Influenza A in Swedish harbour seals (*Phoca vitulina*)

– A serological study

Influensa A hos svenska knubbsälar (Phoca vitulina) – en serologisk studie

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Degree project/Independent project • 30 credits
Swedish University of Agricultural Sciences, SLU
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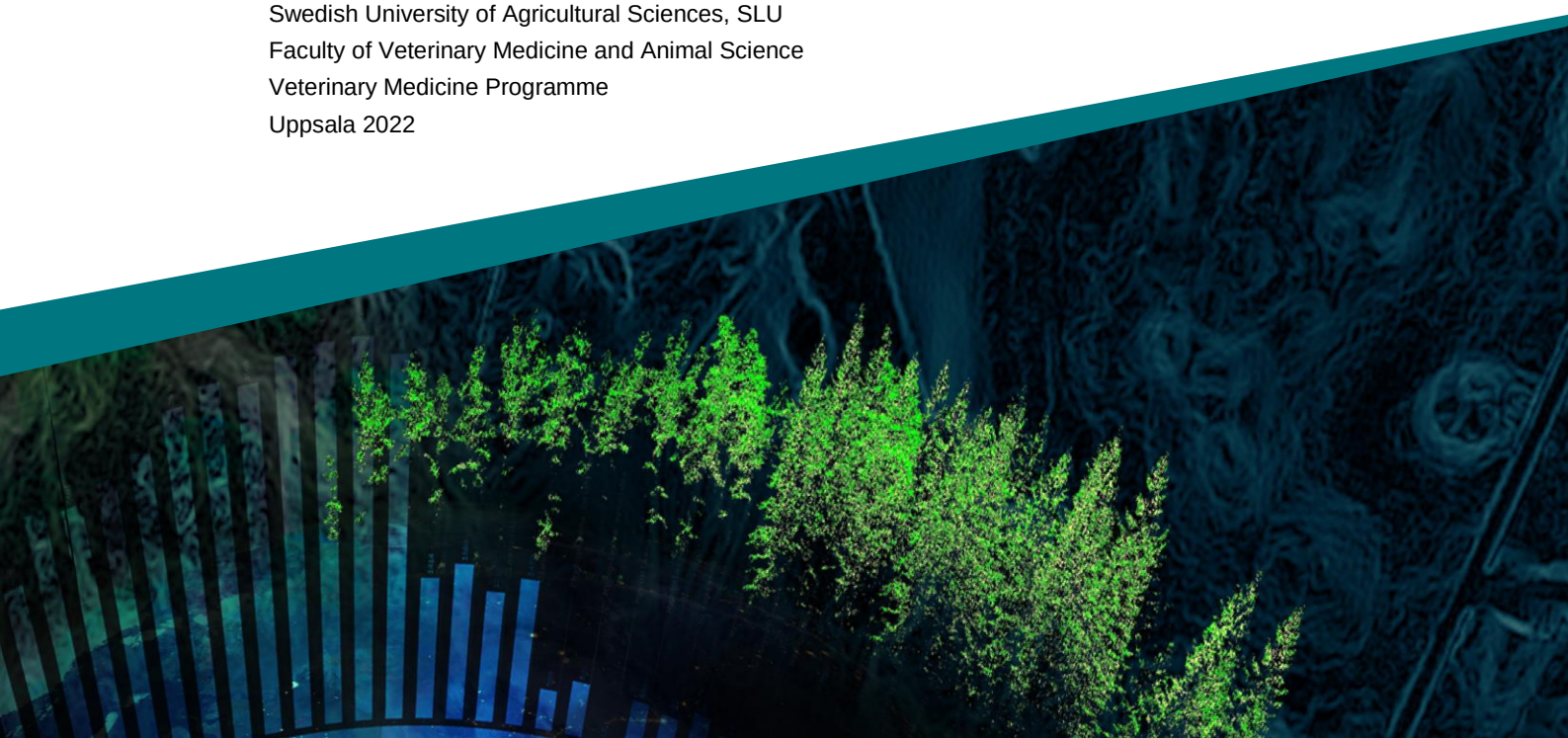
Veterinary Medicine Programme
Uppsala 2022



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Credits:	30 credits
Level:	A2E
Course title:	Independent project in Veterinary Medicine
Course code:	EX0869
Programme/education:	Veterinary Medicine Programme
Course coordinating dept:	Department of Clinical Sciences
Place of publication:	Uppsala
Year of publication:	2022
Cover picture:	
Keywords:	IAV, influenza A, harbour seals, H10N7, marine mammals, Phoca vitulina, serology, Sweden, virus

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Abstract

Evidence of infection with influenza A has been documented in several different marine mammals. However, large outbreaks with mass mortalities have only been seen in harbour seals. In 2014, the first outbreak with influenza A virus (IAV) in harbour seals in Europe was reported. The outbreak started in coastal waters off the Swedish west coast and spread to Denmark, Germany, and the Netherlands. More than 2 000 harbour seals died during the event.

The aim of this study was to do a follow-up on the outbreak with IAV in Swedish harbour seals in 2014. The overall purpose was to increase the knowledge about IAV infections in the Swedish harbour seal populations. More specific objectives included mapping circulation of specific subtypes and evaluating cadaver blood as a sample material instead of pure serum for IAV antibody detection and subtyping.

Serum samples were acquired from live-captured seals in 2014, and cadaver blood samples from hunted or found dead seals from 2014-2021. All samples were screened for anti-nucleoprotein antibodies using a commercial competitive ELISA. Positive and doubtful samples were confirmed and subtyped by hemagglutination inhibition assay (HI). In addition, information from sampled seals was gathered to draw relevant conclusions from test results.

The results show a high level of antibodies against IAV H10N7 in a large proportion of serum samples from 2014, with declining levels of antibodies against IAV in a lower proportion of cadaver blood samples from the west coast in 2015 and 2016. IAV antibodies were not found in samples from the Kalmarsund population or in the population off the west coast after 2016. Half of the ELISA positive or doubtful cadaver blood samples could be confirmed and subtyped by HI. Data from sampled seals made it possible to connect all ELISA positive samples to exposure to the outbreak in 2014. Evidence of circulation of other IAV subtypes was not found.

This study indicates that IAVs do not circulate and persist within the Swedish harbour seal populations, and that the populations most likely are naive in the case of a possible new spillover event. Environmental contaminants, low genetic variation, larger populations, and smaller habitats are identified as important risk factors for increased incidence of disease and spread of infection in a potential new outbreak. Cadaver blood is an accessible sample which according to this study can be useful in IAV surveillance on a population level to determine whether a population has been exposed to the IAV or not. It can therefore be an important tool for understanding disease dynamics, disease control and possibly prevention of pandemic influenza.

Keywords: IAV, influenza A, harbour seals, H10N7, marine mammals, Phoca vitulina, serology, Sweden, virus

Sammanfattning

Hos flera olika arter av marina däggdjur har evidens för infektion med influensa A virus (IAV) dokumenterats, men stora utbrott med hög dödlighet har endast rapporterats hos knubbsälar. Det första utbrottet med IAV hos knubbsälar i Europa inträffade 2014. Utbrottet startade utanför den svenska västkusten och spred sig vidare till Danmark, Tyskland och Nederländerna. Uppskattningsvis dog fler än 2 000 sälar under utbrottet.

Syftet med den här studien var att göra en uppföljning av utbrottet med IAV hos svenska knubbsälar 2014. Den övergripande målsättningen var att bidra till en ökad kunskap om IAV-infektioner hos svenska knubbsälar. Mer specifikt syftade studien till att öka kunskapen om vilka H-subtyper av IAV som svenska knubbsälar har exponerats för, samt att utvärdera användningen av kadaverblod i stället för rent serum för påvisande av IAV antikroppar hos knubbsäl.

Serumprover samlades in under en hälsokontroll av knubbsälar på västkusten 2014. Kadaverblodsprover samlades in från jagade och påträffade döda knubbsälar under åren 2014–2021. Initialt gjordes en screening av samtliga prover med en multispecies ELISA som detekterar antikroppar mot virusets nukleoprotein. Positiva prover bekräftades och subtypades därefter med hemagglutinations inhibitions test (HI). Provsvar sammanställdes därefter med tillhörande metadata för att kunna dra relevanta slutsatser.

Analysresultatet visade på en hög andel antikroppar mot IAV H10N7 i merparten av serumproverna från 2014, med en minskande andel antikroppar i kadaverblodsproverna från 2015 och 2016. Antikroppar hittades inte i prover från Kalmarsundspopulationen eller i prover från västkusten insamlade efter 2016. Hälften av de ELISA-positiva kadaverblodsproverna kunde bekräftas och subtypas med HI. Metadata från provtagna sälar gjorde det möjligt att relatera samtliga prover med antikroppar till exponering för utbrottet med H10N7 2014. Resultaten visade att det inte förekom andra subtyper av IAV.

Studien indikerar att IAV inte cirkulerar hos svenska knubbsälar, vilket även innebär att de svenska knubbsälspopulationerna är naiva och potentiellt mottagliga om IAV skulle introduceras på nytt. Miljöföroreningar, låg genetisk variation, stora populationer och mindre habitat identifieras som riskfaktorer som kan bidra till en ökad sjukdomsförekomst och smittspridning vid ett nytt potentiellt utbrott. Kadaverblod är ett tillgängligt prov som enligt den här studien kan användas för att övervaka IAV hos knubbsälar på en populationsnivå. Kadaverblod kan därför fungera som ett viktigt verktyg för att öka förståelsen kring sjukdomsdynamik, sjukdomskontroll och för att förebygga utbrott med nya IAV.

Nyckelord: IAV, influensa A, H10N7, knubbsäl, marina däggdjur, Phoca vitulina, serologi, Sverige, virus

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Abbreviations

AGID	Agar gel immunodiffusion assay
IAV	Influenza A virus
cELISA	Competitive enzyme-linked immunosorbent assay
ELISA	Enzyme-linked immunosorbent assay
HA	Hemagglutinin
HA	Hemagglutination assay
HAU	Hemagglutination titre units
HI	Hemagglutination inhibition assay
NA	Neuraminidase
NI	Neuraminidase inhibition assay
NP-ELISA	Nucleoprotein enzyme-linked immunosorbent assay
NRM	The Swedish Museum of Natural History
PBS	Phosphate-buffered saline
PCB	Polychlorinated biphenyls
PCR	Polymerase chain reaction
PDV	Phocine distemper virus
rRT-PCR	Real-time reverse transcription polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
SVA	The National Veterinary Institute, Sweden
US	United States
VN	Virus neutralization

1. Introduction

Influenza A viruses (IAVs) are known to cause influenza in a wide range of wild and domestic species of birds and mammals, including humans (Reperant *et al.* 2009). The virus has been detected in marine mammals on several occasions and has at times caused large outbreaks of influenza in harbour seals (Fereidouni *et al.* 2016).

Previously, outbreaks of IAV in harbour seals have been documented in North America (Lang *et al.* 1981; Hinshaw *et al.* 1984; Callan *et al.* 1995; Anthony *et al.* 2012). In 2014 the first known outbreak in Europe with IAV (H10N7) in harbour seals occurred (Zohari *et al*. 2014; Bodewes *et al.* 2015a; b; Krog *et al.* 2015). The outbreak started off the west coast of Sweden and spread to Denmark, Germany, and the Netherlands. An increased number of dead harbour seals was seen during the outbreak, and the most common finding during post-mortem examinations was respiratory tract infection and concurrent bacterial pneumonia.

Wild aquatic birds are known to act as natural reservoirs for IAV, and the viruses documented in harbour seals are of avian origin (Webster *et al.* 1981b; Hinshaw *et al*. 1984; Anthony *et al*. 2012; Bodewes *et al.* 2015a). The fact that viruses of avian origin can spillover and infect marine mammals raises the concern that these viruses may pose a threat to the health of other mammal species, including humans. In fact, the transmission of IAV from seals to humans has been documented, causing conjunctivitis (Webster *et al.* 1981a).

Serological studies can be used to map the exposure of pathogens such as IAVs in a population by detecting and subtyping antibodies in the host. Serology can therefore be used as an important tool for surveillance of IAV in the marine environment. However, serum samples are challenging to obtain from wildlife, whereas cadaver blood can more readily be acquired from hunted or found dead animals.

This study aimed to do a follow-up on the outbreak with H10N7 in Swedish harbour seals. The overall purpose was to increase the knowledge about IAV infections in the Swedish harbour seal populations. More specific objectives included mapping the circulation of specific subtypes and evaluating cadaver blood as a sample material instead of pure serum for antibody detection and subtyping.

2. Literature review

This literature review describes influenza A, the environment in which seals are exposed to IAV, the Swedish seal populations with a specific focus on the harbour seal populations, serologic testing of IAV and previously documented detections of IAV in harbour seals and other marine mammals.

2.1. Influenza A

To begin with, influenza A viruses are described regarding classification, structure and genome, followed by transmission.

2.1.1. Etiology

Influenza viruses are part of the *Orthomyxoviridae* family and form four out of seven genera within this family, each influenza genus is represented by one member; influenza A virus (IAV) represents the genus *Alphainfluenzavirus* and infects humans, other mammals, and birds. The genus *Betainfluenzavirus* consists of influenza B virus and infects humans and seals. Influenza C virus is the only member of genus *Gammainfluenzavirus* and infects humans, pigs, and dogs. Recently described influenza D virus is a member of the genus *Deltainfluenzavirus* and infects pigs and cattle. The differentiation between different genus of influenza is made based on two proteins, the nucleoprotein (NP) and the matrix protein 1 (M1) (Webster *et al.* 1992). Influenza A viruses are further divided into subtypes based on their two major antigens, the glycoproteins hemagglutinin (HA) and neuraminidase (NA). So far, eighteen different HAs and eleven different NAs have been identified. Subtypes H1-H16 and N1-N9 are all found in aquatic birds (Webster *et al.* 1992), whereas H17-H18 and N10-N11 have been isolated from bats in South America (Tong *et al.* 2012, 2013).

Webster *et al.* (1992) described the structure of IAV in their review on the evolution and ecology of influenza A viruses. Starting from the outside, IAVs are structured with a host-derived lipid bilayer membrane, also called the envelope. The envelope is acquired through viral budding when new viruses exit a host cell. The glycoproteins HA and NA are positioned like spikes within the envelope, together with matrix protein 2 (M2). Beneath the envelope, there is a shell of matrix

protein (M1) with a nucleocapsid, consisting of the genome protected by the NP at the centre.

The genome of IAV has been described by many reviews such as Webster *et al.* (1992), Brown (2000) and Tsai & Chen (2011). It consists of eight negative-stranded RNA segments, with each segment coding for at least one protein. The whole genome codes for at least ten different proteins with various functions. Segment 1-3 codes for three proteins: basic polymerase protein 2 (PB2), basic polymerase protein 1 (PB1) and the acidic polymerase protein (PA). These proteins are subunits of the RNA polymerase complex and are involved in the transcription and replication of the viral RNA. The HA glycoprotein is encoded by segment 4 and has several functions; it binds to sialic acid (SA) on the target cells and thus is important for viral attachment and virus entry. HA glycoproteins differ in their affinity for different sialic acid linkages and therefore partly determine the host range of the virus. As an antigen, HA is also targeted by host immunity. Segment 5 codes for the nucleoprotein (NP) which encapsulates the viral genome and aids in transcription. Segment 6 codes for the NA glycoprotein, which cleaves sialic acid and thus is involved in the release of viruses from an infected cell. Like HA, it is also targeted by host immunity. Segment 7 codes for matrix proteins M1 and M2, with the latter working as an ion channel in the envelope aiding in virion uncoating. Segment 8 encodes for the immune modulatory protein non-structural protein 1 (NS1) and nuclear-exporting protein (NEP, also known as NS2 proteins). Some IAVs also codes for an eleventh protein in their second segment, the FB1-F2, an apoptosis-inducing protein (Chen *et al.* 2004). In addition to PB1-F2, several new proteins encoded by different gene segments of IAVs have recently been identified. These additional non-structural proteins, including PB1-N40 (Wise *et al.* 2009) PA-X (Jagger *et al.* 2012), PA-N155 and PA-N182 (Muramoto *et al.* 2013), M42 (Wise *et al.* 2012) and NS3 (Selman *et al.* 2012), are generated either by the splicing, frameshift or truncation of the coding region of the correspondent structural proteins. The exact function of these novel discovered proteins is not entirely understood. Some of these proteins are thought to contribute to the diverse virulence and pathogenicity of IAV strains and are suspected to play roles in the adaptation of influenza to mammalian hosts.

Influenza A viruses are known to have a strong ability to evolve (Webster *et al.* 1992; Brown 2000; Tsai & Chen 2011). Genetic changes arise from mutations and reassortment. Point mutations give rise to antigenic drift and reassortment to antigenic shift. Mutations occur since the proteins involved in transcription are prone to making errors and because the virus lacks a proof-reading mechanism. Reassortment is possible due to the segmented genome, permitting the exchange of whole segments when at least two different IAVs infect the same cell. The capability for genetic evolution by antigenic drift allows the virus to escape the adaptive immune system, and antigenic shift gives rise to new subtypes.

2.1.2. Transmission

Influenza A viruses have been isolated from different species including birds, pigs, horses, minks, seals, whales, bats and humans (Webster *et al.* 1992; Tong *et al.* 2012; 2013). All influenza viruses in these hosts are derived from viruses circulating in natural reservoirs. There has long been a strong consensus that wild aquatic birds from the orders Anseriformes (ducks, swans, geese) and Charadriiformes (shorebirds, gulls, auks) are the natural reservoirs for influenza A viruses (Webster *et al.* 1992; Runstadler *et al.* 2013). More recently, evidence for bats as a possible natural reservoir has also been presented (Tong *et al.* 2012, 2013).

In birds, IAVs mainly replicate in cells lining the intestinal tract and are transmitted by the faecal-oral route (Reperant *et al.* 2009). In mammals, replication occurs primarily in the respiratory tract, and IAVs are therefore transmitted mainly by the respiratory route. Natural infection of mammals by the transmission of viruses from birds could potentially happen through direct contact (e.g., by predation of infected birds), indirect contact (e.g., through an environment contaminated by bird faeces, including water or food) or through airborne transmission (e.g., inhalation of droplets or aerosols). However, not all IAVs possess the ability to be transmitted by airborne transmission.

Receptor binding specificity of the HA antigen has been put forward as one of the key factors for host range and tissue and cell tropism of IAVs (de Graaf & Fouchier 2014). For an IAV to replicate, it needs to enter a host cell. This starts with the HA protein binding to sialic acid receptors (SA) on a cell. It has been shown that avian and human influenza A viruses have different receptor specificities of HA; avian influenza viruses preferably bind to SA linked to galactose in a 2,3 linkage (α2,3-SA), whereas human influenza A viruses prefers a 2,6 linkage between SA and galactose (α2,6-SA). Thus, depending on which receptor is present on the cell membrane, an IAV might or might not have the ability to attach to and enter the cell. Mutations in the HA glycoprotein may lead to a different receptor specificity (Herfst *et al.* 2020), possibly increasing the risk for spillover.

Studies to map receptor distribution in different species and target organs have been carried out. In humans, ferrets and pigs, both α2,6-SA and α2,3-SA have been found in different parts of the respiratory tract, and in chickens α2,6-SA and α2,3-SA have been found in both the respiratory and intestinal tract (de Graaf & Fouchier 2014). A few studies have also investigated the distribution of receptors in marine mammals. Ito *et al.* (1999) presented evidence for the presence of α2,3-SA but not α2,6-SA in the lungs of a seal and a whale, which also corresponded to the receptor specificity of viruses isolated from these animals. Ramis *et al.* (2012) investigated the attachment of avian and human influenza viruses in four different species of marine mammals: harbour seals (*Phoca vitulina*), grey seals (*Halichoerus grypus*), harbour porpoises (*Phocoena phocoena*) and bottlenose dolphins (*Tursiops truncatus*). Harbour seals and grey seals showed a moderate attachment of avian

influenza A viruses in the trachea and bronchi, whereas a lack of attachment to avian influenza A virus was seen in harbour porpoises and bottlenose dolphins. This is partly consistent with outbreaks of IAVs in marine mammals, which have only been reported in harbour seals (Fereidouni *et al.* 2016). Since no major outbreaks with IAVs have been reported in grey seals, the authors concluded that viral attachment is an important, but not the only factor leading to natural infection (Ramis *et al.* 2012). The findings of Ito *et al.* (1999) and Ramis *et al.* (2012) also indicate that transmission by the respiratory route is most likely in marine mammals. However, the exact route of transmission to and between seals is not known.

2.2. Bird-seal interactions in the shoreline habitat

Seals live in the aquatic-terrestrial interface and largely share their habitat with birds, both on land and at sea. Aquatic birds are considered to be the natural reservoir for IAVs and waterfowl, gulls, shorebirds and seabirds are all known to harbour these viruses (Webster *et al.* 1992; Runstadler *et al.* 2013; Lang *et al.* 2016). Also, all viruses identified during previous outbreaks in harbour seals have been of avian origin (Webster *et al.* 1981b; Hinshaw *et al.* 1984; Callan *et al.* 1995; Anthony *et al.* 2012; Zohari *et al.* 2014; Bodewes *et al.* 2015a; Krog *et al.* 2015). Seals can be exposed to IAVs from birds in several ways in their habitat; direct or indirect contact as well as airborne transmission are theoretically possible (Reperant *et al.* 2009).

Birds are known to excrete viruses through their faeces, and some researchers such as Reperant *et al.* (2009) and Measure & Fouchier (2021) have suggested an indirect transmission through water contaminated with bird faeces. Studies of the ability of IAVs to persist in water have shown that under natural conditions, some influenza viruses can remain infectious for months (Brown *et al.* 2009). Viruses are most stable in slightly basic pH, low temperatures and in fresh to brackish salinities, indicating that some habitats and seasons are more favourable for virus maintenance and transmission (Brown *et al.* 2009).

Seals also spend time at haul-out sites (land or ice) where they aggregate to breed, moult and rest, and seabirds may also use the same areas for roosting. This kind of interaction between seals and Terns (*Sterna* sp.) has been suggested as a possible explanation for direct or indirect transmission in previous outbreaks with IAV in harbour seals (Geraci *et al.* 1982).

Food is another possibility for direct or indirect transmission of IAV between seals and birds. Seals are carnivores and predate on aquatic birds such as seabirds (Lucas & Mclaren 1988) and ducks (Tallman & Sullivan 2004), both known to carry IAVs (Webster *et al.* 1992; Runstadler *et al.* 2013; Lang *et al.* 2016). Indirect transmission could also occur through contaminated food, for example, fish feeding on bird faeces. However, there is no clear evidence supporting this theory.

2.3. Pinnipeds in Sweden

Pinnipeds are a clade of aquatic mammals within the Order Carnivora. The clade consists of three extant families: Odobenidae (walruses), Otariidae (fur seals and sea lions) and Phocidae (true seals) (Uhen 2007).

Three different species of Phocidae live and breed in Sweden: harbour seal, grey seal, and ringed seal (*Pusa hispida*). According to the Swedish Species Information Centre at the Swedish University of Agricultural Sciences (SLU), all seal species in Sweden are classified as species of least concern except for a small population of harbour seals in the Baltic Sea (Kalmarsund population), classified as vulnerable (SLU Artdatabanken 2020). This population is isolated and genetically different from the harbour seal population off the west coast (Goodman 1998).

Every year the Swedish Museum of Natural History (NRM) surveys the harbour seal population during the moulting period in August, counting individuals at haul-out sites. For harbour seals, a mean value of two to three counts is reported and it is estimated that 60-80% of the total populations are counted during the survey[1]. In 2020, the hauled-out population were 4703 in Skagerrak and 8023 in Kattegat (ICES 2021). The previous year (2019) 7300 individuals were counted in Skagerrak and 9900 individuals in Kattegat. The lower numbers seen in 2020 were thought to be due to the warm weather and local anthropogenic disturbances during the time of the survey. The population in Skagerrak and Kattegat is increasing, however the annual growth is levelling off compared to previous years, which may indicate that the population is approaching carrying capacity. The smaller population of harbour seals in the Baltic Sea was also surveyed in 2020, with a hauled-out population count of 2056 individuals. The population is increasing and has had an annual population growth of approximately 9% since 1975.

The populations of Swedish harbour seals are exposed to several threats. Olsen *et al.* (2013) listed potential threats to the harbour seal populations in their review on the status of the harbour seal in Southern Scandinavia. In brief the following threats were mentioned; conflicts with fisheries resulting in protective hunting, incidental by-catch and possibly limited food sources for seals; eutrophication resulting in collapse of fish stock and again limited food sources for seals; environmental contaminants such as polychlorinated biphenyls (PCBs) resulting in low reproduction rates and impaired immunity; offshore constructions such as wind farms potentially disturbing seals on land, and altering their behaviour at haul-out sites; human disturbances at land and at sea; interspecific competition with other seal species regarding habitat and food sources; loss of genetic diversity through population declines resulting in inbreeding, which could further lead to reduced immunological response; and lastly epizootics such as exemplified of phocine distemper virus (PDV) in 1988 and 2002, causing mass mortality events within the

[1] Markus Ahola, The Swedish Museum of Natural History, personal communication, 2021-10-26.

population. In 2014, the epizootic with IAV also resulted in an increased number of deaths (Zohari *et al.* 2014)

2.4. Serologic testing of influenza A

There are various methods developed to detect IAV infection targeting either virus and viral antigens or specific antibodies. Serologic testing targets the presence of antibodies in a sample, providing evidence of exposure through either natural infection or vaccination. Whereas studies targeting active infection by demonstration of virus provide a narrow window of infection status, data from serologic testing can be used to estimate the proportion of animals exposed within a population. However, since antibodies naturally decrease with time, detection of all animals ever exposed is most likely not possible. Here, two conventional serologic assays for the detection of antibodies against IAV are presented.

2.4.1. Enzyme-linked immunoassay

Enzyme-linked immunoassay (ELISA) can detect and quantify antibodies in a sample by specific antigen-antibody reactions. There are several different types of ELISAs developed which can either detect antibodies against all IAV or subtype specific IAV antibodies. In ELISAs able to detect all IAV antibodies, influenza nucleoprotein is used as antigen, a protein that is highly conserved in all IAVs (OIE 2021). It is less common to use an ELISA targeting subtype-specific antibodies, although there are assays targeting either HA or NA subtype-specific antibodies (Spackman & Killian 2020).

The most frequently used ELISAs in IAV serologic testing are indirect and blocking or competitive ELISA (Spackman & Killian 2020). They are both based on the same basic principles; antibodies in a sample are allowed to form complexes with antigens coated to a surface and a secondary antibody conjugated with an enzyme specific for either the test antibody or the coated antigen is added. Substrate is added which reacts with the conjugated enzyme, resulting (or not) in a colour change, which can be measured by a spectrophotometer. Optical densities are used to quantify the amount of antibodies in a sample by comparison with controls.

In an indirect ELISA, the secondary antibody is specific for the test antibody (Spackman & Killian 2020). Different species have different antibody proteins, making the assay species-specific. If the test antibody is specific for the coated antigen, it will bind, and the secondary antibody conjugated with an enzyme binds to the test antibody. When substrate is added a colour change reveals a positive reaction. In a blocking or competitive ELISA, the secondary antibody is a monoclonal antibody competing with the test antibody for antigen binding, making the test non-species specific (Spackman & Killian 2020). If the test antibody is specific

for the coated antigen, it will bind and prevent secondary antibodies conjugated with an enzyme from binding. Consequently, a positive sample will have no or little colour change once substrate is added.

ELISA is a fast, inexpensive assay that can easily be used to screen a large number of samples for IAV antibodies. Competitive ELISAs using nucleoprotein as antigen are able to detect antibodies against any IAV subtype, from many different species, with a generally high sensitivity and specificity (de Boer *et al.* 1990; Dundon *et al.* 2007; Lange *et al*. 2009; Busquets *et al.* 2010; De Benedictis *et al.* 2010; Bertran *et al.* 2011; Kittelberger *et al.* 2011; Terregino n.d.).

2.4.2. Hemagglutination inhibition assay

The hemagglutination inhibition assay (HI) is an IAV subtype-specific serologic assay which detects the HA-subtype and antibody titre (OIE 2021). The assay is based on the HA glycoprotein's ability to agglutinate red blood cells. Serum samples are mixed with a known subtype of IAV and chicken red blood cells. If there are antibodies in the serum specific for the virus added, they will bind to the HA-protein and prevent agglutination between the virus and the red blood cells. The highest dilution of serum that prevents agglutination determines the antibody titre in the sample.

HI is simple and inexpensive, although not practical for general large screening of IAV antibodies. It is often used for secondary screening of ELISA positive sera since most ELISAs do not identify against which specific subtype antibodies have been formed. Factors such as the high variation in the HA protein structure and host antibody response might affect the result (Spackman & Killian 2020), and cross-reactions might occur between different subtypes.

2.4.3. Serosurveillance in wild mammals

All diagnostic tests need to be validated for the species they are used for. Some authors such as Gardener *et al.* (1996) and Vandalen *et al.* (2009) have raised concerns on how serological tests are often applied to investigate exposure to pathogens in wild mammals without prior validation in these species. A diagnostic test might not perform correctly outside of its original validation, making results less reliable (Gardner *et al.* 1996). Potential factors influencing the results are differences in host immune response, the difference in pathogenic strains and the risk of antibody cross-reaction due to exposure to similar antigenic structures. Vandalen *et al.* (2009) also points out the need for continued validation since IAVs are prone to genetic evolution and host adaptation, to avoid underreporting of IAVs in wild mammals.

2.5. Cases of influenza A

Cases of influenza A have been reported on multiple occasions in wild and domestic birds and mammals. Here documented outbreaks and evidence of infection in harbour seals are presented, followed by a short overview of detections reported in other species of pinnipeds and marine mammals. Finally, cases of transmission between marine mammals and humans are described.

2.5.1. Influenza A in harbour seals

Sporadic outbreaks with influenza A have been reported in harbour seals on several occasions, of which most have taken place in North America and resulted from cross-species transmission from birds. The first documented outbreak occurred from December 1979 to October 1980 off the east coast of the United States (US) (Lang *et al.* 1981). Biologists recognised an increased number of dead and stranded harbour seals during the period. It was estimated that 500 seals died during the outbreak, which corresponded to 20% of the local population at the time. The main finding at post-mortem examination was acute haemorrhagic pneumonia. Influenza A virus subtype H7N7 was isolated from lung and brain tissue by three independent laboratories (Lang *et al.* 1981; Webster *et al.* 1981b; Geraci *et al.* 1982) and *Mycoplasma* was isolated from the lungs by one study (Geraci *et al.* 1982). Webster *et al.* (1981b) performed an experimental infection of harbour seals with the A/Seal/Mass/1/80 virus (H7N7) isolated from the outbreak. The seals developed clinical signs as well as pathological lesions but not as severe as the harbour seals infected naturally during the outbreak. Antigenic and genetic analyses of the virus indicated that the strain was of avian origin (Webster *et al.* 1981b). However, the virus had a poor ability to replicate in avian species but replicated well in pigs, cats, and ferrets, which indicates an adaptation of the virus towards mammals.

The second documented outbreak with influenza A in harbour seals occurred again off the east coast of the US from June 1982 to March 1983 (Hinshaw *et al.* 1984). Scientists had been monitoring seals in the area for influenza since the previous outbreak in 1979 and 1980 and isolated a new subtype, an H4N5, from one seal with pneumonia in 1982. From January to March 1983, there was an increased number of dead seals in the area. In total, 60 harbour seals were reported dead, which corresponded to 2-4 % of the local population. Animals were examined post-mortem, and 39 of 48 had signs of necrotising bronchopneumonia. Virus isolation from various tissues collected at post-mortem examination was performed, and 16 of 29 seals were positive for influenza A subtype H4N5. Again, the virus was shown to be of avian origin. Unlike H7N7 isolated during the first outbreak, this influenza virus replicated well in the intestinal tract of ducks, which indicates that the virus isolated during the second outbreak in harbour seals was more similar to avian viruses.

Surveillance of marine mammals off the east coast of the US again detected influenza A in harbour seals in January 1991 and January to February 1992 (Callan *et al.* 1995). During these two periods, an increased number of stranded seals were reported, but no severe epizootic was seen as with H7N7 (1979-1980) and H4N5 (1982-1983). In January 1991, influenza A subtype H4N6 was isolated from two stranded harbour seals with pneumonia. From January to February 1992, influenza A subtype H3N3 was isolated from three harbour seals with pneumonia. It was concluded that both the H4N6 and H3N3 subtype were of avian origin.

From September to December 2011, another outbreak occurred off the east coast of the US (Anthony *et al.* 2012). During this outbreak, an increased number of harbour seals were found dead or dying. In total, 162 harbour seals were found, which is about four times as many as compared to a normal year without an outbreak. Five seals were further investigated to determine the cause of the outbreak. A post-mortem examination showed signs of pneumonia and ulcerations of the skin and mucosa in all five seals. The animals were screened by polymerase chain reaction (PCR) for a wide range of pathogens, and influenza A subtype H3N8 was detected and later isolated. Phylogenetic analyses of the virus proved it to be of avian origin, most similar to a virus previously found in North American waterfowl. Mutations known to increase transmissibility and pathogenicity in mammals were also identified, which could indicate a risk for the virus to persist within the population and possibly transmit to other mammals and humans.

In Europe, there has only been one known outbreak of influenza A in harbour seals, starting off the west coast of Sweden in March 2014 (Zohari *et al.* 2014). Throughout the year, the virus continued to spread to Denmark (July), Germany (October) and the Netherlands (November) (Bodewes *et al.* 2015a; b; Krog *et al.* 2015). In Sweden alone, 425 harbour seals were found dead from March to October, a tenfold increase compared to a normal year (Zohari *et al.* 2014). Influenza A subtype H10N7 was identified by real-time reverse transcription polymerase chain reaction (rRT-PCR) in two seals found 4.5 months apart in Sweden, and the virus was later isolated from one of the seals. Phylogenetic analyses of the virus proved it to be most closely related to Eurasian influenza viruses from wild and domestic birds.

In Denmark, 152 harbour seals were found dead between June and August 2014 (Krog *et al.* 2015). Four seals were examined post-mortem, all showing histopathological signs of necrotising bronchopneumonia with extensive growth of *Pseudomonas aeruginosa*. In two of the seals influenza A subtype H10N7 was detected using reverse transcription polymerase chain reaction (RT-PCR), and phylogenetic analysis revealed a high similarity with IAVs detected in birds from Scandinavia and the Republic of Georgia. A high similarity with IAVs detected in seals in Sweden and Germany was also seen, with identities ranging from 99.2-99.7%.

In October 2014, the outbreak reached Germany, where an estimated 1400 dead harbour seals were seen off the coast of Schleswig-Holstein, corresponding to approximately 12% of the local population of harbour seals (Bodewes *et al.* 2015a). Seventeen seals were further investigated and necropsies with histopathological examinations revealed acute necrotising bronchitis and mild interstitial pneumonia in a few cases. Eleven of 17 seals tested positive for influenza A using RT-PCR, and the virus was further characterised as H10N7. Virus isolation was also performed. Genetic analyses showed the virus to be of avian origin, most closely related to wild birds in Georgia, Egypt, and the Netherlands.

The outbreak reached the Netherlands by November 2014, and by January 2015, approximately 180 dead harbour seals were found along the Dutch coast (Bodewes *et al.* 2015b). Compared to the development in Sweden and Germany, the mortality among seals was low. Therefore, a serological study was performed to evaluate the extent of exposure among seals in the Netherlands. Serum samples were collected from 2010 to 2015 from live-captured seals and from seals admitted to a rehabilitation centre. The samples were analysed with blocking ELISA and subtyped using HI. The study showed an increase in antibody titres against H10N7 in 2015 in harbour seals compared to previous years, indicating an exposure in the population during 2014.

2.5.2. Influenza A in other pinnipeds and marine mammals

In addition to the previously described findings of influenza A in harbour seals, there is documentation of some other marine mammals being exposed to influenza A. Here, evidence of active infection and serological findings in pinnipeds are first presented in chronological order, followed by findings in cetaceans and sea otters. This overview is also summarised in table 1 and table 2.

Pinnipeds: virus detection and serological findings

Several studies have reported evidence of IAV infections in different species of pinnipeds. A serological survey in 1978-1995 investigated the exposure to IAV in seven different species of marine mammals in Alaska, and in total 272 serum samples were collected (Danner *et al.* 1998). The only positive sample was from a ringed seal, from a total of 32 samples in this study. The seal was eight years old and had no gross pathology at post-mortem examination. Antibodies were detected using agar gel immunodiffusion assay (AGID).

In another serological study, serum from seals and sea lions in the Bering Sea was collected from 1978-1988 (de Boer *et al.* 1990). IAV antibodies were found in 43 out of 338 samples (12.7%). The same study also analysed serum samples from seals in the North Sea collected in 1988, and 3 out of 757 samples (0.4%) were positive for IAV antibodies. Samples were analysed using a nucleoprotein ELISA (NP-ELISA). The health status of the animals in the study was not reported.

In an experimental study following the second outbreak with IAV in harbour seals (H4N5, 1982-1983) one harbour seal, two ringed seals, and three harp seals (*Pagophilus groenlandicus*) were experimentally inoculated with the virus from the outbreak (Hinshaw *et al.* 1984). The seals showed no signs of clinical disease nor had any histopathological evidence of pneumonia, but virus could be recovered from five of six inoculated pinnipeds during the study. This proved that at least three different species of pinnipeds were susceptible to the virus.

In 1984-1998 another serological study was performed in Arctic Canada (Nielsen *et al.* 2001). Serum samples were collected from 903 ringed seals, of which 23 samples (2.5%) were positive for IAV antibodies using a competitive ELISA. All animals in the study were hunted, the health status was not reported.

In 1991-1992 serum samples were collected from clinically healthy harp seals and hooded seals (*Cystophora cristata*) in Barents Sea (Stuen *et al.* 1994). In harp seals, 33 out of 183 samples (18%) were positive, and in hooded seals, 8 out of 100 samples (8%) were positive for IAV antibodies using NP-ELISA. No pathology was observed in the seals.

In 1993-2000 surveillance of Caspian seals (*Phoca caspica*) was performed in the Caspian Sea (Ohishi *et al.* 2002). Using indirect ELISA, the study identified antibodies against IAV in 28 of 77 serum samples (23%). HI was performed to subtype the antibodies and a strong correlation with the H3N2 strain circulating in humans in 1979-1981 was seen. The seals were sampled within a research program, the health status not reported.

In a serologic study in Alaska 1994-1996, serum or plasma samples were collected from Pacific walrus (*Odobenus rosmarus divergens*) (Calle *et al.* 2002). In 8 out of 38 samples (21%), IAV antibodies were detected against subtypes H10, N2, N3, N5, N6 and N7. Samples were analysed with AGID. The animals included in the study were hunted.

A more recent serologic study in eastern Canada screened serum samples from 394 seals collected during 1994-2005 for antibodies against influenza, using indirect ELISA and HI (Measures & Fouchier 2021). Seals of five different species were included in the study: harp seals, grey seals, harbour seals, hooded seals, and ringed seals. Serum from 86 of 206 harp seals (42%), 19 of 58 harbour seals (33%), 19 of 81 grey seals (23%) and four of 38 hooded (11%) were seropositive for IAV. Ringed seals were seronegative. Subtypes H3, H4, and H10 were identified. The animals were either live-captured or shot. In general, the seals seemed healthy at sampling, and the only diseased individual had lesions of geriatric nature.

A serological study of Kuril harbour seals (*Phoca vitulina stejnegeri*) was carried out between 1998-2005 in Hokkaido, Japan (Fujii *et al.* 2007). Serum samples from 15 of 322 seals (4.7%) were positive for IAV antibodies using ELISA. Samples were further subtyped by HI, and subtype H3 was identified in 10

samples and H6 in two samples. Most of the seals included in the study were by-caught in fishing gear, the health status was not reported.

Serological research in Baikal seals (*Phoca sibirica*) and ringed seals in the central Russian arctic in 2002 once again identified antibodies against the human H3N2 strain in one Baikal seal and four ringed seals using ELISA and HI (Ohishi *et al.* 2004). In total, 2 of 7 Baikal seals (28.6%) and 5 of 6 ringed seals (83.3%) were positive for IAV antibodies. The seals were sampled within a research program, the health status was not reported.

In 2004 another serologic study screened South American fur seals (*Arctocephalus australis*) along the coast of Uruguay for the presence of antibodies against IAV using only HI (Blanc *et al.* 2009). Serum samples from 16 of 37 seals (43.4%) were positive for antibodies against the subtype H1N1. Serum was also tested for H3N2 subtype, and 37 out of 37 serum samples (100%) were positive. All seals were about 10 months old and live-captured, the health status was not reported.

Another surveillance program of marine vertebrates in the Northwest Atlantic 2005-2007 detected IAV subtype H3N8 in one by-caught harp seal using RT-PCR (Bogomolni *et al.* 2008). The health status of the positive seal was not reported.

In April 2010, H1N1 was detected in 2 of 42 (4.8%) tested northern elephant seals (*Mirounga angustirostris*) off the central California coast (Goldstein *et al.* 2013). Both seals were detected through surveillance using nasal swabs analysed with RT-PCR and were without clinical signs of disease. Further genome analyses showed the virus to be of human origin, corresponding well to the pandemic influenza virus circulating in humans in 2009. In total, 305 serum samples were collected from seals admitted to a rehabilitation centre in 2010-2011, and seroconversion against H1N1 was detected in 51 samples, with the first positive sample found in April 2010. Together with the virus detection, it indicated that the virus had recently been introduced to seals in the area. Continued surveillance in 2011 and 2012 could not detect the H1N1 virus in northern elephant seals but 35-40% of the seals tested were seropositive, with declining titres of antibodies in 2012. Further studies of marine mammals off the east coast of the US have also detected antibodies against the pandemic H1N1 in harbour seals and California sea lions, with antibodies detected as early as 2009 in northern elephant seals and harbour seals (Boyce *et al.* 2013). The animals sampled were live-captured or stranded seals admitted to a rehabilitation centre.

In 2012 influenza A subtype H4N6 was detected by RT-PCR from one of 27 sampled free-ranging Caspian seals in the Astrakhan region, Russia (Gulyaeva *et al.* 2018). The seal seemed to be clinically healthy.

Another study sampled live-captured grey seals in Cape Cod US and Sable Island Canada in 2013-2015 (Puryear *et al.* 2016). In total, 402 clinically healthy individuals were sampled and IAV was detected in 9% of the weaned pups and

5.3% of the adults using RT-PCR. Additionally, the study detected IAV antibodies in 19.3% of the weaned pups and 50% of the adults by ELISA and HI.

In contrast, a surveillance program of marine mammals and birds along the central coast of California 2014-2015 detected IAV in only 2 of 1142 (0.2%) sampled pinnipeds using rRT-PCR (Ramey *et al.* 2017). All samples were taken from stranded pinnipeds admitted to a rehabilitation centre and in total, six different species of pinnipeds were sampled: California sea lions (*Zalophus californianus*), Guadalupe fur seals (*Arctocephalus townsendi*), northern elephant seals, northern fur seals (*Callorhinus ursinus*), Pacific harbour seals (*Phoca vitulina richardii*) and Steller sea lions (*Eumetopias jubatus*). The two positive individuals were a California sea lion and a Pacific harbour seal.

Following the European outbreak in harbour seals with H10N7 in 2014, a seroprevalence study was performed in the Netherlands in 2010-2015 (Bodewes *et al.* 2015b). The study found antibodies in harbour seals as previously described, but also in grey seals. In 2014, the study detected seropositivity in one out of 14 samples from adults, and in 2015, 5 out of 19 sampled adults were positive for antibodies against H10N7. The study used a blocking ELISA and HI to detect and subtype antibodies. Grey seals were sampled during live-capture and at a rehabilitation centre.

In 2016 and 2017, two stranded dead grey seals were found five months apart along the Baltic coast of Poland (Shin *et al.* 2019). Both seals were submitted for further investigation, and highly pathogenic H5N8 were detected by rRT-PCR from lung tissue of both seals. Virus isolation from the lung was successful in one of the seals. The viruses showed 99-100% identity with avian IAVs circulating in Europe at the time. At post-mortem examination, the seals did not have signs indicating an influenza infection, although both were in poor nutritional status.

In 2017, an IAV infection with subtype H3N8 was detected by immunohisto-chemistry and rRT-PCR in a 3–4-month-old grey seal pup in Cornwall, England (Venkatesh *et al.* 2020). The pup died during rehabilitation, and post-mortem examination revealed that IAV was most likely not the cause of death. Further investigation of lung tissue even indicated that the IAV infection had cleared naturally. The virus was shown to be of avian origin and had several mutations indicating mammalian adaptation.

In 2020, two different seal species died or were euthanised at a wildlife rehabilitation centre in England due to infection with highly pathogenic IAV H5N8 (Floyd *et al.* 2021). Spillover from swans to four juvenile harbour seals and one juvenile grey seal resulted in encephalitis with neurological signs such as seizures. IAV subtype H5N8 was detected by rRT-PCR and virus was isolated from brain tissue from two harbour seals and one grey seal examined post-mortem.

Cetaceans, virus detection and serological findings

There are only a few documented cases of isolations of IAV in cetaceans. In 1975-1976, H1N3 was isolated from hunted striped whales (Balaenopteridae) in the South Pacific, (Lvov *et al.* 1978). The health status of the sampled whales was not reported. In 1984 H13N2 and H13N9 were isolated from 1 out of 20 long-finned pilot whales (*Globicephala melaena*) along the New England coast, US (Hinshaw *et al.* 1986). During the same period, an increased number of strandings of pilot whales was seen. The positive whale was sick and thus euthanised.

There is also some serologic evidence of influenza A in cetaceans. In serum samples from hunted Beluga whales (*Delphinapterus leucas*) collected in Artic Canada 1984-1998, 5 of 418 samples (1.2%) were positive for IAV using competitive ELISA (Nielsen *et al.* 2001). The health status of the whales was not reported. In serum samples from common minke whale (*Balaenoptera acutorostrata*) and Dall's porpoise (*Phocoenoides dalli)*, 7 of 140 samples (5%) and 2 of 34 samples (5.9%) were positive for IAV antibodies respectively (Ohishi *et al.* 2006). Samples were collected in the Western North Pacific and Antarctic regions in 2000-2003 and were analysed with indirect ELISA. Animals sampled were either by-caught or captured.

Sea otters, serological findings

Antibodies against IAV have been detected in free-ranging sea otters by a few serological studies. In 2011, one study found IAV antibodies in 7 of 30 (23%) sampled northern sea otters (*Enhydra lutris kenyoni*) in Washington, US (White *et al.* 2013). Samples were analysed with an ELISA designed to detect IAV antibodies in serum from multiple avian species. Another study further analysed the same 30 samples and found 22 of 30 (73%) samples to have antibody titres against the pandemic H1N1 virus (Li *et al.* 2014). Samples were analysed by ELISA and HI. The animals were healthy at capture.

Southern sea otters (*Enhydra lutris nereis*) admitted to veterinary care or a research centre were sampled between 1997-2015, California coast, US (Capuano *et al.* 2017). IAV antibodies were detected in 161 out of 661 (24%) sampled otters using a competitive ELISA and virus neutralization (VN). Multiple different H-subtypes were identified, and a strong response to pandemic H1N1 was shown. The health status of the otters was not reported.

Table 1. Serological findings of influenza A virus in marine mammals.

Year sampled	**Location**	**Diagnostic test**	**Species *(Scientific name)***	**No. positive/ No. sampled (%)**	**Reference**	**Comments**
1978-1995	Alaska	AGID[2] HI NI[3]	Ringed seal (*Phoca hispida*)	1/32 (3.1%)	(Danner *et al.* 1998)	Free-ranging 8-year-old seal, no gross pathology observed in post-mortem examination.
1978-1988 1988	Bering sea North Sea	NP-ELISA[4] HI	Seal & sea lion Seal (Not specified)	43/338 (12.7%) 3/757 (0.4%)	(de Boer *et al.* 1990)	Free-ranging, unknown health status.
1984-1998	Arctic Canada	cELISA [5]	Beluga whale (*Delphinapterus leucas)* Ringed seal (*Pusa hispida*)	5/418 (1.2%) 23/903 (2.5%)	(Nielsen *et al.* 2001)	All animals were hunted, unknown health status.
1991-1992	Barents Sea	NP-ELISA	Harp seal (*Pagophilus groenlandicus*) Hooded seal (*Cystophora cristata*)	33/183 (18%) 8/100 (8%)	(Stuen *et al.* 1994)	Free-ranging, no clinical signs or pathological changes observed.

[2] Agar gel immunodiffusion assay
[3] Neuraminidase inhibition assay
[4] Nucleoprotein ELISA
[5] Competitive ELISA

Year sampled	**Location**	**Diagnostic test**	**Species *(Scientific name)***	**No. positive/ No. sampled (%)**	**Reference**	**Comments**
1993-2000	Caspian Sea	Indirect ELISA	Caspian seals (*Phoca caspica*)	28/77 (36.4%)	(Ohishi *et al.* 2002)	Sampled in research program, unknown health status.
1994-1996	Alaska	AGID	Pacific walrus (*Odobensus rosmarus divergens*)	8/38 (21.1%)	(Calle *et al.* 2002)	Free-ranging animals, hunted
1994-2005	Eastern Canada	Indirect ELISA HI	Harp seal (*Pagophilus groenlandicus*) Grey seal (*Halichoerus grypus*) Harbour seal (*Phoca vitulina*) Hooded seal (*Cystophora cristata*) Ringed seal (*Pusa hispida*)	86/206 (41.7%) 19/81 (23.5%) 19/58 (32.6%) 4/38 (10.5%) 0/2 (0%)	(Measures & Fouchier 2021)	Free-ranging, live-captured or shot. All seals clinically healthy at sampling.
1997-2015	California, US	cELISA VN [6]	Southern sea otter (*Enhydra lutris nereis*)	16/661 (2.4%)	(Capuano *et al.* 2017)	Otters admitted to veterinary care and research centre, health status unknown.

[6] Virus neutralization

Year sampled	**Location**	**Diagnostic test**	**Species *(Scientific name)***	**No. positive/ No. sampled (%)**	**Reference**	**Comments**
1998-2005	Hokkaido, Japan	ELISA HI	Kuril harbour seals (*Phoca vitulina stejnegeri)*	15/322 (4.7%)	(Fujii *et al.* 2007)	Mostly by-caught seals, health status unknown.
2000-2003	Western North Pacific and Antarctic regions	Indirect ELISA	Common minke whale (*Balaenoptera acutorostrata)* Dall's porpoise (*Phocoenoides dalli*)	7/140 (5%) 2/34 (5.9%)	(Ohishi *et al.* 2006)	Samples either from by-catch or capture, health status unknown.
2002	Central Russian arctic	ELISA HI	Baikal seal (*Phoca sibirica)* Ringed seal (*Pusa hispida*)	2/7 (28.6%) 5/6 (83.3%)	(Ohishi *et al.* 2004)	Health status unknown, sampled in research program.
2004	Uruguay	HI	South American fur seal (*Arctocephalus australis*)	H1N1: 16/37 (43.3%) H3N2: 37/37 (100%)	Blanc *et al.* 2009)	Live-capture, animals were 10 months old, unknown health status.
2009-2011	California, US	HI	Harbour seal (*Phoca vitulina)* California sea lion (*Zalophus californianus*) Northern elephant seal (*Mirounga angustirostris*)	4/140 (2.9%) 7/183 (3.8%) 98/224 (43.8%)	(Boyce *et al.* 2013)	Live-captured and stranded seals admitted to rehabilitation centre sampled. Human pandemic H1N1.

Year sampled	Location	Diagnostic test	Species *(Scientific name)*	No. positive/ No. sampled (%)	Reference	Comments
2010-2011	California, US	HI	Northern elephant seal (*Mirounga angustirostris*)	51/305 (16.7%)	(Goldstein *et al.* 2013)	Serum from seals admitted to rehabilitation centre. No influenza associated disease. Human pandemic H1N1.
2010-2015	Netherlands	NP-ELISA HI	Harbour seal (*Phoca vitulina)* Grey seal (*Halichoerus grypus*)	25/404 (6.1%) 49/404 (12.1%) 3/119 (2.5%) 8/119 (6.7%)	(Bodewes *et al.* 2015b)	Live-captured healthy seals and seals admitted to rehabilitation sampled, 180 harbour seals dead 2014.
2011	Washington, US	ELISA HI	Northern sea otter (*Enhydra lutris kenyoni*)	7/30 (23.3%) 22/30 (73.3%)	(White *et al.* 2013; Li *et al.* 2014)	Free-ranging, healthy at capture.
2013-2015	Cape Cod, US Sable Island, Canada	ELISA HI	Grey seal (*Halichoerus grypus*)	68/301 (22.6%)	(Puryear *et al.* 2016)	Live-captured, clinically healthy at sampling.

Table 2. Detections/isolations of influenza A virus in marine mammals.

Year sampled	**Location**	**Diagnostic test**	**Species *(Scientific name)***	**No. positives/ No. samples (%)**	**Reference**	**Comments**
1974-1976	South Pacific	Virus isolation	Striped whale (Balaenopteridae)	72 sampled	(Lvov *et al.* 1978)	Whales were hunted, health status unknown. H1N3.
1979-1980	East coast, US	Virus isolation	Harbour seal (*Phoca vitulina)*	6/11 (54.5%)	(Webster *et al.* 1981b)	Outbreak, 500 seals died (20% of population), H7N7
1982-1983	East coast, US	Virus isolation	Harbour seal (*Phoca vitulina)*	16/29 (55.2%)	(Hinshaw *et al.* 1984)	Outbreak, 60 seals died (2-4% of population) H4N5.
1984		Virus isolation	Harbour seal (*Phoca vitulina*) Ringed seal (*Pusa hispida*) Harp seal (*Pagophilus groenlandicus*)	1/1 (100%) 2/2 (100%) 2/3 (66.7%)	(Hinshaw *et al.* 1984)	Experimental study with captive seals, subtype H4N5. All clinically healthy.
1984	New England coast, US	Virus isolation	Long-finned pilot whale (*Globicephala melaena*)	1/20 (5%)	(Hinshaw *et al.* 1986)	Increase number of strandings, sampled whale in poor condition, euthanised. H13N2 and H13N9 detected.

Year sampled	**Location**	**Diagnostic test**	**Species *(Scientific name)***	**No. positives/ No. sampled (%)**	**Reference**	**Comments**
1991-1992	East coast, US	Virus isolation	Harbour seal (*Phoca vitulina*)	H4N6: 3 seals H3N3: 2 seals	(Callan *et al.* 1995)	Increased number of strandings, 450 individuals sampled since 1989.
2005-2007	Northwest Atlantic	RT-PCR	Harp seal (*Pagophilus groenlandicus*)	1/34 (2.9%)	(Bogomolni *et al.* 2008	Positive seal was by-caught, health status unknown. H3N8.
2010	California, US	rRT-PCR Virus isolation	Northern elephant seal (*Mirounga angustirostris*)	2/48 (4.2%)	(Goldstein *et al.* 2013)	Clinically healthy. Subtype pandemic H1N1 circulating in humans 2009
2011	East coast, US	PCR Virus isolation	Harbour seal (*Phoca vitulina)*	5/5 (100%)	(Anthony *et al.* 2012)	Outbreak, 162 seals found dead. H3N8.
2012	Astrakhan region, Russia	RT-PCR	Caspian seal (*Phoca caspica*)	1/27 (3.7%)	(Gulyaeva *et al.* 2018)	Positive seal clinically healthy. Subtype H4N6.
2013-2015	Cape Cod, US Sable Island, Canada	RT-PCT	Grey seal (*Halichoerus grypus*)	34/402 (8.5%)	(Puryear *et al.* 2016)	Live-captured, clinically healthy at sampling.
2014	West coast, Sweden	rRT-PCR Virus isolation	Harbour seal (*Phoca vitulina)*	2/2 (100%) 1/2 (50%)	(Zohari *et al.* 2014)	Outbreak, at least 425 seals died. H10N7.

Year sampled	**Location**	**Diagnostic test**	**Species *(Scientific name)***	**No. positives/ No. sampled (%)**	**Reference**	**Comments**
2014	Denmark	RT-PCR	Harbour seal (*Phoca vitulina*)	2/4 (50%)	(Krog *et al.* 2015)	Outbreak with H10N7, at least 152 seals died.
2014	Schleswig-Holstein, Germany	RT-PCR Virus isolation	Harbour seal (*Phoca vitulina*)	11/17(64.7%)	(Bodewes *et al.* 2015a)	Outbreak with H10N7, at least 1400 seals died (12% of local population).
2014-2015	California, US	rRT-PCR	California sea lion (*Zalophus californianus*) Pacific harbour seal (*Phoca vitulina richardii*)	1/779 (0.1%) 1/133 (0.8%)	(Ramey *et al.* 2017)	Stranded marine mammals admitted to rehabilitation centre sampled.
2016-2017	Baltic coast, Poland	rRT-PCR	Grey seal (*Halichoerus grypus*)	2/2 (100%)	(Shin *et al.* 2019)	Stranded seals, poor nutritional status. Subtype H5N8 identified.
2017	Cornwall, England	rRT-PCR	Grey seal (*Halichoerus grypus*)	1/1 (100%)	(Venkatesh *et al.* 2020)	Subtype H3N8. Pup died during rehabilitation. IAV not the cause of death.
2020	England	rRT-PCR Virus isolation	Harbour seal (*Phoca vitulina*) Grey seal (*Halichoerus grypus*)	2/2 (100%) 1/1 (100%)	(Floyd *et al.* 2021)	Admitted to rehabilitation centre. Five seals affected, neurological signs, died or was euthanised. H5N8.

2.5.3. Cases of transmission between humans and marine mammals

Evidence of transmission of IAV between humans and marine mammals has been reported. The first documented case occurred during the outbreak with H7N7 in harbour seals in 1979-1980 (Webster *et al.* 1981a). Four people working with seal carcasses developed severe purulent conjunctivitis within a couple of days after handling dead seals from the outbreak. Unfortunately, only bacterial cultures were attempted from these human cases, and normal bacterial flora was detected. However, during an experimental infection of seals with the virus isolated from the outbreak, another person developed severe conjunctivitis after being sneezed in the face by one of the infected seals. In this case, high titres of the seal derived virus H7N7 was detected. None of the affected people developed antibodies against the virus.

Furthermore, serological studies of Caspian seals, Baikal seals, and ringed seals have detected transmission of human IAV subtype H3N2 to seals (Ohishi *et al.* 2002, 2004). The animals were sampled in the Caspian Sea (1993-2000), Kara Sea (1998) and Lake Baikal (2002). In 36% of the Caspian seals, 14% of the Baikal seals and 17% of the ringed seals tested, positive reaction with the human strain A/Bangkok/1/79 which circulated in humans in 1979-1981 was seen.

The pandemic H1N1 virus was isolated from northern elephant seals along the central California coast in 2010 (Goldstein *et al.* 2013). The virus was shown to be most closely related to a human isolate from San Diego, US. Further serologic studies of marine mammals in the area also detected antibodies against pandemic H1N1 in harbour seals and northern elephant seals in 2009 and in California sea lions in 2010 (Boyce *et al.* 2013). Furthermore, serological studies in sea otters have detected antibodies against IAV possibly related to the pandemic IAV H1N1 (Li *et al.* 2014; Capuano *et al.* 2017).

3. Material and Methods

3.1. Material

The samples included in this study were collected by the Swedish Museum of Natural History (NRM) and the National Veterinary Institute, Sweden (SVA). Samples were collected either during post-mortem examination or live capture. Seals sampled post-mortem were hunted (n=127), incidentally caught in fishing gear as by-catch (n=25) or found stranded (n=10). Found seals were either already dead or euthanised shortly after being found. Among the seals found dead, the cause of death was in most cases confirmed by post-mortem examination. The main cause of death in this group was drowning.

In total 204 serum and cadaver blood samples from Swedish harbour seals were analysed within this study. Cadaver blood samples (n=162) were collected during post-mortem examination from the heart or from blood vessels. These samples were collected between 2014 and 2021 and consisted of samples from populations off the west (n=137) and east coast (Kalmarsund population, n=25) of Sweden. Serum samples (n=42) were collected during a health control of live captured seals in two different locations along the Swedish west coast (Koster, n=20 and Gothenburg, n=22) in the fall of 2014.

The following sample data were acquired from NRM's and SVA's databases to make relevant conclusions from the ELISA and HI results: date found, place found, geographical coordinates, sea basin, source (hunted, fishery interaction or stranded/ found dead), cause of death, sex, age, age class, total length, weight, and sternal blubber. Not all data were available for all samples. Data could be obtained from most of the cadaver blood samples, but the majority of data from 52.4% (22/42) of the seals sampled during the health control in 2014 was unavailable. Age was determined for only 16.2% (33/204) of the sampled seals. To make relevant conclusions regarding age, animals were assigned to an age class based on the total length. The estimation was based on a system used in a report on the health status of grey seals and harbour seals in the Baltic Sea, conducted by NRM (Bäcklin *et al.* 2021). Three different age classes were used: young of year 0-1 years old (< 115 cm), adult 1-4 years old (115-150 cm), and adult > 4 years old (> 150 cm). However,

many of the seals included in this study were hunted and, in some cases, total length and weight were only estimated by the hunter.

Table 3. Serum samples 2014: date, place and age class.

	Location	**Sampled/age class**		
Date	*(Number of samples)*	*Young of year*	*Adults 1-4 years*	*Adult > 4 years*
Sept. 2014	Koster (13)	7	6	0
Oct. 2014	Koster (7)	2	5	0
	Gothenburg (22)	Age class not identified		
Total	42			

Table 4. Cadaver blood samples 2014-2021: year, population, and age class.

	Population	**Sampled/age class**		
Year	*(Number of samples)*	*Young of year*	*Adults 1-4 years*	*Adult > 4 years*
2014	West coast (3)	1	2	0
	Kalmarsund (8)	2	4	2
2015	West coast (94)	22	48	24
	Kalmarsund (2)	1	1	0
2016	West coast (25)	3	14	8
	Kalmarsund (2)	1	1	0
2017	Kalmarsund (3)	1	2	0
2018	West coast (2)	0	2 adults (age unspecified)	
	Kalmarsund (3)	0	3	0
2019	Kalmarsund (3)	1	2	0
2020	West coast (12)	7	4	1
	Kalmarsund (3)	2	1	0
2021	West coast (1)	1	0	0
	Kalmarsund (1)	1	0	0
Total	(162)	43	84	35

3.2. Methods

Laboratory analyses were performed at the National Veterinary Institute, Sweden (SVA).

3.2.1. Enzyme-linked immunosorbent assay

All 204 samples were initially screened for antibodies against the influenza A virus using a commercial competitive enzyme-linked immunosorbent assay (ELISA). The ID Screen® Influenza A Antibody Competition Multi-Species (ID-vet, France) was used following the instructions by the manufacturer. The test is validated for

birds (Dundon *et al.* 2007; Molia *et al.* 2010; Bertran *et al.* 2011; Terregino n.d.), swine (Lange *et al.* 2009; Busquets *et al.* 2010), horses (Kittelberger *et al.* 2011) and dogs (De Benedictis *et al.* 2010) and detects antibodies against all influenza A subtypes. Competition percentage (S/N %) was calculated for each sample by dividing a sample's (S) optical density (OD) with the negative control's (N) OD and then multiplying by 100. A sample was regarded as positive if S/N % ≤ 45%, doubtful if 45% < S/N% < 50% and as negative if S/N% ≥ 50%. Serum samples were analysed by SVA in 2014 and cadaver blood samples in 2020-2021 by SVA and the author of this report.

3.2.2. Hemagglutination inhibition assay

ELISA positive and doubtful samples were further analysed with hemagglutination inhibition assay (HI) to confirm ELISA results and to classify against which subtype of influenza A antibodies had been formed. HI was preceded by the hemagglutination assay (HA) to define the titre of the reference antigen. Cadaver blood samples were tested against two or four different reference antigens, see table 1. In general, ELISA positive samples were tested against four different antigens, and ELISA doubtful samples were tested against two different antigens, H10N7 and H10N9. The selection of antigens was based on the subtype involved in the outbreak in 2014 (H10N7). One doubtful cadaver blood sample collected in 2020 was additionally tested against H5N8, since circulation of this subtype was reported in wild birds in 2020 in Sweden. Cadaver blood samples were analysed by the author. Serum samples were tested against 19 IAV antigens, see table 2. These samples were analysed by SVA in 2014.

Cadaver blood samples were treated with the hemadsorption technique prior to HI. From each sample, 50 µl was mixed with 150 µl 2.5% washed chicken red blood cell suspension and kept in a refrigerator (4°C) overnight. This was done because spontaneous agglutination had been seen in previous attempts with the samples. Serum samples were not treated with the hemadsorption technique prior to HI.

Table 5. Antigens used in hemagglutination inhibition assay for cadaver blood samples.

IAV antigen subtype	**Strain**
H10N7	A/Seal/Sweden/0546/2014
H10N9	A/Eg.Goose/S.Africa/238/98
H7N7	A/TKY/ENG/647/77 and A/Mallard/Sweden/C99/2008
H7N1	A/African Starling/Eng/989/79
H5N8	A/DK/England/036254/2014

Table 6. Antigens used in hemagglutination inhibition assay for serum samples.

IAV antigen subtype	**Strain**
H1N2	A/DK/HONG KONG/196/77
H2N3	A/DUCK/GERM/1215/73
H3N2	A/Turkey/Eng/69
H4N6	A/DK/CZEH/56
H5N1	A/CK/SCOT/59
H6N8	A/TURKEY/CANADA/63
H7N7	A/TKY/ENG/647/77
H8N4	A/TK/ONT/ 6118/68
H9N2	A/CKKOR/99029/99
H10N9	A/S.AFRICA/EG. GOOSE/238/ 98
H11N6	A/DUCK/ENG/56
H12N5	A/DK/ALBERTA/60/75
H13N6	A/GULL/MARYLAND/704/77
H14N6	A/MALL/GURG/244/82
H16N3	A/ GULL/DK/68110/02
H10N4	A/Mink/Sweden/1984
H10N7	A/Chicken/ Germany/N/1949
H10N7	A/Seal/Sweden/0546/2014
H7N1	A/African Starling/Eng/989/79

Hemagglutination assay

HA was performed using V-bottomed 96-well microplates. Phosphate-buffered saline (PBS) was added into all wells, 25 µl per well, except for the line of wells for blood control were 50 µl was added. Each reference antigen was added into one top well, 25 µl per well, and titrated with 25 µl in 12 steps, with no reference antigen added in wells for blood control. Washed chicken red blood cell suspension (1%) was added into all wells, 25 µl per well. The plate was incubated for 40 minutes in a refrigerator (4°C). After incubation, reading was performed by tilting the plates 60-70° horizontally. The HA-titre was determined by the last dilution where hem-agglutination was complete.

Hemagglutination inhibition assay

Control plates and test plates were prepared for each reference antigen using V-bottomed 96-well microplates. The control plates consisted of four lines of 12 wells with one line each for the positive and negative control, antigen control and blood control. PBS was added into all wells, 25 µl per well, for positive and negative virus control and antigen control. PBS was also added into all wells for blood control, 50 µl per well.

From positive reference sera and negative control sera, 25 µl was added to one top well each and titrated with 25 µl in 12 steps. Antigen control consisting of the reference antigen was added into one top well, 25 µl, and titrated with 25 µl in four steps. Reference antigen was also added into all wells for positive and negative control, 25 µl per well.

At the same time, test plates for the samples were prepared. PBS was added into all wells of a 96-well microtiter plate, 25 µl per well. For the cadaver blood samples treated with hemadsorption, no PBS was added in the first row of wells. Each microtiter plate had a line of wells for blood control, in which 50 µl of PBS was added per well. From serum samples, 25 µl of each sample was added into one top well. From cadaver blood samples, 50 µl of each sample was added into one top well. Cadaver blood samples were titrated with 25 µl in eight steps, and serum samples were titrated with 25 µl in 12 steps. Reference antigen was added into all wells except for the first or two first rows of wells, 25 µl per well. The wells without antigens worked as serum control together with the control plate.

The control and test plates were then incubated at room temperature for 30 minutes. After incubation, 25 µl of washed chicken red blood cell suspension (1%) was added into all wells of the control and test plates. The plates were incubated a second time in a refrigerator (4°C) for 40 minutes. After the second incubation, control plates and test plates were read by tilting the plate 60-70° horizontally. The HI-titre was determined by the last dilution that prevented hemagglutination. HI-titre was regarded as positive if inhibition was seen at serum dilution 1:16 or more against antigen titre four HA-units (HAU).

4. Results

For complete results of ELISA and HI from serum and cadaver blood samples, see appendix 1 and appendix 2.

4.1. Enzyme-linked immunosorbent assay

Here detection of antibodies against influenza A virus nucleoprotein is presented. Results from serum and cadaver blood samples are presented separately. A sample was regarded as positive if S/N % ≤ 45%, doubtful if 45% < S/N% < 50% and as negative if S/N% ≥ 50%. ELISA was performed twice with all serum and cadaver blood samples.

4.1.1. Serum samples

In serum samples collected during the fall of 2014, 24 out of 42 serum samples (57.1%) were positive and 2 out of 42 serum samples (4.8%) were doubtful. In figure 1 ELISA results from all serum samples can be seen.

Location

Serum samples were collected at two different locations, Koster and Gothenburg. In Koster, samples were collected in September and October, and 14 out of 20 serum samples (70%) were positive for antibodies against influenza A virus NP. In addition, 1 out of 20 serum samples (5%) was regarded as doubtful. Samples from Gothenburg were collected during October, and 10 out of 22 serum samples (45.1%) were positive. In addition, 1 out of 22 serum samples (4.5%) from Gothenburg was regarded as doubtful.

Other sample data

Because data regarding date sampled, coordinates, sex, total length, weight and sternal blubber was unavailable from at least 22 out of 42 serum samples, no analysis of these parameters was performed within this study.

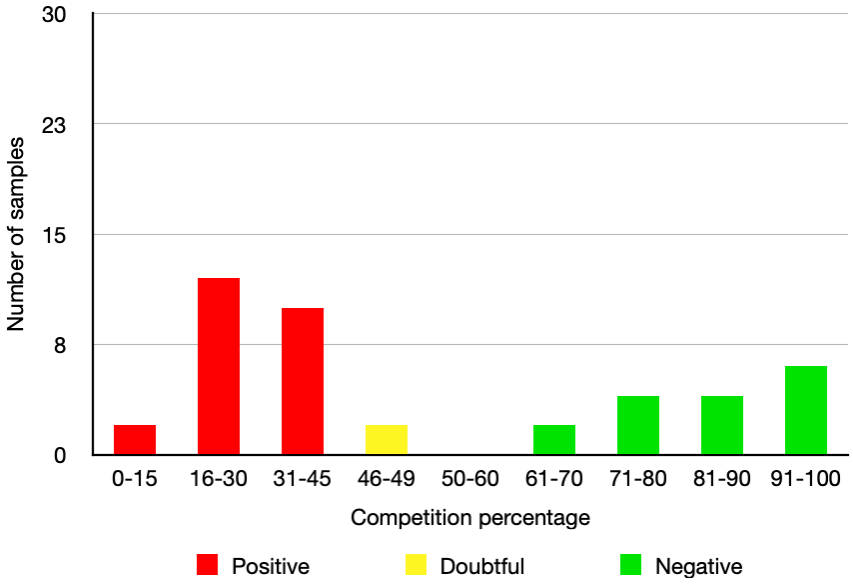

Figure 1. ELISA results from serum samples collected from harbour seals of the Swedish west coast in 2014. Y-axis = number of samples, x-axis = competition percentage (S/N %). S/N % is calculated by dividing a sample's optical density with the negative control's optical density and then multiplying by 100.

4.1.2. Cadaver blood samples

In cadaver blood samples collected from 2014-2021, 23 out of 162 samples (14.2%) were positive for antibodies against influenza A virus NP. In addition, 7 out of 162 samples (4.3%) were regarded as doubtful. In figure 2 ELISA results from all cadaver blood samples can be seen.

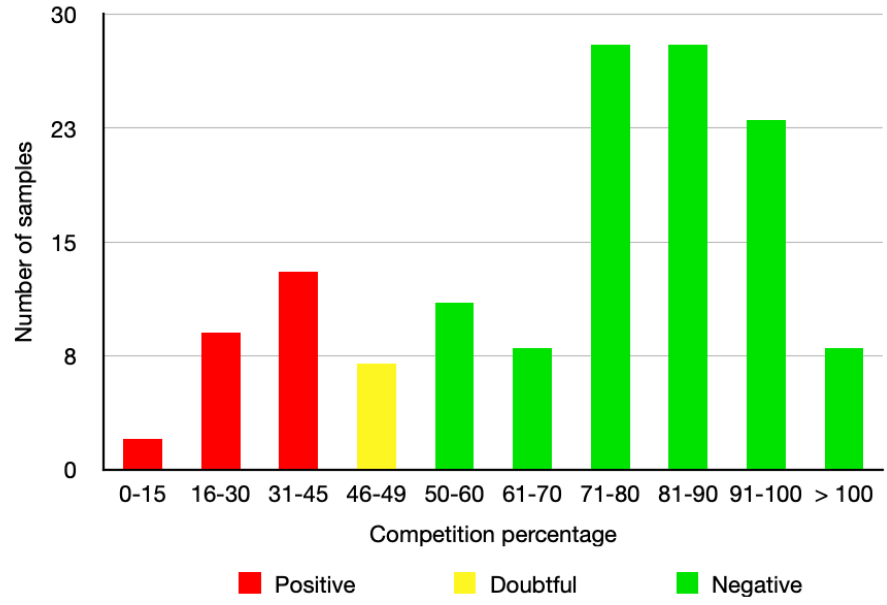

Figure 2. ELISA results from cadaver blood samples collected from harbour seal of the Swedish west coast 2014-2021. Y-axis = number of samples, x-axis = competition percentage (S/N %). S/N % is calculated by dividing a sample's optical density with the negative control's optical density and then multiplying by 100.

Year and month sampled

Cadaver blood samples were collected from seals off the west coast of Sweden in 2014, 2015, 2016, 2018, 2020 and 2021. The number of samples collected per year varies, with most samples collected in 2015, the year after the outbreak with H10N7. In 2015 and 2020, most samples were collected during the second half of the year, and in 2016 samples were only collected during the first two months of that year. The number of samples collected and their ELISA results from each year are summarised below in table 7.

Table 7. ELISA result per year for cadaver blood samples collected from harbour seals from the west coast 2014-2021.

Year	Positive	Doubtful	Negative	Total
2014	1		2	3
2015	20	5	69	94
2016	2	1	22	25
2018			2	2
2020		1	11	12
202			1	1
Total	23	7	107	137

Of the cadaver blood samples analysed each year, the proportion of positive samples decreased from approximately one third in 2014, to one fifth in 2015, and to 8% in 2016. A few doubtful samples were found as well, corresponding to 4-5% of the total number of samples in 2015 and 2016. In addition, one seal sampled in 2020 had a doubtful ELISA result. When considered together with the ELISA results from serum samples, it gives a picture of widespread exposure to IAV and subsequent development of antibodies in 2014 (57.1% of serum samples positive in 2014), with declining seropositivity in 2015 and 2016.

Location

Cadaver blood samples were collected from the east coast (Kalmarsund population, n=25) and west coast (n=137). All cadaver blood samples from the Kalmarsund population from 2014-2021 were negative for IAV antibodies. In cadaver blood samples collected from the west coast of Sweden from 2014-2021, 23 out of 137 samples (16.8%) were positive and 7 out of 137 samples (5.1%) were regarded as doubtful. Almost all cadaver blood samples included in the study can be seen in the map in figure 4. Seven samples out of which one was ELISA positive, did not have information on coordinates and could therefore not be included in the map. Positive samples are marked in red, doubtful in yellow and negative in green. In figure 5, only positive and doubtful samples are presented.

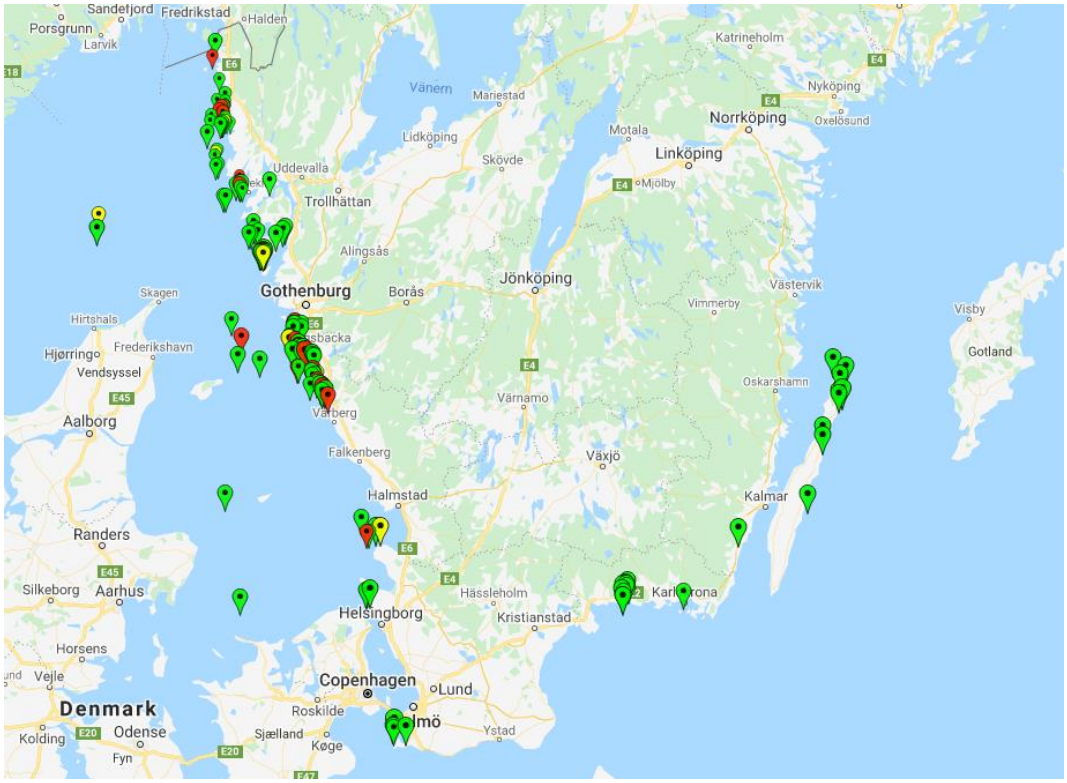

Figure 3. ELISA positive (red), doubtful (yellow), and negative (green) cadaver blood samples 2014-2021. Map data ©2021 Geobasis-DE/BKG (©2009), Google

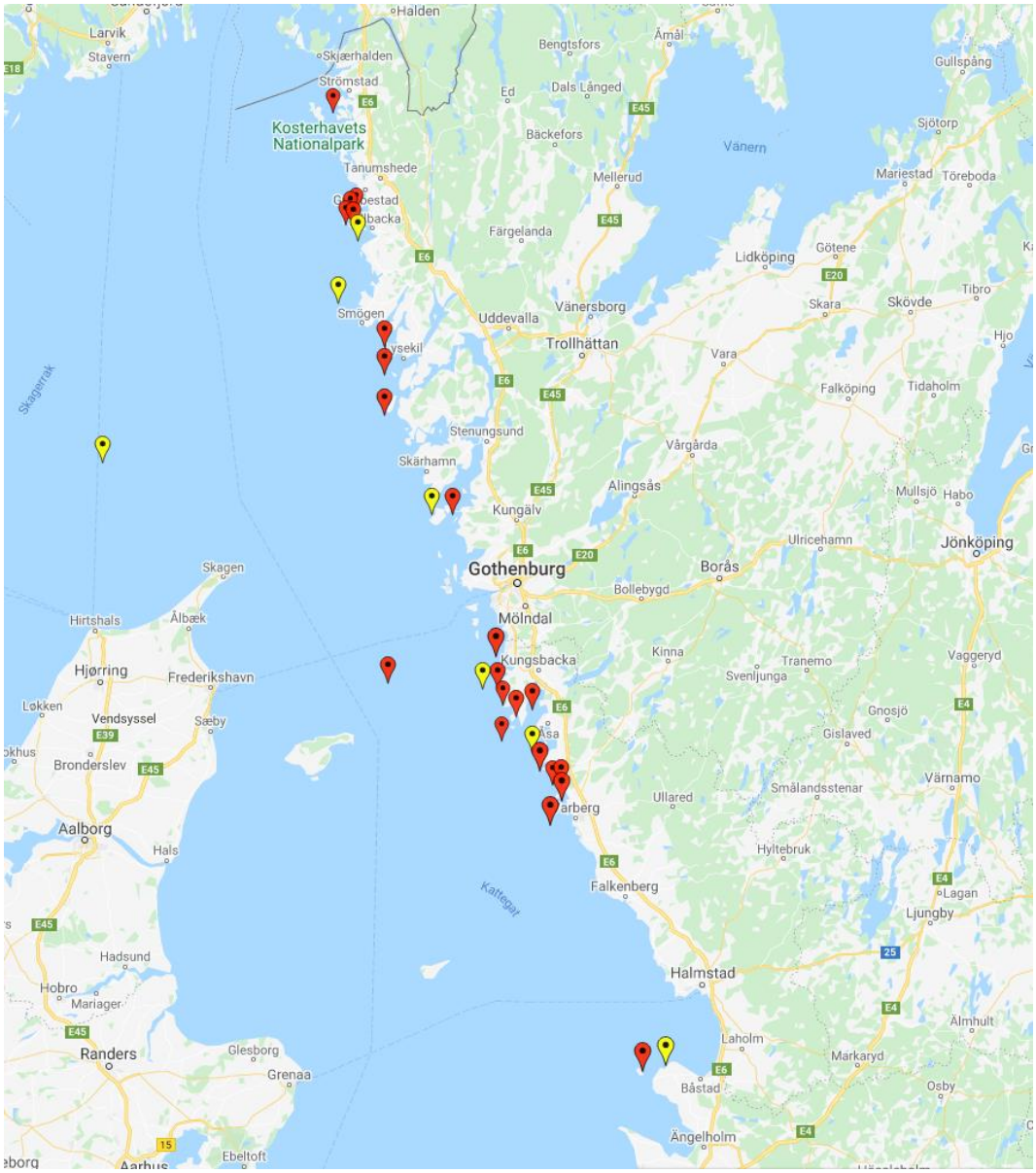

Figure 4. ELISA positive (red) and doubtful (yellow) cadaver blood samples 2014-2021. Map data ©2021 Geobasis-DE/BKG (©2009), Google.

Age class

Seals were divided into three different age groups based on their total length. An overview of the number of positive and doubtful samples per year and age group can be seen in table 8. In 2014, only three cadaver blood samples were collected, with one adult four years or older being ELISA positive. In 2015, 2 out of 22 young of year (9.1%) were positive, and 1 out of 22 young of year (4.5%) was regarded as doubtful. The two positive young of year were sampled in April and July, and the doubtful young of year was sampled in August. In adults, 9 out of 48 adults 1-4 years old (18.8%) were positive, and 3 out of 48 (6.3%) were doubtful in 2015. In adults four years or older, 9 out of 24 samples (37.5%) were positive, and 1 out of

24 samples (4.2%) were doubtful. In 2016, no positive or doubtful young of year was identified. In adults, 1 out of 14 samples (7.1%) from adults 1-4 years old was positive, and 1 out of 14 samples (7.1%) was regarded as doubtful. In adults four years or older, 1 out of 8 samples (12.5%) was positive. In 2020, 1 doubtful sample was identified from the only individual estimated as older than four years sampled that year.

Table 8. ELISA positive and doubtful results from cadaver blood samples per year and age group sampled. All harbour seals from the west coast 2014-2021 are included in the table, except for two adult seals sampled in 2018 (age unspecified, both seals negative).

Year	**Total no. of samples**	**Young of year**			**Adults 1-4 years**			**Adults > 4 years**		
		Pos.	Doub.	Total	Pos.	Doub.	Total	Pos.	Doub.	Total
2014	3			1				1		2
2015	94	2	1	22	9	3	48	9	1	24
2016	25			3	1	1	14	1		8
2020	12			7			4		1	1
2021	1			1						
Total	135			34			66			35

Other sample data

Besides the date found, location and age class, results from cadaver blood samples were also visualised regarding sex, total length, weight, and sternal blubber. However, with these parameters, no apparent differences between ELISA positive and negative individuals were observed.

4.2. Hemagglutination inhibition assay

All ELISA positive and doubtful serum and cadaver blood samples were further subtyped by HI. A sample was regarded as positive if inhibition was seen at serum dilution 1:16 or more against antigen titre four HAU.

Table 9. Interpretation of hemagglutination inhibition assay results.

HI-titre	**Interpretation**
$\leq$ 1:8	No inhibition, negative sample
1:16-1:64	Moderate inhibition, positive sample
$\geq$ 1:128	Strong inhibition, positive sample

4.2.1. Serum samples

All ELISA positive (n=24) and doubtful (n=2) serum samples were further subtyped by HI. HI was performed twice with all ELISA positive and doubtful serum samples. The result from HI is summarised in figure 5 below and the complete result can be seen in appendix 1.

A strong inhibition was seen for all serum samples with H10N7 (A/Seal/Sweden/0546/2014), H10N7 (A/Chicken/ Germany/N/1949), H10N4 and H10N9. However, the highest HI-titre (1:2048) was seen with the antigen H10N7 (A/Seal/Sweden/0546/2014). A moderate inhibition was seen in all samples with antigen H7N7. The result indicates that all ELISA positive and doubtful seals had seroconverted against the A/Seal/Sweden/0546/2014 virus from the outbreak in 2014. Positive reactions with other antigens had a lower HI-titre and is likely a result of cross-reaction between antibodies against seal IAV H10N7 and influenza A viruses of similar subtypes.

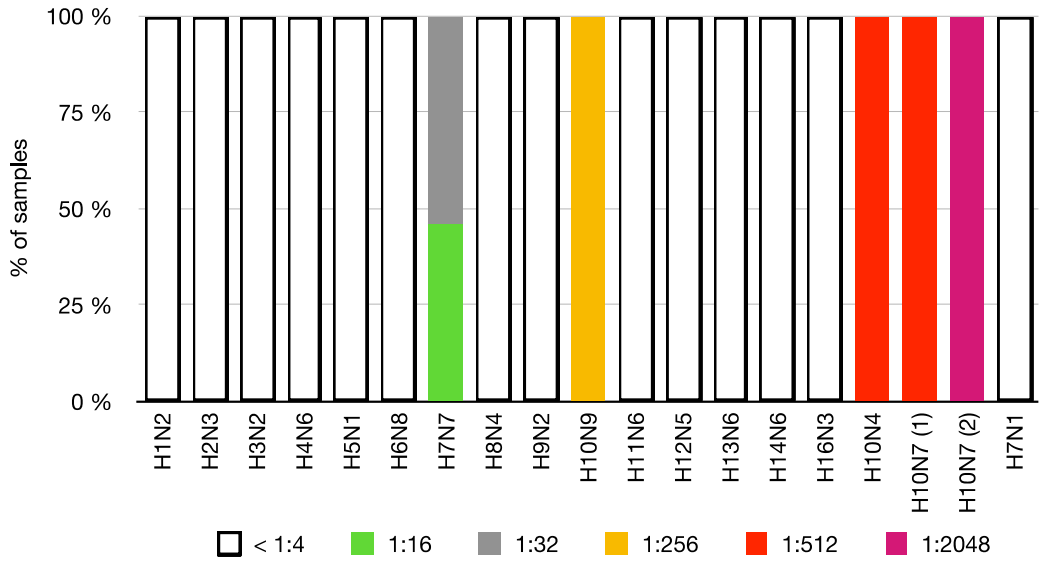

Figure 5. Results from hemagglutination inhibition assay on ELISA positive and doubtful serum samples from 2014. H10N7 (1) = A/Chicken/ Germany/N/1949; H10N7 (2) = A/Seal/Sweden/0546/2014.Y-axis = percentage of total number of samples, x-axis = virus subtypes.

4.2.2. Cadaver blood samples

All ELISA positive (n=23) and doubtful (n=7) cadaver blood samples were analysed with HI. HI was performed twice, once with hemadsorption and once without. Without hemadsorption, only spontaneous agglutination was seen. An overview of the results from samples treated with hemadsorption can be seen in figure 6, and complete results can be seen in appendix 2.

With antigens H7N1 and H7N7 all 24 analysed cadaver blood samples failed to inhibit hemagglutination. With antigen H10N7, no inhibition was seen in 20 out of

30 samples (66.7%) and a moderate level of inhibition was seen in 10 out of 30 (33.3%) samples. With antigen H10N9, no inhibition was seen in 15 out of 30 samples (50%) and a moderate level of inhibition was seen in 15 out of 30 samples (50%). One ELISA doubtful sample collected in 2020 was additionally tested with antigen H5N8; the sample showed no inhibition with all five antigens. No clear correlation between strong positive ELISA results and high HI-titres was seen. However, most of the samples with no inhibition for H10 also had an ELISA result in the higher reference span, which indicates a weaker positive response. ELISA doubtful samples also showed no inhibition for H10 (≤1:8) in general.

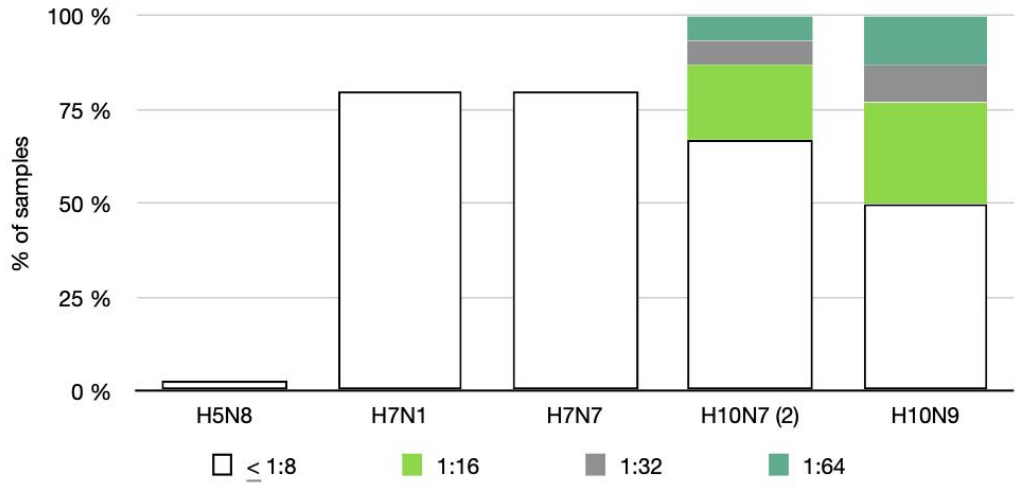

Figure 6. Results from hemagglutination inhibition assay on ELISA positive or doubtful cadaver blood samples from 2014-2020. H10N7 (2) = A/Seal/Sweden/0546/2014. Y-axis = percentage of total number of samples, x-axis = virus subtypes.

5. Discussion

5.1. Follow-up outbreak with H10N7

The results from serologic testing of serum and cadaver blood samples from 2014-2021 from the west coast showed a pattern with high levels of antibodies against seal IAV H10N7 in a large proportion of serum samples from the health control in 2014, with declining levels of antibodies in a lower proportion of cadaver blood samples from the two years following the outbreak. Based on serological results, there is no evidence to suggest that the isolated Kalmarsund population of harbour seals was exposed to the outbreak.

All ELISA positive or doubtful samples collected after 2014 were from seals which most likely were exposed to the virus during the outbreak, and therefore most probably represents persisting antibodies after exposure to the virus. In 2015, two positive and one doubtful sample from seals estimated as young of year (0–1-year-old) were found, and none from that age class was positive or doubtful in 2016. The two positive samples were collected in April and July, whereas the doubtful sample was collected in August. Since pups are generally born in June, it is very likely that the seal sampled in April was exposed to the outbreak in 2014. The positive seal sampled in July had a total length of 114 cm, which indicates that the seal is more likely to be one year old rather than one month old, since the estimated age class 1-4 years old was based on the total length 115-150 cm. It is more difficult to interpret if the ELISA doubtful seal estimated as young of year and sampled in August 2015 was exposed to the outbreak in 2014. To begin with, it is a doubtful sample, which means it could be either true positive or true negative. The seal had a total length of only 86 cm, which could indicate that it was born in June 2015, and not exposed to the outbreak. Low levels of antibodies resulting in a doubtful sample could also be from maternal immunity. Another seroprevalence study following the outbreak with H10N7 in harbour seals also had weak positive samples from young individuals sampled in 2015, and concluded it to be a result of maternal immunity (Bodewes *et al.* 2015b). The transfer of maternal antibodies to harbour seal pups through colostrum intake has been described previously with antibodies to PDV (Ross *et al.* 1994).

After 2016, only one doubtful sample, collected in 2020, was identified in a seal estimated to be four years or older (total length > 150 cm). Again, it is a doubtful sample, which means it could either be true positive or true negative. The sample was additionally tested against H5N8, which circulated in wild birds in Sweden in 2020 (SVA, 2020), but could not be subtyped within the study. However, since the sample came from an older seal (> 4 years) and possibly shows low levels of antibodies, it is not unlikely that the seal lived during the H10N7 outbreak and was exposed to the virus. The sample would then represent persisting antibodies from the outbreak. However, since age classes were estimated based on total length and since the hunter estimated the total length in some cases, and in addition it is a quite rough measure for age, it is difficult to draw any sure conclusions regarding exposure of certain individuals to the outbreak.

The fact that all ELISA positive or doubtful results within the study likely are related to exposure to the outbreak in 2014, indicates a solitary outbreak with IAV in 2014, which did not persist within the harbour seal population of the Swedish west coast. Since ELISA positive and doubtful samples were only found from the population off the west coast and in connection with the outbreak, and not later on, it is unlikely that other IAV subtypes circulated within the Swedish harbour seal populations. However, few samples were collected from the Kalmarsund population 2014-2021 (n=25) and very few from the west coast population in 2017-2021 (n=15). To compare, haul-out counts in 2020 indicate that the west coast population consists of about 16 000-21 000 harbour seals, and the Kalmarsund population of 2500-3400 harbour seals. The low number of samples and the uneven temporal distribution makes it difficult, if not impossible, to predict if more positive or doubtful samples would have been found, had more samples been collected.

Of cadaver blood samples, 50% could be subtyped within the study, which leaves some uncertainty regarding ELISA positive and doubtful samples that could not be subtyped by HI. However, the distinct pattern with high levels of antibodies in a large proportion of serum samples in 2014, with declining levels of antibodies in a lower proportion of cadaver blood samples in 2015 and 2016, indicates that ELISA positive and doubtful results, which could not be subtyped by HI, are consistent with antibodies developed during the outbreak with H10N7. Also, 50% of the samples could be subtyped and showed a positive reaction for H10. Most of the samples that could not be subtyped were from ELISA weak positive or doubtful samples, and it is generally agreed that ELISA is a more sensitive assay than HI, and therefore it is expected that more positive samples will be identified by ELISA. Several studies on IAV in marine mammals have shown a similar pattern with more samples being positive by ELISA than HI (Nielsen *et al.* 2001; Ohishi *et al.* 2002, 2004; Fujii *et al.* 2007).

5.2. Cadaver blood as a sample material

Another aim of the study was to evaluate cadaver blood as a sample material instead of pure serum. As stated by Gardener *et al.* (1996) and Vandalen *et al.* (2009), a diagnostic test might not perform correctly out of its original validation. In this study, a competitive ELISA and HI have been applied for both a species and a sample material which they have not been validated for, although several studies have used ELISA (Stuen *et al.* 1994; Nielsen *et al.* 2001; Ohishi *et al.* 2002, 2006; Capuano *et al.* 2017), HI (Danner *et al.* 1998; Blanc *et al.* 2009; Goldstein *et al.* 2013; Capuano *et al.* 2017) or both (de Boer *et al.* 1990; Ohishi *et al.* 2004; Fujii *et al.* 2007; White *et al.* 2013; Bodewes *et al.* 2015b; Puryear *et al.* 2016; Measures & Fouchier 2021) to investigate the presence of antibodies for IAV in marine mammals previously. Also, both assays used within this study are not species-specific, with the competitive ELISA targeting IAV nucleoprotein and HI targeting hemagglutinin.

On the other hand, the author of this report has not found any studies evaluating cadaver blood collected during a necropsy as a sample material for IAV antibody detection by ELISA or HI in marine mammals. The distinct pattern with positive samples only found in connection with the outbreak indicates that results from ELISA with cadaver blood samples are not false positive. Also, half of the cadaver blood samples could be confirmed and subtyped by HI as stated previously, suggesting that the ELISA results from samples from 2015 and 2016 are not false positive.

Cadaver blood samples can be obtained in different ways from dead animals, and the quality of the sample might differ depending on several factors, for example how and when it is collected and stored. In this study, cadaver blood samples also had to be treated with hemadsorption technique overnight prior to HI, which brings an additional step into the diagnostics. The material can also be more difficult to handle in the lab compared to a pure serum, since it may obtain coagulated blood, making aspiration and titration of the sample more challenging. However, cadaver blood is an accessible sample which can be obtained from hunted or found dead animals, whereas pure serum samples from individual animals are difficult to obtain from wildlife. According to this study, it is possible to detect and subtype IAV antibodies in cadaver blood by ELISA and HI. The result of both ELISA and HI in this study showed a tendency for lower antibody levels in positive cadaver blood samples compared to positive serum samples. This pattern is expected since antibody levels naturally decrease with time; most cadaver blood samples were collected later on and not in direct connection with the outbreak like the serum samples were. An additional explanation could be that antibodies most likely are present at a lower level in cadaver blood compared to serum. Further studies to directly compare serum with cadaver blood in the same animals are needed to investigate this further. However, the results of this study show that cadaver blood

samples can be useful in surveillance on a population level to determine whenever a population has been exposed to the IAV or not.

5.3. Cut-off value for hemagglutination inhibition assay

Within this study, the cut-off value used to interpret HI result was serum or cadaver blood dilution 1:16, with samples inhibiting hemagglutination at dilution 1:16 or more against antigen titre four HAU considered as positive. This cut-off value for HI is used for poultry. Some laboratories prefer to use a higher antigen titre of 8 HAU, which affects the interpretation of HI results, moving the cut-off to serum dilution $\geq$ 1:8 (OIE 2021). However, in this study we investigated a very different species. With poultry there is more experience and research supporting this cut-off, for example research on immune system response to IAV infection or re-infection, and antibody kinetics. In addition, results are usually re-evaluated on a herd basis. In harbour seals, less is known about the immune system and how it responds to an IAV infection, for example how soon circulating antibodies can be detected, the level of antibody response, and for how long antibodies can be detected in a sample post an infection.

However, it is reasonable to assume that the immune system of a seal reacts in a similar way as other mammals' immune systems, when infected with influenza A viruses. Two mammalian species where influenza A is thoroughly studied are horses and swine. Unfortunately, in these two species, no clear cut-off value for HI is established. Instead paired serum samples collected two weeks apart are used, and a four-fold increase or more between samples indicate a recent infection with influenza A (OIE 2015; 2019) With wild mammals, especially hunted or found dead, there is not the option to take paired samples. In other serosurveys of harbour seals, different cut-off values for HI have been used. Bodewes *et al.* (2015b) and Measures & Fouchier (2021) used the cut-off value $\geq$ 1:20 against antigen titre four HAU. Fujii *et al.* (2006) studied Kuril harbour seals and used the cut-off value $\geq$ 1:10 against four HAU, and Boyce *et al.* (2013) used cut-off value $\geq$ 1:40, unknown HA titre, to interpret positive samples. To determine a standard cut-off level for HI in harbour seals, more studies on the harbour seal's immune system and its response to an IAV infection is required. Zohari *et al.* (2014) documented the outbreak with IAV H10N7 from March to October 2014 and the last ELISA positive sample in this study was collected at the end of February 2016, which suggests that antibodies against IAV can persist in harbour seals for at least 1,5 years.

5.4. Influenza A in harbour seals in the future?

The Swedish harbour seal populations are exposed to several threats. Viral epizootics in the past has been one of the major causes of harbour seal mortality in Scandinavia, with up to a 50% population decline seen during the epizootics with PDV in 1988 and 2002 (Härkönen *et al.* 2006). Increased mortality rate was also observed during the outbreak with IAV in 2014 (Zohari *et al.* 2014). In this study, no antibodies against IAV were detected in the Kalmarsund population, and in recent years, no IAV antibodies were detected in the population off the west coast. This implies that the Swedish harbour seal populations are most likely naive and susceptible to new IAV spillovers. In addition, exposure to environmental contaminants present in the Baltic Sea has been revealed as a risk factor, resulting in higher susceptibility to viral infections (Ross *et al.* 1995). Low genetic diversity due to population declines might also have an effect on individual fitness, possibly leading to an impaired immunity (Olsen *et al.* 2013). The level of genetic diversity of the west coast population is similar to other observed populations in Europe (Goodman 1998). However, the isolated Kalmarsund population experienced a severe bottleneck with approximately only 10 reproductive females in the 1970s, resulting in a low genetic variation within the population (Härkönen *et al.* 2005; Härkönen & Isakson 2010), which could indicate a higher susceptibility to an IAV spillover in the future. In addition, the Kalmarsund population in the Baltic Sea is likely more heavily exposed to environmental contaminants than the seals off the west coast.

In the case of a new spillover event, increased contact rate is likely one of the most important factors for increased transmission resulting in a new outbreak. Factors likely to influence contact rate between harbour seals are increased population size, decreased habitat, and seasonal behaviour. Annual surveys of the Swedish harbour seal populations has lately shown a decrease in population growth in both Skagerrak and Kattegat, suggesting that the population of the west coast is approaching carrying capacity (ICES 2021). Such a pattern has not been seen in the Kalmarsund population, which is still increasing annually by 9%. In their review on the status of harbour seals in Southern Scandinavia, Olsen *et al.* (2013) describes a situation where harbour seals have disappeared from areas close to human developments and aggregate in protected areas such as sanctuaries. Large population sizes in smaller habitats paves the way for transmission of an infectious disease such as IAV. In addition, seasonal behaviour might have an effect since harbour seals spend more time ashore during breeding season and the annual moult. Harbour seals are the only known marine mammals were IAV infections have resulted in severe outbreaks with mass mortalities, why other marine mammal species are not as severely affected is still largely unknown.

5.5. Why surveillance IAV in harbour seals?

Surveillance of IAV in harbour seals is also of interest from a public health perspective. Spillover from aquatic birds to marine mammals indicate that these viruses may pose a threat to the health of other mammal species, including humans. Cases of direct transmission of IAV from seals to humans have been documented causing conjunctivitis in humans (Webster *et al.* 1981a). Several authors, such as White (2013) and Ohishi *et al.* (2002; 2004), have also raised a concern about the possibility of seals acting as mixing vessels for influenza, combining genes from human and avian influenza A viruses through genetic reassortment, and thus creating new strains with pandemic potential. In the avian influenza H3N8 virus causing an outbreak in harbour seals in 2011, the ability to use both avian and mammalian receptors for cell entry and thus viral replication was found (Anthony *et al.* 2012). In addition, the virus had mutations which are known to increase transmission and pathogenicity.

Circulation of IAVs is not solely a problem for the health of marine mammals, it also might have implications for public health through spillover to wild and domestic animals, as well as humans. Cadaver blood from hunted and found dead seals yields the possibility to survey populations which are otherwise difficult to sample, providing an important tool for understanding the disease dynamics, disease control and possibly prevention of pandemic influenza.

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Acknowledgements

First, I would like to thank my supervisor Sanna Sternberg Lewerin and assistant supervisors Aleksija Neimanis and Siamak Zohari for their invaluable support with this degree project. Especially I would like to thank Siamak Zohari for his support, patience, and cheerfulness during the many hours of laboratory work. I would also like to thank Tero Härkönen who performed the health check of harbour seals and collected the serum samples in 2014. Lastly, I would like to thank everyone else who were involved in the sample collection and data gathering at the Swedish Museum of Natural History and the National Veterinary Institute, Sweden.

Popular science summary

The aim of this study was to do a follow-up of the influenza outbreak in Swedish harbour seals in 2014. The overall purpose was to increase the knowledge about influenza infections in the Swedish harbour seal populations. More specific objectives included to investigate the presence of antibodies against different types of influenza and to evaluate cadaver blood as a sample material instead of a pure serum for detection of antibodies.

Influenza is caused by a group of influenza viruses. There are four different kinds of influenza virus; influenza A which infects humans, other mammals, and birds; influenza B which infects humans and seals; influenza C which infects humans, pigs, and dogs; and influenza D which infects pigs and cattle. Influenza A viruses (IAV) can be further divided into different types based on two proteins, which are exposed on the surface of the virus. These come in many variants and in various combinations and can create up to almost 200 different types of IAV.

Evidence of infection with IAV has been detected in several different marine mammals. However, large outbreaks with mass mortalities have only been seen in harbour seals. In 2014, the first documented outbreak with IAV in harbour seals in Europe was reported, starting in coastal waters off the Swedish west coast. The outbreak further spread to Denmark, Germany, and the Netherlands, and more than 2000 harbour seals died during the event.

IAVs are usually carried by wild aquatic birds, which seals largely share their habitat with. The fact that marine mammals such as harbour seals can get infected with a virus from birds, indicates that the virus might have acquired certain traits, possibly making them able to infect other mammals and even humans. A so-called spillover of influenza from birds to marine mammals is a health problem for the seals, but it could also pose a threat to public health.

When infected with a virus, the immune system is activated. One of the responses of the immune system is to create specific antibodies to the virus. Antibodies remains for some time after an infection, and detection of antibodies can thus provide evidence of past infection. In serological studies, the presence of antibodies is investigated. This can be used to map the exposure to, for example, certain viruses within a population, which is important for disease control. However, a blood sample is required which can be challenging to obtain from wild

animals such as harbour seals. Cadaver blood is a sample material which more readily can be obtained from hunted or found dead animals.

In Sweden, three different seal species live and breed; harbour seal, grey seal and harp seal. The harbour seal is found in southern Sweden, with one population on the west coast and another on the east coast (the Kalmarsund population). All seal species are classified as species of least concern, except for the Kalmarsund population, which is isolated and classified as vulnerable.

In this study, serum and cadaver blood samples were analysed for antibodies against IAV, and positive samples were further analysed to detected the IAV type. Serum samples were collected during a health control of the harbour seal population off the west coast in the fall of 2014. Cadaver blood samples were mostly collected from hunted seals, but also from seals found as incidental by-catch in fishing gear, or from stranded seals. These samples were collected from the west and east coast from 2014 to 2021, with most samples collected in 2015.

The results show a high level of antibodies against IAV in a large proportion of serum samples from 2014, with declining levels of antibodies against IAV in a lower proportion of cadaver blood samples from the west coast in 2015 and 2016. IAV antibodies were not found in samples from the Kalmarsund population, and not in the population of the west coast after 2016. All samples with antibodies for IAV could be related to exposure to the virus from the outbreak in 2014. In addition, no evidence of infection with other IAV types was found.

Since no antibodies were detected in samples collected after 2016, we know from this study that there is most likely no specific protection against IAV in the Swedish harbour seal populations in the case of a new influenza outbreak. According to this study, cadaver blood samples from hunted or found dead animals can be used in surveillance to detect and type IAV antibodies in harbour seals. Cadaver blood samples could therefore be an important tool for disease control and possibly prevention of new influenza outbreaks.

Appendix 1

Here, both ELISA and HI results are presented from ELISA positive and doubtful serum samples. For ELISA, the numbers represent competition percentage, and a sample was regarded as positive if S/N % ≤ 45% and doubtful if 45% < S/N% < 50%. For HI result, numbers indicate the level of inhibition. A sample was regarded as negative for ≤ 1:8 and positive for ≥ 1:16. HI-titre 1:16-1:64 was interpreted as moderate inhibition, and ≥ 1:128 as strong inhibition.

Table 10. ELISA and hemagglutination inhibition assay results from positive and doubtful serum samples 2014.

No	*ELISA*	*H1N2*	*H2N3*	*H3N2*	*H4N6*	*H5N1*	*H6N8*	*H7N7*	*H8N4*	*H9N2*	*H10N9*	*H11N6*	*H12N5*	*13N6*	*H14N6*	*H16N3*	*H10N4*	*H10N7*[7]	*H10N7*[8]	*H7N1*
1	13	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:32	< 1:4	< 1:4	1:256	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:512	1:512	1:2048	< 1:4
2	36	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:16	< 1:4	< 1:4	1:256	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:512	1:512	1:2048	< 1:4
3	19	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:32	< 1:4	< 1:4	1:256	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:512	1:512	1:2048	< 1:4
4	35	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:16	< 1:4	< 1:4	1:256	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:512	1:512	1:2048	< 1:4
5	17	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:32	< 1:4	< 1:4	1:256	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:512	1:512	1:2048	< 1:4
6	22	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:32	< 1:4	< 1:4	1:256	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:512	1:512	1:2048	< 1:4
7	25	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:32	< 1:4	< 1:4	1:256	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:512	1:512	1:2048	< 1:4
8	43	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:16	< 1:4	< 1:4	1:256	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:512	1:512	1:2048	< 1:4
9	29	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:32	< 1:4	< 1:4	1:256	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:512	1:512	1:2048	< 1:4
10	26	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:32	< 1:4	< 1:4	1:256	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:512	1:512	1:2048	< 1:4

[7] Strain: A/Chicken/ Germany/N/1949

[8] Strain: A/Seal/Sweden/0546/2014

No	*ELISA*	*H1N2*	*H2N3*	*H3N2*	*H4N6*	*H5N1*	*H6N8*	*H7N7*	*H8N4*	*H9N2*	*H10N9*	*H11N6*	*H12N5*	*H13N6*	*H14N6*	*H16N3*	*H10N4*	*H10N7*	*H10N7*	*H7N1*
11	49	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:16	< 1:4	< 1:4	1:256	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:512	1:512	1:2048	< 1:4
12	22	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:32	< 1:4	< 1:4	1:256	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:512	1:512	1:2048	< 1:4
13	43	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:16	< 1:4	< 1:4	1:256	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:512	1:512	1:2048	< 1:4
14	31	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:16	< 1:4	< 1:4	1:256	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:512	1:512	1:2048	< 1:4
15	12	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:32	< 1:4	< 1:4	1:256	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:512	1:512	1:2048	< 1:4
16	38	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:16	< 1:4	< 1:4	1:256	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:512	1:512	1:2048	< 1:4
17	41	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:16	< 1:4	< 1:4	1:256	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:512	1:512	1:2048	< 1:4
18	29	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:32	< 1:4	< 1:4	1:256	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:512	1:512	1:2048	< 1:4
19	23	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:32	< 1:4	< 1:4	1:256	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:512	1:512	1:2048	< 1:4
20	25	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:32	< 1:4	< 1:4	1:256	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:512	1:512	1:2048	< 1:4
21	42	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:16	< 1:4	< 1:4	1:256	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:512	1:512	1:2048	< 1:4
22	20	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:32	< 1:4	< 1:4	1:256	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:512	1:512	1:2048	< 1:4
23	24	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:32	< 1:4	< 1:4	1:256	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:512	1:512	1:2048	< 1:4
24	34	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:16	< 1:4	< 1:4	1:256	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:512	1:512	1:2048	< 1:4
25	40	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:16	< 1:4	< 1:4	1:256	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:512	1:512	1:2048	< 1:4
26	48	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:16	< 1:4	< 1:4	1:256	< 1:4	< 1:4	< 1:4	< 1:4	< 1:4	1:512	1:512	1:2048	< 1:4

Appendix 2

Here, both ELISA and HI results are presented from ELISA positive and doubtful cadaver blood samples. For ELISA, the numbers represent competition percentage, and a sample was regarded as positive if S/N % ≤ 45% and doubtful if 45% < S/N% < 50%. For HI result, numbers indicate the level of inhibition, and a sample was regarded as negative for ≤ 1:8 and positive for ≥ 1:16. HI-titres 1:16-1:64 were interpreted as moderate inhibition, and ≥ 1:128 as strong inhibition.

Table 11. ELISA and hemagglutination inhibition assay results from positive and doubtful tissue cadaver blood samples 2014-2021.

Sample number	*ELISA (S/N)*	*HI H10N7*	*HI H10N9*	*HI H7N7*	*HI H7N1*	*HI H5N8*
1	27	1:8	1:8	< 1:4	< 1:4	
2	44	1:16	1:16	< 1:4	< 1:4	
3	45	1:8	1:16	< 1:4	< 1:4	
4	27	1:16	1:16	< 1:4	< 1:4	
5	21	1:16	1:32	< 1:4	< 1:4	
6	39	1:8	1:16	< 1:4	< 1:4	
7	26	1:16	1:32	< 1:4	< 1:4	
8	46	≤ 1:8	≤ 1:8	< 1:4	< 1:4	< 1:4
9	37	1:32	1:16	< 1:4	< 1:4	
10	37	< 1:4	1:8	< 1:4	< 1:4	
11	35	1:64	1:64	< 1:4	< 1:4	
12	34	1:8	< 1:4	< 1:4	< 1:4	
13	26	1:8	1:32	1:8	< 1:4	
14	22	1:32	1:64	< 1:4	< 1:4	
15	30	1:16	1:16	< 1:4	< 1:4	
16	37	1:16	1:64	< 1:4	< 1:4	
17	32	1:8	1:16	< 1:4	< 1:4	
18	36	< 1:4	< 1:4	< 1:4	< 1:4	
19	22	1:64	1:64	1:8	< 1:4	
20	37	< 1:4	< 1:4	< 1:4	< 1:4	
21	44	≤ 1:8	< 1:4	< 1:4	< 1:4	

Sample number	*ELISA (S/N)*	*HI H10N7*	*HI H10N9*	*HI H7N7*	*HI H7N1*	*HI H5N8*
22	22	1:8	< 1:4	< 1:4	< 1:4	
23	43	≤ 1:8	≤ 1:8	< 1:4	< 1:4	
24	13	1:8	1:16	< 1:4	< 1:4	
25	49	≤ 1:8	≤ 1:8			
26	46	≤ 1:8	≤ 1:8			
27	49	≤ 1:8	≤ 1:8			
28	49	≤ 1:8	≤ 1:8			
29	49	≤ 1:8	≤ 1:8			
30	46	≤ 1:8	≤ 1:8			