

U. PORTO



INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

Relatório Final de Estágio
Mestrado Integrado em Medicina Veterinária

SCREENING OF DISEASES IN SWEDISH MUSKRATS

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Co-Orientadores

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Abstract

The muskrat (*Ondatra zibethicus*) is a large semi-aquatic rodent native to North America. The population in Sweden has decreased over the years and it has been speculated that Tularaemia could be a contributing factor. In August 2017 the muskrat was listed as an invasive species and during 2018-2019 an eradication campaign was held in Västernorrland. The muskrats that were killed were sent to the SVA for post-mortem examination.

The aim of this project was to analyse the 238 muskrats received, focusing on the seroprevalence of *Francisella tularensis*, occurrence of lesions in the organs of seropositive animals, as well as documenting any other signs of disease and the general health and reproductive status of the muskrat population.

To that end, necropsies were performed, completed with seroagglutination tests for *F. tularensis*, parasitological screenings, histopathology of organs of seropositive animals and PCR screening of mandibular lymph nodes and spleens of seropositive animals.

These individuals were found to be generally healthy, with a few, most likely subclinical, infections. There was no macroscopic evidence of Tularaemia. The seroprevalence of Tularaemia was 14,35%. There were two incidental findings in the lungs, 22,3 % of the animals were infected with adiaspiromycosis, from light to heavy infections, and 30,1% exhibited ectopic bone formations with no apparent consequences to their health.

It can be concluded that Tularaemia is unlikely to be the main cause of the observed decrease of the local muskrat population, although muskrats may be of importance for the maintenance of *F. tularensis* in wild aquatic ecosystems and the contamination of arthropod vectors, such as mosquitos, and other carriers.

Acknowledgements

During my traineeship at the National Veterinary Institute (SVA) I had the opportunity and privilege to experience a completely different field of work from what I was introduced to in my academic journey, in the company of an entire team of wonderful professionals and individuals. I am truly grateful to everyone that made this possible, but I would particularly like to thank:

My supervisor, Professor Alexandra Müller, for the tireless support and help, from choosing the ideal traineeship, to assuring me that success can best be achieved by doing what one is really passionate about, from countless proof-readings to inspiring life discussions.

Dolores Gavier-Widén for helping me get accepted in this traineeship and welcoming me into the Department of Pathology and Wildlife Diseases (POV) with open arms.

Erik Ågren for being a mentor in the truest sense of the word and making me feel like the SVA and Uppsala were my second home. Thank you for all the help, for pushing me to always strive for more and for all the knowledge shared.

Caroline Bröjer for being the best mentor I did not know I would have, for accepting me into your project and teaching me the reality of being a researcher. I am extremely grateful for all your patience, company and kindness, for always making time for my numerous questions and guiding me through this journey.

Ulrika Larsson Pettersson for being a lovely necropsy and laboratory partner, for preparing hundreds of cassettes and histology slides and for all the delightful fikas and conversations.

Aleksija Neimanis, Henrik Uhlhorn, Neele Doose, Gustav Averhed, Jasmine Stavenow, Janna Nises, Emil Wikström and everyone in POV for making it a joy to come to work every day. Thank you for being wonderful colleagues, doing rounds in English even if it took much longer, and teaching me so much, I hope I get the chance to work with you again. A special thanks to Gete Hestvik for trusting me with your project and for the expertise shared. I would also like to thank everyone in obduktionssektionen and the histology laboratory for all the help and kindness.

Pedro for keeping me sane and motivated even on the worst days. Thank you for being so understanding and supportive.

My mother, for making all of this possible and always believing in me and my dreams.

Friends and family for the unconditional support, care, patience and inspiration. Thank you for all the moments shared, far and near.

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Abbreviations

SVA - Statens Veterinärmedicinska Anstalt / National Veterinary Institute
POV - Patologi Och Viltjukdomar / Department of Pathology and Wildlife Diseases
CWD - Chronic Wasting Disease
PCR - Polymerase Chain Reaction
RT-PCR - Real Time Polymerase Chain Reaction
DNA - Deoxyribonucleic acid

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Introduction

The entirety of this 16 week traineeship was spent at the Swedish National Veterinary Institute (Statens Veterinärmedicinska Anstalt, SVA, <https://www.sva.se/en>), mostly in the wildlife section of the Department of Pathology and Wildlife Diseases (Patologi Och Viltsjukdomar, POV), following the daily routine and working on the “Screening of Diseases in Swedish Muskrats” project. However, there was also a day spent in the molecular diagnosis laboratory and a day in the trichinella and parasitology laboratory, as well as a visit to the bacteriology laboratory and a day at the Swedish Museum of Natural History, assisting in a necropsy of a ringed seal.

Most of the Wildlife section of POV’s efforts are focused on the National Wildlife Surveillance Program in conjunction with several research projects exploring various diseases such as West Nile/ Usutu Virus in birds, Chronic Wasting Disease (CWD) in cervids, African Swine Fever, Large Carnivore Project and recently, the development of a new Marine Mammal Surveillance Program.

The National Wildlife Surveillance Program aims to monitor, analyse and report on the disease status of Swedish wildlife in a detailed and organized manner which can serve as a basis for investigative efforts. This centralized system allows for a more efficient management of wild populations and ecosystems.

Table 1: The pillars of Wildlife surveillance (SVA 5 May 2018, <https://www.sva.se/om-sva/publikationer/vilda-djur/sjukdomslaget-hos-vilt-i-sverige>)

| Wildlife surveillance | | | | |
|---|------------------------------------|------------------|-------------------|------------------------------|
| Information gathering/ field surveillance | Necropsies and laboratory analysis | Targeted surveys | External analysis | Dissemination of information |

The Swedish Wildlife disease surveillance is based on 5 steps, described in Table 1, and relies on a joint effort between the SVA, several governmental agencies, various hunter’s associations, and of course the help of the general public. There is also a heavy focus on a One Health approach, with added importance on zoonoses and other diseases that can be transmitted to domestic animals, resulting in large economic, emotional or any other impact on human lives.

The weekly routine of wildlife pathologists and administrative staff starts with a meeting, every Monday morning, where the work for the week is outlined, tasks are distributed, projects are assessed, and meetings and lectures are booked. After that, work starts in the necropsy room. For the first few days I mainly watched the ongoing necropsies, having started taking my own cases and writing those internal reports in the SVA’s software, SVALA, after I got my account opened. There was always a pathologist responsible for the necropsies, who would clarify my doubts and supervise my work. These necropsies are accounted for in Table 2.

In the afternoon there were rounds, where everyone would gather around each of the 9 necropsy tables and describe and discuss each case. These were done for both the wildlife and the domestic animals. The main findings were debated and when necessary possible causes of death and additional diagnostic tests were suggested. Because of these rounds, I was able to follow, discuss and learn from hundreds of different post-mortem examinations in various animal species. In my opinion this dynamic was highly beneficial for everyone involved, allowing us to challenge our first assumptions and learn from extremely experienced professionals.

Table 2: Necropsies performed and observed during the 4-month traineeship

| Necropsies | | Carried out myself | Watched |
|----------------------------|----------------------------|-----------------------------|---------|
| Mammals | <i>Ondatra zibethicus</i> | 43 | 3 |
| | <i>Alces alces</i> | 6 | 7 |
| | <i>Lynx lynx</i> | 2 | 4 |
| | <i>Ursus arctos</i> | 2 | 4 |
| | <i>Lutra lutra</i> | 2 | 2 |
| | <i>Capreolus capreolus</i> | 2 | 2 |
| | <i>Canis lupus</i> | 1 | 3 |
| | <i>Sciurus vulgaris</i> | 1 | 1 |
| | <i>Gulo gulo</i> | 1 | - |
| | <i>Mustela putorius</i> | 1 | - |
| | <i>Phocoena phocoena</i> | - | 7 |
| | <i>Sus scrofa</i> | - | 3 |
| | <i>Erinaceus europaeus</i> | - | 1 |
| | <i>Pusa hispida</i> | - | 1 |
| | <i>Plecotus auritus</i> | - | 1 |
| | Birds | <i>Haliaeetus albicilla</i> | 6 |
| <i>Strix uralensis</i> | | 4 | - |
| <i>Chloris chloris</i> | | 3 | 3 |
| <i>Strix nebulosa</i> | | 3 | - |
| <i>Phalacrocorax carbo</i> | | 3 | - |
| <i>Turdus merula</i> | | 3 | - |
| <i>Accipiter gentilis</i> | | 2 | 1 |
| <i>Accipiter nisus</i> | | 2 | - |
| <i>Falco peregrinus</i> | | 2 | - |
| <i>Bubo bubo</i> | | 2 | - |

| | | | |
|--------------|----------------------------------|------------|-----------|
| | <i>Buteo buteo</i> | 1 | 1 |
| | <i>Aegolius funereus</i> | 1 | - |
| | <i>Strix aluco</i> | 1 | - |
| | <i>Falco tinnunculus</i> | 1 | - |
| | <i>Circus aeruginosus</i> | 1 | - |
| | <i>Milvus milvus</i> | 1 | - |
| | <i>Dendrocopos major</i> | 1 | - |
| | <i>Picus viridis</i> | 1 | - |
| | <i>Corvus cornix</i> | 1 | - |
| | <i>Cuculus canorus</i> | 1 | - |
| | <i>Cyanistes caeruleus</i> | 1 | - |
| | <i>Turdus philomelos</i> | 1 | - |
| | <i>Turdus pilaris</i> | 1 | - |
| | <i>Passer montanus</i> | 1 | - |
| | <i>Anas platyrhynchos</i> | 1 | - |
| | <i>Fringilla coelebs</i> | - | 1 |
| | <i>Columba livia</i> | - | 1 |
| | <i>Anser anser</i> | - | 1 |
| Reptiles | <i>Trachemys scripta elegans</i> | - | 2 |
| Total | | 106 | 51 |

Regarding the necropsies in mammals I would like to point out that there was a “Large carnivore project” ongoing, where all lynxes, brown bears, wolves and wolverines were sent to the Swedish Museum of Natural History after examination, and their skeletons were left intact to the best of our ability, unless there was a clinical history or signs that would indicate lesions to the Central Nervous System, in which case we were allowed to open the cranium and/or spine. There were also some forensic cases, usually related to hunting accidents and handled by the senior staff. The CWD project required at times the collection of samples from the brain stem and mandibular lymph nodes. Some of the moose necropsies described in Table 1 were just heads sent to the SVA for sampling for CWD, although some conclusions could be made about the general health status of the animals by examining their teeth, the presence of fat deposits, the opacity of their lenses, their ears, skin and fur, etc. Since the diet of the Swedish brown bear across the seasons was being studied, their stomach contents were carefully sorted and measured. Dozens of hares were also examined in this 4-month period, but this was done in a high-security room due to the risk of Tularaemia.

During the summer and autumn of 2019 all birds necropsied at the SVA were screened for Avian Influenza and West Nile/ Usutu Virus. As a result of this surveillance program, two Usutu positive birds were found in Sweden this summer, a blackbird and a red kite.

Several species of birds of prey are considered “Statens vilt”, or wildlife of the State, and their skeletons, skins and feathers were preserved and sent to the Swedish Museum of Natural History too (Figure 1). These include White-tailed Eagles which often die of severe trauma or by lead poisoning when eating animals shot with lead pellets and left for scavengers.

Other than these specific cases where particular care should be taken, all necropsies were performed according to a general protocol, with some species-specific details and alterations. Whenever necessary, samples were taken for histopathology, bacteriology, parasitology, virology and toxicology. From all cases, samples of liver, lungs, kidney, spleen, muscle, intestine and brain (unless it was “Statens vilt”) were taken and saved at -20°C in SVA’s Biobank for future research.

After collecting histopathology samples in 10% formaldehyde, these had to be trimmed in and placed in cassettes to be impregnated with paraffin, sectioned and stained. Fortunately, I had the chance to accompany the entire process and prepare over 200 of these samples, which I then studied under the microscope (Figure 2).

Table 3 depicts a summary of the necropsy reports I completed by myself, with the main causes of death and most significant lesions in mammals and birds necropsied by me. Most animals perished from either trauma of various sorts (predation, car and train accidents, hunting, flying into objects, etc) or emaciation.

Table 3: Summary of necropsy reports (n=37)

| | Cause of death | Nº | Other findings |
|----------------|----------------------------|-----------|---|
| Mammals | Trauma | 3 | Endoparasites, ectoparasites, cysts in uterus, cysts in spermatic cords, nodular hyperplasia of the spleen |
| | Emaciation | 1 | - |
| | Unknown | 2 | Splenomegaly, pale spleen, muscle atrophy, atelectasis, endoparasites, bad body condition, fractures, white spots in heart |
| | Enteritis | 1 | Ectoparasites, yeast, parasite eggs |
| Birds | Trauma | 17 | Hepatic lipidosis, bumble foot, abscesses, uralites, aerosaculitis, endoparasites, nail overgrowth, splenomegaly, pale spleen |
| | Emaciation | 7 | Endoparasites, oedematous lungs, granulomas, dehydration, pale kidneys, blood clots, small pale spleen, uralites |
| | Unknown | 3 | Acute enteritis, haemorrhagic lungs, haemorrhages in the cranium, hepatomegaly, aerosaculitis, dehydration, endoparasites |
| | <i>Mycobacterium avium</i> | 1 | Hepatomegaly, granulomas in liver, spleen, duodenum and lungs, endoparasites |
| | <i>Herpesvirus</i> | 1 | Hepatomegaly with white spots, splenomegaly pale and granular, pneumonia, haemorrhagic enteritis with white masses |

In addition to the normal routine, I was included in the previously started “Screening of Diseases in Swedish Muskrats” project (Figure 3). This project was a result of the continuation of the work done by the SVA in Tularemia in hares and other mammals, combined with an eradication campaign of muskrats in Sweden and mostly, scientific curiosity due to the evidence of a steep decline in numbers of the free living muskrat population in Sweden over the years, possibly because of some infectious disease.

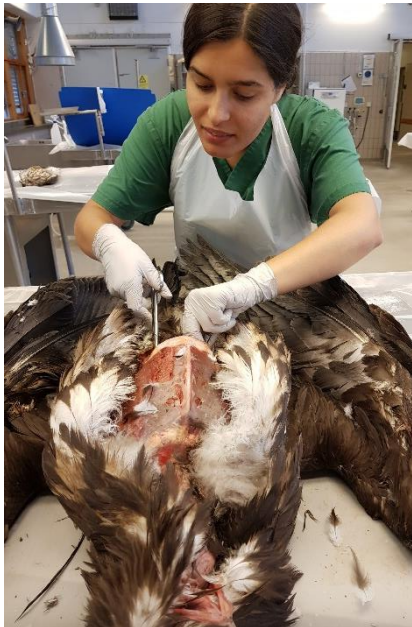


Figure 1: Necropsy of a juvenile White-tailed eagle



Figure 2: Microscope observation of muskrat's lung sections



Figure 3: Muskrat necropsy

Briefly, the muskrat (*Ondatra zibethicus*) is a large semi-aquatic rodent native to North America. It was introduced to Europe in the beginning of the 1900s, for fur farming, and has since migrated from Finland to Sweden in the middle of the 1950s, spreading southward to Västernorrland (Danell, 1977). The population has decreased over the years and it has been speculated that it could be due to Tularemia. Since this disease, caused by the bacteria *Francisella tularensis*, is fairly common in the muskrats' home range, it could be a contributing factor to their decrease. In August 2017 the muskrat was listed by the European Union as an invasive species and during 2018-2019 a group of hunters participated in a project, aiming to eradicate muskrats from their southernmost home range in Västernorrland. The muskrats that were killed were sent to the SVA to detect any possible diseases. The aim of this project was to analyse the 238 muskrats received, focusing on the seroprevalence of *F. tularensis*, occurrence of lesions in the organs of seropositive animals, as well as documenting any other signs of disease and the general health and reproductive status of the muskrat population in Sweden.

Background

The muskrat is a common species, widespread in the Northern Hemisphere. It was first introduced to Europe, to the Czech Republic, due to their highly valued pelts, from where it spread across most of the Eurasian continent (Skyriene, 2012; Solari & Baker, 2007). Muskrats are found in lakes, ponds, streams, rivers and marshes, usually with a population density of about 40 individuals per hectare (Feldhamer, 1999). They have developed a series of particular characteristics for aquatic life, such as lips that close behind incisors, partially webbed hind feet, and they are capable of staying submerged up to 20 minutes. Their diet consists mostly of aquatic vegetation (cattails and horsetails) and occasionally mussels, turtles, mice, birds, frogs and fish (Willner et al., 1980). They live in burrows or houses built out of vegetation, leaving the main chambers above water level and the entrance through underwater tunnels (Feldhamer, 1999).

Their main threat is trapping and hunting for pelts, and the fact that they are many times considered pests. Being one of the species most often responsible for “eatouts”, or consuming plants faster than they can replace themselves, muskrats in high population densities eliminate vegetation (aquatic vegetation and garden crops growing near water) and destroy habitats for many species (Little & Webb, 2013). The most serious damage is washouts and cave-ins of pond dams, levees and irrigation canals (Little & Webb, 2013) caused by burrowing of riverbanks (Cassola, 2016).

Nowadays abundance is reduced in some countries, including Sweden. It has been speculated that this could be due to some diseases, mainly Tularaemia, or other ecological factors such as availability of food, parasites and carnivore predators (Skyriene, 2012).

Health status

The list of diseases transmitted by rodents, including muskrats, is long, counting with Hantavirus Pulmonary Syndrome, Haemorrhagic Fever with Renal Syndrome, Lassa Fever, Leptospirosis, Lymphocytic Choriomeningitis, Omsk Haemorrhagic Fever, Plague, Rat-bite Fever, Salmonellosis, South American Arenaviruses and Tularaemia (CDC, 2017).

Muskrats in particular have been shown to be infected with several pathogens and parasites transmissible to wildlife, livestock, pets and humans (Umhang et al., 2013). There have been reports of muskrats infected with *Cryptosporidium* muskrat genotype I and genotype II (Danišová et al., 2017), *Campylobacter jejuni* (Pacha et al., 1985), several *Giardia* species (Kirkpatrick & Benson, 1987; Pacha et al., 1985), *Echinococcus multilocularis* (Umhang et al., 2013), Omsk haemorrhagic fever, Adiaspiromycosis and Tyzzer's disease (Williams & Barker, 2001). In

addition, the following non-zoonotic parasites have been identified: *Taenia taeniaeformis*, *Taenia mustelae*, *Taenia polyacantha* and *Taenia martis* (Umhang et al., 2013).

Muskrats can also act as reservoirs for puumala-like hantavirus strains (Vahlenkamp et al., 1998) and leptospira serovars linked to Weil's disease, which is a serious and life threatening condition in humans (Hurd et al., 2017). However, the most problematic zoonotic diseases potentially transmitted by these animals probably are Rabies and Tularaemia (Dyer et al., 2013; CDC, 2017).

Tularaemia

Tularaemia is an important, potentially fatal, multisystemic zoonosis, affecting more than 300 animal species, including mammals, birds, amphibians and invertebrates across the Northern Hemisphere (Foley & Nieto, 2010; Williams & Barker, 2001) and recently found in Australia (Eden et al., 2017). However, the most often infected species belong to the orders Lagomorpha and Rodentia (OIE, 2018).

This disease is caused by the highly contagious bacterium *Francisella tularensis*, described as a small gram-negative facultative intracellular coccoid rod (Tärnvik & Berglund, 2003). This bacteria is classified into several different species and subspecies, namely *F. tularensis* subsp. *tularensis* (Type A) which occurs in North America, *F. tularensis* subsp. *holarctica* (Type B), found all over the Northern Hemisphere, *F. tularensis* subsp. *mediasiatica* found in Central Asia as the name suggests, and *F. tularensis* subsp. *novicida* which can be considered as a separate species, *F. novicida* (Eden et al., 2017; Foley & Nieto, 2010). The subspecies most commonly found responsible for disease in both humans and animals are the first two mentioned above, Type A generally being considered more virulent (OIE, 2018).

Transmission

Tularaemia can be transmitted in various ways, resulting in different clinical presentations. Some of these include arthropod vectors, direct contact with infected animals, inhalation of contaminated dust, water exposure, and eating undercooked meat of infected animals (Dryselius et al., 2019; Foley & Nieto, 2010; CDC, 2018). The severity of the disease varies with animal species. For example, in humans who are intermediate susceptible to develop disease, the main symptoms include fever, depression and often septicaemia. In addition, humans sometimes portray ulcers or abscesses at the site of inoculation (rarely seen in animals, probably because of the difficulties in detecting the site due to fur obscuring the skin), and regional lymphadenomegaly (OIE, 2018). In animals very susceptible to develop disease, e.g. hares and many small rodent

species, the disease process is short with acute symptoms and often septicaemia leading to death.

In North America the main mode of transmission seems to be by various ticks (*Dermacentor variabilis*, *Dermacentor andersoni* and *Amblyomma americanum*) and deer flies (*Chrysops* spp.) (CDC, 2018). In Norway and throughout Europe, ingestion of contaminated water is the most frequent cause of infection, whereas in Sweden mosquito bites are the main culprits (Dryselius et al., 2019). Nonetheless, transmission by ticks and rain flies was also reported in Sweden (Dryselius et al., 2019). In general, hematophagous arthropods are considered to play an important role in the maintenance in nature and transmission of *F. tularensis* (OIE, 2018).

Less frequent, but still worth considering, are infections through pets, particularly cats and hamsters have proven to be susceptible and capable of infecting their tutors. These have only been described with Type A Tularaemia (CDC, 2018).

When it comes to clinical disease, infections due to handling infected animals (hunting or skinning) and bites of arthropods like ticks and mosquitos can result in glandular, ulceroglandular and oculoglandular Tularaemia. Drinking contaminated water leads to oropharyngeal Tularaemia while inhalation of aerosols, although rare, results in one of the most severe manifestations, pneumonic Tularaemia (CDC, 2018).

Due to its low infectious dose, high aerosol-related infection rate and ability to induce fatal disease if no medical assistance is provided, *F. tularensis* was declared as a potential agent of biological warfare (Foley & Nieto, 2010).

Distribution

Tularaemia is widespread across the northern hemisphere including North America, Europe, and Asia. Recently it was even reported in Australia (Eden et al., 2017). In the Iberian Peninsula, *F. tularensis* subsp. *holarctica* and several *F. hispaniensis* like DNA sequences were identified in ticks, lagomorphs and small mammals in 2015 (Lopes de Carvalho et al., 2016). The exceptions are Iceland, Ireland and the United Kingdom, where to this date, Tularaemia has not been described. The highest incidence is reported in some parts of Sweden and Finland, where it is endemic (Dryselius et al., 2019).

Historically, some of the most relevant outbreaks were recorded in Japan in 1820, in Western Siberia in the late 19th century, in the USA in 1911 and in Norway in 1911 (Williams & Barker, 2001).

When it comes to infections linked to direct contact with aquatic mammals, the largest outbreak occurred in the spring of 1968 in Vermont, USA, which had no previous reports of Tularaemia. There were reports of 47 cases, all in hunters who had trapped or handled muskrats. Later studies revealed a 5% detection rate of *F. tularensis* in muskrats in the area of most intensive trapping (Young et al., 1969).

In 2019, Sweden experienced its largest outbreak of Tularaemia in over 50 years. From July to October a total of 979 human cases (734 laboratory-confirmed) have been reported in central Sweden. This number exceeds the amount of cases usually reported in the whole of Europe in a normal year. The reason behind it might be the weather conditions in 2019, consisting of a relatively wet spring and a mild summer and autumn, creating a favourable environment for mosquito populations (Dryselius et al., 2019). In addition to the human cases, 58 hares (both European brown hares, *Lepus europaeus*, and mountain hares, *Lepus timidus*) necropsied at the SVA in 2019, were confirmed positive for Tularaemia, by macroscopic lesions and PCR testing (<https://www.sva.se/smittlege/karta-over-harpest>).

Knowledge is scarce regarding possible reservoirs for *F. tularensis*, in which the bacteria may survive and be a source of infection. It is theorized that these could be water courses, ticks and certain animal species (Hestvik, 2017). Since muskrats have been proven to contribute to water contamination as well as being a direct source of infection in its original habitat in North America, it was considered extremely relevant to study the available specimens in this regard.

Identification

F. tularensis is highly fastidious and particularly hard to culture. Therefore, polymerase chain reaction (PCR) is often the best option, when dealing with clinical samples. The agent can also be identified in impression smears and/or fixed organ samples using the fluorescent antibody test or immunohistochemistry (OIE, 2018).

Serological tests, commonly agglutination tests, can be a useful option too, mainly in humans, in carnivores and omnivores, and in some species of lagomorphs and rodents, like European brown hares and muskrats, where it allows to carry out epidemiological studies (OIE, 2018). In less resistant animal species, the utility of serology is limited, as animals frequently die before developing antibodies.

Materials and Methods

Samples

From February 2018 to May 2019, 238 wild muskrats were either shot dead or trapped and euthanised from 8 lakes, in the Västernorrland county, located in the south-eastern part of northern Sweden (Figure 4). The animals were placed in marked plastic bags, frozen and transported to the SVA for post-mortem examination. The identification of each individual included a code number, place where it was killed, type of trap, date and weight (Figure 5).

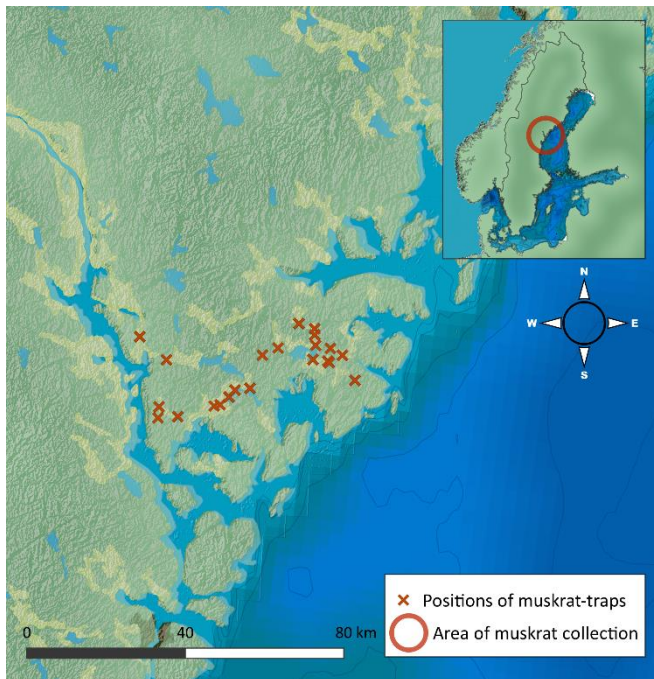


Figure 4: Trapping and hunting areas for muskrat eradication (courtesy of Jasmine Stavenow)



Figure 5: Muskrat in marked plastic bag

Necropsy examination

In March and September 2019, 216 complete necropsies were performed, after discarding 22 individuals in which the autolytic changes were too advanced for reliable results (Annex I). Samples from liver, lungs, kidney, spleen, muscle, brain, intestine and submandibular lymph nodes were taken and stored in the biobank of the SVA, as well as a blood sample and a small muscle sample in alcohol for future genetic analysis. Furthermore, additional serological, microbiological, parasitological and histopathological investigations were performed.

All the remaining 216 individuals underwent serological screening using a slide agglutination Test (Bioveta® a.s., Ivanovice na Hané, Czech Republic) for *F. tularensis*-specific antibodies with *Francisella tularensis* antigen. The assay was performed at room temperature. A drop of blood (approx. 0.04 ml), preferably from the thoracic cavity, was mixed on a slide with a slightly smaller

drop of the antigen solution, using a disposable pipet. As recommended by the manufacturer, the result was determined within three minutes, against a white backlight, to identify flakes/clusters and clearing of the blood, in which case the animal was considered seropositive.

Microbiological testing for *Salmonella* spp. was performed on tissue samples from colon of 11 muskrats, chosen by their origin, to represent the different hunting sites. After enrichment in BPW (Buffered Peptone Water) medium, the samples were transferred to MSR/V (Modified Semi-solid Rappaport-Vassiliadis) medium and cultivated for 48 hours with a control after 24 hours.

The parasitological screening of these same 11 individuals included macroscopic morphological examination of small and large intestinal contents as well as Sedimentation and Flotation of the contents following standard protocols (Britain. & Ministry of Agriculture, 1986; Thienpont et al., 1986), with some modifications. In brief, for sedimentation 3-5 g faeces were diluted in 10 ml of 2% formalin, sieved and mixed with an equal volume of ether. After centrifugation for 3 minutes at 214 g, the supernatant was discarded, and the sediment examined by microscopy. For flotation, 3-5 g faeces were homogenized in 10-15 ml saturated sucrose/sodium chloride solution, poured into a Clayton-Lane tube which was filled to the rim and a cover slip was placed on top of it. After centrifugation for 5 minutes at 214 g, the coverslip was placed on a glass slide and examined by microscopy.

Moreover, 7 randomly chosen specimens were cultured for *Erysipelothrix* spp as described by Eriksson et al, 2014. Briefly, small pieces of soft palate were placed in Sodium-azide crystal-violet broth (SACVB) for 48 h, at 37°C to minimize growth of other bacteria, then approximately 10 µl of the broth was plated on horse blood agar with and without antibiotics for 48 h at 37°C.

Histopathology

Histopathology of liver, lung, kidney, spleen, and submandibular lymph node was completed for every serologically positive case as well as for those which showed lesions during the post-mortem examination and two healthy muskrats, a total of 54 animals. As adiaspiromycosis was identified, lungs for all animals were sectioned to screen for this fungus (Figure 6). Tissue samples were fixed in 10% neutral buffered formalin, processed routinely and embedded in paraffin wax. All sections (4 µm) were standardly stained with haematoxylin and eosin (HE).



Figure 6: Sectioning of muskrat lungs saved in SVA's Biobank

Polymerase Chain Reaction

The serologically positive samples for *F. tularensis*-specific antibodies were examined by real time PCR (RT-PCR) of the submandibular lymph nodes and/or spleens for detection of *F. tularensis*. The same protocol was used for both organs. Thawed samples were swabbed using sterile cotton swabs. The swabs were incubated in 380 μ l G2 buffer and 20 μ l proteinase K solution (EZ1 Tissue DNA Extraction Kit, Qiagen, Sollentuna, Sweden) at 56°C for 15 minutes under continuous agitation, followed by 5 minutes incubation at 95°C. DNA was extracted from 200 μ l 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 μ l elution buffer and 1 μ l was used as template for each PCR. *Francisella tularensis* subsp. *holarctica* genotyping was performed on 22 frozen submandibular lymph node samples and 15 frozen spleen samples (from animals where the submandibular lymph nodes were not available and those tested positive) using two indel markers (Ftind49 and Ftind38) and nine canSNP markers (B.13, B.19, B.23, B.26, B.39, B.40, B.41, B.42 and B.43) 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). Whole genome sequenced *F. tularensis* subsp. *holarctica* strains with known genotypes were used as controls, together with no template controls.

Results

Necropsy examination

The general data regarding sex, age, body condition and autolytic changes of the 216 muskrats necropsied is described in Table 4. The majority of the population sample consisted of adults (82.87%), in good body condition and with moderate to severe autolytic changes. There was a significant sex imbalance, with one female for each 1,64 males. Two of the individuals were trapped and, since their bodies were not recovered before being scavenged, it was not possible to determine their sex.

Table 4: General data and biometrics of the necropsied muskrats (n=216, %); Body condition 2, 3, 4, 5 = thin, average, overweight; obese respectively; Autolytic changes 2 to 5 = light to very severe

| | | |
|-------------------|--------|--------------|
| Sex | male | 133 (61,57%) |
| | female | 81 (37,5%) |
| Age | young | 37 (17,13%) |
| | adult | 179 (82,87%) |
| body condition | 2 | 17 (7,87%) |
| | 3 | 172 (79,63%) |
| | 4 | 25 (11,57%) |
| | 5 | 2 (0,93%) |
| autolytic changes | 2 | 40 (18,52%) |
| | 3 | 136 (62,96%) |
| | 4 | 38 (17,59%) |
| | 5 | 2 (0,93%) |



Figure 7: Reproductive organs of muskrats. (A) Pregnant uterus (B) Testicles

When it comes to their sexual physiology, 50 females had not been pregnant, and 21 females were found to be pregnant with four to ten fetuses. The fetuses' sizes varied from 2x2mm to 30x35mm (Figure 7 (A)). Of the remaining females, 10 showed signs of a former pregnancy with one to fourteen implantation scars in the uterus. The reproductive status of the males was harder to determine but the size of the testicles varied from 5x3mm to 26x15mm (Figure 7 (B)).

In 31 muskrats (14.35%) an agglutination reaction was detected during the serological testing for *F. tularensis*-specific antibodies at the time of necropsy and were thus considered positive.

Regarding the additional testing of random individuals/ muskrats chosen by their origin, to be representative of the population from different hunting sites, all the 11 screened for *Salmonella spp* and general gastrointestinal parasites were reported as negative. The same was true for the 7 specimens tested for *Erysipelothrix spp*.

The main macroscopic findings are described briefly in Table 5. These consisted mostly of alterations such as enlarged or reduced organ size and mild changes in coloration, like petechiae in the kidney. Some livers were found to have small white spots, miscolorations, hepatomegaly, microhepatopathy, soft consistency and cysts with tapeworms. Often the bladders were filled with a yellowish, moderately hard concrement. The spleens were either enlarged, up to two times their normal size, with rounded edges, or reduced in size. Some muskrats exhibited ulcers in their stomachs, and one had small black spots in the mucosa. There were also a few cases of lymphadenomegaly and some fractures of limbs, craniums, spines or incisor teeth (most likely caused by method of euthanasia), as well as overgrown incisor teeth. Some animals had severely tainted blood. Due to the advanced decomposition of many animals, more minute descriptions were difficult.

Table 5: Main found macroscopic lesions and organs affected (n=216)

| Bladder | Spleen | Cuts in the tail | Fractures | Liver | Leukoderma | Stomach | Kidney |
|---------|--------|------------------|-----------|-------|------------|---------|--------|
| 22 | 11 | 11 | 10 | 9 | 7 | 3 | 1 |

Most lungs were severely oedematous and haemorrhagic but were not recorded as a macroscopic lesion since it was probably a result of the method of euthanasia (trapped or shot) and not a sign of disease.

Histopathology

Slides containing samples of liver, lung, kidney, spleen, submandibular lymph node and other organs with macroscopic lesions of 54 animals were analysed. Having found adiaspiromycosis in five of these lungs, a total of 152 more lungs were processed. The results are displayed in Table 6. Few microscopic changes were identified, partially due to the good health status of the trapped animals and partially due to moderate to marked autolytic changes in most cases.

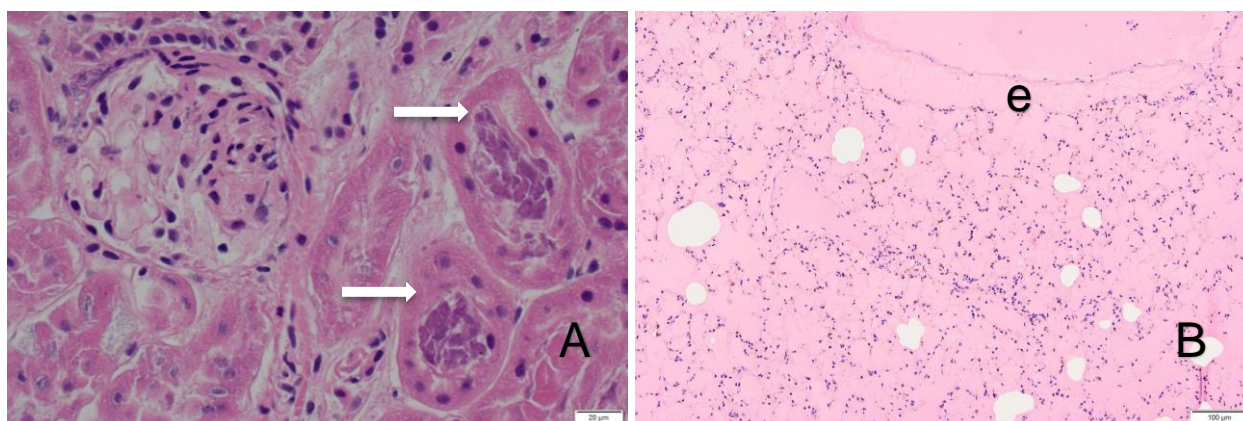


Figure 8: (A) Concrement in renal tubules (arrow) 20x; (B) Severe oedema in lung (e) 2x.

Table 6: Main histopathological findings

| Organ | Description | Number of animals (%) |
|----------------|--------------------------------------|-----------------------|
| Liver (n= 54) | Brown pigment in Kupfer cells (bile) | 1 (1,85%) |
| Kidney (n= 54) | Concrement in tubules | 8 (14,81%) |
| | Oedema | 170 (82,52%) |
| | Ectopic bone formations | 62 (30,10%) |
| | Mild inflammation | 55 (26,70%) |
| Lung (n= 206) | Adiaspiromycosis | 46 (22,33%) |
| | Foamy macrophages | 25 (12,13%) |
| | Bronchitis | 6 (2,91%) |
| | Small granulomas | 4 (1,94%) |
| Spleen (n= 54) | Mild mixed inflammation | 5 (9,26%) |
| | Yellow-brown pigment (hemosiderosis) | 4 (7,41%) |
| Stomach (n=1) | Ulcer with connective tissue | 1 |

Adiaspiromycosis

Focusing on the lung slides, there were two incidental findings, the main one being adiaspores of *Emmonsia spp* and the second one being ectopic bone formations. The adiaspores were found in mild as well as in extremely heavy infestations, ranging from 91,86 to 370,71 μm (Figure 9 (A)). Roughly half of these were a stand-alone finding, with no inflammatory reaction whatsoever surrounding it, and the other half triggered severe reactions, with mixed inflammatory cells, but mainly a large halo of foamy macrophages as seen in Figure 9 (B). The small ectopic bones ranged from 88,24 to 251,65 μm (Figure 10 (B)).

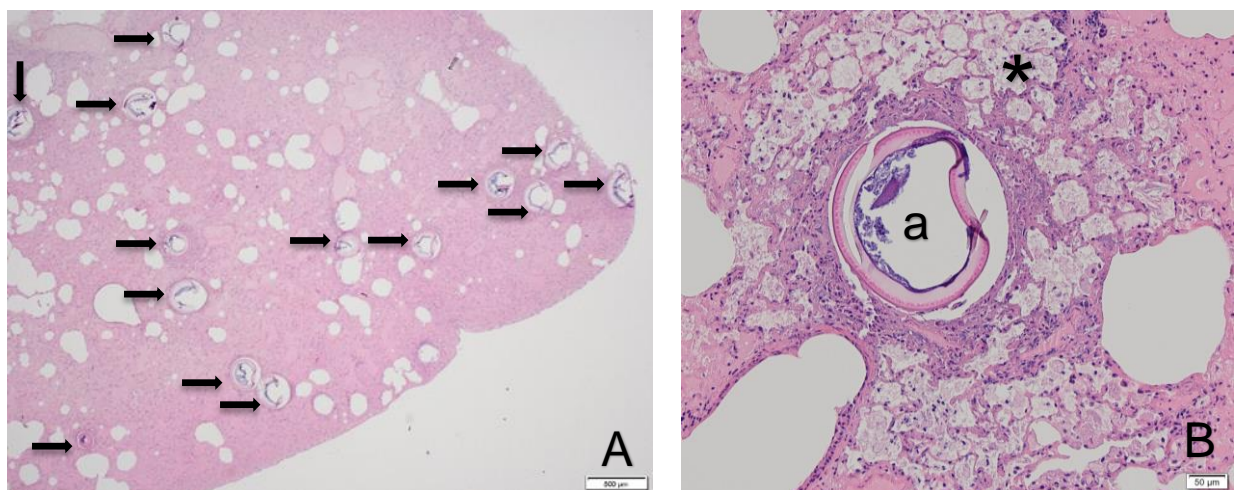


Figure 9: (A) Heavy infection of *Emmonsia spp* adiaspores with no surrounding inflammatory response in lung, (arrows) 2x. (B) Cross-section of adiaspore (a) surrounded by halo of foamy macrophages (*) in lung, 10x.

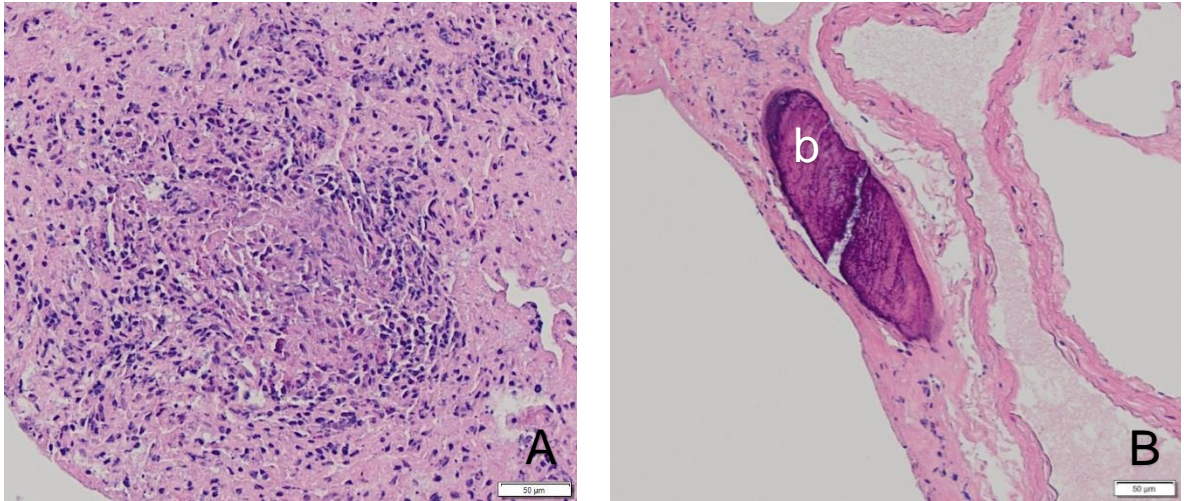


Figure 10: (A) Unencapsulated granuloma in lung, 20x; (B) Ectopic bone formation in lung, (b), 10x

Polymerase Chain Reaction

Of the 31 animals that were seropositive for *F. tularensis*, there were only 22 submandibular lymph nodes available. These were analysed by PCR and 8 (36.4%) were found positive for the bacteria. Of these 8, half were found positive both for *F. tularensis* spp. and *F. tularensis* type B, the other half were only positive for *F. tularensis* spp.

For the 9 seropositive animals whose submandibular lymph nodes were not available, the spleens were tested by PCR and found negative. The spleens of the 8 previously PCR positive muskrats were also tested, for evidence of systemic disease, but they were all found to be negative as well. A lung slide from one of these animals that displayed lesions compatible with Tularaemia such as general mixed inflammation with foci of necrosis was also tested by PCR for *F. tularensis* spp and found positive.

It is worth noting that immunohistochemistry for Tularaemia is pending. In the future, attempts will be made to demonstrate the presence of the bacteria in the tissues of seropositive animals.

Discussion

Although the Swedish muskrat population is declining, the animals sent to the SVA were hunted and trapped, in the context of an eradication program, and did not die of natural causes. As such the specimens making up this sample were generally considered to be healthy, in good body condition and with no major anomalies. The only biometric finding worth noting is an unbalanced sex ratio. No specific reason was found other than the assumption that the males move about more during the reproductive season making them easier to spot and shoot.

The macroscopic examination revealed a small number of animals (3,24%, n=7) with leucodermic areas in their tails, possibly vitiligo, similar to that found in otters necropsied at the SVA and the Swedish Museum of Natural History, in Stockholm (personal communication with Dr. Anna Roos). This was an accidental finding that was not explored histopathologically and did not seem to impact their lives, other than possibly making them easier to spot for potential predators, mostly humans. However, to the best of our knowledge there is no published studies on this subject and more research would be necessary to determine the cause of this alteration.

In addition, the concrement found in the bladder and the renal tubules, 10,18% (n=22) and 14,81% (n=8) respectively was not associated with inflammation or degenerative changes that could be observed histologically. As such, we propose it is a natural variation in this species.

Regarding the presence of Tularaemia, the expected classical lesions described by the OIE and found in diseased hares are caseous necrosis of the lymph nodes, foci of necrosis in the liver, spleen, lungs, pericardium and kidneys, and splenomegaly in septicaemic cases (OIE, 2018). However, none of these were present in muskrats, or were impossible to access due to the advanced decay and/or bullet wounds. Therefore, the only indication of *F. tularensis* in these muskrats were the positive responses to the serological screening, howbeit it is worth mentioning that there is no antigenic difference between Type A and Type B strains and cross-reactions with some *Brucella* and *Legionella* species are possible (OIE, 2018). Considering the current panorama of Tularaemia in Sweden there is no reason to suspect anything other than *F. tularensis* subsp. *holarctica* (Type B) (Tärnvik & Berglund, 2003). It is also important to note that several animals had severe autolytic changes, with heavily contaminated blood (outside particles, coagulated lumps, fragments of various internal organs, etc). In this case, the results of the seroagglutination were often unreliable and were registered as negative. As such it is possible that the seroprevalence could be slightly higher.

Most sectioned organs were not very informative, either because the animals were likely healthy/ asymptomatic or because the autolytic changes were too severe to draw any reliable conclusions. Regardless, there were some interesting incidental findings, mainly in the lungs.

There was a surprisingly high percentage of moderate to severe oedema (82,52%), additionally almost a quarter of all examined lungs showed the animal carried or suffered from adiaspiromycosis, and almost half showed signs of inflammation, either in the form of general mild mixed inflammation, bronchitis, small granulomas or areas of foamy macrophages. Some of these were intrinsically related to the adiaspores, in others it was not possible to determine their origin.

Adiaspiromycosis is a rare self-limited pulmonary mycotic infection found in several small animals, caused by *Emmonsia parva* and *Emmonsia crescens*. *E. crescens* has the widest host range and is more broadly distributed than any other mycotic pathogen. It can cause sporadic human infections and disease in domestic animals (Morandi et al., 2012; Williams & Barker, 2001).

There is no multiplication in the host species though massive infections have been reported in small mammals. These are usually members of the orders Carnivora, Rodentia and Insectivora with the most commonly and heavily affected being water voles, muskrats and several species of the Mustelidae family (Williams & Barker, 2001). However, this fungus has not been previously described in Swedish muskrats. There is only a report of one European beaver necropsied at the SVA where similar lesions were found although the inflammatory reaction was different, consisting mostly of mononuclear leukocytes and giant cells around the adiaspores (Mörner et al., 1999), whereas in the muskrats the main cell group found was foamy macrophages.

There were also ectopic bone formations found in about 30% of the lungs, similar to what has been described previously in minks (Borst et al., 1976). These ectopic bones have been found as well in birds, guinea pigs, cattle, goats, dogs and wolves. There seems to be no relation between the age of the animals and the presence of bone, and it does not seem to cause disease or affect the animals in any way. The same cannot be said for humans, where the presence of ectopic bone in varied organs is associated to a large range of diseases (Borst et al., 1976).

Only 3,7% of the total number of muskrats examined were confirmed to be positive for *F. tularensis* by PCR and seroagglutination. However, this is not a strong indicator of the prevalence of Tularaemia in the general population since the lymph nodes of several seropositive animals were not available (9 to be exact) for PCR testing and as such were not included in this percentage.

Of the 8 lymph nodes considered positive to *F. tularensis*, 4 were also positive for *F. tularensis* Type B, but in the other 4 the subspecies could not be determined. This incongruence was most likely due to the last 4 animals having had a lighter infection, with not enough bacteria for the less sensitive type B test to pick up on. However, since it is the only type known to exist in Sweden to

this date, it is most likely Type B (Tärnvik & Berglund, 2003). Moreover, if it were Type A the PCR protocol used should have been able to identify it as such.

The existence of lymph node positive, spleen negative animals would indicate exposure to the bacteria without development of systemic infection and consequently clinical disease. Therefore, Tularaemia is unlikely to be the cause of the decreasing population of wild muskrats in Sweden.

Regardless, muskrats cannot be excluded as a natural reservoir, contributing to the maintenance of the bacteria in the wild ecosystems and subsequently the direct or indirect infection of definite vulnerable hosts such as hares and people.

To this end, it would be interesting to necropsy a naturally dead muskrat. However, none have been sent to the SVA in the recent past. There are also very few beavers sent in for necropsy, which could provide a general idea of the possible diseases spreading in their shared ecosystem.

It is important to note that humans are highly susceptible to contracting Tularaemia from direct contact with infective materials. It is recommended taking special precautions when handling sick or dead animal (CDC, 2018) such as wearing gloves, masks and eyeshields. Laboratory manipulations of live cultures or potentially contaminated material must be performed following strict biosafety protocols (OIE, 2018) as it was done in the SVA.

Conclusion

The wild muskrat population in Västernorrland, Sweden, is generally healthy, with a few, mostly likely, subclinical infections. This sample population has definitely been exposed to the agent of Tularaemia with at least 14.35% of the animals having developed antibodies against *F. tularensis*. Although uncommon, it seems to be possible for muskrats to be infected systemically and to develop the typical tularaemic lesions. It is not known whether muskrats in general are relatively resistant to Type B Tularaemia or whether the seropositive muskrats in this study were individuals that managed to survive a clinical infection and others have died. This is hard to determine due to the circumstances in which they die. Traditionally the biggest threat to these animals were hunters but they do not spot muskrats anymore. The muskrat's habitat is nowadays severely restricted to a very small area, and they tend to die in or around water, where they are not found and are scavenged fairly quickly and thus are not sent in for necropsy.

Based on the present study, it is not possible to determine whether Tularaemia has contributed to the decrease in the muskrat population. However, the study does show that muskrats are very likely an optimum reservoir for the maintenance of this bacteria in wild aquatic ecosystems and for contamination of arthropod vectors such as mosquitos, other carriers like water voles and other small mammals, hares and even directly or indirectly humans.

The incidental finding of adiaspiromycosis does not seem to carry much importance other than scientific curiosity since it is often a self-limited infection with a few cases of chronic inflammation surrounding it that did not impair the animal from living normally. Even so it did affect a large portion of the population with seemingly no relation to their place of origin. This fungus also has a certain zoonotic potential although significantly less relevant than Tularaemia.

In summary, *Ondatra zibethicus* is an invasive alien species, introduced in Europe for their pelts, that causes significant damage to their environments and serves as a reservoir for several pathogenic agents, some of them potentially zoonotic like *F. tularensis*. Further investigations are necessary to determine to what extent muskrats are responsible for the maintenance of this pathogen in semi-aquatic ecosystems and whether they can be clinically affected. It would be extremely helpful for this purpose to necropsy some naturally dead individuals as well as specimens caught in the height of the human outbreak during the summer of 2019.

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Annexes

Annex I – Step-by-step diagram of muskrat necropsy



Figure A1: Preparing the table and identifying the animal



Figure A2: Skinning the animal



Figure A3: Identifying and removing the mandibular lymph nodes

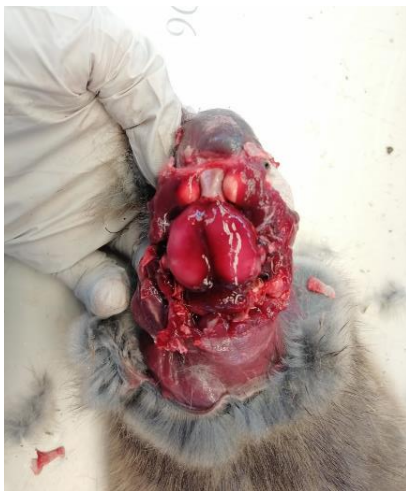


Figure A4: Skinning the head and removing the brain

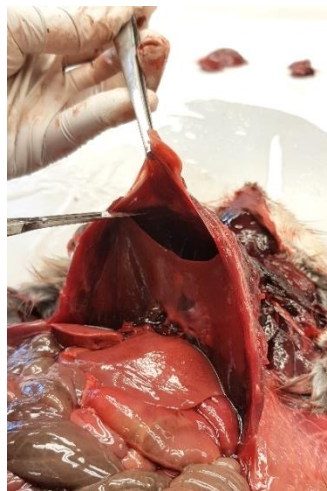


Figure A5: Opening the abdominal wall and sectioning the diaphragm ventrally



Figure A6: Removing approximately 3ml of blood from the thorax with a disposable pipet

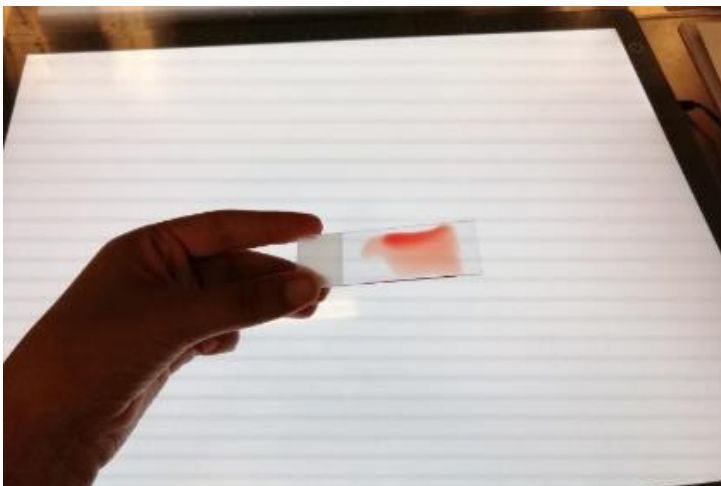


Figure A7: Mixing a drop of blood and a drop of antigen in a glass slide and checking for agglutination against a white light

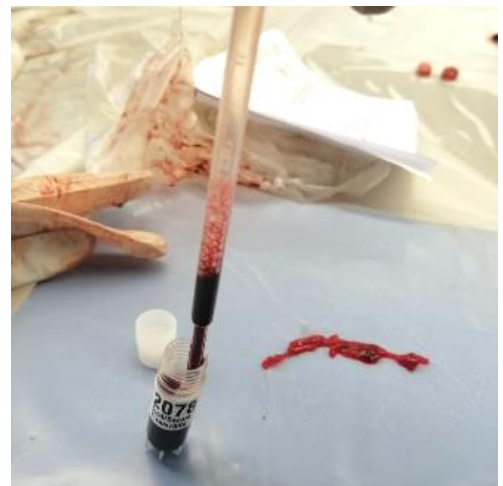


Figure A8: Collecting the remaining blood in a duly identified, rubber sealed, tube



Figure A9: Collecting a portion of liver, lung, kidney, spleen, muscle, brain and intestine (respectively) for SVB's biobank

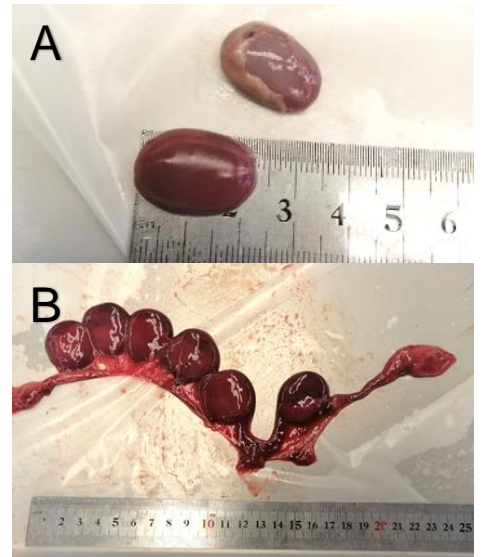


Figure A10: (A) Measuring the testicles. (B) Checking the uterus for foetus and implantation scars

Incidental macroscopic findings:



Figure A11: Leucodermic area in the extremity of the tail

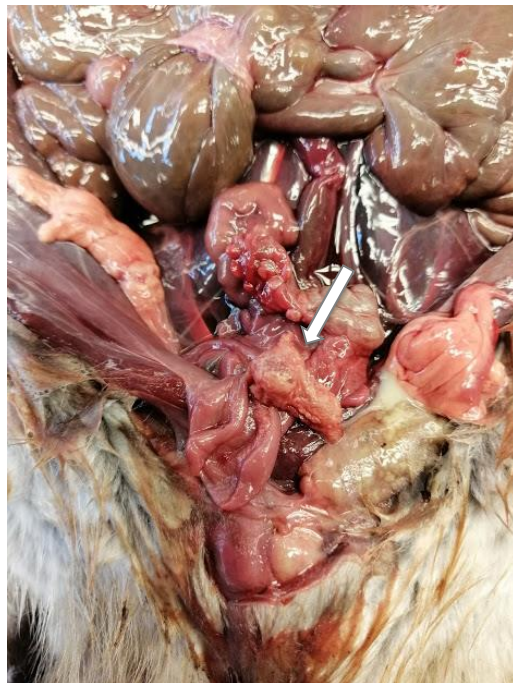


Figure A12: Concrement in the bladder (arrow)

Annex II - Birds of prey

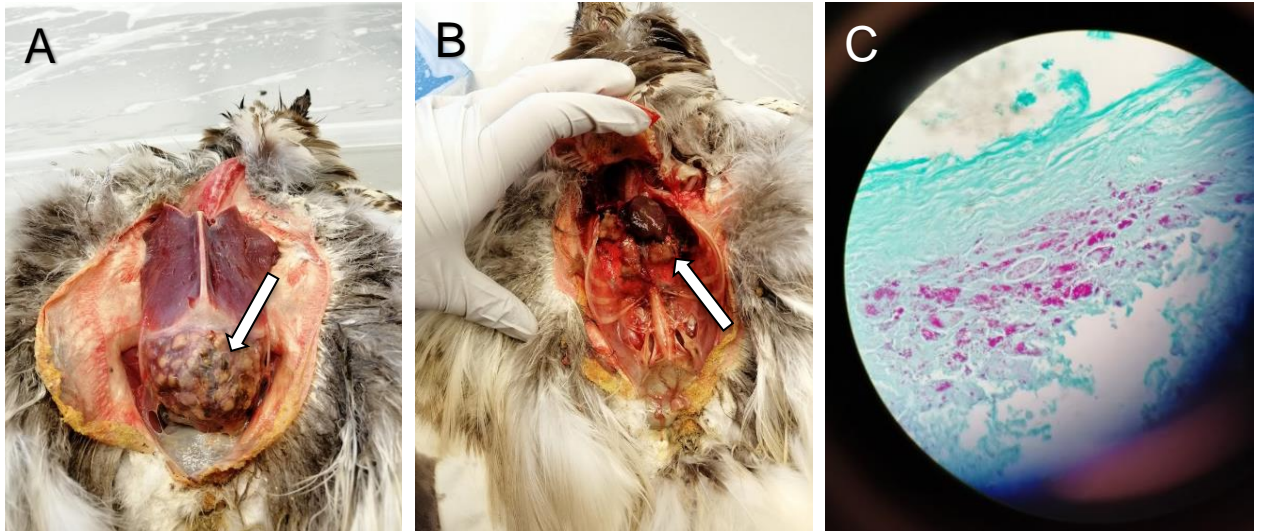


Figure B1: Common buzzard; (A, B) Multiple granulomas in its liver, spleen, lungs and smaller granulomas in other organs (arrows); (C) Ziehl-Neelsen staining of liver slide (*Mycobacterium avium*), 20x



Figure B2: Eagle owl, liver with herpesvirus



Figure B3: Eagle owl, young, probable congenital joint malformation in the right leg (arrow)



Figure B4: Marsh harrier, crop granuloma



Figure B5: White-tailed eagle, spine fracture (train accident) (arrow)



Figure B6: White-tailed eagle, feathers with ectoparasites (lice) (arrows)

Annex III - Mammals

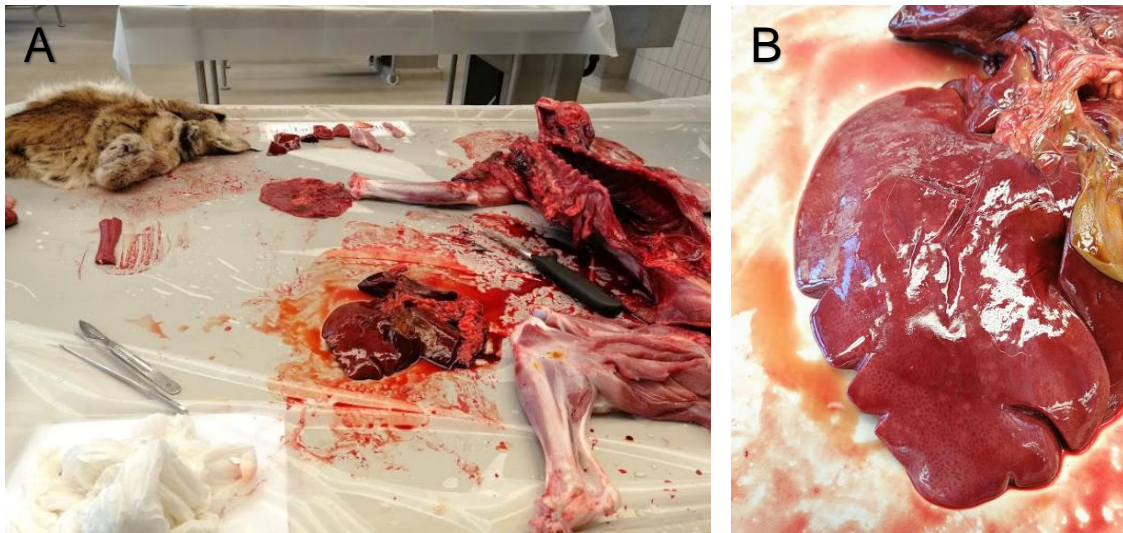


Figure C1: Lynx, killed by trauma; (A) General aspect; (B) Liver with small milk spots

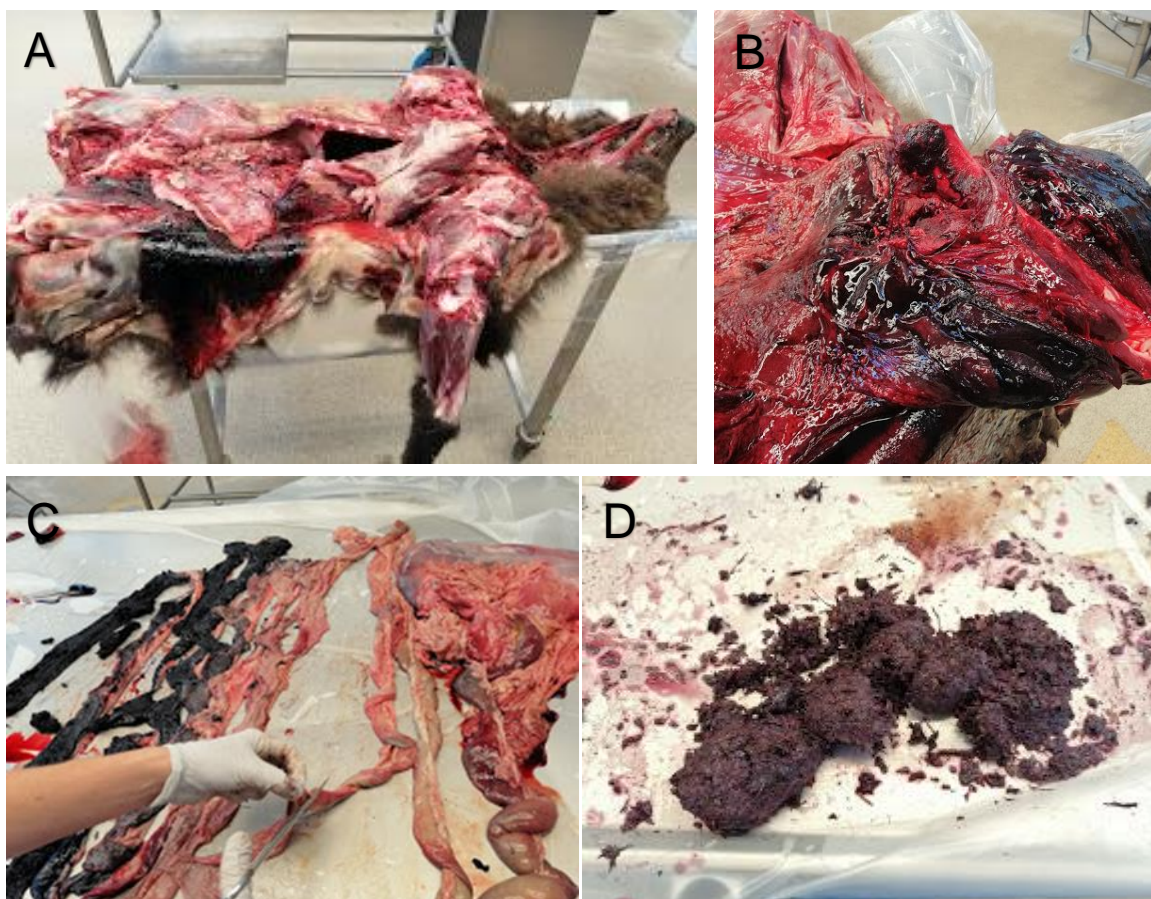


Figure C2: Brown bear killed by trauma; (A,B) Subcutaneous hematomas; (C) Intestines with purple content; (D) Stomach content, this diet consisted mainly of blueberries



Figure C3: Wolf with bone trapped between the roots of upper molar teeth



Figure C4: Wolverine killed by trauma; (A) Pelt; (B) Cysts in the uterus



Figure C5: European polecat, cervical subcutaneous hematoma



Figure C6: Wild boar, oedematous lungs



Figure C7: Wild boar, juvenile, congenital malformation with no cervical vertebrae



Figure C8: Roe deer, large ventral cervical hematoma (arrow)



Figure C9: Roe deer, erosion at the base of the antlers (in life)



Figure C10: Moose, lens opacity in senior animal (compared to a normal lens)



Figure C11: (A) *Strongyloides* spp. eggs in moose's lungs, 20x (B) Observation under the microscope



Figure C12: Ringed seal foetus



Figure C13: Porpoise; (A) Respiratory tract; (B) Lungs with severe lungworm infection