Bats as hosts of zoonotic pathogens

– potential conflicts between nature conservation and public health

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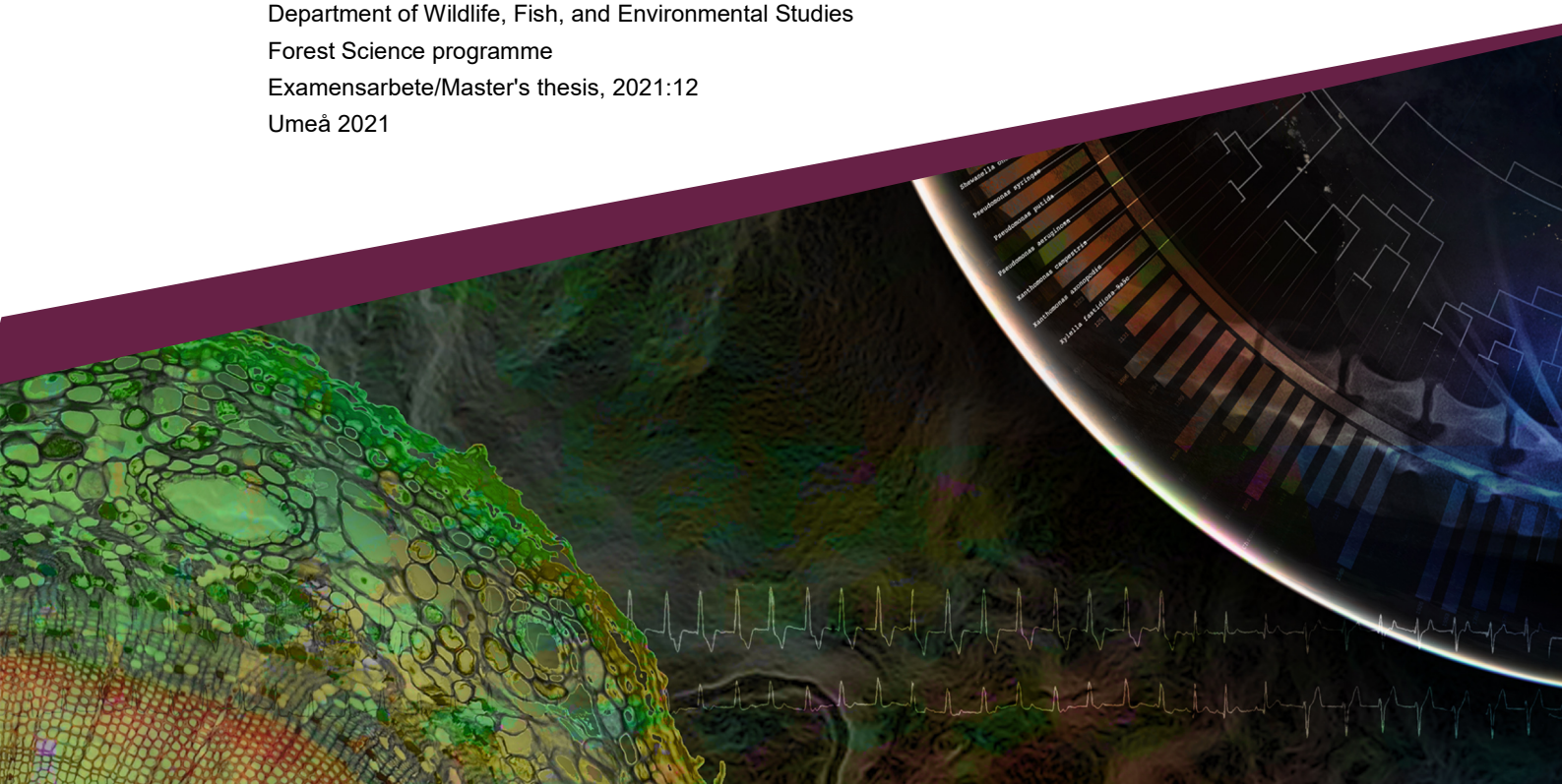

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”Applying more advanced laboratory analyses (TWIST) complemented with analysis of antibodies did not confirm the presence of lyssavirus / Analys med avancerad TWIST metod kompletterad med antikroppsanalys resulterade i negativa resultat. Därför kan fyndet av lyssavirus inte bekräftas”

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Abstract

Bats belong to the order Chiroptera, one of the most species rich mammalian groups of Sweden, but it is also the most threatened one. Nineteen different bat species have been found in Sweden, of which 12 are red-listed. Globally rodents and bats are the animal groups known to host most zoonotic pathogens. Bats are known to carry rabies, even though there has been no discovery of cases in Sweden where humans have been infected. Bats in Sweden have previously been found to carry rabies specific antibodies, thus indicating that rabies is circulating among the Swedish bat populations. Various hantaviruses have been found in insectivorous bats, however in Sweden so far only rodents are identified hosts. In Sweden, the knowledge of pathogens hosted by bats is generally low. Thus, the aim of this study was to explore and answer the following three questions: (I) To which extent are Swedish bats, focusing on the Daubenton's bat, reservoirs of zoonotic pathogens? (II) Do bats in Sweden host puumala orthohantavirus (PUUV)? (III) If bats host zoonotic pathogens, is there a conflict between nature conservation and public health?

In this study, bats were trapped using mist nets mounted perpendicular to small streams in summer 2020. Blood, saliva and feces were sampled from 77 bats and tested for lyssavirus, flavivirus and Puumala orthohantavirus using PCR (polymerase chain reaction) techniques, sequencing, IFA and ELISA. Bats were found to host flavivirus and lyssavirus, but no hantaviruses. However, further tests are necessary to determine what kind of lyssavirus and flavivirus they carry. Furthermore, more tests are necessary to conclude if Swedish bats carry PUUV or not as the results were unclear. Conclusively, Bats living close to humans may bring some potential risks to human health. However, evicting, or culling bats may increase the transmission risk of the zoonotic disease's bats host. Bats in Sweden are protected by law; thus, people should focus on measures to mitigate transmission risk without harming bats. Furthermore, bats provide ecosystem services benefiting human health, for example by preying on mosquitoes and hence suppressing other potential vector-borne diseases. Hence, bat conservation and public health may have a common goal.

Keywords: Bats, conservation, Daubenton's bat, Myotis daubentonii, public health, vector, zoonotic pathogens

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Abbreviations

BBLV = Bokeloh bat lyssavirus

CR = Critically endangered

DBS = Dried blood spots

DD = Data deficient

DENV = Dengue virus

EBLV-1 = European bat lyssavirus-1

EBLV-2 = European bat lyssavirus-2

ELISA = Enzyme-linked immunosorbent assay

EN = Endangered

FOI = Swedish defence research agency

IPC = Internal positive control

JEV = Japanese encephalitis virus

KFDV = Kyasanur Forest disease virus

LC = Least concern

LGTV = Langat virus

LIV = Louping ill virus

NE = Nephropathia epidemica

NT = Near threatened

OHFV = Omsk hemorrhagic fever virus

POWV = Powassan virus

PUUV = Puumala orthohantavirus

RABV = Rabies virus

TBEV = Tick-borne encephalitis virus

VU = Vulnerable

WCBV = West caucasian bat lyssavirus

WNV = West nile virus

YFV = Yellow fever virus

1. Introduction

Bats belongs to the mammalian order of Chiroptera and are the only mammals capable of flight. The Latin name Chiroptera means hand – "chir" and wing – "optera" and refers to the wing structure, which is constituted by a thin membranous tissue that generally spans over the elongated arm and fingers but that in some species also connects legs and tail. The Chiroptera order contains over 1000 species, 39 bat species can be found in Europe, all of them insectivorous (Blank et al, 2008). In Sweden, 19 reproducing species occur of which 12 species are included in the red list (SLU & Artdatabanken, 2020).

Sweden is part of the *Eurobats* 1994 agreement and are therefore obliged to identify and protect important habitats for bats (Eurobats & UNEP, 2021). The agreement also states that bats are not to be deliberately killed, captured, or disturbed (Hutson et al, 2019), which is also found in Sweden's national *species protection ordinance* (SFS 2007:845).

The biggest threats towards bats are habitat loss and loss of foraging grounds or roosting sites, which is often connected to increase of human population (Jung & Threlfall, 2016; Mickleburgh et al, 2002). However, there are also cases in the world where bats come in direct conflict with humans and public health such as the vampire bats in Latin America, or fruit bats and fruit farmers in other parts of the world. There are also areas where bats are persecuted because of misinformation, and in other areas bats are exploited as a food resource (Mickleburgh et al, 2002).

Bats provide essential ecosystem services. Insectivorous bats can suppress populations of pest insects and vectors. By foraging they may help decrease the spread of zoonotic diseases by feeding on insects acting as vectors towards humans, for example eating mosquitos carrying dengue, yellow fever, and chikungunya fever (Andrianaivoarivelo et al, 2006; Kemp et al, 2019). Insectivorous bats may also help farmers by decreasing pest insects (Maas et al, 2016; Puig-Montserrat et al, 2015; Russo et al, 2018; Wanger et al, 2014). In other parts of the world bats are important pollinators (Fleming et al, 2009) and dispersers of seeds (Abedi-Lartey et al, 2016). Furthermore, bat excrements can be used as a fertilizer or to produce soaps, gasohol and even antibiotics (Calisher et al, 2006). Additionally, bats could also be used as an indicator of environmental changes as they respond quickly to changes in insect biomass, land use or vegetation structure, which will in turn impact the remaining community (Blank et al, 2008).

Bats harbor numerous zoonotic viruses, many more than any other mammalian group (Olival et al, 2017). The aim of this study is to explore and answer the following three questions: (I) To which extent are bats in Sweden reservoirs of zoonotic pathogens, focusing on the Daubenton's bat? (II) Do bats in Sweden host Nephropathia epidemica? If yes, can the southern Swedish case of NE 2018 have been caused by bats? (III) If bats host zoonotic pathogens – is there a conflict between nature conservation and public health?

1.1. Bat ecology

Bats are nocturnal. They use echolocation to navigate in the dark by creating sounds, beyond the audible wavelength of the human hearing, through their nose or mouth and then receive

feedback from the returning echo. Bats also use different sounds to communicate with each other. Furthermore, bats have small hairs on their wings capable to feel air movements around them helping them during their flight (Bjärvall & Ullström, 2010).

The majority of the bats in the world are insectivores, as are all species occurring in Sweden. They hunt using the echolocation and insects may be caught using their mouth or parts of their skin membrane. Insects are consumed in the air or after the bat has landed (Bjärvall & Ullström, 2010).

1.1.1. Yearly life cycle of temperate bats

A bat's body temperature varies, during flight it can be 40 °C and after it has landed the body temperature may drop to 30 °C or further down to that of its environment. Bats in colder climate have adapted by hibernating. During hibernation, the body temperature of bats is usually slightly above 0 °C, but it may drop a few degrees below 0°C without adverse effects for the bat (Bjärvall & Ullström, 2010). Hibernation for temperate bats may begin in September and last until April. Bats live solitary until colonies are formed by the females in June to July, while male bats usually live solitary. The females give birth to one young and raise the young in the colony (Bat Conservation Trust, 2020b).

Mating takes place in September, but fertilization is delayed and takes place in spring. The young will be born during the summer when conditions are favorable, and the mother will be able to produce enough milk. Consequently, the gestation may be prolonged by bad weather. If the conditions worsen in spring, the mother can pause gestation and go back into dormancy, thus prolonging the gestation (Bjärvall & Ullström, 2010).

1.2. The bats of Sweden

All of the 19 bat species in Sweden belong to the family *Vespertilionidae*, which contains over 300 species. These species are found all over the tropical and temperate part of the globe. The scientific name *Vespertilionidae* comes from the Latin "*vesper*" meaning night and it alludes to the *Vespertilionidae* being nocturnal. All species belonging to *Vespertilionidae* release the echolocation sound from the mouth. Their flight membrane reaches their tail. Furthermore, in this family, the shape of the tragus can be used to distinguish between the genera (figure 1(Bjärvall & Ullström, 2010).

Figure 1. Tragus shape distinguishing the genera within the *Vespertilionidae* family.

In Sweden, there are 12 bat species on the red-list 2020 (table 1). For a species to be added to

the red-list it has been evaluated to be at least “near threatened” or there may be a deficiency of data. The abbreviations of the status for the red-list are explained in figure 2 (SLU & Artdatabanken, 2020).

Table 1. The Swedish bat species included in the red-list and their status 2020.

Latin name	English name	Status
Myotis nattereri	Natterer’s bat	NT
Myotis bechsteinii	Bechstein's bat	EN
Myotis myotis	Greater mouse-eared bat	EN
Myotis dasycneme	Pond bat	NT
Myotis alcathoe	Alcathoe bat	EN
Pipistrellus pipistrellus	Common pipistrelle	VU
Nyctalus leisleri	Leisler's bat	VU
Eptesicus nilssonii	Northern bat	NT
Eptesicus serotinus	Serotine	NT
Plecotus auritus	Brown long-eared bat	NT
Plecotus austriacus	Grey long-eared bat	CR
Barbastella barbastellus	Barbastelle	NT

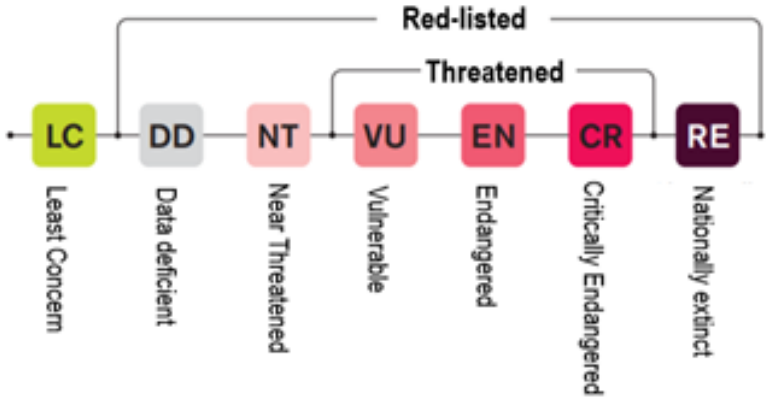

Figure 2. Explanation of the symbols of endangered status used in the red-list.

1.2.1. Daubenton's bat – *Myotis daubentoni*

The Daubenton’s bat is common in all of Europe. In Sweden it can be found from the south up to Jämtland and Ångermanland (Bjärvall & Ullström, 2010). It has also been found higher up north along the coastline (Artdatabanken, 2020; Schneider, 2020). It is one of the smaller species in the *Myotis* genus and has a length of 40-50 mm and a weight of 6-14 g. The forearm has a length of 34-41 mm. The ears are short barely reaching the nose when folded forward. The feet are long and free of skin membrane. The tragus has a straight, forward pointing, convex trailing edge. The nose is light reddish-brown and the fur on its back is dark with a hint of a red tone (Bjärvall & Ullström, 2010). It can be heard around 45 kHz (Natursidan.se, 2018).

Daubenton's bats reside close to water, both natural and man-made. They hunt flying insects in the treetops of the forest and the swarming insects flying above the water. Occasionally, it may even catch small fish. In winter, they squeeze into underground spaces and the first individuals to wake up during spring are the older males (Bjärvall & Ullström, 2010). In summer, colonies are formed by both males and the females, and the ratio between males and females in a colony may vary (Dietz et al, 2006; Encarnaçao, 2012). Dietz et al. (2006), found three colonies in Germany, one with a greater number of males, one with a greater number of females and one colony where the sexual ratio between males and females was around 50/50. The sexual segregation can also be seen at feeding sites, the distance between sex-specific feeding sites being larger in late spring and early summer compared to late summer. Also, the number of mixed sexes at feeding sites increased during the summer (Encarnaçao, 2012). Over 180 individuals can be found in a colony, but the colony could also be smaller than 10 individuals (Lučan, 2009). The colonies formed by females can be located far away from the water. The Daubenton's bat tends to be stationary, but a migration distance of more than 200 km is known (Bjärvall & Ullström, 2010)

1.3. Bats as a vector

Bats are one of the oldest mammalian orders and it is possible that their immune system has attributes different from other mammals. Currently, little is known about bats immune system, but some studies have found that the bats immune system is similar to that of other mammals (Calisher et al, 2006; Chakravarty & Sarkar, 1994; McMurray et al, 1982; Sarkar & Chakravarty, 1991). However, an example of a difference found, is that the interferon (IFN) response (interferons are proteins which help regulate the immune system) varies in mammals and has also been found to differ in bats compared to other mammals, and the IFN response may even differ between various bat species. There are several antiviral genes found in bats which are IFN induced, while in other mammals these genes are not IFN induced (Irving et al, 2021).

Chiropteran is an old order which hosts many viruses, due to the co-evolution between bats and viruses (Dobson, 2005; Han et al, 2016). The origin of the chiropterans has been dated to the Eocene period (56-33.9 million years ago) using the co-evolution between bats and viruses, through an analysis of 17 nuclear genes together with certain zoonotic viruses which have been maintained in bats until today. Henipaviruses and lyssaviruses have a long history of coexistence (Badrane & Tordo, 2001; Gould, 1996), in addition to coronaviruses and paramyxoviruses (Cui et al, 2007; Drexler et al, 2012). The viruses could have evolved and adapted to the cellular receptors and biochemical pathways in bats to facilitate the transmission of pathogens between mammalian hosts with similar cellular receptors and biochemical pathways (Calisher et al, 2006).

Bats fly when hunting every night and may also fly great distances when migrating (Bjärvall & Ullström, 2010; Griflin, 2012). Thus, it is possible to transmit and contract pathogens over large areas. In France, the geographical range of the Nathusius' pipistrelle (*Pipistrellus nathusii*) migratory routes have been identified to be connected to rabies infections (Brosset, 1990). In America, the Silver-haired bat (*Lasionycteris noctivagans*) has a seasonal range from Alaska to south Texas and is found all over Canada. The Silver-haired bats geographical range have been found to overlap with the various rabies viruses these bats may host (Calisher et al, 2006). Thus, different migratory patterns in bats may allow exchange of viruses between migrating and stationary bat populations of the same, or different, species (Rohde et al, 2004).

Additionally, because of their seasonal patterns, bats might cause periodic or seasonal outbreaks of diseases (Calisher et al, 2006).

1.4. Host and viruses

A host is the organism that the disease-causing pathogen reside in (Farlex, 2021a). A reservoir host or reservoir is the organism where the pathogen (for example virus) live and multiply in without the reservoir host becoming pathogenic. The pathogen is dependent on the reservoir to be maintained in nature when there is no active transmission to other hosts (Farlex, 2021a). Lastly, a vector is the organism which may transmit the pathogen to a potential host, either from a reservoir or a host (Farlex, 2021b).

1.4.1. Immunogenetics

Reproductive success, predation and infectious diseases are evolutionary drivers in nature. The pathogenic selective pressure may shape the genetics for host immunity and result in resistance to infectious diseases (Acevedo-Whitehouse & Cunningham, 2006). These immune-related genes can be found in reservoirs, explaining why the reservoir does not become pathogenic from harboring the pathogen (Howick & Lazzaro, 2017; Mandl et al, 2015; Råberg et al, 2007).

1.4.2. Rabies

Rabies is a disease caused by lyssavirus. Potential reservoirs and vectors for rabies are bats, foxes, raccoons, skunks, jackals, mongooses and other carnivores. However, in Europe and North America transmission of rabies to humans are rare from any of these vectors. The most common vector transmitting rabies to humans are dogs, which causes over 99% of all human cases (WHO, 2018).

The virus is excreted through the saliva during the diseased period (period with apparent symptoms) but may also be excreted 13 days before any visible symptoms. The most common method rabies is known to spread is through bites, alternatively licking, with the saliva (Folkhälsomyndigheten, 2018a). However, it can also spread by inhalation of the virus (aerosol) (Davis et al, 2007; Johnson et al, 2006) and through transplantation of infected organs (Maier et al, 2010; Srinivasan et al, 2005). Rabies is a fatal disease, without a cure once symptoms have emerged. The virus usually has an incubation period of three to six weeks, but sometimes as short as five days or as long as a year, depending on the transferred viral load. Because rabies usually has a long incubation time it is possible to prevent the disease, even after the patient have been infected, by immediately vaccinate and provide specific immunoglobulin. The risk of infection could also decrease by thoroughly washing the wound for 15 minutes, followed by disinfecting with alcohol. People who have previously vaccinated themselves have a good protection, but should vaccinate themselves again after being infected, however they don't need immunoglobulin (Folkhälsomyndigheten, 2018a).

There are four different rabies types known to circulate in European bat species. Bokeloh Bat Lyssavirus (BBLV) is associated with Natterer's bat. West Caucasian Bat Lyssavirus (WCBV) is associated with Schreiber's long-fingered bat (Schatz et al, 2013), a bat species found in southern Europe, North Africa and the Middle East (Eurobats & UNEP, 2021). European Bat Lyssavirus-1 (EBLV-1) is carried by the Serotine bat and European Bat Lyssavirus-2 (EBLV-2) is the lyssavirus the Daubenton's bat is commonly found carrying, but the pond bat has also been found hosting this virus (Schatz et al, 2013). In Europe, there

have been two known cases with human infections with EBLV-2 and both patients were bat researchers who had not been vaccinated (Fooks et al, 2003; Lumio et al, 1986).

Sweden is believed to be free from the classic rabies virus (RABV) since 1886 (Folkhälsomyndigheten, 2018a). However, rabies specific antibodies can still be found in bats, indicating that the disease is present within the Swedish bat populations (Folkhälsomyndigheten, 2018a; Hammarin et al, 2016; Schatz et al, 2013). Many studies in Europe have found bats hosting lyssavirus and/or carrying antibodies against rabies (Brookes et al, 2005; Ceballos et al, 2013; Freuling et al, 2008; Jakava-Viljanen et al, 2010; Moldal et al, 2017; Nokireki et al, 2017; Van der Poel et al, 2005; Whitby et al, 2000). In Sweden, there has been one study where 452 bats were trapped and tested. The saliva was tested for virus and the blood was tested for antibodies. No virus was found in the bats, but 14 Daubenton's bats were found to carry rabies specific antibodies (Hammarin et al, 2016).

1.4.3. Hantaviruses

Orthohantaviruses belong to the *hantaviridae* family (Adams et al, 2017), which consist of over 300 viruses. Among the genus hantaviruses there are at least 21 viruses infectious to humans (Jonsson et al, 2010). In America hantaviruses are known to cause the hantavirus cardiopulmonary syndrome (HCPS), and in Europe and Asia hantaviruses may cause the hemorrhagic fever with renal syndrome (HFRS) (Krüger et al, 2011). Rodents are widely known as reservoirs for hantaviruses. However, various studies have found that insectivorous bats host different hantaviruses (Witkowski et al, 2016; Xu et al, 2015; Zhang, 2014) as do other insectivores such as shrews and moles (Kang et al, 2011; Kang et al, 2009; Klempa et al, 2007; Radosa et al, 2013).

1.4.3.1. Nephropathia epidemica

The disease nephropathia epidemica (NE) is caused by the Puumala orthohantavirus (PUUV). The disease can give symptoms of hemorrhagic fever and kidney disorder. The symptoms can be both mild and severe. Thirty percent of the diagnosed patients are treated at the hospital and the disease has a death rate of 0.5% (Ahlm, 2018; Pettersson et al, 2008b; Vapalahti et al, 2003).

The bank vole (*Myodes glareolus*) is the only known host of PUUV and the disease is usually contracted by humans through inhalation of infected dust (Ahlm, 2018; Vapalahti et al, 2003). The virus has been found in human saliva, but there has been no confirmation of cases where the disease has spread from human to human (Pettersson et al, 2008b). NE causes a chronic infection (a persistent infection) in humans and once cleared immunity is attained for the rest of an individual's lifespan once they had NE (Ahlm, 2018). The risk of infection is higher when the bank vole population peaks, but it also depends on the climate (Khalil et al, 2019; Khalil et al, 2014; Pettersson et al, 2008a).

NE can be found in central and northern Europe. In Sweden it is found in the northern parts, especially above the latitude of 59ºN, in the Nordic countries Sweden, Norway and Finland (Ahlm, 2018; Borg et al, 2017; Lõhmus et al, 2016; Olsson et al, 2003). The disease is notifiable, which makes it easy to generate data for each municipality. In 2018 there was a particular case in Skåne which is in southern Sweden at a latitude around 55ºN. The patient had not previously visited the endemic area of NE. There have been two previous cases of NE

in Skåne in 2013 and 2014, but the patients probably contracted the disease during their visit to respectively Jämtland and Finland (Mälardalen–men & tycks, 2019). There. has been no other cases of patients with NE in Skåne 2019 (Folkhälsomyndigheten, 2020).

1.4.4. Flavivirus

The genus flavivirus include 53 known virus species. Many of them infectious to humans, such as the dengue (DENV), yellow fever (YFV), Japanese encephalitis (JEV) and West Nile (WNV) which are all carried by mosquitos (Mackenzie et al, 2004). There is also the tick-borne encephalitis (TBEV) carried by ticks (Gaunt et al, 2001). The flavivirus have been categorized into three groups based on their antigenic properties and vector association, mosquito-borne, tick-borne and unknown-vector viruses (Cook & Holmes, 2006; Gaunt et al, 2001). The mosquito vector viruses can further be subdivided into Cluex-clade which have birds as reservoirs, are neurotrophic and usually cause meningo-encephalitis. There are also the Aedes-clade viruses which have primate reservoirs, are neurotrophic and usually cause hemorrhagic diseases (Gaunt et al, 2001; Solomon et al, 2000). The tick-borne viruses can be subdivided into two groups. The first group appears to circulate among seabirds and the second group is associated with mammals. Whereof the mammalian group can cause encephalitic and hemorrhagic diseases in humans, such as Kyasanur Forest disease virus (KFDV), Langat virus (LGTV), Louping ill virus (LIV), Omsk hemorrhagic fever virus (OHFV), Powassan virus (POWV) and TBEV (Chambers & Monath, 2003).

1.4.4.1. Flaviviruses in Europe

Several Tick-borne flaviviruses are of medical importance in Europe. TBEV infects over 10,000 humans each year (Dobler, 2010). Louping-ill virus (LIV) is a disease with zoonotic potential found to infect sheep (M'Fadyean, 1894), is most common on the British islands, but is also found in Ireland, Norway, Spain, Turkey, and Bulgaria (Dobler, 2010). Omsk hemorrhagic fever virus (OHFV) is found in Russia and can cause hemorrhagic diseases in rodents and humans (Lvov, 1988). Other tick-borne flaviviruses have also been found in Europe, but the pathogenicity of these viruses is relatively unknown (Beck et al, 2013). An example of these viruses is the Meaban virus (MEAV) carried by sea birds (Chastel et al, 1985) and the Tyuleniy virus (TYUV) also carried by sea birds. There have been three suspected human cases of TYUV infections (Hubálek & Halouzka, 1996).

The mosquito-borne flaviviruses WNV and Usutu virus (USUV) can be found throughout Europe. Among the mosquito-borne flaviviruses WNV has the most widespread geographical range and hosts (Weissenböck et al, 2010). WNV can infects and be pathogenic to both birds and mammals (Bakonyi et al, 2013; Bakonyi et al, 2006; Malkinson et al, 2002; Murray et al, 2010; Papa et al, 2010). In 2012 alarming outbreaks of WNV occurred in Several countries in Europe. In Grece 261 confirmed human cases were reported and 34 deaths occurred (Papa et al, 2010). In Romania 57 cases and five deaths were reported (Sirbu et al, 2011). In Russia 480 cases and six deaths were reported (Onishchenko et al, 2011). Also, Italy have had several outbreaks of WNV 2008 and 2010, caused by genetically divergent isolated of the virus (Calistri et al, 2010; Calzolari et al, 2013).

USUV is a virus closely related to WNV. It was most likely brought into Europe from Africa by migrating sea birds. The virus was first noticed in 1996 as I caused multiple bird deaths in

Italy (Weissenböck et al, 2013). In contrast to WNV, USUV rarely causes dangerous neurological symptoms in humans (Meister et al, 2008; Weissenböck et al, 2013).

1.4.4.2. Mitigation means and vaccines.

There is a vaccine against TBE which is recommended to be used by people who spend a loat of time outdoors in the forest or other risk areas. If you have been outside in the forest it is important to check your clothes and body for ticks afterwards and remove any ticks if found (Folkhälsomyndigheten, 2018b). There are also other measures one could take, such as using insect repellent and wearing protective clothing with the long pants tucked into the socks or the boots (ecdc, 2015).

There are no vaccines to prevent WNV infections, therefore the best way to prevent infection is to protect oneself from mosquito bites. This could be done by using insect repellents, cover yourself with clothes, using insect nets and prevent the mosquitoes from accessing indoor space. If there is still water outside in your yard, the water may be changed once a week to prevent the mosquito eggs from hatching (CDC, 2020).

2. Material and Methods

2.1. Field study

Bats were trapped at nine localities in in Skåne (see table 2) during 13 July to 24 July 2020. During the day potential catching sites were identified. We searched for shallow streams with a bridge to place the net, and with little or floating vegetation. Because bats releases ultrasound for echolocation when flying, a heterodyne, which will pick up the inaudible ultrasound and filter it with an ultrasonic signal to generate an audible sound of lower frequency audible to the human ear (Ahlén, 2004; Bat Conservation Trust, 2020a), was used to check the sites for bat activity during the evening.

Bats were trapped using mist nets mounted perpendicular to small streams, starting at sunset roughly until midnight. When a bat was caught it was immediately disentangled from the net and kept in a cotton bag, inside a field workers shirt, until the time of sampling to be kept warm. First the bat was checked for ectoparasites; wings and ears were checked for mites and the fur was blown on to collect mites. All ectoparasites found on the same individual were stored in the same tube at -20ºC. Then following information of the individual bat was recorded: species, age (adult or juvenile), weight (g) and the forearm length (mm). Feces was collected from the cotton bag or directly from the bat. The feces was covered with RNAlater, which is an aqueous, nontoxic solution reagent used to store tissues which rapidly imbue the tissue to protect and stabilize the cellular RNA (Thermo-Fisher-Scientific, 2020) and allows the samples to be stored in room temperature without damaging the cellular RNA (Bachoon et al, 2001; Grotzer et al, 2000; Mutter et al, 2004).The fecal samples were first kept on ice, then stored at a -20ºC freezer during transport to the lab and then stored at a -80ºC freezer until analysis. The bat was swabbed to collect saliva and the saliva sample were kept on dry ice until stored at a -20ºC freezer during transport to lab and then stored at -80ºC freezer until analysis. A small amount of hair (less than 5 mg) was collected from the interscapular region of the bats' back. The hair was stored in a small plastic tube, kept at ambient temperature for isotopic analysis. Lastly, a 100 µl blood sample was taken from the tail membrane-vein and

stored dry on a Nobuto blood filter, a paper strip used to collect dried blood spots (DBS) samples (Nobuto, 1963), at room temperature until further processing (Dusek et al, 2011).

2.2. Laboratory work

The saliva, DBS and fecal samples was processed and analyzed, at Umeå University in Sweden at the Department of Clinical Microbiology, Virology, as described in this section.

2.2.1. Saliva

Saliva samples are pure enough for the RNA to be extracted without any previous preparations.

2.2.1.1. RNA Extraction

The QIAamp viral RNA Mini was used for the RNA extraction and the procedure was done following the manufacturer's instructions. 310 µL buffer AVE was added to 310 µg lyophilized carrier RNA, creating a solution of 1 µL/µg. Then the amount of AVL needed for *n* samples was calculated with the formula: *n * 0.56 mL = y mL.*
Then, the amount of RNA-buffer AVE which should be added to the y mL AVL was calculated with the formula: *y mL * 10 µL/mL = z µL.*
Then the AW1 and AW2 buffer was prepared by adding the amount of ethanol described on the bottles.

With the preparations done, in the first step 560 µL of the buffered AVL containing carrier RNA AVE buffer was added to a 1.5 microcentrifuge tube. In step two, 140 µL of the saliva was added to the tube in an infectious room. The tube was pulse-vortexed for 15 s for efficient lysis and then the samples were incubated for 10 minutes at room temperature for the viral particle lysis to complete. Step three, after the lysis, the tubes were centrifuged at 8000 rpm for 1 min. Step four, 560 µL of 96% ethanol was added and mixed by pulse-vortex for 15 s. After mixing the tubes were centrifuged again at 8000 rpm for 1 min. Step five, 630 µL of the solution in the tube was transferred to a QIAamp column placed in a 2 mL collection tube, and then it was centrifuged at 8000 rpm for 1 min. After centrifugation, the QIAamp mini column was placed into a clean collection tube and the tube containing the filtrate was discarded. Step five was repeated until all of the lysate in the microcentrifuge tube was emptied. Step six, 500 µL buffer AW1 was added and then the tubes were centrifuged at 8000 rpm for 1 min. The QIAamp mini column was then placed in a clean collection tube and the used one was discarded. Step seven, 500 µL buffer AW2 was added and then the tube was centrifuged at 14 000 rpm for 3 min. Afterwards it was centrifuged again at 8000 rpm for 1 min. Step eight, the QIAamp mini column was placed in a clean 1.5 mL microcentrifuge tube. Lastly step nine, 60 µL buffer AVE was added and then it was centrifuged at 8000 rpm for 1 min. The AVE brings the RNA with it as it passed the QIAamp mini column down to the microcentrifuge tube, thus the QIAamp mini column can be discarded. The microcentrifuge tube was then labeled and stored at -80*C until further analysis.

2.2.2. Dried Blood Spots (DBS)

The DBS was first eluted as described in section 2.2.2.2. and then RNA could be extracted from the eluted samples as described in section 2.2.1.1. above. The RNA was used to test for PUUV and Lyssavirus, but cDNA is required to test for flavivirus. Thus, as described in section 2.2.2.2. the RNA was turned into cDNA.

2.2.2.1. Elution

The DBS was cut with a scissor and put in an Eppendorf tube. Then 500 µL phosphate buffered saline containing 0.05% Tween and 0.08% sodium azide was added. The DBS was left to elute on a laboratory shaker overnight. The next morning the elution was pipetted into microcentrifuge tubes and centrifuged for 2 min at 10 500 rcf to free the supernatants from debris which may have formed during the elution. The dried blood samples were stored in -80°C until the RNA extraction (see section 2.2.1.1.).

2.2.2.2. cDNA

The "Revert Aid kit" by Thermo scientific Vilnius Lithuania was used to convert RNA to cDNA. The Master mix was prepared by adding the following amount for each sample, 9 µL nucleus-free water, 4 µL 5x reaction buffer, 2 µL dNTP mix, 1 µL Random hexamer primer, 1 µL Roblock RNase and 1 µL Revert Aid RT. The Master mix was spun to be mixed evenly. 18 µL of the master mix and 2 µL sample was put into each of the wells on a 0.1 mL 96 well optical well plate. The plate was sealed with an optical well plate cover and centrifuged at 1000 rpm for 2 min. After spinning the plate was put through a PCR. The hold stage consisted of 25°C for 5 min. The PCR stage consisted of 42°C for 60 minutes. The melt curve stage consisted of 70°C for 5 minutes and then the PCR finished with 4°C for ∞. The cDNA was stored at -80°C until further use.

2.2.3. Feces

1.5 mL Eppendorf tubes were prepared with three crushing beads and filled with 500 IU penicillin per mL, 500 µg streptomycin, 3 µg amphotericin B per mL and then the tubes were filled up with hanks buffer. The fecal samples were thawed on ice and the fecal pellets were put in the prepared tubes while avoid adding the RNA-later which the pellets were preserved in. Homogenization of the pellet was performed using *FastPreps 120 (Q-BIOgene, Irvine, CA, US)* at 6.5 m/s for 20 s. After Homogenization, the samples were briefly centrifuged. After the centrifugation, the samples were filtered using 0.2 µm sterile filters attaining a clear liquid. The samples were stored in -80°C until the RNA extraction (see section 2.2.1.1.).

2.3. Testing for Lyssavirus

The saliva and blood samples were used to test for the virus through qPCR as described in section 2.3.1. The blood samples were used to test for antibodies by Enzyme-Linked Immunosorbent Assay (ELISA) as described in 2.3.2. bellow.

2.3.1. qPCR RABV

The "ViroReal Kit Rabies Virus" by Ingenetix Vienna Austria was used to test for lyssavirus following the manufacturers instruction. The Master mix was prepared by adding the following amount for each sample, 2 µL nuclease-free water, 5 µL RNA reaction Mix, 1 µL Rabies Virus Assay Mix, 1 µL RNA IPC Assay Mix and 1 µL freshly diluted RNA IPC Target into a clean tube. The Master mix was then spun to be mixed evenly. 18 µL of the master mix was put into each of the wells on a 96 well optical well plate, then 2 µL sample was added. Nucleus free water was added to the negative controls instead of a sample. The negative controls were always added first to detect any contamination and the positive control was added last to decrease the risk of contamination when adding the samples. The plate was sealed with an optical well plate cover and centrifuged at 1000 rpm for 2 min. After spinning, the plate was run in the qPCR machine. The hold stage consisted of 50°C for 15 min, the PCR stage consisted of 95°C for 20 seconds, the melt curve stage consisted of 95°C for 5 seconds and then 60°C for 1 min, which was repeated through 45 cycles, the qPCR finished with 4°C for ∞.

2.3.2. ELISA

The "BioPro antibody rabies kit" by O.K. SERVIS BioPro, s.r.o. Czech Republic was used to test for antibodies following the manufacturers instruction. The control and samples were diluted according to the instructions with 60 µL of the control to 60 µL sample diluent, and 15 µL sample was diluted with 135 µL sample diluent. Then 100 µL of the samples and controls were dispensed into the wells of the 96-well ELISA plate provided by the kit. The positive control was added to A1 and B1, the negative control was added into A2 and B2, the serum controls provided by the samples was dispensed into A3, A4 and A5. Then the 77 bat blood samples were added into the remaining of the wells. The plate was then incubated overnight in a cold-room at 2-8 °C. The next step was the incubation with the biotinylated anti-rabies antibody. The biotinylated anti-Rabies antibody was prepared by adding 11 mL diluent for biotinylated antibody to110 µL concentrated biotinylated anti-Rabies antibody, thus creating a diluent of 1/100. The overnight incubated plate was washed six times with washing solution and then 100 µL of the diluted biotinylated anti-Rabies antibody was added to all the wells. The plate was then incubated at 37°C for 30 minutes. The third step was incubation with streptavidin peroxidase conjugate. The streptavidin peroxidase conjugate was diluted to 1/100. The wells were washed four times and then 100 µL of diluted streptavidin peroxidase conjugate was added to all the wells. The plate was then incubated in 37°C for 30 minutes. The wells were washed four times with washing solution and then 100 µL of TMB substrate was added to the wells. The plate was incubated for 20 minutes at room temperature (18 - 25°C) and the reaction was stopped by adding 50 µL of stop solution. A Tecan reader was used for measuring the OD at the wavelength 450 nm, the results were then calculated and interpreted according to manufacturer's instructions.

2.4. Analysis of Puumala orthohantavirus (PUUV)

The blood, saliva and fecal samples were tested for the virus with a qPCR (see section 2.4.1.) and some of the samples were sent in for sequencing at Swedish Defence Research Agency (FOI) (see section 2.4.2.). The blood samples were tested for antibodies by an immunofluorescent assay (IFA) (see section 2.4.3). After the IFA, the blood samples were

tested for antibodies using ELISA (see section 2.4.4.), since the ELISA is more sensitive than an IFA.

2.4.1. qPCR

The primers targeting the S-segment PuKzSF2: GAARTGGACCCGGATGACGTTAAC and PuKzSR2: CKGGACACAGCATCTGCCATTC (Byström et al, 2018) was used with the kit "2x qPCR SyGreen 1-Step Go Hi-Rox" by PCRBIOSYSTEMS to test the DBS, saliva and fecal samples for NE following the manufacturer's instructions. The Master mix was prepared by adding the following amount for each sample, 4.5 µL nuclease-free water, 10 µL 2x SyGreen 1-Step Go Hi-Rox mix, 0.8 µL 10 µM S-segment F primer and 0.8 µL 10 µM S-segment R primer and 1 µL 20x RTAse Go enzyme into a clean tube. The Master mix was then spun to be mixed evenly. 18 µL of the master mix was put into each of the wells on a 96 well optical well plate. Then 2 µL sample was added. For the negative controls nucleus free water was added instead of sample and the negative controls was added first. The positive control was added last. The plate was sealed with an optical well plate cover and centrifuged at 1000 rpm for 2 min. After spinning the plate was run in the qPCR. The hold stage consisted of 45°C for 10 min and 95°C for 2 minutes. The PCR stage consisted of 95°C for 5 seconds and 60°C for 25 seconds, which was repeated through 40 cycles. The melt curve stage consisted of 95°C °C for 15 seconds, 60°C for 1 minute, 95°C for 15 seconds and then the qPCR finished with 4°C for ∞.

The samples were then put through electrophoresis on a 3% standard agarose gel, using a low range DNA ladder and run on 60 V. This was done to control the results from the qPCR and see if there is any background.

2.4.2. Sequencing

The positive samples one, two, five, seven, 11 and 12 were purified using the "ExoSAP-IT kit" by Thermo Fisher Scientific, Vilnius, Lithuania, and sent in for sequencing. 10 µL sample and 4µL ExoSAP-IT was added to the wells and put through PCR, the program consisting of 37 degrees for 15 minutes followed by 80 degrees for 15 minutes. After the PCR, 5 µL of 5 pcM F-primer and R-primer, 95 µL nucleus free water and 5 µL PCR product was added to a 1.5 ml eppendorf tube. The tube was spun for even mixing and then labeled with a sequencing id before sent for sequencing at FOI.

2.4.3. IFA

The 25 µL of eluted DBS was added to a prepared glass coated with NE virus. The samples were then incubated for one hour in a moist chamber at 20ºC. After the incubation, unbound particles were washed away using two cups of PBS and then the glass was placed in a cuvette holder filled with PBS and left on a shaker for 10 minutes. The glass was dried lightly so that it is still moist, and then 25 µL of diluted conjugate 2:2000 was added to each sample (goat anti-bat antibody and a human antibody for the human positive control). The samples were incubated for one hour in a moist chamber at 37ºC. Lastly, the unbound particles were washed away using four cups and then left in a cuvette holder filled with PBS and then left on a shaker for 10 minutes. The finished glasses can be stored in a dark fridge until examination. One to two drops of glycerin were added to each sample before the glass was examined using

a fluorescent microscope, looking for a fluorescent signal indicating the presence of antibodies (Wang-Shick, 2017).

2.4.4. ELISA

The goat anti-Bat antibody was also used for an ELISA. A ready coated ELISA plate was washed with PBS tween, then incubated for 30 minutes with 25 µL milk. Five µL sample was added to 25 µL milk and was then incubated at 4ºC over night. The plate was washed with PBS tween the next morning. The conjugate (secondary antibody) was diluted to 1:1000 by adding 40 µL conjugate to 40 mL milk. Then 100 µL of the diluted conjugate was added to the wells. The plate was incubated at room temperature (20ºC) for one hour. The plate was washed with PBS tween and then 100 µL TMB was added to the wells and incubated at room temperature in the dark for 15 minutes. The reaction was stopped by adding 100 µL 1M sulfuric acid. If there are antibodies the conjugate would have bound to the antibodies, and the enzyme linked to the conjugate will produce a blue colored product when provided the substrate TMB (Shomu's Biology, 2015; Racaniello, 2010). Therefore, the result can be seen by the color change, a blue color indicates a positive sample, and no color indicates a negative sample.

2.5. Testing of Flavivirus

The cDNA created from the extracted DBS RNA was used to test for flavivirus using the "Phusion Green Hot Start kit" by Thermo Scientific. The Flavivirus genus specific primers targeting the NS5 gene FU1: TACAACATGATGGGAAAGAGAGAGAA and CDF2: GTGTCCCAGCCGGCGGTGTCATCAGC (Bryant et al, 2005) was used. Whereof, the expected amplicon size is 276 bp. The Master mix was prepared by adding the following amount for each sample, 4.9 µL nuclease-free water, 10 µL 2x phusion Green hot start mix, 1.25 µL F primer and 1.25 µL R primer and 0.6 µL DMSO into a clean tube. The Master mix was then spun to be mixed evenly. 18 µL of the master mix was put into each of the wells on a 0.1 mL 96 well optical well plate. Then 2 µL sample was added. For the negative controls nucleus free water was added instead of sample and the negative controls was added first. The positive control was added last. The plate was sealed with an optical well plate cover and centrifuged at 1000 rpm for 2 minutes. After spinning the PCR was performed. The hold stage consisted of 98°C for 30 Seconds. The PCR stage consisted of 98°C for 7 seconds, 60°C for 15 seconds and 72°C for 20 seconds, which was repeated through 35 cycles. The melt curve stage consisted of 72°C for 7 minutes and then the PCR finished with 4°C for ∞.

After the PCR the samples was run on a 3% standard agarose gel using a low range DNA ladder and 60 V. The samples suspected to be positive were put through a re-PCR after purification. 1µL of the suspected positive PCR product sample was added to 9 µL nucleus free water, and then vortexed for even mixing. 2 µL of the DNA template and 18 µL of the master mix, described previously in this section, was added to the PCR plate. Samples that tested positive on the gel after the re-PCR were purified and sent for sequencing at FOI (see section 2.4.2.).

3. Results

A total of 77 bats were sampled from ten different locations, 74 Daubenton's bats, two soprano pipistrellus and one whiskered bat. Saliva and blood samples were collected from all 77 bats, whilst fecal samples were only collected from 75 bats since there were two Daubenton's bats which didn't leave any feces. Among the 77 bats caught, 76 were adults and only one juvenile was caught. 28 of the trapped bats were male and 49 are female. The number of bats caught at each location and the coordinates of the location is presented in table 2 bellow.

Table 2. Table of all the bats caught at the different locations.

		Coordinates		Species caught			Number of Male/female		Number of Adult/Juvelie	
Locality	Stream	Longitude	Latutude	M. daubetoni	P. pygmaeus	M. mystacinus	Male	Female	Adult	Juvelie
Tollarp, Malmvägen	Vramsån	13.975432	55.929812	16	1	0	1	16	16	1
Ängsbo, Haväng	Verkaån	014.15537	55.72065	7	0	0	1	6	7	0
Bosarp, easto of Brösarp	Verkaån	014.12410	55.72818	1	0	0	1	0	1	0
Allevadsmölla	Nybro ån	13.904590	55.505064	3	0	0	0	3	3	0
Rålambsdal	Vinne å	014.033311	56.086835	12	0	0	6	6	12	0
Röverkulans naturreservat	Bråån	13.497462	55.794195	6	0	0	6	0	6	0
Everöd	Mjöån	14.105448	55.892231	5	1	1	5	2	7	0
Tobisviks camping, Simrishamn	Tommarpa ån	14.335166	55.570009	17	0	0	5	12	17	0
Vinslöv, south of the pond	Vinne å	13.908735	56.108372	6	0	0	3	3	6	0

3.1. Lyssavirus

The saliva and blood samples were tested for lyssavirus using the *ViroReal Kit Rabies Virus Kit*. All the 77 saliva samples were negative. However, for the blood sample 76 tested negative and one sample tested positive for lyssavirus. The positive sample belongs to an adult male Daubenton's bat caught at Tobisviks camping, Simrishamn. The BioPro Rabies ELISA Ab Kit was used to test for antibodies in the DBS samples. All 77 samples came out negative. The results of the qPCR and the ELISA are presented in table 3 and table 4 bellow.

Table 3. Results of the qPCR analysis for lyssavirus.

qPCR: tested positive for Lyssavirus							
	Species			Sex		Age	
Locality	M. daubetoni	P. pygmaeus	M. mystacinus	Male	Female	Adult	Juvenile
Tollarp, Malmvägen	0	0	0	0	0	0	0
Ängsbo, Haväng	0	0	0	0	0	0	0
Bosarp, easto of Brösarp	0	0	0	0	0	0	0
Allevadsmölla	0	0	0	0	0	0	0
Rålambsdal	0	0	0	0	0	0	0
Röverkulans naturreservat	0	0	0	0	0	0	0
Everöd	0	0	0	0	0	0	0
Tobisviks camping, Simrishamn	1	0	0	1	0	1	0
Vinslöv, south of the pond	0	0	0	0	0	0	0

Table 4. Results of the ELISA testing for rabies antibodies.

ELISA: tested positive for rabies antibodies							
	Species			Sex		Age	
Locality	M. daubetoni	P. pygmaeus	M. mystacinus	Male	Female	Adult	Juvenile
Tollarp, Malmvägen	0	0	0	0	0	0	0
Ängsbo, Haväng	0	0	0	0	0	0	0
Bosarp, easto of Brösarp	0	0	0	0	0	0	0
Allevadsmölla	0	0	0	0	0	0	0
Rålambsdal	0	0	0	0	0	0	0
Röverkulans naturreservat	0	0	0	0	0	0	0
Everöd	0	0	0	0	0	0	0
Tobisviks camping, Simrishamn	0	0	0	0	0	0	0
Vinslöv, south of the pond	0	0	0	0	0	0	0

3.2. PUUV

The saliva, blood and fecal samples were tested for the virus PUUV by qPCR using the *2x qPCR SyGreen 1-Step Go Hi-Rox* kit, then the suspected samples were sent in for sequencing at FOI. There were no bat samples found positive for PUUV. A IFA and an ELISA was used to test for PUUV specific antibodies, but no samples tested positive. The results are seen in table 5 to 7 bellow.

Table 5. Sequencing results for PUUV using twist sequencing.

Sequencing results for PUUV							
	Species			Sex		Age	
Locality	M. daubetoni	P. pygmaeus	M. mystacinus	Male	Female	Adult	Juvenile
Tollarp, Malmvägen	0	0	0	0	0	0	0
Ängsbo, Haväng	0	0	0	0	0	0	0
Bosarp, easto of Brösarp	0	0	0	0	0	0	0
Allevadsmölla	0	0	0	0	0	0	0
Rålambsdal	0	0	0	0	0	0	0
Röverkulans naturreservat	0	0	0	0	0	0	0
Everöd	0	0	0	0	0	0	0
Tobisviks camping, Simrishamn	0	0	0	0	0	0	0
Vinslöv, south of the pond	0	0	0	0	0	0	0

Table 6. IFA result for PUUV.

IFA: tested positive for PUUV antibodies							
	Species			Sex		Age	
Locality	M. daubetoni	P. pygmaeus	M. mystacinus	Male	Female	Adult	Juvenile
Tollarp, Malmvägen	0	0	0	0	0	0	0
Ängsbo, Haväng	0	0	0	0	0	0	0
Bosarp, easto of Brösarp	0	0	0	0	0	0	0
Allevadsmölla	0	0	0	0	0	0	0
Rålambsdal	0	0	0	0	0	0	0
Röverkulans naturreservat	0	0	0	0	0	0	0
Everöd	0	0	0	0	0	0	0
Tobisviks camping, Simrishamn	0	0	0	0	0	0	0
Vinslöv, south of the pond	0	0	0	0	0	0	0

Table 7. ELISA result for PUUV.

ELISA: tested positive for PUUV antibodies							
	Species			Sex		Age	
Locality	M. daubetoni	P. pygmaeus	M. mystacinus	Male	Female	Adult	Juvenile
Tollarp, Malmvägen	0	0	0	0	0	0	0
Ängsbo, Haväng	0	0	0	0	0	0	0
Bosarp, easto of Brösarp	0	0	0	0	0	0	0
Allevadsmölla	0	0	0	0	0	0	0
Rålambsdal	0	0	0	0	0	0	0
Röverkulans naturreservat	0	0	0	0	0	0	0
Everöd	0	0	0	0	0	0	0
Tobisviks camping, Simrishamn	0	0	0	0	0	0	0
Vinslöv, south of the pond	0	0	0	0	0	0	0

3.3. Flavivirus

cDNA extracted from blood samples were tested for flavivirus using the *Phusion Green Hot Start kit* with genus specific primers. Out 77 samples eight samples tested positive for flavivirus and all of the bats were adult Daubenton's bats. Whereof, two of the bats are male and six of the bats are female. The bats were caught at four different locations in Skåne, which is presented in table 8 bellow.

Table 8. PCR results of Flavivirus test.

PCR: tested positive for Flavivirus							
	Species			Sex		Age	
Locality	M. daubetoni	P. pygmaeus	M. mystacinus	Male	Female	Adult	Juvenile
Tollarp, Malmvägen	2	0	0	0	2	2	0
Ängsbo, Haväng	0	0	0	0	0	0	0
Bosarp, easto of Brösarp	0	0	0	0	0	0	0
Allevadsmölla	0	0	0	0	0	0	0
Rålambsdal	2	0	0	0	2	2	0
Röverkulans naturreservat	0	0	0	0	0	0	0
Everöd	0	0	0	0	0	0	0
Tobisviks camping, Simrishamn	1	0	0	1	0	1	0
Vinslöv, south of the pond	3	0	0	1	2	3	0

4. Discussion

This is one of the few virus studies on Swedish bats. Here the meaning of the results from this study will be discussed and compared to other studies, especially studies in Europe.

4.1. Lyssavirus

In this study one Daubenton's bat tested positive for the lyssavirus virus using qPCR. This is the first time in Sweden where any bat has tested positive for the actual virus, as previously only antibodies towards a lyssavirus (EBLV-2) has been found (Hammarin et al, 2016). However, it should be noted that in this study we tested both the blood and saliva for lyssavirus and only the blood samples tested positive, whilst Hammarin et al, only tested the saliva for virus. However, in this study, there were no rabies specific antibodies found in any of the bats. In Europe there have been studies in several countries where Daubenton's bats

have tested positive for the both EBLV-2 specific antibodies and the actual virus (Brookes et al, 2005; Ceballos et al, 2013; Fooks et al, 2003; Freuling et al, 2008; Hammarin et al, 2016; Jakava-Viljanen et al, 2010; Moldal et al, 2017; Nokireki et al, 2017; Van der Poel et al, 2005; Whitby et al, 2000). This study confirms Swedish bats carry lyssavirus.

In this study we used the *BioPro Rabies ELISA Ab Kit* to test eluted DBS samples for antibodies. It is possible that this kit is not compatible with eluted DBS samples as the concentration of the samples may be too low. According to the manufacturers instruction tissue samples obtained from the hearth and blood vessels should be used. The *BioPro Rabies ELISA Ab Kit* ability to detect the antibodies using DBS should be tested by testing and comparing different concentrations of the tissue samples and the DBS samples from the same individuals.

From the human health perspective, it is nothing new that bats in Europe, including Sweden, host lyssavirus. The highest risk group of contracting rabies from bats are people who work with bats. People who handle bats in their profession should always be vaccinated and wear gloves when handling bats to mitigate the transmission risk. The transmission risk between bats and normal civilians is low. To mitigate the transmission risk people should be advised to keep their distance. If handling a bat is necessary the handler should wear gloves, also vaccination against rabies is recommended. For people having a bat colony in their home, or close to their home, it is best to keep away from that area for the time the colony has settled, and more importantly keep the cat or the dog away from the bats, especially in nursing colonies where the young bats are learning to fly. To remove the colony from the house an exemption from the species protection ordinance is required, but preventing the bats from moving to other parts of the house doesn't require an exemption (Naturvårdsverket, 2020). Furthermore, evicting bats from houses is not only a threat to the bats but also to human health as it may increase the transmission risk to humans (Amman et al, 2014; Blackwood et al, 2013; Ellison et al, 2007; Olival, 2016; Streicker et al, 2013; Streicker et al, 2012), since the evicted bats will search for a new roost, thus moving around a larger area, and the old roost is likely be taken over by other bats (Ellison et al, 2007; Naturvårdsverket, 2020; Streicker et al, 2013). In short, it is best to leave the bats alone and keep a distance.

4.2. PUUV

The PUUV results for this study are unreliable. There were some samples who tested positive for PUUV during the qPCR, but when sequenced none of the samples were found PUUV positive. Also, there were no antibodies against PUUV found in any of the bats. There have been no previous studies of bats carrying PUUV, but bats have been found to host other hantaviruses (Witkowski et al, 2016; Xu et al, 2015; Zhang, 2014). In this study no hantavirus was found in any of the bats and further studies are required to determine if Swedish bats host PUUV or other hantaviruses.

Both IFA and an ELISA was performed to test for antibodies. However, because there were no positive bat samples, it is impossible to know if the tests worked or not. Since there is no positive bat control available, it is possible that the bat conjugate did not work. Another problem is the uncertainty of the DBS concentration which may have been too low for the test. However, because a human positive control was used, we know that there is nothing

wrong with the other reagents or the virus-coated plate and glass. It is also possible that the test worked, thus the bats do not carry any PUUV specific antibodies.

The bank vole is the known vector for PUUV in northern Sweden and people often become infected through exposure of infected dust in our homes (Ahlm, 2018; Vapalahti et al, 2003). Measures to prevent the inhalation of dust in suspected areas should be taken to mitigate the transmission risk of NE. This could be done by using masks when cleaning and clean using a wet cloth or a mop to avoid the swirling of dust. Also, raking of leaves and grass outside should be done after it has rained (Gherasim et al, 2015; PraktiskMedicin, 2020).

4.3. Flavivirus

In this study eight Daubenton's bats tested positive for flavivirus using qPCR, however, the type of virus has yet to be identified. In a previous study bats infected by TBE has been found in China (Lu et al, 2008). In Sweden ticks are known to carry TBE and it is possible that bats may be infected when bitten by ticks (Folkhälsomyndigheten, 2018b). Another notorious flavivirus in Europe is WNV, as there have been several outbreaks of WNV in humans since the 2000s (Calistri et al, 2010; Calzolari et al, 2013; Onishchenko et al, 2011; Papa et al, 2010; Sirbu et al, 2011). Earlier in previous studies, bats have been found carrying different types of WNV (Constantine, 1970; Hubálek & Halouzka, 1999; Paul et al, 1970). It is possible that the bats been infected by mosquitoes, as mosquitoes are the vector for WNV (CDC, 2020; Mackenzie et al, 2004). Thus, the flaviviruses found in the bats could be an indication that mosquitoes in Sweden carry some type of WNV, or it could be a result of the ticks carrying TBE and having bitten the bats. Therefore, a study examining if Swedish mosquitoes carry WNV may be of interest.

Bats may also spread the disease across a larger area by transporting TBE infected ticks (Hasle, 2013). It is already known that TBE exist in Sweden and people spending time outdoors in risky areas should protect themselves from ticks and TBE. There is an effective vaccine against TBE (Folkhälsomyndigheten, 2018b), however one could also use insect repellent and wear protective clothing with long pants tucked into the socks or boots (ecdc, 2015).

It is also possible that non-infected mosquitoes may become infected with WNV through biting a bat carrying WNV, thus it is possible that bats help spread WNV between European countries. However, bats also mitigate the spread of WNV by preying on the mosquitoes (Andrianaivoarivelo et al, 2006; Hubálek & Halouzka, 1999; Kemp et al, 2019). Furthermore, oneself can protect yourself against mosquitoes using insect repellents, cover yourself with clothes, using insect nets and prevent the mosquitoes from accessing the indoor space. Also, if there is still water outside in your yard, the water may be changed once a week to prevent the mosquito eggs from hatching (CDC, 2020). Furthermore, bats may help mitigate the disease spread by suppressing the mosquito population (Andrianaivoarivelo et al, 2006; Hubálek & Halouzka, 1999; Kemp et al, 2019).

4.4. Conservation

Bats are protected by Sweden's national species protection ordinance (SFS 2007:845), and by the Eurobat agreement. Sweden is therefore obliged to identify and protect important bat habitats, also bats are not to be deliberately killed, captured, or disturbed (Hutson et al, 2019;

SFS 2007:845) Today 12 out of Sweden's 19 bat species are found on the red-list, and the biggest threats towards bats are habitat loss and loss of foraging grounds or roosting sites (Jung & Threlfall, 2016; Mickleburgh et al, 2002). The transmission risk between human and bats are considered low, but people who learn about bats hosting zoonotic diseases may shift to a more negative attitude towards bats. Consequently, people who had been willing to put up bat boxes may become unwilling to do so, and people with bats in their house may try to evict the bats and not only increase the transmission risk to themselves, but also increase the spread of the zoonotic diseases (Amman et al, 2014; Blackwood et al, 2013; Ellison et al, 2007; Olival, 2016; Streicker et al, 2013; Streicker et al, 2012). Therefore, it is important to inform people of how to handle the risks and mitigate the disease spread.

5. Conclusions

Bats in Sweden were found to host flavivirus and lyssavirus, but further analysis is required to determine what type of virus. This study confirms bats in Sweden carry lyssavirus and as antibodies had been found in bats in a previous study, there were already indications that lyssavirus circulates among Swedish bats. In general, the transmission risk of lyssavirus between bats and humans is low as there is little contact. However, people who handle bats should always wear gloves and be vaccinated. No orthohantavirus was found in any of the bats and further studies are required to determine if Swedish bats host PUUV or other orthohantaviruses.

Bats that live close to humans may bring some potential risks to human health. However, evicting, or culling bats may increase the transmission risk of the zoonotic diseases bats host. Bats in Sweden are protected by law; thus, people should focus on measures to mitigate transmission risk without harming bats. Furthermore, bats provide ecosystem services benefiting human health, for example by preying on mosquitoes and hence suppressing other potential vector-borne diseases, or by functioning as an indicator of zoonotic diseases in mosquitoes or other creatures in their environment. Hence, bat conservation and public health may have a common goal.

6. References

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