Tularaemia in Swedish wildlife – a One Health perspective

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Cover:
European brown hare and wolverine (Photo: Karin Bernodt).
Meat-eating woman and liver with a macrophage containing red-staining *F. tularensis* in the cytoplasm (Photo: Gete Hestvik).

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Abstract

Tularaemia is a zoonotic disease caused by the bacterium *Francisella tularensis*, which may infect a wide range of hosts. The subspecies known to cause disease in Europe is *F. tularensis* subsp. *holarctica*. The susceptibility to develop disease varies between animal species. For example, mountain hares (*Lepus timidus*) and many small rodent species succumb to fulminate disease, while many carnivores and omnivores show no signs of clinical disease.

This thesis has investigated the pathology and serology of tularemia in selected wildlife hosts. It has also reviewed the status of tularaemia in Europe in a One-Health perspective.

Tularaemia is widely distributed throughout Europe. Differences in surveillance and reporting between countries, different ecosystems, the presence of different species of arthropod vectors and wildlife species, present difficulties in making direct comparisons across all of Europe.

The pathology of tularaemia in European brown hares (*Lepus europaeus*), mountain hares and two yellow-necked mice (*Apodemus flavicollis*) were similar, all presenting with acute disseminated disease. However, some of the European brown hares, in addition to the acute lesions, also had subacute or chronic changes. This raises the question of whether European brown hares in Sweden might play an epidemiological role as reservoir of *F. tularensis*. *F. tularensis* was also demonstrated in muscles of infected hares, which highlights the risk of acquiring infection through consumption of under-cooked meat.

Many predators and omnivores develop antibodies upon infection and therefore may be suitable sentinels of the presence of tularaemia. The study revealed seropositivity in brown bear, red fox, wild boar and wolverine, for the first time reported in Sweden.

This thesis contributes to the knowledge of tularaemia in Europe, its pathology in European brown hares and yellow-necked mice, and its possible routes of infection and shedding. Additionally, it contributes to the understanding of the role of predators and scavengers. The results of our studies highlight the importance of further investigations of different wildlife species to explore their role in the epidemiology of tularaemia, as possible sources of infection, transmitters of disease and potential reservoirs.

Keywords: Francisella tularensis, hare, omnivore, pathology, predator, serology, tularaemia, yellow-necked mouse, wildlife.

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Harpest hos svenska vilda djur ur ett “One Health”-perspektiv

Sammanfattning

Tularaemia (harpest) orsakas av bakterien *Francisella tularensis* och är en zoonos som kan infektera ett stort antal värdar. Det subspecies som orsakar sjukdom i Europa är *F. tularensis* subsp. *holarctica*. Känsligheten för att insjukna varierar mellan djurslag. Skogshare (*Lepus timidus*) och många smågnagararter är exempel på djurarter med hög känslighet och dessa dör ofta snabbt i akut sjukdom. Många karnivorer och omnivorer visar inga sjukdomstecken efter infektion.

I denna avhandling har patologiska och serologiska undersökningar utförts på några vilda djurarter. En översikt av kunskapsläget om tularemi hos människa, djur och vektorer i Europa har sammanställts ur ett One Health-perspektiv.

Tularemi finns i majoriteten av Europas länder, men variationer i sjukdomsövervakning och rapportering, ekosystem, vilda djurarter och arthropoda vektorer gör det svårt att göra direkta jämförelser mellan länderna.

Patologin var likartad hos fälthare (*Lepus europaeus*), skogshare och två större skogsmöss (*Apodemus flavicollis*). De hade alla akut harpest involverande många organ. Några av fälthararna hade, förutom akuta, även subakuta eller kroniska förändringar. Detta väcker frågan huruvida fälthare i Sverige kan vara potentiell reservoar för *F. tularensis*. *F. tularensis* kunde även påvisas i muskulaturen hos infekterade harar, medförande en risk för infektion vid konsumtion av otillräckligt tillagat harkött.

Många rovdjur bildar antikroppar vid infektion, vilket kan göra dem lämpliga som biologiska indikatorer för förekomsten av tularemi i ett område. Blodprover som togs i fält och förvarades och transporterades under bristfälliga förhållanden var ofta av dålig kvalitet, vilket försvårade de serologiska undersökningarna. I studien påvisades antikroppar hos brunbjörn, järv, vildsvin och rödräv. Det är första gången antikroppar mot *F. tularensis* påvisas hos dessa djurarter i Sverige.

Denna avhandling bidrar med kunskap om tularemi i Europa, sjukdomens patologi hos fältharar, skogsharar och större skogsmöss, samt möjliga infektions- och utsöndringsvägar. Därtill bidrar arbetet till förståelsen av den roll rovdjur och asätare kan spela. Resultatet av våra studier visar vikten av att fortsätta studera vilda djurslag för att utröna deras roll i tularemins epidemiologi, och huruvida de kan utgöra potentiella infektionskällor, smittspridare och reservoarer.

Nyckelord: Francisella tularensis, hare, harpest, omnivorer, patologi, rovdjur, serologi, större skogsmus, tularemia, vilda djur.

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Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I G. Hestvik*, E. Warns-Petit, L.A. Smith, N. J. Fox, H. Uhlhorn, M. Artois, D. Hannant, M. R. Hutchings, R. Mattsson, L. Yon and D. Gavier-Widén (2015). The status of tularaemia in Europe in a one health context: a review. *Epidemiology and Infection*, 143(10), pp. 2137-60

II G. Hestvik*, H. Uhlhorn, F. Södersten, S. Åkerström, E. Karlsson, E. Westergren and D. Gavier-Widén (2017). Tularaemia in European Brown Hares (*Lepus europaeus*) and Mountain Hares (*Lepus timidus*) Characterized by Histopathology and immunohistochemistry: Organ Lesions and Suggestions of Routes of Infection and Shedding. *Journal of comparative pathology*, 157, pp. 103-114.

III G. Hestvik*, H. Uhlhorn, F. Södersten, R. Mattsson, E. Westergren, S. Åkerström and D. Gavier-Widén. Tularaemia in two naturally infected yellow-necked mice (*Apodemus flavicollis*) (submitted manuscript).

IV G. Hestvik*, H. Uhlhorn, T. Jinnerot, S. Åkerström, F. Södersten and D. Gavier-Widén (2017). *Francisella tularensis* in muscle from diseased hares – a risk factor for humans? *Epidemiology and Infection* (accepted).

V G. Hestvik*, H. Uhlhorn, M. Koene, S. Åkerström, A. Malmsten, F. Dahl, P-A. Åhlén, A-M. Dalin and D. Gavier-Widén. *Francisella tularensis* serology in Swedish predators and scavengers (manuscript).

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1 Introduction

1.1 General background

Tularaemia is an important zoonotic disease caused by *Francisella tularensis*, a small gram negative and facultative intracellular bacterium. *F. tularensis* subsp. *tularensis* and subsp. *holarctica* are the subspecies causing significant disease in humans and other animals. This zoonotic disease can infect over 300 species, including mammals, birds, amphibians and invertebrates (Morner and Addison, 2001). The concept "one health" recognizes that the health of people is connected to the health of animals and the environment, and this is to a high degree true for tularaemia.

F. tularensis subsp. *holarctica* is found all over the Northern hemisphere, and *F. tularensis* subsp. *tularensis* historically has been found only in North America (Ellis *et al.*, 2002). However, recently *F. tularensis* subsp. *tularensis* has been found in the environment and in arthropods in Slovakia and Austria (Chaudhuri *et al.*, 2007), and in Australia, *F. tularensis* subsp. *holarctica* has been detected in ringtail possums (*Pseudocheirus peregrinus*), and in a woman bitten by a wild ringtail possum (Eden *et al.*, 2017; Jackson *et al.*, 2012). Symptoms of tularaemia are related to the site of entry of the bacteria, the virulence of the *F. tularensis* strain, and the immune status of the host (Anda *et al*. 2007).

There are two subspecies responsible for disease in humans and animals, *F. tularensis* subsp. *tularensis* (type A) and *holarctica* (type B). The infective dose varies between animal species depending on their natural sensitivity and the route of infection, in humans the infective dose can be as low as ten bacteria. (Sjostedt, 2007). Type A is considered to be the more virulent subspecies of *F. tularensis*. However, investigations have shown that the virulence also differs between subpopulations within type A (Petersen and Molins, 2010). In a study in mice, comparing two type B strains and four type A strains, all type A strains

were more virulent than the type B strains (Molins *et al.*, 2014). The phylogeography of *F. tularensis* type B has been extensively studied (Afset *et al.*, 2015; Gyuranecz *et al.*, 2012; Karlsson *et al.*, 2013; Pilo *et al.*, 2009; Sissonen *et al.*, 2015; Vogler *et al.*, 2009). Investigations regarding differences in the virulence between type B strains are fewer. One interesting finding is the difference in presentation of the disease in European brown hare when comparing *F. tularensis* type B genotypes B.12 and B.FTNF002-0. Subacute or chronic lesions were associated with B.12 and acute lesions with B.FTNF002-00. (Gyuranecz *et al.*, 2012; Kreizinger *et al.*, 2017; Origgi and Pilo, 2016). Experimental infection of laboratory rats has confirmed a difference in virulence between the genotypes, B.FTNF002-00 being the more virulent one (Kreizinger *et al.*, 2017).

1.1.1 Clinical presentations of tularaemia

In humans, tularaemia has several clinical presentations, including ulceroglandular, glandular, oculoglandular, oropharyngeal, respiratory, and typhoidal. The type of disease is dependent on the route of infection. In the ulceroglandular form, a local skin lesion is often considered to be the route of entrance (scratch, cut, insect bite) and the disease progresses to the swelling of the regional lymph node, which may ulcerate and suppurate. The glandular form of this disease is similar to the ulceroglandular form, but no primary skin lesion is detected in these cases. The respiratory form results from inhalation of the bacterium and affects the lungs. The oropharyngeal form of tularaemia is linked to ingestion of contaminated food or water. A more infrequent clinical presentation is typhoidal tularaemia where no route of infection is possible to establish. (Anda *et al*., 2007). The more virulent type A may cause fulminant fatal disease. Generally, type A may cause death in 5-15% of diseased humans if left untreated. The more severe forms, often respiratory, caused death in 30-60% of diseased humans before antibiotic treatment was available. Nowadays the fatality rate is 2%. (Dennis *et al.*, 2001). If septicaemia develops it is frequently very severe and may lead to septic shock. (Anda *et al*. 2007). Type B is less virulent and causes less severe disease, and is seldom fatal.

In animals, less is known about the presentation of disease caused by *F. tularensis* type B, and most information derives from experimental studies. In one study of experimental infection of mountain hares (*Lepus timidus*), the only clinical signs were slight depression (Mörner 1988), but in another study the hares were apathic and showed inappetence, polydipsia and ataxia (Borg *et al.*, 1969). Signs in common hamsters (*Cricetus cricetus*) ranged from apathy to moribund (Gyuranecz *et al.*, 2010a), and in a study of field voles (*Microtus*

agrestis) and bank voles (*Myodes glareolus*) general malaise was observed (Rossow *et al.*, 2014). The clinical symptoms described in a case report of a dog were lethargy, in-appetence and fever (Nordstoga *et al.*, 2014).

1.1.2 Francisella tularensis as a bioterrorist agent

Due to the low infection dose and its potential to cause severe disease and death if not treated, *F. tularensis* is a potential biological weapon. One important characteristic for this purpose is that the bacteria can be made airborne, and the infectious aerosol would cause severe respiratory disease in humans. Aerosols could also cause infection through the eye, broken skin or oropharynx. Since *F. tularensis* is ubiquitous in the nature it is possible to isolate and multiply the bacteria under laboratory conditions. (CDC, 2015 and Dennis *et al.*, 2001).

1.1.3 Differences in susceptibility to develop tularaemia

The susceptibility to develop disease, and the severity of disease, varies between animal species and species are divided into sensitive (class 1), intermediate (class 2) and resistant (class 3). In class 1, several small rodent species are found (e.g. *Microtus* spp. and *Apodemus* spp), while rats (*Rattus* spp.) are confined to class 2. Many lagomorphs belong to class 1, and insectivores can be found in class 1 and 2. Carnivores, like the genera *Canis*, *Felis*, *Vulpes*, *Nyctereutes* and *Mustela*, are classified into class 3, as suggested by Olsufjev and Dunayeva (Olsufjev 1970 see Sjöstedt 2007). The European beaver (*Castor fiber*) is placed in class 3, while the American beaver (*Castor canadensis*) is more sensitive and belongs to class 2. (Sjostedt, 2007).

1.1.4 Tularaemia – an endemic disease with outbreaks

Tularaemia is endemic in many areas, and outbreaks occur with variable time intervals. In highly endemic areas, or natural foci of tularaemia, disease is often yearly recurrent and cases appear in higher numbers than in the less affected endemic areas. In Sweden, tularaemia currently occurs in areas that previously had not shown evidence of presence of the infection. (Petersen and Schriefer, 2005; Tarnvik *et al.*, 1996). The European Centre for Disease Prevention and Control (ECDC) reports that confirmed human cases in EU/EFTA countries increased by 70% in 2014 compared to 2013. Sweden was the country that reported the highest number of cases (150) (ECDC 2016). Tularaemia outbreaks occur regularly in Sweden, the more recent ones in 2010, 2012 and 2015. During the outbreak in autumn 2015 859 human cases, 582 of these from the counties Norrbotten and Västerbotten, were reported to the Public Health Agency of

Sweden (Folkhälsoinstitutet). This is the highest number reported since the 1960´s. In 35 of 434 confirmed cases (7%), contact with animals was reported as the cause. (The Public Health Agency of Sweden, 2017). During the outbreak, the National Veterinary Institute (SVA) received reports of more than 150 dead mountain hares in the outbreak area. Thirty-one hares were submitted to SVA for necropsy, and tularaemia was confirmed in 24 of these. (Unpublished data). During outbreaks, and to a lesser extent also in the years between outbreaks, reports of disease in humans and hares are common. The surveillance system is passive, and diseased humans and hares are relatively easy to detect, but presumably also other wildlife species, e.g. small rodent species, succumb to tularaemia unnoticed by surveillance. There is a lack of knowledge about the potential effect of tularaemia on wildlife populations in Sweden. During and after the outbreaks, the public often reports a decline of the hare population in the affected area. The year after a large outbreak in humans and hares 1966-67, the mountain hare population in some areas declined to 1/5-1/10 of the population before the outbreak (Borg *et al.*, 1969).

1.2 History

In 1911, McCoy described a disease in ground squirrels in Tulare County in California in the USA, during an investigation of plague in squirrels. The squirrels had changes similar to plague, but the plague bacteria could not be detected. In 1912, McCoy and Chapin isolated the bacterium behind the disease, naming it *Bacterium tularense*. It was later re-named *Francisella tularensis*. During the following years, tularaemia was linked to deer-fly fever in humans, wild hares and rabbits in North America. In the United States, Scandinavia and Russia, the existence of the disease in 18th and 19th century was discovered retrospectively by medical historians (Francis, 1919 and Olsen, 1975). In Europe and Russia, disease outbreaks in humans were associated with high abundance of rodents as early as in the16th century. Jacob Ziegler already in 1532 described that lemmings died in an epidemic disease in Norway, and also transferred the disease to humans. In 1653, Olaus Wormius, described humans with a disease causing swelling of glands, and he connected this to the Norwegian´s fear of lemming invasion. In the later years of the 19th century the disease was called lemming fever. (Pearson, 1998). Extensive work on tularaemia in a large number of animal species was performed during the mid-20th century, and a few examples are mentioned below. Jellison described tularaemia in North America including the geographical distribution, investigating and discussing the role of lagomorphs, rodents, insectivores, carnivores, mustelids, non-human primates, birds and sheep (Jellison, 1974). In the former Soviet Union, Olsufjev, among

others, investigated and reviewed tularaemia in many wild and domestic animal species including lagomorphs, rodents, insectivores, herbivores, omnivores, carnivores, birds and amphibians, performing bacterial culture, necropsies, parasitology and serology. The studies were of both field and experimental type (Olsufjev, 1963). These studies contributed with abundant information on the vast number of animal species that can be infected and their differences in susceptibility to develop disease. During the 1980´s, Pfahler-Jung reviewed the global distribution of tularaemia including where the disease was endemic or sporadic. Extensive information regarding wildlife species involved in different countries, and the importance of ecosystems including vectors was also reviewed (Pfahler-Jung, 1989).

In Sweden, the first confirmed cases of tularaemia were reported in a mountain hare and three humans who had been in contact with the infected hare (Granström, 1931). The number of diagnosed human cases increased the following years and in 1938 an outbreak in the north of Sweden was linked to abundance of lemmings (*Lemmus lemmus*). Tularaemia was diagnosed in the lemmings, and it was assumed that humans were infected by mosquitoes transferring *F. tularensis* from the rodents rather than by direct contact with the lemmings. (Olin, 1942). During the following two decades, several more outbreaks occurred with tularaemia reported in humans, hares, squirrels, lemmings and voles. In a large outbreak in 1966-1967 involving humans, hares and voles, *F. tularensis* was isolated from 18 of 49 investigated voles, and in a study of several small rodent species in Fennoscandia in the early 1970s antibodies were detected in some of them (Borg *et al.*, 1969; Omland *et al.*, 1977). During the outbreak in 1966-1967, the mountain hare population was reduced (Borg *et al.*, 1969). Pathological examination of naturally and experimentally infected mountain hares showed an acute disease quickly leading to death (Borg *et al.*, 1969). Acute disease has also been described in naturally infected mountain hares during the 1980s and later on in Sweden and Norway (Morner *et al.*, 1988; Vikoren and Djonne, 2008; Vikoren *et al.*, 2008). Serology has been performed in cattle (Bos sp), moose (*Alces alces*), European beaver and mountain hare in Sweden. Approximately 50% of the beavers had positive antibody titers, but a few cases were also found serologically positive in cattle, moose and mountain hares. (Morner and Sandstedt, 1983).

1.3 Life cycles, transmission and reservoirs

1.3.1 Life cycles

Two principal life cycles of tularaemia are recognized, one terrestrial and one aquatic. Previously, *F. tularensis* subsp. *tularensis* (type A) was associated with the terrestrial, and *F. tularensis* subsp. *holarctica* (type B) with the aquatic, but nowadays both life cycles are suspected to be present in association with type B tularaemia in Europe. The terrestrial cycle is common in central and southern Europe, while the aquatic predominates in northern Europe. The terrestrial cycle involves the terrestrial environment. Lagomorphs (rabbits and hares) and rodents are vertebrate hosts, and ticks are invertebrate hosts and vectors which are sources of infection for other hosts. In the aquatic cycle, centred around lakes, rivers and water wells, the vertebrate hosts involved are rodent species such as voles, muskrats and beavers, and mosquitos act as invertebrate hosts and vectors. (Carvalho *et al.*, 2014; Maurin and Gyuranecz, 2016).

1.3.2 Reservoirs and transmission

The epidemiology of tularaemia is complex and involves mammals, birds, invertebrates such as mosquitos and ticks, and the environment. There is insufficient knowledge regarding the disease epidemiology and the interplay between the multiple hosts and environmental factors involved. Species that act as reservoirs for *F. tularensis* and their role are incompletely understood. Haydon et al. defined reservoirs as "populations or environments where a pathogen is permanently maintained and from which the pathogen is transmitted to a target population" (Haydon *et al.*, 2002).

The European brown hare (*Lepus europaeus*) is considered a reservoir species of tularaemia in central and eastern Europe (Gyuranecz *et al.*, 2010b; Maurin and Gyuranecz, 2016). These hares show subacute and chronic lesions (Gyuranecz *et al.*, 2010b; Sterba and Krul, 1985). In other parts of Europe, for example in France and Sweden, the role of the European brown hare as possible reservoir of tularaemia is less clear, since the hares mostly present acute forms of disease (Decors *et al.*, 2011; Hestvik *et al.*, 2017), thus limiting the time of bacterial shedding. Also, these acute septicaemic presentations appear to be fatal in the hares so that hares are not able to maintain the infection for prolonged periods to act as reservoirs. Full understanding of possible different disease patterns in European brown hares has not been achieved. For example, in European brown hares necropsied in Sweden it was observed that a few of the hares that died of acute tularaemia also had chronic tularaemic lesions in the

lung and/or kidney, probably these hares had a chronic disease that was reactivated and caused their death. (Hestvik *et al.*, 2017 and unpublished data SVA). These findings warrant further investigations to elucidate if the European brown hare also might be a reservoir species in Sweden. Rodent species differ in their susceptibility to develop disease, for example species of *Apodemus* are very susceptible, while *Rattus* spp. have an intermediate degree of susceptibility. Therefore, some rodent species might function as reservoirs while others might not.

Tick and mosquito bites, ingestion of contaminated water and direct contact with infected animals are important sources of infection. (Maurin and Gyuranecz, 2016). *F. tularensis* have been found in ticks in several European countries (Hestvik *et al.*, 2014; Morner *et al.*, 1988), and there is evidence of the presence and replication of the bacterium in the midgut and salivary glands of the ticks, and also of transstadial transmission. Ticks can transmit bacteria during a blood meal and through interrupted feeding. (Petersen *et al.*, 2009; Reif *et al.*, 2011). Transmission via mosquitos has been less well investigated, but in a Swedish study the bacterium was detected in several mosquito species (Lundstrom *et al.*, 2011). Experimental studies have shown that mosquitos may aquire infection when feeding on infected mammals, but transmission in the opposite direction has not been experimentally proven (Thelaus *et al.*, 2014; Triebenbach *et al.*, 2010). Some studies have found evidence of transstadial transmission (Backman *et al.*, 2015; Thelaus *et al.*, 2014). In a recent study, bacteria have been shown to be transferred between mosquitos when feeding on flower nectar (Kenney *et al.*, 2017). Since mosquitos have a strong connection to water sources, an association between *F. tularensis*, mosquitos and natural waters is stipulated. Investigations of water samples from natural waters in endemic areas have detected DNA from *F. tularensis* subsp. *holarctica*, and in experimental studies the bacterium has been able to survive in water for several months (Broman *et al.*, 2011; Forsman *et al.*, 2000). Free-living amoebas can interact with different bacteria, and in experimental studies of *Acanthamoeba castellanii* co-cultured with *F. tularensis*, bacteria were found intracellularly in the amoebas. It has also been shown that the bacteria can survive inside the amoebas for at least three weeks. It is speculated that amoebas could be environmental reservoirs for *F. tularensis* (Abd *et al.*, 2003; El-Etr *et al.*, 2009).

Chronically infected animals, as well as acutely infected animals, before they succumb to disease, may contaminate the environment with infected urine and faeces if the bacterial load is sufficiently high (Bell and Stewart, 1975; Gyuranecz *et al.*, 2010b). Carcasses of infected animals may contaminate both the terrestrial environment and water sources, and may also be a source of infection for predator and scavenger species. Ticks have been postulated as

reservoirs of tularaemia. *F. tularensis* subsp. *holarctica* was found to persist in ticks in an endemic area in Slovakia for six years (Gurycova *et al.,* 1995). The finding of *F. tularensis* DNA in natural waters, and the survival of the bacterium in water experimentally may also be a sign that natural waters might be environmental reservoirs (Broman *et al.*, 2011; Forsman *et al.*, 2000).

The information regarding wildlife as reservoirs in Sweden is scant, hares and small rodents may play a reservoir role, but this must be further explored.

1.4 Pathogenesis and immunity

At the site of infection, *F. tularensis* invades the host cells, most commonly macrophages. *F. tularensis* has a particular predilection for macrophages, even though it can also infect other cell types. (Oyston, 2008). *F. tularensis* avoid the host´s immune system by blocking or weakening signalling in macrophages and other immune cells when interacting with the cells´ receptors (Bosio, 2011). To gain entry into the macrophage, *F. tularensis* interacts with several cell surface molecules resulting in phagocytosis. Inside the cell cytoplasm the engulfed bacteria are taken up by phagosomes. (Barel and Charbit, 2013; Clemens and Horwitz, 2007). The membrane of the phagosome is degraded and bacteria are free to replicate into high numbers in the cytoplasm of the cell. When the bacteria are free in the cytoplasm, they are vulnerable to the cell´s defence mechanisms, such as reactive oxygen and nitrogen. *F. tularensis* has the ability to inhibit or interfere with these mechanisms, promoting its own survival in the cytosol. (Barel and Charbit, 2013; Bosio, 2011). *F. tularensis* may induce cell death by several pathways. Apoptosis-induced cell death is achieved by induction of caspases without an inflammatory response. In pyroptosis, a process induced by other types of caspases than in apoptosis, pro-inflammatory cytokines are released, inducing an inflammatory response. The signalling complex that activates caspases in pyroptosis are called inflammasomes, and they have proved to be crucial for host defence against *F. tularensis*. (Barel and Charbit, 2013; Parmely *et al.*, 2009). Unlike other intracytoplasmic pathogens, *F. tularensis* re-enter membrane-bound compartments after replication, and this may induce autophagocytosis, a process where the cell eats its own content, which may trigger cell death (Barel and Charbit, 2013). Pathogen-induced cell death can result in the elimination of infected cells and thereby halt the infection, but on the other hand it can also be advantageous to the bacteria since immune cells are eliminated, thereby suppressing the immune response. By activating the different host cell death pathways, bacteria are released and can spread further in the body. (Barel and Charbit, 2013; Parmely *et al.*, 2009).

The normal incubation period of tularaemia in humans is 3-5 days, but spans between 1 and 21 days (Anda, 2007). It is difficult to know the incubation time in wild animals, but experimental studies in mountain hares (Borg *et al.*, 1969), common hamsters (Gyuranecz *et al.*, 2010a), and field and bank voles (Rossow *et al.*, 2014) indicate similar incubation times as in humans. Observable symptoms appeared within 2-8 hours in the mountain hares, on day 7 and 8 in the hamsters, and days 6-9 in the voles. In a Norwegian study, a dog showed symptoms two days after killing an infected hare (Nordstoga *et al.*, 2014).

The first line of defence is provided by the innate immune response where macrophages, dendritic cells, granulocytes, mast cells and natural killing cells are activated. Various cytokines are produced that activate humoral and cell-mediated responses. Around 6-10 days after infection, IgM, IgG and IgA serum antibodies are detected, and they reach their highest level approximately one to two months after infection. The antibodies persist for about a decade. (Cowley and Elkins, 2011). In contrast to humoral immunity, cell-mediated immunity may persist for 25 years. (Ericsson *et al.*, 1994). Compared to cell-mediated immunity, humoral immunity is often of less importance for protection against intracellular bacteria, but since studies have shown that a large part of *F. tularensis* bacteria are located extracellularly in the blood, humoral immunity might contribute more than previously assumed. (Cowley and Elkins, 2011; Forestal *et al.*, 2007).

1.5 Pathology of *F. tularensis* subsp. *holarctica* – a brief overview

In humans, the lesions of tularaemia are often focal. Macroscopically in ulceroglandular tularaemia the skin lesions range from erythematous to ulcerative at the site of bacterial entrance and enlargement of draining lymph nodes, followed by suppurative lymphadenitis (Syrjälä *et al.*, 1984, and Anda, 2007). Histopathology reveals that the skin ulcerates and there is infiltration of lymphocytes, plasma cells, macrophages and neutrophils, and formation of granulomas. The lymph nodes may present with sinus histiocytosis and follicular hyperplasia, small abscesses, granulomas and/or casseous necrosis (Asano, 2012; Syrjala *et al.*, 1984). In oropharyngeal tularaemia, the disease macroscopically presents as an ulcerative-exudative stomatitis and pharyngitis, and tonsillitis may be present. (Anda, 2007). Histopathological investigations show variably appearing morphological changes, such as suppurative inflammation, granulomatous inflammation, or extensive areas of necrosis that resemble casseous necrosis (Turhan *et al.*, 2013). Pulmonary lesions have been less well investigated histologically and is described from diagnostic imaging as

pulmonary consolidation and enlargement of hilar lymph nodes (Anda, 2007). In one case in the USA where the subspecies of *F. tularensis* could not be determined, histopathology showed multiple necrotic foci and mixed inflammatory infiltrate with a large number of macrophages, lymphocytes and neutrophils. In the pleura there was chronic inflammation, granulation tissue and fibrosis. (Navarro *et al.*, 2011).

The pathology in animals is dependent on the susceptibility of each respective species to develop disease. In wildlife species that are prone to develop acute disease, frequently observed gross changes are enlarged spleen, and pinpoint necrotic lesions in the spleen, liver and bone marrow. Often, gross lesions are inapparent. At histopathological examination, abundant coagulative or lytic necrosis with no or minimal inflammation is frequently seen; lesions are often described in additional organs and sepsis is apparent (Borg *et al.*, 1969; Hestvik *et al.*, 2017; Morner *et al.*, 1988; Rossow *et al.*, 2014; Vikoren and Djonne, 2008). Acute lesions are often observed in several small rodents, mountain hares and European brown hares. In the European brown hare, subacute and chronic lesions have also been described (Gyuranecz *et al.*, 2010b; Origgi and Pilo, 2016), constituting grossly visible inflammatory changes, most frequently in the lungs, pericardium and kidneys. Histopathology shows a predominance of granulomatous inflammation. Domestic animals have rarely been investigated using pathology, most reports are limited to clinical examinations. One diseased dog developed enlargement of pharyngeal, prescapular and popliteal lymph nodes after killing a tularaemic hare (Nordstoga *et al.*, 2014). No reports on the pathology of cats infected with *F. tularensis* subsp. *holarctica* could be found.

1.6 Diagnosis

There are several methods that can be used to diagnose an infection by *F. tularensis*, only a few will be mentioned here.

In humans, and wild and domestic animals, the same methods may be applied. Serology is used to detect antibodies in the blood. Commonly used serological methods are microagglutination, tube agglutination and enzyme-linked immunosorbent assay (ELISA). If possible, paired samples are used to detect a rise in antibody-titres, ensuring that the infection is ongoing. Polymerase chain reaction (PCR) is used on samples from wounds, biopsies from lymph nodes or other organs, bronchiolar lavage or blood, to detect the bacteria. A less often used method nowadays is to culture for *F. tularensis*. (The Public Health Agency of Sweden, 2007 and Tärnvik, 2007).

In wildlife, serology is used both in active surveillance of hunted, often apparently healthy, animals and in animals found dead. In animals that are

diseased and found dead, or euthanized due to disease, necropsy and histopathological investigations can be performed. In these cases, common diagnostic methods are PCR and immunohistochemistry (IHC) on tissue samples to detect the bacteria.

2 Aims of the thesis

The main aim of this thesis was to acquire knowledge of *Francisella tularensis* subsp. *holarctica* infection in a one health perspective, mainly focusing on the disease in Swedish wildlife.

More specific aims were:

- To review recent information on tularaemia in Europe in humans, wildlife, domestic animals and vectors (paper I).
- To describe the pathology in Swedish European brown and mountain hares and yellow-necked mice, with the purpose of learning more about possible routes of infection and shedding (paper II and III).
- To elucidate the risk for humans and other animals to contract infection and to investigate possible sources of infection (paper I, II and IV).
- To investigate the seroprevalence of *F. tularensis* in Swedish wild predators and scavengers, and to evaluate their suitability as biological indicators for occurrence, spread and/or increase of tularaemia prevalence (paper V).

3 Materials and methods

3.1 Data collection (study I)

In study I, information on tularaemia in Europe in humans, wild and domestic animals and vectors during a 20-year period was compiled. Data from 38 European countries was retrieved and reported data and publications from 1992 to 2012 were included. For humans and animals, the study was based on relevant databases and publications, while for vectors publications were the only source of data.

3.1.1 Human data

The database of the European Centre for Disease Prevention and Control (ECDC, 2014) was queried for data on reported human cases of tularaemia between 2006 and 2012, and the WHO-CISID database (World Health Organization, 2014) for 1992-2012. Case data by county/region and by year were also extracted from several National Public Health Institute websites. Population data produced by Eurostat (European Commission, 2014) was used for incidence calculations. A literature review was conducted in order to find additional case data and information on surveillance systems, type of clinical presentation, and source of infection and exposure, in order to assess the disease risk in humans. Information on type of study, event description, frequency of clinical forms, routes and sources of infection, exposures and/or identified risk factors was extracted.

3.1.2 Wild and domestic animal data

Information about tularaemia and *F. tularensis* infection in wild and domestic animals was compiled from the World Organization for Animal Health (OIE) databases and from published articles. Because the OIE databases only included data from 1996 onward, data for the period from 1992 to 1995 was only available from published articles. The OIE databases used for this review were the World Animal Health Information Database Handistatus II (OIE Handistatus II, 2017) and the World Animal Health Information Database (OIE WAHIS, 2017). Data on the geographical range of tularaemia are available only as notification or no notification of tularaemia at the country level. Information on type of surveillance, outbreaks, disease status in wild and domestic animal species, estimated prevalence, source of infection, route of shedding and diagnostic methods used was extracted.

3.1.3 Vector data

For vectors, there are no official databases or obligations to report, information was solely extracted from studies investigating different types of arthropod species. For the purpose of this review, a vector was defined as any arthropod which can introduce *F. tularensis* into a susceptible host. Data extracted included arthropod species, country, number of arthropods sampled, number of arthropods for which the test gave a positive response, prevalence of *F. tularensis* in arthropods studied, and the diagnostic method used. All studies regarding arthropod vectors were of the type active surveillance.

3.1.4 Mapping of data

Maps were compiled to compare the reported distribution of *F. tularensis* in Europe in humans, wildlife, domestic animals, and arthropod vectors, and the different types of *F. tularensis* surveillance in humans across Europe. Due to restrictions in the resolution of the available data, all information is shown at the country level. Country base layers were downloaded from Global Administrative Areas (Global Administrative Areas, 2013), and maps were created in ArcGIS.

3.2 Study animals

All animals used in study II, III, IV and V were wild animals collected as part of the National Wildlife Disease Surveillance in Sweden. No experimental infections were conducted.

3.2.1 European brown and mountain hares

In study II, all European brown hares (EBH) and Mountain hares (MH) necropsied 2000-2013 and diagnosed with tularaemia were investigated, in total 49 EBHs and 37 MHs. From all, findings from the necropsies were compiled, but only 27 EBHs and three MHs had mild to moderate post-mortem changes and were suitable for histopathological and immunohistochemical examinations. In paper III, 32 EBHs and 10 MHs necropsied during the period 2007-2015, and one hunted EBH, were included.

3.2.2 Yellow-necked mice

Two yellow-necked mice (*Apodemus flavicollis*) found dead were necropsied and diagnosed with tularaemia (study III).

3.2.3 Predators and scavengers

Predator and scavenger species commonly found in Sweden were investigated in study V. Blood was sampled from all, tonsils and submandibular lymph nodes from a subset of the animals, details on animal species and samples are given in table 1. Sampling was performed in the period 2010-2015.

Table 1. *Number of animals of each species of predators and scavengers studied from which samples of blood and lymph nodes were obtained.*

Animal species	Blood (no. of animals)	Lymph node (no. of animals)
Brown bear	50	50
Eurasian lynx	34	33
Raccoon dog	126	20
Red fox	119	109
Wild boar	248	27
Wolf	59	59
Wolverine	20	20
Total no. of samples	656	318

All brown brown bears (*Ursus arctos*), raccoon dogs (*Nyctereutes procyonides*) and wolverines (*Gulo gulo*) had died of trauma. Of the Eurasian lynx (*Lynx lynx*), red fox (*Vulpes vulpes*), wild boar (*Sus scrofa*) and wolf (*Canis lupus*), 9, 10, 7 and 1% respectively, had died from disease or were euthanized due to disease. The remaining animals were hunted, killed in traffic or euthanized in restraining traps.

3.3 Laboratory diagnostics

3.3.1 Histopathology and immunohistochemistry

Histopathology and immunohistochemistry (IHC) were used in study II, III, IV and V. Pieces of tissue from internal organs (paper II, III and IV), muscles (paper III) and lymph nodes (study V) were fixed in 10% neutral buffered formalin, processed routinely, embedded in paraffin wax, sectioned at approximately 4 µm thickness, and stained with haematoxylin and eosin. Lesions were described and subjectively scored, based on their severity and extension, as negative, mild, moderate, marked or severe. In paper II, the area affected by necrosis was estimated as 0–20%, 20–50%, 50–75% or 75–100%. The abundance of each type of inflammatory cell (i.e. lymphocyte, plasma cell, heterophil and macrophage) was graded subjectively as not observed, few, moderate or numerous. IHC was applied on duplicate sections to visualize *F. tularensis* (study II, III, IV and V), and the presence of T-cells and B-cells (study II). The location of the labelled *F. tularensis* in paper II, III and IV was described as: present in lesions and/or in healthy tissue, present in blood vessels, intra- or extracellularly located, and in type of cells when located intracellularly.

IHC for *F. tularensis* was performed on formalin-fixed tissues from all histopathologically examined organs using a mouse primary monoclonal antibody FB11 (Meridian Life Science Inc., Nordic Biosite AB, Täby, Sweden) directed against *F. tularensis* spp. lipopolysaccharide antigen. Sections were dewaxed and incubated with H_2O_2 in Tris-HCL, pH 7,6 for 15 min to block endogenous peroxidase activity. Antigen was retrieved by proteinase K (Dako, Agilent, Glostrup, Denmark) treatment for 6 min; thereafter the sections were incubated at room temperature (RT) with 2% bovine serum albumin (BSA) (Sigma-Aldrich, Stockholm, Sweden) for 20 min to block non-specific labelling. The primary antibody was applied at a 1 in 3,500 dilution and the sections were incubated at RT for 45 min. Antibody binding was detected by use of the anti-mouse EnVisionTM polymer detection system (Dako) and the sections were counterstained with Mayer's haematoxylin. A serial section incubated with 2% BSA was used as negative control, and a known positive tularaemia sample from an EBH was used as positive control. Additionally, for some cases in paper II, a serial section was incubated with normal mouse serum and mouse IgG1 (Dako). This was also performed on sections known to be tularaemia negative. In paper III, where IHC was performed on yellow-necked mice, a modified method with an additional step using Vector®M.O.M™ (Vector Laboratories, Burlingame, CA) was applied to enable staining of *F. tularensis* with the mouse primary antibody FB11 in mouse tissue. Sections were incubated in the primary antibody

diluted in M.O.M Diluent at 1:3000 for 30 minutes, followed by incubation in a working solution of M.O.M. biotinylated Anti-Mouse Ig Reagent for 10 minutes. As detection system, the VECTASTAIN®elite ABC standard kit (Vector Laboratories, Burlingame, CA) was used.

Immunohistochemistry for detection of T lymphocytes (mouse primary antibody CD3 monoclonal; Dako) and B lymphocytes (mouse primary antibody CD79; BioCare Medical, Pacheco, California, USA) was performed on selected cases and organs to identify cell types (study II). Sections were dewaxed and antigen was retrieved in a microwave oven (in Tris EDTA buffer, pH 9.0) at 750 W for 7 min followed by 350 W for 14 min. To block endogenous peroxidase activity, sections were incubated with H_2O_2 in Tris-HCL, pH 7,6 for 20 min; thereafter the sections were incubated at RT with 2% BSA for 20 min to block non-specific labelling. The primary antibody was applied at 1 in 75 (CD3) or 1 in 30 (CD79) dilution and the samples were incubated at RT for 45 min. Antibody binding was detected by use of the anti-mouse EnVisionTM polymer detection system (Dako) and the sections were counterstained with Mayer's haematoxylin. A serial section incubated with 2% BSA was used as a negative for control.

3.3.2 Immunofluorescence

Indirect immunofluorescence (IIF) (study III) was performed on unstained formalin fixed tissue sections using an in-house *Francisella tularensis*-positive rabbit serum (polyclonal antibodies), (SVA, Uppsala, Sweden) for detection. Sections were incubated in a 37°C moist-chamber for 30 minutes with the rabbit primary polyclonal antibody diluted 1:20 in 0.05 M phosphate buffer, pH 7.9 (PBS). After rinsing the slides in PBS, a secondary fluorescein-labelled goat anti-rabbit IgG (H+L) (Vector, Laboratories, Burlingame, CA) diluted 1:20 in PBS was applied. The slides were incubated in a 37°C moist-chamber for 30 minutes. The slides were rinsed in PBS and mounted with a cover glass and phosphate-buffered glycerol, pH 8.6. To exclude false negative staining, a tissue section from a known tularaemia-positive sample was examined in the same way. To exclude false positive staining, serum from a known tularaemia-negative rabbit was used as primary antibody. The slides were examined in a fluorescence microscope, excitation 490 nm and emission 530 nm. A bright green fluorescence and a morphology consistent with the bacterium was considered as positive.

3.3.3 Slide agglutination and microagglutination

To detect *F. tularensis* antibodies, slide agglutination and microagglutination were performed on all blood samples in study V.

In the slide agglutination test, a *F. tularensis* antigen was used (Bioveta a.s., Ivanovice na Hané, Czech Republic). 40 µl of the sample was mixed with 200 µl of the containing *F. tularensis*-containing solution and the result was read after 1-3 minutes. A positive result was assessed as presence of agglutination flakes and clarification of the surrounding solution.

For the microagglutination, a protocol from Wageningen Bioveterinary Research (WBVR), Wageningen, the Netherlands was used. *F. tularensis* non-viable bacteria in 0,5% formaldehyde with addition of crystal-violet (Becton Dickinson AB, Stockholm, Sweden) was used as antigen. The antigen was diluted in saline solution in the ratio 1:15 to create a working solution. Eighty microliters of antigen working solution was mixed with 20 µl of serum in the first row of a 96-well V-bottom microtiter plate (VWR International, Stockholm, Sweden). 50 µl of the mixture was transferred to the next row, containing 50 µl of the working solution. This procedure was repeated to the last row, causing a two-fold dilution in each step and the titres to be tested 1:5, 1:10, 1:20, 1:40, 1:80, 1:160, 1:320 and 1:640. A known positive serum (Germaine Laboratories, San Antonio, United States), and a negative rabbit serum (Bio Jet Service, Uppsala, Sweden) was used as positive and negative controls for each plate. The plate was sealed (VWR International, Stockholm, Sweden) and incubated for 21 +/- 1 hour at 37°C. The wells were examined visually using a light box.

3.3.4 Polymerase chain reaction (PCR)

Real-time PCR to detect *F. tularensis* subsp. *holarctica* was used in study II-V. Tissues sampled were internal organs from hares (study II) and yellow-necked mice (study III), muscles from hares (study IV), and lymph nodes from predators and scavengers (study V). Material from the tissue samples was retrieved using sterile cotton swabs. The swabs were incubated in 380 µl G2 buffer and 20 µl proteinase K solution (study II, III and V) or 570 µl G2 buffer and 30 µl proteinase K solution (study IV). (EZ1 Tissue DNA Extraction Kit, Qiagen, Sollentuna, Sweden) at 56°C for 15 min under continuous agitation, followed by 5 min incubation at 95°C. DNA was extracted from 200 µl of the resulting lysate using the EZ1 Tissue DNA Extraction Kit and the EZ1 Advanced Instrument (with the Bacteria Card) (Qiagen). The DNA was eluted in 50 µl elution buffer and 1 µl was used as template for each PCR (study II, III and V), or in 100 µl elution buffer and with 2 µl used as template (study IV). In study II, *Francisella tularensis* subsp. *holarctica* genotyping was performed in 23 hares for typing of

the three major canSNP-groups B.4, B.6 and B.12, and the subgroups B.7, B.10, B.20, B.23 and B.39 (Karlsson *et al*., 2013, Svensson *et al*., 2009;) according to the qPCR-based method described previously (Karlsson *et al*., 2013).

3.3.5 Bacteriological culture

In study IV, 14 muscle samples were analysed by aerobic bacterial culture to investigate if *F. tularensis* were able to grow after variable length of storage in a freezer at -20°C. The samples had been stored for approximately 0.5 (one sample), 1 (two samples), 2 (two samples), 3 (two samples), 4 (three samples), 5 (two samples), 7 (one sample) or 8 years (one sample). The samples were cultured on T4 medium as described previously in Harmonization of methods at BSL-3 laboratories (Thelaus *et al*., 2012). T4 medium contains 37 g/L brain-heart infusion (Oxoid, Hampshire, UK), 15 g/L Bacto-agar (Neogen, Auchincruive, UK), 10 g/L sodium chloride (Honeywell, Morris Plains, NJ), 90 mL/L sheep blood (Håtunalab, Bro, Sweden), 1 g/L L-cysteine, 10 g/L glucose, 2.7 mg/L amphotericin B, 5 mg/L vancomycin, 2.5 mg/L trimethoprim and 2.8 mg/L cefsulodin (Sigma-Aldrich, St-Louis, MO) making it selective for *Francisella*. The culture plates were incubated at 37ºC for four days and checked for growth daily.

In study II, aerobic general bacterial culture was performed on selected organ samples (seven lungs, two kidneys, nine livers, three spleens, one uterus and two testicles) from 13 tularaemic hares suspected to be co-infected with additional bacteria. A few samples were submitted for culture in conjunction with the necropsy examination, while the rest had been stored at –20°C for up to 7 years. The samples were transferred to horse blood agar (in house) and a purple agar plate (in house). Plastic loops were changed between primary, secondary and tertiary streaks. The plates were incubated in an aerobic atmosphere at 37°C and analyzed after 1 and 2 days.

4 Results and discussion

4.1 Tularaemia in Europe

In study I, information was retrieved from databases and publications for the period 1992-2012, aiming to compile the information on tularaemia in Europe in humans, wild and domestic animals, and vectors during the recent two decades. The level of reporting for humans and animals differs between countries leading to gaps in the retrieved information, but it can be concluded that tularaemia is widely distributed in Europe. In humans, an overall seasonal pattern with most cases occurring during summer and early autumn was seen. However, at country level the seasonality varied with peaks in spring, summer, summer/autumn and winter. In European wildlife in our study I, a seasonality pattern was not obvious, but other studies describe outbreaks associated with the mosquito season in hares in Scandinavia (Josefsen *et al.*, 2012; Morner *et al.*, 1988; Vikoren and Djonne, 2008; Vikoren *et al.*, 2008). In study I, published information regarding bacterial detection and serology in wild and domestic animals was reviewed for the chosen time period. In wildlife, *F. tularensis* was detected in hares, rabbits, several small rodent species and birds. Antibodies against *F. tularensis* was reported in hares, red fox and wild boar. Fewer studies investigated domestic animals, but antibodies have been reported in ruminants, dogs and cats. Studies on vectors mostly concern ticks and mosquitos, and we found that the bacterium has been detected in several species. Tularaemia occurs with outbreaks in both endemic and new geographic areas. Different geographical areas have different ecosystems, which influence the epidemiology and disease presentation. Such factors include temperature and humidity, the presence of different kinds of arthropod vectors, and the variety of wildlife species present. There are highly endemic areas, or natural foci, where cases occur most years and in higher numbers compared to other endemic areas

(Gurycova *et al.*, 2001; Olsufjev, 1963; Tarnvik *et al.*, 1996). The differences in the level of reporting of tularaemia, type and level of surveillance, and presence of different hosts and vectors, makes it difficult to make direct comparisons across all of Europe. Understanding of the disease epidemiology would benefit from coordination between countries, for example for level of reporting and type of surveillance.

4.2 Wildlife

4.2.1 Pathology

European brown hares and mountain hares

Tularaemia is often considered to be acute in MH (Josefsen *et al.*, 2012; Morner *et al.*, 1988; Vikoren and Djonne, 2008; Vikoren *et al.*, 2008), and subacute/chronic or acute in EBHs (Decors *et al.*, 2011; Gyuranecz *et al.*, 2010b). However, it should be noted that in the studies reporting subacute/chronic lesions in EBH the hares had been hunted, thus were apparently (clinically) healthy. Conversely, the reports of acute forms of disease in EBH as well as in MH were conducted on animals that had been found dead or moribund. In the former disease presentation (subacute/chronic) the animals had seemingly survived the acute phase of disease while in the latter (acute or per-acute) tularaemia was fulminant. One could assume that a hare that has a longer course of disease would be in a poorer nutritional state, losing weight during the course of the infection, when compared to hares that die from acute disease. In study II, the pathology of EBH and MH found dead or moribund was described. When comparing the nutritional state in EBH and MH, no statistically significant difference between the two hare species could be found. Organ enlargement and grossly visible necroses were frequently found in the spleen, bone marrow and liver in both hare species, but lung lesions were found only in the EBH. There was a statistically significant difference in the presence of grossly visible necroses between MH and EBH in the bone marrow (41% in MH, 12% in EBH), and in the lung (none in MH, 14% in EBH). In general, gross lesions were present in a higher number of organs in each individual in the EBH than in the MH. In 30% of the hares no or minimal grossly visible lesions were seen. The histopathology could not be statistically compared between species since only three MH, but 27 EBH, were suitable for examination. Acute lesions were present in both species, and necroses were visible in liver, spleen, bone marrow, lymph nodes and adrenal glands in the majority of the hares, most often

with an associated mild inflammation consisting of macrophages, lymphocytes and plasma cells, and lesser numbers of heterophils (Fig 1a).

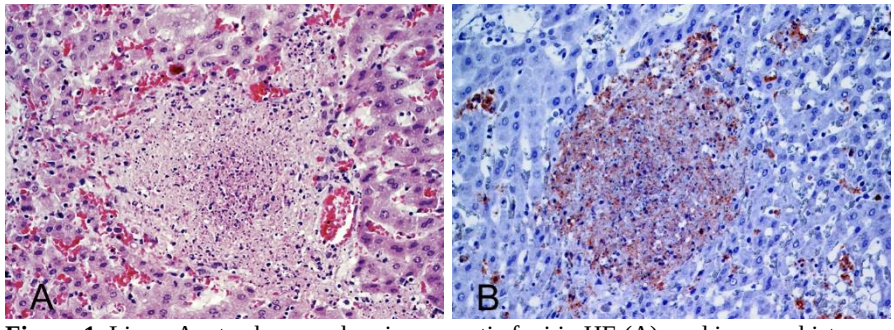

Figure 1. Liver. Acute changes showing necrotic foci in HE (A), and immunohistochemical staining of intralesional *F. tularensis* (B).

Many other organs, investigated in fewer animals, were also involved, such as gonads, lactating mammary glands, heart and intestine. Several of the EBHs had meningitis with occasional adjacent encephalitis, and pneumonia. Encephalitis has also been described in a Japanese hare (*Lepus brachyurus augustidens*) found dead (Park *et al.*, 2009). In study II, more severe and chronic lesions were seen in the lungs of five EBH and the kidneys of three EBH (Fig 2a).

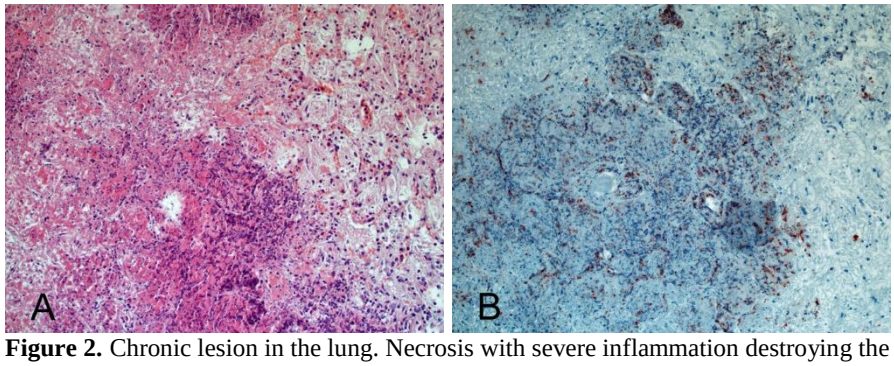

Figure 2. Chronic lesion in the lung. Necrosis with severe inflammation destroying the tissue, HE (A). Immunochemical staining showing *F. tularensis* (B).

Lesions of this type have also been described in hunted EBH in Germany and Hungary (Gyuranecz *et al.*, 2010b; Sterba and Krul, 1985). In our study II, dual infection by *Klebsiella pneumoniae* and *F. tularensis* was confirmed in the lungs of one hare. One other hare presented with dual infection *Escherichia coli* and *F. tularensis* infection in the kidney, but in the remaining hares with suspected dual infections the results were inconclusive. Dual infection with *K. pneumoniae* has previously been described in man (Givham, 1965). Immunohistochemistry

(IHC) for *F. tularensis* in our hares detected the bacterium extra- and intracellularly in association with necrosis and inflammation, with the most common intracellular location being in macrophages (Fig 1b, 2b). This finding, together with the dissemination of *F. tularensis* in many organs, show that the hares died of an acute disseminated disease, which is in accordance with the pathology in EBH found dead in France (Decors *et al.*, 2011) and in MH found dead in Sweden and Norway (Josefsen *et al.*, 2012; Morner *et al.*, 1988; Vikoren and Djonne, 2008; Vikoren *et al.*, 2008). In conclusion, since the hares in our study were all found dead or moribund, this increased the likelihood of finding acute lesions of tularaemia, that actually caused the death of the animals. Including hunted healthy hares in the study, would have increased the chances of finding more chronic lesions. Further research is warranted to characterize the range of pathological presentations since this will help to elucidate if the EBH may serve as reservoir for *F. tularensis* subsp. *holarctica* also in Sweden. The presence of chronically infected hares may also increase the risk of exposure to hunters and hunting dogs, since the hares may be apparently healthy, particularly since gross pathological lesions were absent in 30% of infected hares.

Yellow-necked mice

In study III, the pathology in two yellow-necked mice is described. The only gross lesion was an enlarged spleen in one mouse. By histopathology, both mice had necroses and associated mild inflammation. Due to marked post-mortem changes the remaining organs were difficult to assess, but there was a strong suspicion of necroses in both mice´s spleen and lung. To detect and visualize the bacteria, real-time PCR, IHC and indirect immunofluorescence (IIF) was used. *F. tularensis* was detected in the liver, spleen, bone marrow, lung and kidney (Fig. 3).

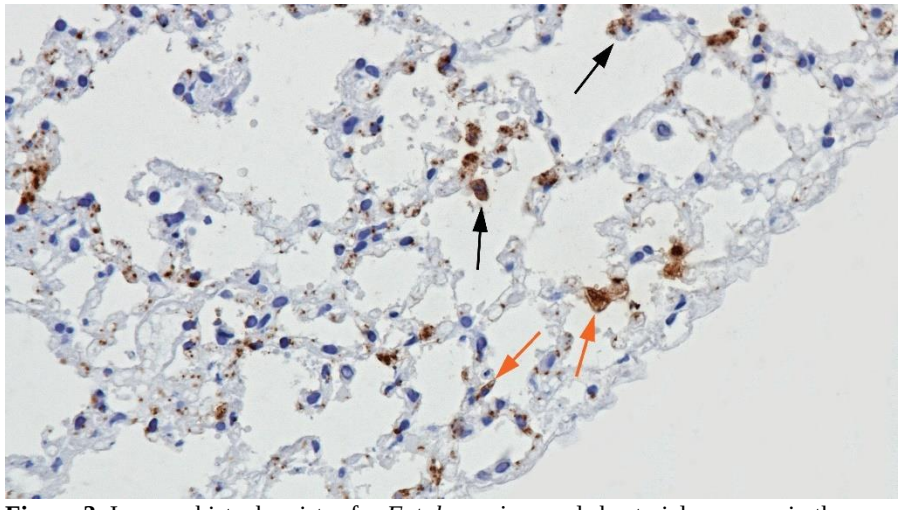

Figure 3. Immunohistochemistry for *F. tularensis* reveals bacterial presence in the cytoplasm of pneumocytes (orange arrow) and alveolar macrophages (black arrow).

Bacteria were found in necroses and blood vessels, and intracellularly in macrophages and pneumocytes. The pathological findings in the yellow-necked mice were similar to those described in laboratory mice (Conlan *et al.*, 2003), wild common hamsters (*Cricetus cricetus*) (Gyuranecz *et al.*, 2010a) and laboratory-born field voles (*Microtus agrestis*) and bank voles (*Myodes glareolus*) experimentally infected with *F. tularensis* subsp. *holarctica* (Rossow *et al.*, 2014). In naturally infected field voles in Finland and free-ranging house mice in Switzerland, necrosis in spleen, liver and/or lung were found (Origgi *et al.*, 2015; Rossow *et al.*, 2014).

The role of small rodents in the epidemiology of tularaemia in Sweden has not been thoroughly investigated. Wildlife disease surveillance is mainly passive and small rodents are seldom submitted for investigation. Among the few rodents investigated at SVA during the last decade, tularaemia has only been diagnosed in the two yellow-necked mice in study III, and in a few lemmings (unpublished data). More information about the disease in small rodents is necessary to better understand their role in the epidemiology. In the literature it is frequently discussed whether small rodent species die of acute disease or if they may survive and act as reservoirs or transmitters of the bacteria. There is a high diversity among the rodent species, and the sensitivity to develop disease differs among them.

Hare meat

Examination of muscle samples from 43 tularaemic EBH and MH with general tularaemic lesions as described in study II, showed that *F. tularensis* may also infect muscle tissue (study IV). The meat may contain *F. tularensis*, either due to transportation via the blood stream during the disease process (sepsis), or due to post-mortal contamination from infected internal organs. *F. tularensis* was detected by PCR and/or IHC in 40 of the 43 hares. The most common location was in the connective tissue of the perimysium surrounding medium-sized arteries. *F. tularensis* was frequently seen intracytoplasmic in macrophages, but also in the cytoplasm of fibroblasts and lymphocytes, or extracellulary. In the endomysium the bacteria were mostly intracytoplasmic in spindle cells, presumably fibroblasts (Fig. 4a). In three of the samples *F. tularensis* was detected intravascularly (Fig. 4b).

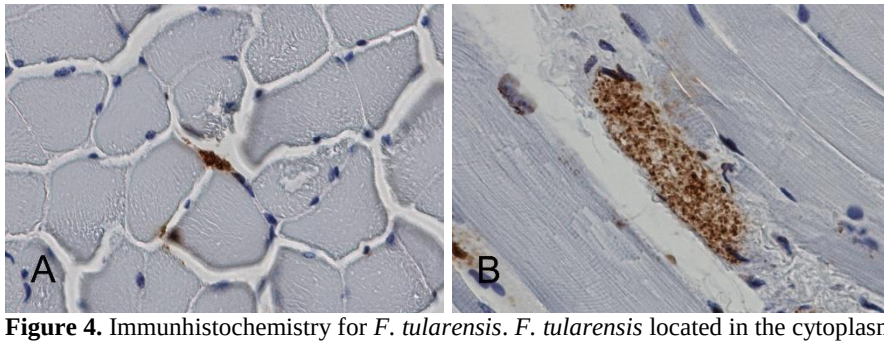

Figure 4. Immunhistochemistry for *F. tularensis*. *F. tularensis* located in the cytoplasm of a spindle cell (A), and in high numbers intravascularly (B).

By routine histopathology the only signs of infection in the muscle tissue were mild infiltration of macrophages and a few lymphocytes and plasma cells, mostly associated with the presence of *F. tularensis* shown by IHC. In two samples, small areas of necrosis were evident in the perimysium with mild infiltration of macrophages and lymphocytes. Bacterial culture for *F. tularensis* was positive in two out of 14 cultured samples, both stored in -20°C for approximately one year. This is in accordance with a Russian study where sheep were experimentally infected with *F. tularensis* and the meat still contained viable bacteria after storage at -20°C for 60-75 days (Airapetyan and Khachatryan, 1956). Our findings in study IV, show that even if hunters and others who handle the hares are very careful not to contaminate the meat, there is still a risk to contract infection when ingesting under-cooked meat since *F. tularensis* may infect the muscles during the disease process. The risk is

accentuated by the findings in study II, that showed that 30% of the hares did not have visible gross changes in their organs.

4.2.2 *F. tularensis* subsp. *holarctica* genotypes

In study II, *F. tularensis* subsp. *holarctica* genotypes were investigated in samples from 23 hares to see if any pathological differences were associated with infection by different genotypes. Two genotypes, B.6 (subgroup B.7), and B.12 (subgroups B.20, B.23 and B.39), were found but pathological presentation did not differ between them. This is in contrast with findings in tularaemic EBHs in Switzerland where two canSNP groups, B.6 (subgroup B.10) and B.12 (subgroup B.13) were found to have different disease presentations (Origgi and Pilo, 2016). The authors concluded that canSNP group B.6 (subgroup B.10) was associated with splenitis and hepatitis, while canSNP group B.12 (subgroup B.13) was associated with lesions in the pleura, pericardium and kidney, as described in Hungary (Gyuranecz *et al.*, 2010b; Origgi and Pilo, 2016). The majority of the hares in our study presented with lesions in the spleen, liver and many other organs, regardless of which of the two canSNP groups the strains belonged to. Hares with a more severe and chronic disease presentation could be found in both canSNP groups. Since the subgroups found for each canSNP group differed between our study II and the studies performed in Hungary (Gyuranecz *et al.*, 2010b) and Switzerland (Origgi and Pilo, 2016), the possibility of a difference in disease presentation on that level cannot be excluded.

4.2.3 Serology in wild predators and scavengers

Many predator and scavenger species are considered to be relatively resistant to develop disease when infected by *F. tularensis* (Olsufjev, 1970 see Sjöstedt 2007). As other animal species, they might get infected by vector-bites (mosquitos, ticks), by direct contact or through ingestion and inhalation. One further mean of infection is predation or scavenging on infected prey such as hares and small rodents. The predators and scavengers produce antibodies against *F. tularensis* upon infection, and might also harbour bacteria in their organs. European studies have shown that raccoon dogs, red foxes and wild boar may be useful as biological indicators for tularaemia (Hoflechner-Poltl *et al.*, 2000; Hubalek *et al.*, 1993; Hubalek *et al.*, 2002; Kuehn *et al.*, 2013; Otto *et al.*, 2014). Antibodies have also been detected in Japanese black bears (*Ursus thibetanus japonicas*) and Japanese raccoon dogs (*Nyctereutes procyonides viverrinus*) (Sharma *et al.*, 2014). In study V, an investigation of the Swedish predator or scavenger species brown bear, Eurasian lynx, raccoon dog, red fox,

wild boar, wolf and wolverine was performed. In total, 656 blood sera were investigated by slide agglutination and microagglutination. In the slide agglutination test, one brown bear and one wild boar were positive. In the microagglutination test, one brown bear (titer 1:80), one wolverine (titer 1:40), one red fox (titer 1:20) and nine wild boar (titers 1:20, 1:40 and 1:80 in six, two and one animals respectively) were positive. The slide agglutination test is a simple and quick method that can be used on whole blood. Since our study only managed to detect titers at 1:80 and above, our assessment is that this method is not suitable for these species since 10 animals positive in microagglutination, all with titers 1:20 or 1:40, were not detected by the slide agglutination test. On the other hand, the microagglutination method is more dependent of good quality sera and due to haemolysis 559 samples were readable at titer 1:20, and only 357 samples at titer 1:10. Examples of testing of good and haemolytic sera respectively are seen in figure 5.

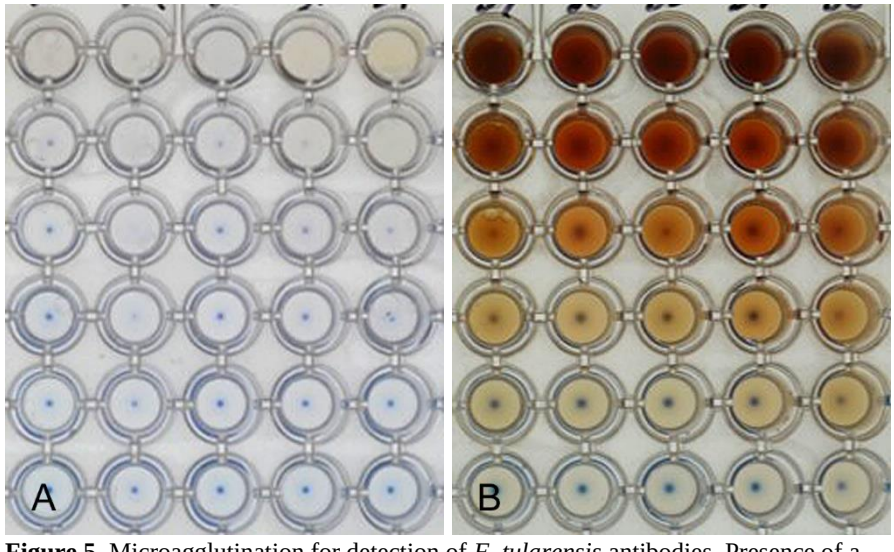

Figure 5. Microagglutination for detection of *F. tularensis* antibodies. Presence of a blue dot indicates a negative titer. A shows the readability with good quality sera and, B with haemolytic sera (B).

This indicates that the true number of positive animals is probably higher than what is shown in this study. This is exemplified by the fact that nine of the positives were hunted wild boar from which good quality sera were available. For seroprevalence studies it is important to apply a method that is sensitive enough to detect low antibody titers despite poor quality sera. In Sweden, larger hunting events only take place in the southern and central parts of the country.

In areas where tularaemia is more frequent, hunting is mostly executed by one or a few hunters and spread over the whole hunting season. Sampling opportunity is dependent on the voluntary participation of hunters taking and submitting the samples themselves, which often results in haemolysis during transportation of the blood sample. In our study, to elucidate if *F. tularensis* was present in lymphoid tissues in serological positive animals, tonsils and submandibular lymph nodes were investigated separately by real-time PCR and IHC. From the twelve positive animals, lymphoid tissues were available only from three, a brown bear, a red fox and a wolverine, and no bacteria could be detected. In a study conducted by Hofer et al. 2010, *F. tularensis* was found in the submandibular lymph nodes of red foxes (Hofer *et al.*, 2010). An active serologic surveillance of indicator species on the borders surrounding endemic areas of tularaemia, could be a useful tool to enable early detection of spread of disease.

4.3 Routes of infection and shedding

The routes of infection of *F. tularensis* are through bites of vectors (frequently mosquitos and ticks), direct or indirect contact, ingestion of infected food or water, and inhalation of infected aerosols or dust. In Sweden, mosquitos are frequently linked to infection in humans (Ryden *et al.*, 2012), and it could be assumed that this is true also for animals. One study found *F. tularensis* in several mosquito species (Lundstrom *et al.*, 2011). Since the 1980´s there have been milder winters, which has allowed the ticks to expand further north in Sweden (Lindgren and Jaenson, 2006), presumably being an increasingly important source of infection for humans and animals. In central and southern Europe *F. tularensis* has been detected in several tick species, including *Ixodes ricinus*, the most common tick species in Sweden (study I). During the necropsies in study II, ticks were found still attached to the skin bite-lesion in two EBH, and IHC showed presence of *F. tularensis* in the skin lesion, proving it to be the route of infection (Fig 6).

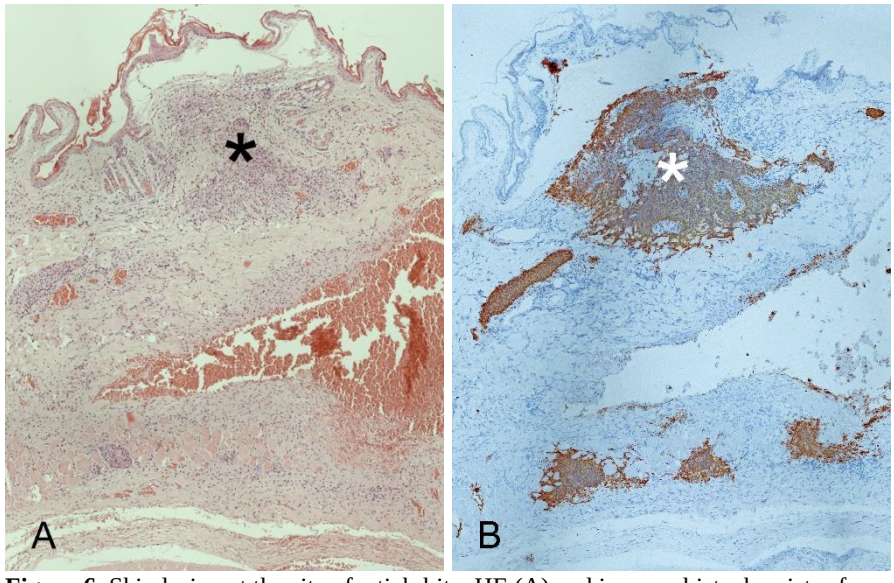

Figure 6. Skin lesion at the site of a tick-bite, HE (A) and immunohistochemistry for *F. tularensis* (B). Necrosis and inflammation with numerous intralesional *F. tularensis* (*).

This has also been described in a mountain hare (Morner *et al.*, 1988) and a Japanese hare (Park *et al.*, 2009). Wildlife, since living in the nature, could be more prone to acquire infection through contaminated soil and water. Contamination may be from live or dead tularaemic animals, urine, faeces, blood and other secretions from infected animals (Larssen *et al.*, 2011). There are also studies showing the presence of the bacterium in water and sediment, these elements being possible reservoirs in the environment (Broman *et al.*, 2011). In study II, several routes of shedding were described based on the pathology and associated IHC-labelling. By IHC, *F. tularensis*, were detected in exudate in the milk of lactating mammary glands, in exudate in airways and in exudate in the renal pelvis (Fig 7).

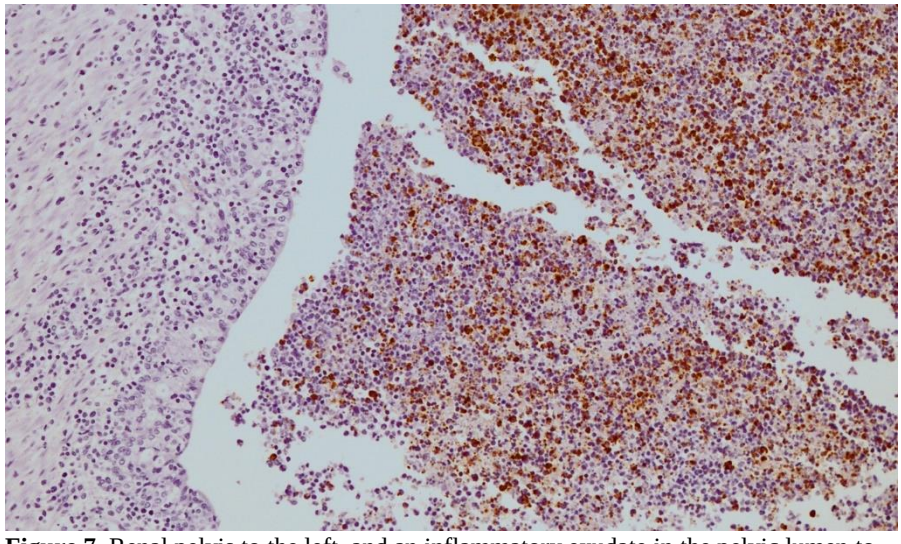

Figure 7. Renal pelvis to the left, and an inflammatory exudate in the pelvic lumen to the right containing numerous *F. tularensis* bacteria, shown by immunohistochemistry.

F. tularensis were also found in superficial mucosal lesions in the uterus and intestine of a few hares. However, most of the investigated hares did not have any of these lesions, and they presumably died of sepsis before lesions in these organs had time to develop. Hares presenting with chronic lesions, for example in the lungs and kidney, might be more common than we are aware of. If so, these hares could be of importance for the epidemiology of tularaemia, e.g, serving as shedders and reservoirs of *F. tularensis*. In hunted EBH in Hungary, chronic lesions in the kidney and lung were common, and EBH is considered to be a reservoir species (Gyuranecz *et al.*, 2010b). Small rodents are often implicated as sources of infection and/or reservoirs. Carcasses of infected animals, like the yellow-necked mice in study III, may contaminate the environment, e.g. water wells (Larssen *et al.*, 2011). If the rodent species are more resistant to develop fulminant disease, such as rats (*Rattus* spp.) (Sjöstedt 2007), bacteria may be shed and contaminate the environment through their faeces and urine (Larssen *et al.*, 2011; Reintjes *et al.*, 2002).

4.4 Risk factors

Common risk factors for humans are consumption of contaminated water and food, direct contact with infected wildlife, arthropod bites and exposure to infected environments via air (study I). This means that hunters, farmers and people taking part in outdoor activities are at greater risk to contract infection. In study II, 30% of the 86 investigated hares had no or minimal visible lesions.

Hunters dressing hunted hares may not notice that the hare is diseased, and if they do not use protective gloves they are at risk to get infected through skin contact. As shown in study IV, bacteria may also be found in the meat. If internal organs, for example the liver, or the meat are undercooked there is a risk to get infected through ingestion. In Sweden, there are no reports of people contracting tularaemia after consumption of hare meat, but there are reports from France and the Netherlands (Benlyazid *et al.*, 1997; Maurin *et al.*, 2011; Warris-Versteegen and van Vliet, 2003). Likewise, hunting dogs fed uncooked morsels may contract infection. Another example is a Norwegian hunting dog that was infected after killing a diseased mountain hare (Nordstoga *et al.*, 2014). Infected small rodents may contaminate food, water and dust such as hay on a farm, causing infection in humans and animals. In Kosovo, human tularaemia cases were connected to small rodents in food storage, and in Norway lemmings contaminated private water wells (Larssen *et al.*, 2011; Reintjes *et al.*, 2002). More seldom, infection through inhalation occurs. During outbreaks in Sweden 1966-1967, and in Finland 1982, hay dust was implicated as the source for 140 and 53 diseased humans respectively (Syrjala *et al.*, 1985; Tarnvik and Berglund, 2003). Further investigations, including pathology, of different wildlife species such as hares and small rodents would help to elucidate and further understand different risk factors, and to evaluate their importance.

5 Conclusions

Tularaemia involves animal health, human health and the environment and it is therefore best approached from a One Health perspective. This thesis has focused on a review of the epidemiology, pathology of key species, and serology studies of *F. tularensis* infection. The aim of the studies was to contribute to the understanding of the epidemiology of this complex zoonotic disease. In summary:

- Tularaemia is widely distributed throughout most of Europe and has repeatedly shown signs of local emergence and re-emergence in humans and wildlife. There are considerable differences in surveillance and reporting between countries. Additionally, there are geographical and regional differences connected to ecosystems, the presence of different species of arthropod vectors, and the variety of small rodent and other wildlife species involved. Therefore, it is difficult to make direct comparisons across all of Europe.

- The pathology of tularaemia was similar in Swedish European brown hares and mountain hares, both presenting with acute disseminated disease in most individuals. One important difference between the two hare species was the finding of more severe and chronic lesions in the lung and kidney in a few of the European brown hares.

- The pathology of two necropsied yellow-necked mice showed acute lesions and disseminated disease.

- Routes of shedding from infected hares that might be of importance are, in addition to biting insects, via urine, milk, uterine secretions and intestinal content, and dead animals. The finding that some European brown hares had

chronic lesions indicate that these hares may shed *F. tularensis* for longer times than previous recognized.

- The finding of chronic lesions in some of the European brown hares raises the question of hares being a possible reservoir of *F. tularensis* in Sweden.

- The findings of chronic disease in some European brown hares, the absence of readily visible pathological changes in up to 30% of the hares and the finding of *F. tularensis* in the muscle of hares shows that hunted hares and particularly under-cooked hare meat, could be a risk factor for contracting tularaemia.

- Active surveillance in combination with passive surveillance of small rodent species is needed to characterize tularaemia in these species in order to better understand the role of rodents in the epidemiology of *F. tularensis* infection.

- Active serologic surveillance of indicator species in areas surrounding endemic areas of tularaemia could enable early detection of spread of disease. In endemic areas, a variation in seroprevalence between years will contribute to the knowledge of disease dynamics.

6 Future perspectives

Since tularaemia is a complex disease involving man, many different species of animals and arthropod vectors, the knowledge and understanding of its epidemiology would benefit from studies involving several aspects simultaneously. One part of this would be active surveillance of indicator species, animal species that survive infection since they are less susceptible to develop disease. To make this possible, simple and robust field-based diagnostic methods, applicable to multiple animal species, are needed. There are limited existing data on the pathobiology of the infection in different small rodent species. The knowledge gaps of natural infection in small rodents include the range and variability in pathological presentation, and the distribution and abundance of bacteria in organs and excretions. Pathological investigations of different species of naturally infected small rodents would contribute to the understanding of the different species´ roles as potential transmitter or carriers of the infection. The present findings of lesions of a chronic type in some diseased European brown hares in Sweden, together with findings of chronically infected hares in for example Hungary and Germany, warrant further investigations of this hare species and its potential role as a reservoir of tularaemia in Sweden.

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Popular science summary

Tularaemia is a vector-borne disease caused by the bacterium *Francisella tularensis*. Tularaemia is a zoonotic disease, meaning that it may cause disease in both humans and animals. *F. tularensis* may infect a wide range of hosts including mammals, birds and arthropods such as ticks and mosquitos. There are several subspecies of the bacterium but only *F. tularensis* subsp. *holarctica* causes significant disease in humans and animals in Europe. The susceptibility of various hosts to develop disease is variable. For example, certain lagomorphs as mountain hares and many small rodent species succumb to fulminate disease, while many species of carnivores and omnivores, e.g. red fox and wild boar, show no or mild signs of disease upon infection. Tularaemia is a complex disease involving many different animal species, but also vectors such as ticks and mosquitos, and the environment. Knowledge is lacking regarding possible reservoirs for *F. tularensis*, where the bacterium may survive and be a source of infection. There are theories that possible bacterial reservoirs could be water courses, ticks and certain animal species. *F. tularensis* has several infection routes, e.g. skin, respiratory organs and pharynx. The bacterium may be shed from diseased individuals, for example through urine and faeces, and dead infected animals can infect the environment.

This thesis reviewed the literature for the time-period 1992-2012 regarding the presence of tularaemia in humans, wild and domestic animals, and vectors such as ticks and mosquitos, in Europe. Through autopsies and microscopic investigations (pathology) of European brown hares, mountain hares and yellow-necked mice, organ lesions caused by the disease were investigated. The presence of antibodies against *F. tularensis* (serology), produced by the infected individuals immune defence, was investigated in selected Swedish predator and/or scavenger species.

Tularaemia is widely distributed throughout most of Europe. Differences in surveillance and reporting of the disease, different ecosystems, the presence of different kinds of arthropod vectors (e.g. ticks and mosquitos), and the variety

of small rodent species and other wildlife species make direct comparisons across all of Europe difficult.

The pathology of tularaemia in European brown hares, mountain hares and two yellow-necked mice was similar. The infection involved many organs and had a short course that rapidly caused death of the animals. However, some of the European brown hares, in addition to the acute lesions, also had chronic changes in the lungs and/or kidneys. This shows that these hares had been sick for a longer period of time before the disease fulminated, and raises the question if the European brown hare in Sweden could function as a bacterial reservoir, as described elsewhere in Europe. *F. tularensis* infection was demonstrated in muscles of infected hares, which highlights the risk of acquiring infection through consumption of under-cooked meat.

Many predators and omnivores do not develop disease after infection but may develop antibodies, thus serological investigations show if they are, or have been, infected with *F. tularensis*. In wildlife disease surveillance, this could inform on whether tularaemia is spreading to new areas, and if there are seasonal or annual variations in areas where tularaemia is endemic, i.e. where it is always present. In our study, antibodies towards *F. tularensis* were detected in the brown bear, red fox, wild boar and wolverine.

The results of our studies contribute to the current knowledge of tularaemia in Europe, its pathology in European brown hares and yellow-necked mice, and its possible routes of infection and shedding. Additionally, it contributes to the understanding of the role of predators and scavengers in the epidemiology of tularaemia. The results of our studies highlight the importance of further investigations of different wildlife species to explore their role in the epidemiology of tularaemia, as possible sources of infection, transmitters of disease and potential reservoirs.

Populärvetenskaplig sammanfattning

Harpest (tularemi) är en viktig zoonotisk sjukdom, vilket innebär att den kan infektera och överföras mellan ett stort antal djurslag, varav människa är ett. Harpest orsakas av bakterien *Francisella tularensis*, och i Europa är det underarten *F. tularensis* subsp. *holarctica* som orsakar sjukdom hos människor och djur. Känsligheten för att bli sjuk efter infektion varierar mellan olika djurslag. Som exempel kan nämnas att skogsharar och ett flertal smågnagararter är mycket känsliga och ofta drabbas av akut sjukdom som snabbt leder till döden, medan många kött- och allätare, t.ex. brunbjörn, järv, räv och vildsvin, inte insjuknar alls eller drabbas av mild sjukdom. Människors känslighet ligger däremellan. Om infektionen sker genom huden så uppkommer ett sår, och de lymfkörtlar som dränerar infektionsområdet svullnar. Detta åtföljs av influensaliknande symptom med feber. Harpest är en komplex sjukdom som involverar ett stort antal värddjur, men även vektorer såsom fästingar och myggor, samt omgivande natur. Mycket kunskap saknas om möjliga bakteriereservoarer, d.v.s. källor där bakterien kan överleva och varifrån den kan spridas vidare. Teorier finns om att möjliga reservoarer för bakterien kan vara vattendrag, fästingar och vissa djurarter. Infektion med *F. tularensis* kan erhållas via flera vägar, t.ex. hud, luftvägar eller svalg. Bakterien kan utsöndras från sjuka individer via t.ex. urin och avföring, och infekterade döda djur kan kontaminera den omgivande naturen.

I denna avhandling har vi genom litteraturstudier undersökt förekomsten av harpest hos människa, vilda och tama djur, och vektorer i Europa under åren 1992-2012. Undersökningar av vilka förändringar harpesten orsakar i kroppens organ (patologi) hos harar och större skogsmöss, samt förekomsten av antikroppar som kroppens immunförsvar bildat mot bakterien (serologi) hos vilda rovdjur och asätare har också utförts.

Harpest förekommer i flertalet av Europas länder. Olikheter i sjukdomsövervakning och rapporteringsplikt, ekosystemens variation,

förekomst av olika vektorarter och arter av vilda djur gör det svårt att göra direkta jämförelser mellan länderna.

I våra undersökningar var de förändringar som harpesten orsakade, likartade hos fältharar, skogsharar och två undersökta större skogsmöss. Infektionen hade ett snabbt förlopp med spridning till många organ och en snabbt inträdande död. Några av de undersökta fälthararna hade utöver de akuta förändringarna, kroniska förändringar i lungor och/eller njurar. Detta tyder på att de varit sjuka en längre tid innan sjukdomen akutiserades och ledde till döden. Detta väcker frågan om fältharar kan vara en reservoar för *F. tularensis* i Sverige, något som beskrivits i andra delar av Europa. Vid undersökningar av harmuskulatur konstaterades att bakterien även infekterade detta organ. Därmed finns det en risk att infekteras om man äter otillräckligt tillagat harkött.

Många arter av rovdjur och asätare verkar inte bli sjuka när de infekteras. Eftersom de vid infektion kan bilda antikroppar som svar på bakterieinfektionen kan serologiska undersökningar av dessa djurarter ge svar på om de är eller har varit infekterade med *F. tularensis*. I övervakningen av viltsjukdomar kan resultaten ge svar på om harpesten är på väg att sprida sig till ett nytt område, och det ger också möjlighet att ta reda på hur förekomsten av harpesten varierar över tid. I vår studie fanns individer som bildat antikroppar mot *F. tularensis* hos brunbjörn, järv, rödräv och vildsvin.

Resultaten av våra studier bidrar med kunskap om harpest i Europa, sjukdomsbilden hos fältharar och större skogsmöss, samt möjliga infektions- och utsöndringsvägar. Resultaten bidrar också till förståelse för att rovdjur och asätare kan ha betydelse för spridning av *F. tularensis*. Vidare undersökningar av olika vilda djurslag är viktiga för att se vad de har för betydelse i harpestens epidemiologi, för att ta reda på mer om möjliga infektionskällor, samt för att lära mer om vilka djurslag som kan tjänstgöra som smittspridare och potentiella reservoarer.

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