heterakis spumosa, Aonchotheca murissylvatici, Trichuris muris, Nippostrongylus brasiliensis* en *Strongyloides* sp.), drie cestoden (platwormen) soorten (*Hymenolepis diminuta*, *H. nana* and *Hymenolepis* (=*Rodentolepis*) *fraterna*) en tenslotte vier soorten trematoden (zuigwormen) (*Plagiorchis muris, Plagiorchis proximus, Echinostoma chloropodis* and *Notocotylus imbricatus*). De laagste variatie in intestinale wormsoorten (6 soorten) werd gevonden in zwarte ratten op boerderijen, die gemiddeld 0,93 soorten tegelijk droegen. In bruine ratten op boerderijen werden zeven verschillende wormsoorten gevonden, met gemiddeld 1,91 soorten per rat, terwijl de hoogste variatie werd gezien in bruine ratten uit buitenwijken met toegang tot sloten (11 verschillende soorten, gemiddeld 1,82 soorten tegelijk per rat). In ratten die gevangen werden op boerderijen, werden geen trematoden aangetroffen, wat er op kan wijzen dat er geen of zeer weinig uitwisseling plaats vindt tussen ratten van boerderijen en de omgeving er omheen. We vonden vier helminthensoorten die van veterinair (*Syphacia muris*) of zoönotisch belang zijn (*Hymenolepis diminuta*, *Hymenolepis nana* and *Plagiorchis muris*).

Er werden geen *Trichinella* spierlarven gevonden in de onderzochte ratten, wat overeen komt met de lange termijn prevalentie in Nederlandse ratten.
Tenslotte werd in **Hoofdstuk 9** een kwantitatief microbiologisch risico analyse (QMRA) model ontwikkeld voor *Trichinella*, om te onderzoeken wat het geschatte risico op besmetting en ziekte is voor de consument, na het eten van niet goed verhit varkensvlees afkomstig van verschillende productie typen. Cruciale factoren werden geïdentificeerd en opgenomen in het QMRA model: verdeling van *Trichinella* larven in en tussen varkens of wilde zwijnen, testeigenschappen van de kunstmatige digestiemethode, inactivering van *Trichinella* door het koken van vlees, voorkeur van consumenten voor het eten van vlees met verschillende gaarheid, en een dosis-respons model voor *Trichinella* infecties bij de mens.
In veel landen over de hele wereld is individuele karkascontrole op *Trichinella* verplicht, maar daarmee zijn grote bedragen gemoeid en tegelijkertijd worden geen Trichinella positieve dieren gevonden, die afkomstig zijn van bedrijven met gecontroleerde huisvesting. Voor deze groep dieren voorziet EU Verordening 2015/1375 in een alternatieve, risico gestuurde aanpak. Daarnaast zijn er richtlijnen gepubliceerd voor controle op *Trichinella* in vlees van varkensachtigen, door de Codex Alimentarius, in samenwerking met de OIE *Terrestrial Animal Health Code*. Om een risico gestuurde aanpak te onderbouwen, hebben we het risico op humane trichinellose gemodelleerd voor consumptie van vlees dat afkomstig is van varkens die onder niet-gecontroleerde huisvesting worden gehouden en van wild zwijnen, gebruik makend van de ontwikkelde QMRA voor *Trichinella*.
Ons QMRA-model kwantificeert de verdeling van *Trichinella* larven in en tussen varkens (of wilde zwijnen), de testgevoeligheid van vleeskeuring met behulp van de kunstmatige digestiemethode, portionering van eetbare delen van het varken, verdeling van *Trichinella* spierlarven over en tussen porties vlees, hitte-inactivering en tenslotte portie grootte. De resulterende schatting van de blootstelling werd gecombineerd met een dosis-responsmodel voor *Trichinella* soorten, wat resulteerde in een schatting van de incidentie van humane trichinellose door het eten van besmet vlees. Parameterschattingen zijn gebaseerd op data uit de literatuur en experimentele data.
In Polen, dat diende als voorbeeld, werd een gemiddelde incidentie geschat van 1,56 (95% betrouwbaarheidsinterval 0 – 5.52) gevallen van humane trichinellose, ten gevolge van het eten van varkensvlees van niet-gecontroleerde huisvesting, en 1.97 (95% betrouwbaarheidsinterval 0.83 – 4.00) van het eten van wilde zwijnenvlees per miljoen personen per jaar. De totale geschatte incidentie van humane trichinellose komt in de buurt van de geobserveerde incidentie in Polen, zoals die gerapporteerd werd door EFSA.
Voor varkens onder gecontroleerde huisvesting werd over de hele EU een maximale incidentielimiet geschat van 5.3×10^{-5} per miljoen personen per jaar, ofwel minder dan één voorspeld geval van humane trichinellose per 39,4 jaar voor de hele EU. Hieruit mag geconcludeerd worden dat het testen van varkens van gecontroleerde huisvesting niet verder bijdraagt aan bescherming van de volksgezondheid.

In de Algemene Discussie van dit proefschrift worden detectie van *Trichinella* soorten in varkens en wilde zwijnen, *Trichinella* soortenidentificatie, infectiedynamiek en controle, evenals transmissie risico en ontwikkeling van een QMRA-model bediscussieerd in een breder perspectief. Het laatste deel van de Discussie laat zien hoe de resultaten van dit proefschrift kunnen worden toegepast. We laten zien dat de prevalentie van *Trichinella* in Nederlands wild zeer laag is op dit moment en dat er geen aanwijzingen zijn dat *Trichinella* presentie in Nederlands wild effect heeft op Nederlandse varkens, die bijna uitsluitend gehouden worden onder gecontroleerde huisvesting. Echter, varkens met vrije uitloop, lopen mogelijk risico, waarvoor *Trichinella* controle van wild van belang blijft.
Het door ons ontwikkelde QMRA-model voor *Trichinella* laat zien dat het rest-risico op infectie door het eten van varkensvlees van varkens van gecontroleerde houderij praktisch afwezig is. Onder deze voorwaarde kunnen moderne kookstijlen, waarbij varkensvlees opzettelijk minder gaar gekookt wordt om de textuur en de smaak te verbeteren (*medium done*), parasitologisch gezien veilig beschouwd worden. Echter, het in de praktijk brengen van dergelijke kookmethoden in endemische landen, zoals in het Poolse voorbeeld in dit proefschrift, verhoogt het risico op humane trichinellose met een factor vijf, vergeleken met het eten van gaar vlees van varkens en wilde zwijnen, die gemist werden bij de slachthuiscontrole. Consumptie van rauw vlees (rauwe worstjes, carpaccio van wild zwijn) is helemaal een riskante bezigheid onder deze omstandigheden.
Onze QMRA laat zien dat in het Poolse voorbeeld, consumptie van wild zwijn verantwoordelijk is voor 55% van alle gemodelleerde gevallen van humane trichinellose, terwijl het aantal geteste wilde zwijnen 168 keer lager ligt dan het aantal varkens van niet-gecontroleerde houderijen. Tegelijkertijd is de prevalentie in wilde zwijnen 4100 keer hoger dan in varkens van niet-gecontroleerde houderijen in het Poolse voorbeeld. EU Verordening 853/2004 laat toe dat vlees van wild dat 'rechtstreeks wordt geleverd aan de eindverbruiker of aan de plaatselijke detailhandel die rechtstreeks aan de eindverbruiker levert', niet getest wordt op *Trichinella*. Speciaal in endemische gebieden, waar thuis geproduceerde vleesproducten traditioneel worden gemaakt en geconsumeerd, kan verplichte keuring van wild dat gevoelig is voor *Trichinella* infectie, trichinellosis uitbraken voorkomen. Dergelijke uitbraken worden nog steeds gezien in endemische landen, maar ook over hun landsgrenzen. Er zijn geen aanwijzingen dat de aanwezigheid van *Trichinella* in Nederlands wild een bedreiging vormt voor Nederlandse varkens, die praktisch allemaal worden gehouden in geïsoleerde stallen, maar varkens die contact hebben met de buitenwereld vormen een risico en daarom blijft monitoring van wild van belang.
Met een slachtvolume van 140 miljoen *Trichinella*-negatieve varkens uit gecontroleerde houderij in Nederland in de afgelopen tien jaren, is de kans om een Trichinella-positief varken te vinden in het daarop volgende jaar niet meer dan 0.0007, als een steekproef van 10% van de varkens getest wordt, inclusief zeugen en beren, zoals aangetoond in dit proefschrift. In het Nederlandse voorbeeld is testen van ongeveer 7,3 miljoen varkens nodig (50 – 60% van het totale jaarlijkse slachtvolume) om een kans van 5% te verkrijgen op het vinden van een *Trichinella*-positief varken. Dit voorbeeld laat zien dat onder

gecontroleerde houderij, het testen van varkens op *Trichinella* geen toegevoegde waarde heeft voor de bescherming van de volksgezondheid. Desondanks wordt nog steeds 100% van alle Nederlandse slachtvarkens getest op *Trichinella* om exportbelangen te beschermen.

Dierwelzijn en milieubewegingen in geïndustrialiseerde landen propageren natuurlijker productiemethoden voor vlees. Daarbij wordt gewezen op het belang van vrije uitloop en dierenwelzijn en is het streven gericht op de traditionele kleinschalige gemengde veehouderij, of totale uitbanning van grootschalige veehouderij in geïsoleerde stallen (Davies, 2011). Echter, beslissingen hierover kunnen alleen genomen worden als beleidsmaker wetenschappelijk geïnformeerd zijn over de risico's voor de volksgezondheid.

Curriculum vitae

Frits Franssen was born on 4th of November 1957 in Schaesberg, the Netherlands, where he graduated from the Eijkhagen College in 1976. Having a broad interest in biology and technique, he chose to start at the Zuid-Limburgse Laboratorium School in Sittard (HBO-A), where he graduated in 1978. In his second year, he found his prime interest in parasitology. In January 1979, he started working as technician at the Institute for Tropical Veterinary Science and Protozoology (TVS&P) of the Utrecht University, where he was trained in the *in vitro* culture of *Theileria* species and microscopical determination of protozoan parasites. In 1980, he continued a part-time Research Technician study at the Avondschool voor Laboratoriumpersoneel in Eindhoven (HBO-B), where he graduated in 1982, with a graduation thesis on 'The in vitro titration of sporozoites of *Theileria parva*, the causative agent of African theileriosis in bovines'. In the mean time, he was trained to assist in practicals on tropical protozoan parasites. In 1988 he continued with a part-time study at the Haagsche Hogeschool (The Hague University for Applied Sciences) in The Hague, the Netherlands, from which he graduated in 1990 in Medical Chemistry.

He developed into a specialist on the morphological identification and in vitro culture of protozoan parasites, and provided practical courses on the morphology and microscopical pathology of protozoan parasites for veterinary students at the Department of Infectious Diseases and Immunology. In 1997, he organised and conducted an international course on the in vitro culture of *Plasmodium falciparum*, at the Escuela de Química Farmacéutica, Facultad de Ciencias Químicas y Farmacia, San Carlos University, Guatemala City, Guatemala.

Additional to his appointment as research technician at the Parasitology department, he was responsible for the daily supervision and quality control of the Facility Unit of the Department Infectious Diseases and Immunology, from 2004 – 2007.

In 2007 he switched to the Centre for Zoonoses and Environmental Microbiology, Centre for Infectious Disease Control of the National Institute for Public Health and the Environment, Bilthoven, the Netherlands, where he was appointed at the National Reference Laboratory for Parasites. In his work for the NRL for Parasites, he combined quality control and supervision on *Trichinella* laboratories at slaughterhouses, with research on zoonotic helminth parasites in meat and fish.

In 2012, he was admitted to the PhD programme Infection and Immunity of Utrecht University, as PhD candidate on *Trichinella* in wildlife and evaluation of risk-based monitoring, which led to this thesis.

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