Endemic Aleutian Disease

A strategy for diagnosis and control

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Endemic Aleutian disease. A strategy for diagnosis and control.

Abstract

Aleutian disease (AD) is a common global disease in farmed mink caused by infection with Aleutian disease virus (ADV). This thesis investigated the use of ADV antibody ELISA as a tool to reduce the impact on welfare and reproduction of mink in ADV endemic areas. First, two ELISA systems were compared. The ELISA system based on VP2 antigen detected ADV antibodies in serum with high sensitivity and specificity when compared to counter immunoelectrophoresis (CIEP). In contrast, the ELISA system based on ADV-G antigen did not perform as well and had a low sensitivity compared to CIEP. The VP2 ELISA also detected antibodies to ADV in dried whole blood eluted from filter paper (DBS) with a preserved high sensitivity and specificity and with good correlation to antibody levels in serum. The ADV antibody levels were comparable over a period of two weeks which may correspond to the time needed to sample all mink in large herds.

The correlation between the estimated antibody level in DBS and the ratio of albumin: gamma globulins (A:γG) in serum in individual mink was generally high but superior for DBS compared to serum samples from the same mink. The use of DBS VP2 ELISA was therefore concluded to be the preferable indirect method for estimating hypergammaglobulinemia in mink. Further, the association between the reproductive performance and the antibody level was also investigated at an individual level. For the primiparous mink, the risk of being barren was associated with the AD status prior to mating. Diseased mink had a higher risk of being barren than non-diseased mink. In addition, the litter size of the primiparous non-barren female mink was also associated with the AD status prior to mating, where diseased mink had a 5% reduced litter size. For older mink, on the other hand, no difference due to AD status was found but they had approximately 5 % larger litters than primiparous mink irrespective of AD status.

To aid selection of future breeders, the female mink could be categorized into two or three different disease categories depending on the mean antibody level in the herd. Selection of healthy mink for breeding can be expected to be beneficial for the welfare of the female mink, the overall breeding performance of the herd, as well as the production economy.

Keywords: Aleutian disease, ADV, plasmacytosis, mink, CIEP, ELISA, hypergammaglobulinemia, breeding result, reproduction, litter size, barren percentage

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Dedication

To my family – love you to the moon and back!

A goal without a plan is just a wish…
Antoine de Saint Exupéry

Contents

List of publications

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

I Andersson, AM.* and Wallgren, P. (2013). Evaluation of two enzyme-linked immunosorbent assays for serodiagnosis of Aleutian mink disease virus infection in mink. *Acta Veterinaria Scandinavica*, 55(1): 86.

II Andersson AM.*, Reineck HB., Nyman AK., and Wallgren P. (2015). Quantitative detection of antibodies to Aleutian disease virus in dried blood spots as an estimation of hypergammaglobulinemia in mink. *Virology & Mycology* 4: 147.

III Andersson, AM.*, Nyman AK., and Wallgren, P. (2016). Serodiagnosis of Aleutian disease virus infection in mink – Short term stability and long term consistency of antibody levels measured by VP2 ELISA. *Veterinary Sciences: Research and Reviews*, 2(1): pp. 23-30.

IV Andersson, AM.*, Nyman AK., and Wallgren, P. (2017) A retrospective cohort study estimating the individual Aleutian disease progress in female mink using a VP2 ELISA and its association to reproductive performance. *Preventive Veterinary Medicine*, 140C: pp. 60-66 (In press).

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The contribution of Anna-Maria Andersson (AMA) to the papers included in this thesis was as follows:

I Designed the study, applied for its funding, sampled mink with support from farm staff, compiled all the data, performed statistical analyses, and wrote the manuscript with input from the co-author.

II Designed the study, sampled mink with support from farm staff, collected data from databases, compiled all the data, performed statistical analyses with support from the supervisors, and wrote the manuscript with input from the co-authors.

III Designed the study, sampled mink with support from farm staff, collected data from databases, compiled all the data, performed statistical analyses with support from the supervisors, and wrote the manuscript with input from the co-authors.

IV Designed the study, collected data from databases, compiled the data, performed statistical analyses in collaboration with the supervisors, and wrote the manuscript with input from the co-authors.

Abbreviations

A:γG	Albumin: Gamma globulin ratio
AD	Aleutian disease
ADV	Aleutian disease virus
AMDV	Aleutian mink disease virus
AUC	Area under the curve
BCR	B cell receptors
CI	Confidence interval
CIEP	Counter immunoelectrophoresis
CV	Coefficient of variation
DBS	Dried blood spots
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
Fc	Fragment, crystallisable (region of the antibody)
HRP	Horseradish peroxidase
IAT	Iodine agglutination test
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IRR	Incidence risk ratio
LCA	Latent class analysis
MCMC	Markov Chain Monte Carlo
MHC	Major histocompatibility complex
n	Number
NK	Natural killer cells
NP	Non-progressive
NS	Non-structural proteins
OD	Optical density
OD_{450}	Optical density at 450 nm wavelength
OIE	Office International des Epizooties

OR	Odds ratio
PBS	Phosphate-buffered saline
PCI	Posterior credibility intervals
PCR	Polymerase chain reaction
ROC	Receiver-operating characteristics
SD	Standard deviation
TOST	Two one-sided test for equivalence
VP	Capsid viral protein
ZIP	Zero-inflated Poisson

1 Background

1.1 Mink production

Mink, *Neovison vison*, have been held for pelt production since the early 20th century (Joergensen, 1985b) and today, more than 50 million skins are produced in the world each year (Kopenhagen Fur, 2016; Fur Europe, 2015). The largest mink producing countries in the world are Denmark, Poland, the Netherlands and China, and together they account for approximately two thirds of the total global production (Fur Europe, 2015). In Sweden, the production is relatively small and in 2015, the number of breeding female mink were approximately 170 000 and about 1 million pelts were produced (Svensk Mink, 2016; Svensk Mink, 2015). There are today around 50 mink herds in Sweden, all located in the Southern parts of Sweden and with most farms situated at Listerlandet, Blekinge, and around Skara, Västra Götaland (Svensk Mink, 2017) (Figure 1).

All mink are kept in wire netting cages with an adjacent nest box. From weaning, juvenile mink are kept in small groups and before the time for sexual maturity, they are moved into separate cages. Thereafter, adult mink only share cage during mating (Fur Europe / European Fur Breeders' Association, 2015; Joergensen, 1985a). In contrast to many other farm animals, mink production in general is completely integrated and the complete cycle takes place at the same farm (Fur Europe / European Fur Breeders' Association, 2015). Consequently, the number of animals in a herd varies greatly during the year (Fur Europe / European Fur Breeders' Association, 2015).

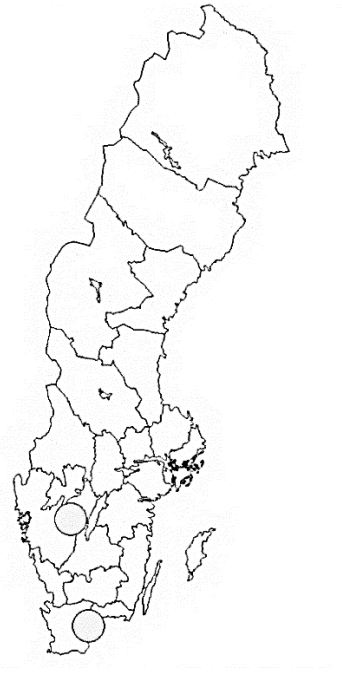

Figure 1. Areas in Sweden where mink herds are primarily located

The production year at a mink herd (Figure 2) is highly predictable since the reproduction in mink is strictly seasonal (Murphy, 1992; Hansson, 1947). The breeding season starts with mating in early March (early spring in the Northern hemisphere) and gestation ends with parturition in late April/early May. At eight weeks of age (late June/early July), the mink kits are weaned and moved two and two into new cages. During the summer, the mink kits grow rapidly and in September, they have reached full body length. Thereafter, the winter fur develops and in October, breeding animals for the next season are selected while the remaining mink are pelted during November and December (Joergensen, 1985a).

The key to success in mink production is to produce a maximum number of large, high quality pelts of a, by the market, desirable colour (Joergensen, 1985b). Hereditary characteristics, in combination with welfare of the animals as well as environmental factors such as climate, feed, hygiene and management, will determine the net production (Joergensen, 1985b). Selection of breeding animals is primarily focused on resistance to disease, reproductive performance and different fur characteristics (Joergensen, 1985b). The past decades, welfare of the animals has rendered more and more focus (Fur Europe / European Fur Breeders' Association, 2015).

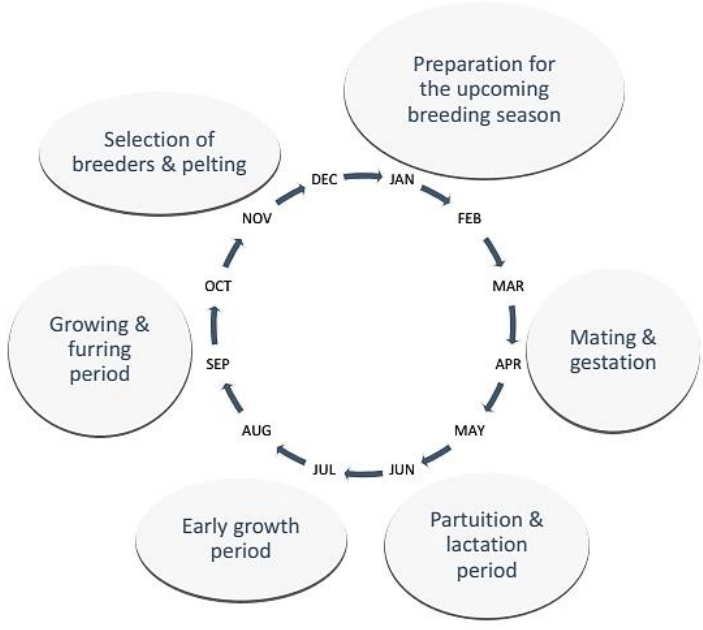

Figure 2. The production year at a mink herd

1.2 The farmed mink

The farmed mink (*Neovison vison*) originates from several subspecies of the North American mink (Nes, 1988; Shackelford, 1957). Originally, the farmed mink had dark brown coat colour and larger areas with white fur were not uncommon (Nes, 1988). In 1931, a new mink colour type evolved due to gene mutation and was named *Silver blue* (Nes, 1988). With time, an array of different dominant and recessive gene mutations has resulted in the wide variety of mink colour types that exist today (Nes, 1988).

Each colour type mutation has its own positive and negative characteristics. For instance, one of the recessive gene mutations, the Aleutian gene, will in homozygous mink not only lead to a dark blue coat colour but also to development of Chediak-Higashi syndrome (Nes, 1988). This syndrome is characterized by a leukocyte defect leading to for instance immunodepression (Nes, 1988). Hedlund white mink, on the other hand, are born deaf leading to a calmer mink but with risk of impaired maternal ability due to impaired kit – female vocal communication (Nes, 1988; Joergensen, 1985b). The choice of colour distribution in the herd is highly specific for each farmer but often the aims are to allow for both market trends and long-term stability which will

decrease the economic risk and enable genetic progress at the same time (Joergensen, 1985b).

1.3 Mink reproduction

1.3.1 General description

Since mink are housed under the influence of natural light and the reproductive cycle is dependent on the day length, reproduction in mink is strictly seasonal (Murphy, 1992; Hansson, 1947). In the Northern hemisphere, female mink accept to be mated during approximately the first three weeks of March (Murphy, 1992). Ovulation of the female mink is induced by mating, and take place within three days after mating (Lagerkvist, 1992; Sundqvist *et al.*, 1989). After ovulation, the oocysts are transported to the oviduct where the fertilization takes place. The fertilized ova then develops into blastocysts that reach the uterus within a week after mating (Lagerkvist, 1992; Hansson, 1947). The embryo development then ceases and the blastocysts float around freely in the uterus during what is called the *embryonic diapause* (Lopes *et al.*, 2004; Sundqvist *et al.*, 1989). The actual length of the embryonic diapause is not fixed but is determined mainly by the increasing day length (Lopes *et al.*, 2004; Allais & Martinet, 1978; Murphy & James, 1974). It is the change in day length associated with the vernal equinox in the Northern hemisphere which leads to hormonal changes of the female mink and initiates the *delayed embryo implantation* (Lefevre *et al.*, 2011; Desmarais *et al.*, 2004; Lopes *et al.*, 2004; Murphy *et al.*, 1981).

Interestingly, this embryonic diapause and the delayed embryo implantation enable mink to have a second ovulation before implantation. A new set of follicles develop within a week after the first ovulation and these follicles will also ovulate if the female mink is re-mated (Lagerkvist, 1992; Murphy, 1992). For this reason, female mink can give birth to kits from different ovulations (*superfetation*) (Shackelford, 1952). At the time of such a second mating 7-8 days after the first mating (“*1+8 or 1+9 system”),* the first set of embryos are floating free in the uterus and the second set of embryos will join the first set of embryos in the uterus. Studies have shown that the relative contribution to the litter born is higher for the second compared to the first mating (Murphy, 1996; Lagerkvist, 1992). An alternative way of repeated mating worth mentioning is mating on two consecutive days (“*1+1 system”).* Here the first mating will induce ovulation but due to the delay of a couple of days until the ovulation occurs, the second mating will also occur prior to the arrival of ova to the

oviduct. The ova can therefore be fertilized by sperm from both matings (*superfecundation*) (Murphy, 1996; Einarsson, 1992; Lagerkvist, 1992)

Parturition occurs 30±3 days after implantation (late April and the beginning of May) (Murphy, 1992; Hansson, 1947). The female mink usually give birth to a litter between one to 12 kits (Svensk Mink, 2017) but unfortunately, some female mink will not give birth (i.e. *barren females*). In Sweden, the mean number of kits born per mated female mink for the years 2012-2016 was 4.8 – 5.1 and the mean percentage of barren females was 8 – 12% (Svensk Mink, 2017).

1.3.2 Factors associated with reproductive performance

Reproductive performance of the female mink can be expressed in terms of percentage of barren females and/or litter size at different times *post-partum* (e.g. at birth, three weeks *post-partum*, six weeks *post-partum*, at weaning or at pelting). Due to kit mortality, this will influence the reported reproductive performance (Svensk Mink, 2017; Joergensen, 1985b) and needs to be taken into account when comparing different studies. In addition, the number of kits born per mated female mink can be reported even though this is a joint measure of two separate processes i.e. being barren and litter size of non-barren female mink. This further complicates comparisons between different studies but some general factors that have been shown to affect the net reproductive performance are the genetic predisposition, presence of disease, age of the female mink, nutrition and feeding as well as mating regimes.

Genetic predisposition

There are many different sub-traits involved in reproductive performance where litter size is a commonly used trait in genetic selection for better reproductive performance. The heritability for litter size in mink is approximately 0.1 – 0.2 (Hansen *et al.*, 2010; Einarsson, 1992). Different genetic lines of mink (colour types, for instance) can therefore differ in reproductive performance (Seremak, 2013; Brzozowski *et al.*, 2012; Dziadosz, 2010; Felska & Sulik, 2000), and intentional or unintentional inbreeding can lead to differences in reproductive performance (Demontis *et al.*, 2011; Berg, 1996).

Diseases

Diseases can negatively affect the reproductive organs and have a detrimental effect on the female reproductive performance. Abortions and death of neonatal kits due to infections with *Campylobacter jejuni*, *Salmonella Dublin*, and

Toxoplasma gondii have been described (Dietz *et al.*, 2006; Frank, 2001; Hunter *et al.*, 1983). Another important disease which can have a significant impact on the reproductive performance is Aleutian disease (AD) (Hansen & Lund, 1988; Kangas & Smeds, 1983).

Age

Reports of the effect of age on reproduction have not been consistent. Some reports have indicated that two-year-old female mink have the best reproductive performance in terms of kits per mated female mink as well as litter size, and that the reproductive performance decreases from the third litter (Tauson, 1985; Venge, 1973; Hansson, 1947). Another study reported no significant difference between primiparous and two-year old female mink, but a decreased number of live kits born from the age of three years (Dziadosz, 2010). In addition, that study reported that the primiparous mink had the highest number of live born kits for some of the colour types (Dziadosz, 2010). This highlights the idea that the various factors most likely interact and together influence the reproductive performance of the mink (Dziadosz, 2010; Tauson, 1985)

Nutrition and feeding

Nutritional factors that can have an effect on the reproductive performance include the amount of nutrients (Matthiesen & Tauson, 2015; Matthiesen *et al.*, 2010; Tauson, 1992; Helgebostad, 1980), the energy supply/female body condition (Tauson, 1985), the hygienic quality and the chemical quality of the feed (Juokslahti, 1980; Moeller & Nordstoga, 1978; Jarosz & Barteczko, 1976). In addition, feeding regimes can play a role and strategic flushing just before mating has been reported to be positively associated with an increased litter size in both primiparous and older female mink (Tauson, 1992; Tauson, 1985).

Mating regimes

Knowledge about the reproduction physiology of the female mink is strongly reflected in the mating regimes practiced with purpose of maximizing the number of kits. Mating only once is known to result in an inferior reproductive performance compared to repeated matings (Lagerkvist, 1992; Venge, 1973). The most commonly used repeated mating system is mating 7-8 days after the first mating (the *1+8 or 1+9 system)* which has been reported to improve the net number of kits compared to both single matings, as well as mating on two consecutive days (the *1+1 system* (Murphy, 1996; Tauson, 1985; Venge, 1973)). In addition, with the aim of further maximizing the net number of kits born, a

combination of the above two repeated mating systems where mink are allowed to mate twice with 6-7 days apart and then thirdly one day after the second mating has been practiced (Murphy, 1996; Venge, 1973). However, recent publications have presented contradictory results where the highest number of kits weaned was documented for female mink mated twice with an interval between the first and the second mating between one and four days (Ślaska & Rozempolska-Rucińska, 2011). Being aware that this contradicted most previous publications, the authors concluded that other factors than the mating system probably also contributed to the reproductive performance. Such factors could include date of first mating, colour type and age which all had previously been demonstrated to have an effect on the reproductive performance (Ślaska & Rozempolska-Rucińska, 2011). Though, for the scope of this thesis, it is enough to conclude that management around mating can influence the reproductive performance.

1.4 Aleutian disease in mink

Infection with Carnivore Amdoparvovirus 1 cause AD in mink and other mustelids worldwide (Farid & Rupasinghe, 2016; Porter *et al.*, 1982). A complex interaction between the virus and the immune system of the mink causes an immune complex mediated disease with diffuse symptoms such as inappetence, impaired growth, and reduced reproductive performance (Reichert & Kostro, 2014; Hansen & Lund, 1988; Eklund *et al.*, 1968). At farm level, AD can have a significant negative impact on both animal welfare and production economy. It has been estimated that the loss of kits due to AD until weaning is up to 1 kit per litter (Kangas & Smeds, 1983) while the mortality from weaning to pelting is slightly increased in AD seropositive herds compared to AD seronegative herds (Svensk Mink, 2017; De Rond. & Kleyn Van Willigen, 2012). Translating this into economic terms, a pelted mink represented in average approximately 20 € in 2015-2016 (Saga Furs Oyj, 2016) leading to an estimated production loss of up to 50,000 € per year due to AD for an average Swedish farmer with 2,500 female mink.

Currently there is no treatment for the disease and traditionally in countries where AD is less prevalent and the pathogen load is low to moderate, stamping out has been used to control AD (Cho & Greenfield, 1978). Stamping out has, however, been less successful in areas where AD is highly prevalent and the pathogen load is high (Themudo *et al.*, 2011). In countries with endemic AD, the focus has instead shifted towards the idea that some individuals seem to be more resistant to development of clinical disease with the aim to develop strategies to control the negative impact of AD (Thomsen, 2016; Farid &

Segervall, 2014). Every year, in conjunction with pelting and/or prior to the breeding season, all mink are screened for AD usually one to three times (Svensk Mink, 2017) to ensure that no clinically diseased animals are bred.

1.4.1 Characteristics of Carnivore amdoparvovirus 1

The Carnivore Amdoparvovirus 1, formerly known as Aleutian Disease Virus (ADV)[1], is a small, non-enveloped DNA virus belonging to the genus Amdoparvovirus, subfamily Parvovirinae and family Parvoviridae (Canuti *et al.*, 2015; Cotmore *et al.*, 2014; International Committee on Taxonomy of Viruses) (Figure 3). Many different strains of ADV have been described (Alexandersen *et al.*, 1994; Aasted *et al.*, 1984a) and often multiple strains can be detected within a herd as well as within an individual mink (Olofsson *et al.*, 1999; Gottschalck *et al.*, 1994; Hadlow *et al.*, 1984)

The ADV genome encodes for two capsid viral proteins (VP) and three non-structural (NS) proteins (Huang *et al.*, 2014; Qiu *et al.*, 2007; Qiu *et al.*, 2006). The capsid proteins VP1 and VP2 form the nucleocapsid together with the genome (Quinn *et al.*, 2011; Lodish H, 2000). By binding to receptor proteins on the host cells, they initiate the infection process (Lodish H, 2000) and this interaction also determines the pathogenicity, host range and cellular tropism of ADV (Kontou *et al.*, 2005; Lodish H, 2000; McKenna *et al.*, 1999). Moreover, it has been suggested that it is one part of the VP2 which is essential for the immune complex formation typical to AD (Bloom *et al.*, 2001). The NS proteins, on the other hand, are not a part of the virus itself. They are instead coded for by the viral genome, expressed in the infected host cells and involved in various processes associated with the replication of the virus (Huang *et al.*, 2014; Uversky & Longhi, 2011; Bloom *et al.*, 1982).

[1] In lack of a well-established abbreviation, the carnivore amdoparvovirus 1 will be referred to as ADV in this thesis for simplicity and in order to avoid confusion even though the taxonomy has been revised.

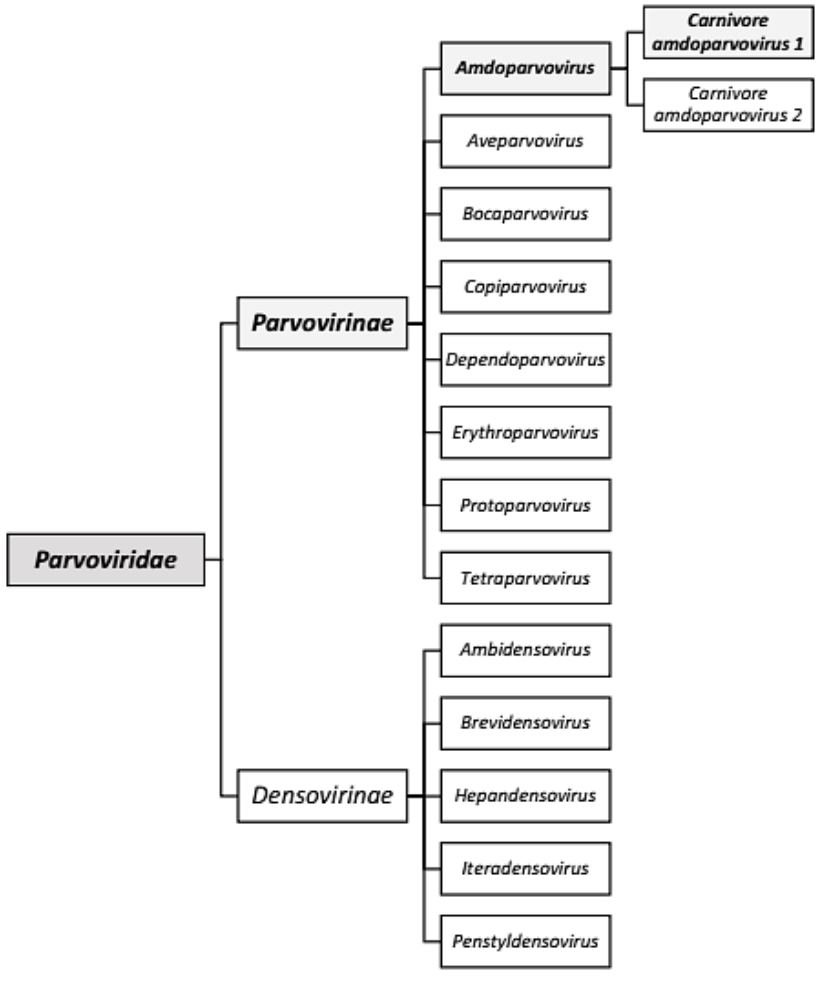

Figure 3. Members of the virus family Parvoviridae (Canuti *et al.*, 2015; Cotmore *et al.*, 2014; International Committee on Taxonomy of Viruses)

1.4.2 Transmission of virus

As seen in Figure 4, ADV can spread both horizontally (Jackson *et al.*, 1996; Gorham *et al.*, 1976; Gorham *et al.*, 1964; Kenyon *et al.*, 1963) and vertically (Broll & Alexandersen, 1996; Gorham *et al.*, 1976; Henson *et al.*, 1976; Padgett *et al.*, 1967a). Depending on the virus strain and genotype of the mink, the rate of transmission will vary (Oie *et al.*, 1996; Gorham *et al.*, 1976; Gorham *et al.*, 1964).

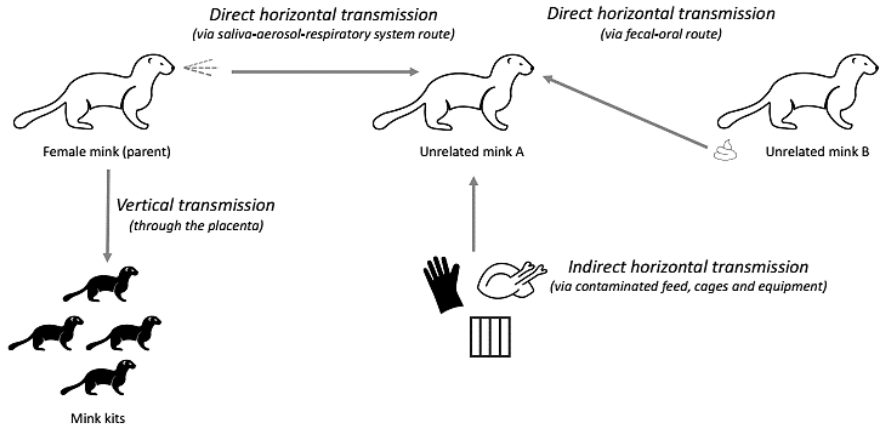

Figure 4. ADV transmission routes within a mink herd: Vertical transmission between the female mink and her kits or horizontal transmission either direct or indirect from one mink to another[2].

1.4.3 The immune response upon infection with ADV

When an individual is infected with a virus, the innate immune system normally induce the initial protection through recognition and killing of the invading microorganism e.g. by phagocytosis and apoptosis by neutrophils, macrophages and natural killer (NK) cells (Abbas *et al.*, 2012; Tizard, 2012a). In addition, signals are sent to the adaptive immune system (Abbas *et al.*, 2012) where the cell-mediated adaptive immune system works through different T cell mediated activities (Abbas *et al.*, 2012). An infected host cell presents foreign peptides (antigens) via the major histocompatibility complex (MHC) proteins on the cell surface and this activates the cytotoxic T cells which then will destruct the infected cell by either cell lysis or apoptosis (Abbas *et al.*, 2012).

With time, the humoral immune response develops where the antimicrobial activity instead is mediated by antibodies produced by plasma cells originating from the B cells (Abbas *et al.*, 2012). Viral antigens bind to the B cell receptors (BCR) leading to selection of the clones of immature B cells with the appropriate BCR (*clonal selection*) and the selected clones begin to divide repeatedly (*polyclonal expansion*) (Abbas *et al.*, 2012; Tizard, 2012a). The B cells then develop further into plasma cells (*differentiation*) resulting in an increased concentration of antigen specific antibodies. Initially, the antibodies produced

[2] Illustration by: Anna-Maria Andersson with graphical contributions as follows: "Mink" icon from Anna Bearne, "feces" icon from Douglas Santos, "cage" icon by corpus celicti, "glove" icon by Dolly Vu and "roast chicken" icon from Emilegraphics – all from the Noun Project, thennounproject.com

by plasma cells are of IgM type, but the production is switched to for instance IgG within a few days. Alternatively, the B cells can differentiate into memory cells which are essential for the immunological memory (Tizard, 2012a).

In general, antibodies aid removal of the infection in four different ways (Abbas *et al.*, 2012): (i) through neutralization where antibodies bind to circulating virus and thereby hinder entrance into the host cells, (ii) through opsonization where antibodies (or complement fragments) coat the viral particles and thereby enhance phagocytosis of the microorganism by neutrophils and macrophages, (iii) through antibody-dependent cellular cytotoxicity where antibodies bind to virus infected cells and thereby aid the lysis of these cells by cells of the innate immune system, and (iv) through activation of the complement system leading to both lysis of microorganisms and indirect enhancement of phagocytosis through opsonization.

However, there are viruses that have evolved methods to resist the immune system. Such viruses manage to invade the host cells where they survive, replicate and further evade the immune system (Abbas *et al.*, 2012). One example is ADV where the antibodies produced bind to the virus but fail to neutralize it. Instead, the entrance of ADV into the host cells is facilitated by the opsonization and Fc-receptor-mediated endocytosis (Tizard, 2012c; Bloom *et al.*, 2001; Lodish H, 2000; Kanno *et al.*, 1993). Once the virus has entered the host cells, the infection commonly becomes non-cytopathic due to an intricate mechanism (Knuuttila, 2015; Alexandersen *et al.*, 1989; Alexandersen *et al.*, 1988; Hadlow *et al.*, 1985; Eklund *et al.*, 1968). Consequently, the virus becomes sequestered, has the possibility to replicate and can persist within the mink for a very long time (Knuuttila, 2015; Alexandersen *et al.*, 1988; Porter, 1986; Hadlow *et al.*, 1985; Porter *et al.*, 1980; Eklund *et al.*, 1968).

The incessant virus replication leads to a continuous stimulation of the humoral immune response, which in turn results in elevated levels of serum gamma globulin (hypergammaglobulinemia) and an accumulation of plasma cells in different organs (plasmacytosis) (Reichert & Kostro, 2014; Bloom *et al.*, 1994; Porter *et al.*, 1980; Porter *et al.*, 1969). At the same time, immune complexes are formed between viral antigens and antibodies that activates the complement system (Maclachlan & Dubovi, 2016; Tizard, 2012b). These immune complexes then circulate in the body and deposit in various tissues. In the tissues, deposition of a large amount of immune complexes and accompanying activated complement components attract neutrophils and lead to a severe inflammatory reaction called type III hypersensitivity reaction (Maclachlan & Dubovi, 2016; Tizard, 2012b). The severity of the hypersensitivity reaction depends on both the affected organ as well as the net amount of immune complexes and activated complement components (Tizard,

2012b). The pathological lesions due to ADV infection is therefore a result of a maladjusted host immune response to the virus rather than the virus infection itself (Porter, 1986), and the degree of tissue lesions has been shown to correlate to the degree of hypergammaglobulinemia (Kenyon *et al.*, 1963). In mink with AD, organs such as kidneys, liver, spleen, bone marrow, uvea and arteries are subject to immune complex mediated tissue damage (Porter *et al.*, 1980) leading to vasculitis (Tizard, 2012b; Porter *et al.*, 1980; Porter *et al.*, 1973), membranoproliferative glomerulonephritis (Tizard, 2012b; Alexandersen *et al.*, 1988; Porter *et al.*, 1973; Henson *et al.*, 1969), splenomegaly, hepatomegaly, lymphadenopathy, CNS and mucosal haemorrhages (Tizard, 2012b; Henson *et al.*, 1976) (Figure 5).

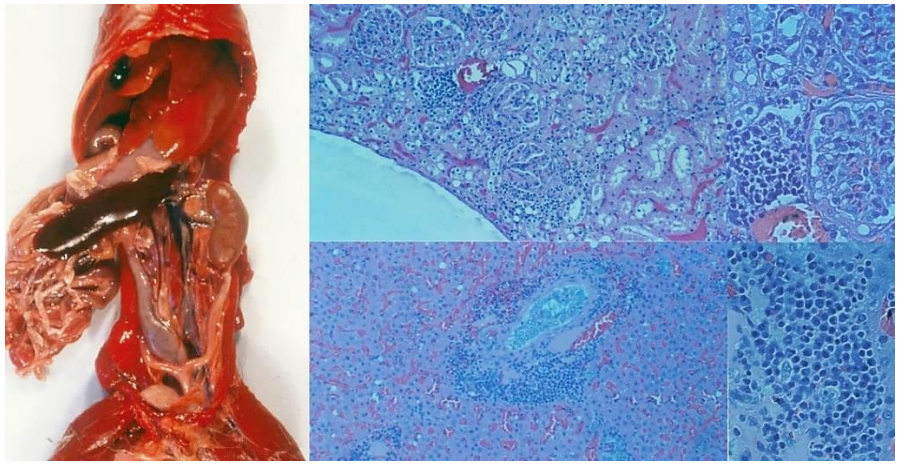

Figure 5. Pathological lesions associated with AD: Left: splenomegaly and petechial haemorrhages on the surface of the kidney. Upper middle & right: interstitial plasma cell infiltration of the renal cortex and mesangial thickening of the glomeruli [kidney]. Lower middle & right: plasma cell infiltration in the portal areas [liver]. Photo by SVA.

The hypergammaglobulinemia in AD mink is essentially a consequence of an increased amount of specific ADV antibodies (Aasted & Bloom, 1984; Porter *et al.*, 1984a; Henson *et al.*, 1976; McGuire *et al.*, 1971) produced against both structural and non-structural viral proteins (Costello *et al.*, 1999; Aasted & Bloom, 1984; Bloom *et al.*, 1982). However, estimations of the correlation between the amount of ADV specific antibodies and the hypergammaglobulinemia have shown varying results with correlation coefficients ranging from -0.39 to 0.82 (Cepica, 2016; Aasted & Bloom, 1984; Porter *et al.*, 1984a). Part of these discrepancies can be due to differences in the different studies concerning the method to determine the serum proteins as well

as the method used for ADV antibody detection (Cepica, 2016; Aasted & Bloom, 1984; Porter *et al.*, 1984a). However, a lack of correlation between the ADV antibody levels and gamma globulin levels of an individual can, just as the clinical manifestation, also possibly be attributed to differences in virus strains and host factors. For instance, infection with highly pathogenic strains of ADV induce high levels of gamma globulins more rapidly than infections with less pathogenic strains (Knuuttila, 2015; Aasted & Bloom, 1984) and the correlation between the antibody level and the level of gamma globulins can then be expected to be high. The genotype of the mink can also play a role for the increase in gamma globulin levels (Johnson *et al.*, 1975; Porter *et al.*, 1973; Padgett *et al.*, 1968; Padgett *et al.*, 1967b). Moreover, during an ADV infection, B cell clones responding to other substances than ADV antigens are also expanding and antibodies to those substances are formed (Hahn & Hahn, 1983). Therefore, the gamma globulin levels can increase although the increase in ADV antibody level has levelled out (Bloom *et al.*, 1994; Bloom *et al.*, 1975). An example is autoantibodies to DNA which can be detected in both normal and infected mink but at higher concentrations in AD mink (Hahn & Kenyon, 1980; Barnett *et al.*, 1969). The amount of autoantibodies has been reported to be proportional to the amount of serum gamma globulins in adult mink with AD (Aasted *et al.*, 1984b; Porter *et al.*, 1980; Porter *et al.*, 1965a; Henson *et al.*, 1961).

1.4.4 Pathogenesis of disease and clinical manifestations

The general target cells for parvovirus infections are actively dividing cells since parvoviruses are dependent upon the S-phase of mitosis (the DNA synthesis phase) to replicate (Maclachlan & Dubovi, 2016; Cotmore & Tattersall, 2014; Quinn *et al.*, 2011; Oleksiewicz & Alexandersen, 1997). Consequently, tissues with dividing cells will be susceptible to parvovirus-induced cell damage and this influences the outcome of AD infections (Maclachlan & Dubovi, 2016).

When adult mink are infected (*classical AD*), cells of the immune system will be the primary locations for replication (B cells, macrophages, and dendritic cells) (Kanno *et al.*, 1993; Aasted & Leslie, 1991; Mori *et al.*, 1991; Alexandersen *et al.*, 1988; Porter, 1986; Porter *et al.*, 1980). The virus crosses the placental barrier in mink infected both prior to and during pregnancy irrespective of the female mink has previous ADV antibodies or not (Broll & Alexandersen, 1996; Clewley *et al.*, 1987; Mengeling, 1978; Mengeling & Cutlip, 1976). Infections during gestation will therefore affect the foetuses since they have a large amount of actively dividing cells. Subsequently, there will be an increased risk for abortions, embryonic death, stillbirth and birth of weak kits

(Hansen & Lund, 1988; Padgett *et al.*, 1967a). The decreased reproductive performance is often one of the most apparent clinical symptoms at herd level both in terms of an increased percentage of barren females as well decreased mean litter size (Broll & Alexandersen, 1996).

In neonatal mink which have not been transplacentally infected or are born without maternal antibodies to ADV, the type II cells of the lungs will be the primary target cells leading to interstitial pneumonia, surfactant deficiency and an acute form of disease with high mortality (*neonatal AD*) (Alexandersen, 1986). In contrast, AD has a chronical progression in neonatal mink with maternal antibodies since the maternal antibodies partially restrict the viral replication and limit tissue damage. The disease thereby resembles *classical AD* in adult mink (Alexandersen *et al.*, 1989; Alexandersen, 1986).

Apart from the direct cytopathic effects of ADV infection, most of the clinical signs of *classical AD* are diffuse and relate to the immune complex mediated organ damage: lethargy, anorexia, cachexia, polyuria, polydipsia, dehydration, uraemia, neurological symptoms, anaemia, thrombocytopenia, melena, and eventually death (Porter *et al.*, 1980; Henson *et al.*, 1976; Eklund *et al.*, 1968).

The degree of pathological lesions/clinical symptoms/mortality due to ADV infection is the net result of a combination of virus factors (strain and dose), host factors (genotype and age) and individual factors (immune status of the mink). In addition, the environment and different physiological determinants may have an effect (Gorham *et al.*, 1976; Eklund *et al.*, 1968):

- Virus factors: There are hundreds of different strains of ADV described (Knuuttila, 2015; Christensen *et al.*, 2011; Knuuttila *et al.*, 2009b; Olofsson *et al.*, 1999) which range from being highly virulent to avirulent (Knuuttila, 2015; Knuuttila *et al.*, 2009b; Alexandersen *et al.*, 1994; Aasted *et al.*, 1984a). Example of highly pathogenic strains are ADV-Utah 1, ADV-Ontario and ADV-TR (Oie *et al.*, 1996; Hadlow *et al.*, 1983; Porter *et al.*, 1969) while strains such as ADV-Montana and ADV-Pullman are known to be moderately – low virulent strains (Hadlow *et al.*, 1983; Bloom *et al.*, 1975). Alternatively, it has been argued that it is rather a difference in time for onset of pathological lesions where some strains cause higher levels of hypergammaglobulinemia and severe pathological lesions faster than other strains (Alexandersen *et al.*, 1994; Johnson *et al.*, 1975; Porter *et al.*, 1973; Padgett *et al.*, 1967b).
- Genotype of the mink: Mink with the Aleutian gene (Aleutian mink and colours crossbred with these) are more susceptible to infections, since the blue-grey pigmentation of the fur is linked to a lysosomal disorder causing immune deficiency and bleeding anomalies (*the Chediak-Higashi*

syndrome) (Anistoroaei *et al.*, 2013; Hadlow *et al.*, 1983; Bell *et al.*, 1980; Bloom *et al.*, 1975; Larsen & Porter, 1975; Padgett *et al.*, 1967b). However, there is an interaction between the virus strain and the genotype of the mink. With the highly pathogenic strains, the genotype of the mink seems subordinate and mink of any colour type develop disease. In contrast, with a few exceptions, only mink with the Aleutian gene will develop severe disease when infected with low pathogenic strains of ADV (Hadlow *et al.*, 1984; Hadlow *et al.*, 1983; Porter *et al.*, 1969; Eklund *et al.*, 1968). Thus, it can be concluded that different strains of ADV differ in their ability to cause disease in Aleutian and non-Aleutian mink genotypes. Aleutian mink have been reported to typically die within a couple of months *post-infection* (Eklund *et al.*, 1968) whilst non-Aleutian mink rarely die earlier than five months after infection and may survive for several years depending on virus strain (Hadlow *et al.*, 1983; Porter *et al.*, 1980; Gorham *et al.*, 1965).

- Individual factors: In general, disease develops faster in Aleutian mink than in non-Aleutian mink, but also among non-Aleutian mink the severity and rate of clinical signs vary greatly between individual mink (Eklund *et al.*, 1968).

Two principally different forms of ADV infection in adult mink have been reported:

- Progressive persistent AD (classical AD): With time after infection with ADV, the levels of IgG continue to increase and the clinical disease progresses (Porter *et al.*, 1984a).
- Non-progressive AD (NP-AD): This form of AD can further be differentiated into (i) *persistent NP-AD* characterized by low antibody titres, low levels or absence of hypergammaglobulinemia, no pathological lesions but ADV in limited amount in internal organs (Bloom *et al.*, 1994; An & Ingram, 1977), and (ii*) transient NP-AD* where the mink have a normal cellular immune response (An & Wilkie, 1981), low and decreasing antibody titres, transient increase of serum gamma globulin and no viremia (Jackson *et al.*, 1996; Porter, 1986; Hadlow *et al.*, 1984; An & Ingram, 1977; Henson *et al.*, 1976; Larsen & Porter, 1975; Eklund *et al.*, 1968). It has been estimated that the non-progressive form of AD may correspond to 25-40% of the infections in non-Aleutian mink (An *et al.*, 1978; Larsen & Porter, 1975).

1.5 Detection of ADV and diagnosis of AD and hypergammaglobulinemia

In general, diagnostic tests for viral infections can be based on identification of cytopathic cellular changes, virus isolation, detection of viral components or evaluation of the immune response to the infection (Murray *et al.*, 2015). While histopathology is a good method for diagnosing AD in euthanized mink, methods that can be performed on live mink are preferable when screening for AD in a mink herd. Since field strains of ADV are challenging to grow in cell culture (Bloom *et al.*, 1980), two important diagnostic methodologies for ADV infection and AD are identification of viral components and evaluation of the immune response.

1.5.1 Identification of viral components

Identification of ADV components can be performed with polymerase chain reaction (PCR) or real-time PCR (Prieto *et al.*, 2014; Jensen *et al.*, 2011). PCR has three main advantages: (i) it detects presence of virus DNA, (ii) it is an extremely sensitive technique, and (iii) with real-time PCR it can also be used to estimate the viral load in the sample (Maclachlan & Dubovi, 2011). However, there are also some drawbacks: (i) the method is so sensitive so that contamination during sampling in a farm environment can easily occur and lead to a false positive result (Maclachlan & Dubovi, 2011), (ii) since viremia and faecal excretion is intermittent, samples from spleen and/or mediastinal lymph nodes are preferred and this means in reality that the mink need to be euthanized (Jensen *et al.*, 2014; Jensen *et al.*, 2011; Maclachlan & Dubovi, 2011), and (iii) PCR for ADV is substantially more expensive than other available diagnostic methods (SVA, 2016) and therefore not suitable for mass screening purposes. Consequently, PCR is best used for confirmation of dubious results or for virus sequencing (Jensen *et al.*, 2011).

1.5.2 Detection of the immune response

The immune response following ADV infection is a key factor in the development of disease and can be mirrored by detection of ADV specific antibodies (the ADV infection) or by detection of increased level of serum gamma globulins (the hypergammaglobulinemia).

For detection of specific ADV antibodies, several different serological methods have been developed (Farid & Segervall, 2014; Knuuttila *et al.*, 2009a; Aasted *et al.*, 1986; Alexandersen & Hau, 1985; Aasted & Cohn, 1982; Crawford *et al.*, 1977) based on the fact that in ADV, antibodies are directed at both the

structural (VP2 and VP1) and the non-structural proteins (primarily NS1) (McKenna *et al.*, 1999; Bloom *et al.*, 1997; Porter *et al.*, 1984a; Porter *et al.*, 1984b; Bloom *et al.*, 1982). In *counter immunoelectrophoresis (CIEP)*, an electrophoresis is performed with the ADV antigen on one side and plasma samples from the mink on the opposite side of an agarose gel (Cho & Ingram, 1972). When the electrical voltage is applied, the negatively charged antigen will move towards the anode (Figure 6a). If the plasma sample contains ADV antibodies, which are positively-charged, they will move towards the cathode and meet the antigen on its way. As a result, antigen-antibody immune complexes will form and can be visualized as a white precipitation line on the agarose gel (Figure 6b; (Cho & Ingram, 1972)). When CIEP was first introduced in the early 1970's by Cho and Ingram(Cho & Ingram, 1972), the antigen used was whole virus propagated in mink. During the early 1980's, cell culture derived virus antigen was introduced (Aasted & Cohn, 1982). Since then, one of the most widely strains for diagnostic purposes has been the ADV-G strain which is a non-pathogenic strain derived from an initially highly pathogenic strain (Utah 1) (Bloom *et al.*, 1980). Even though CIEP has not been completely validated according to the standards presented by OIE, it has been used for diagnosing ADV infections in mink in many countries and has since long been regarded as the gold standard test for ADV infection (Knuuttila, 2015; Bloom *et al.*, 1997; Uttenthal, 1992; Cho & Greenfield, 1978). The sensitivity has been estimated to 79% - 99% and the specificity to 90% - 100% (Dam-Tuxen *et al.*, 2014; Knuuttila *et al.*, 2014; Aasted *et al.*, 1986; Wright & Wilkie, 1982).

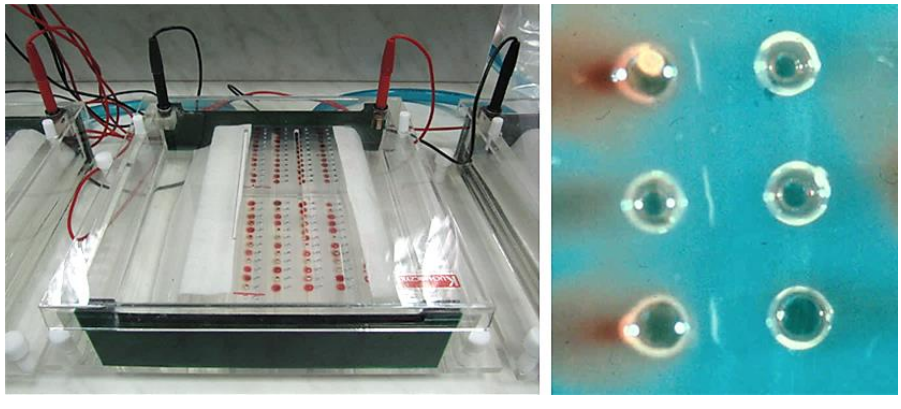

Figure 6. Counter immunoelectrophoresis – (a) the set-up (to the left), and (b) the white precipitation line formed when antigen and ADV antibodies meet (to the right). Photo by SVA.

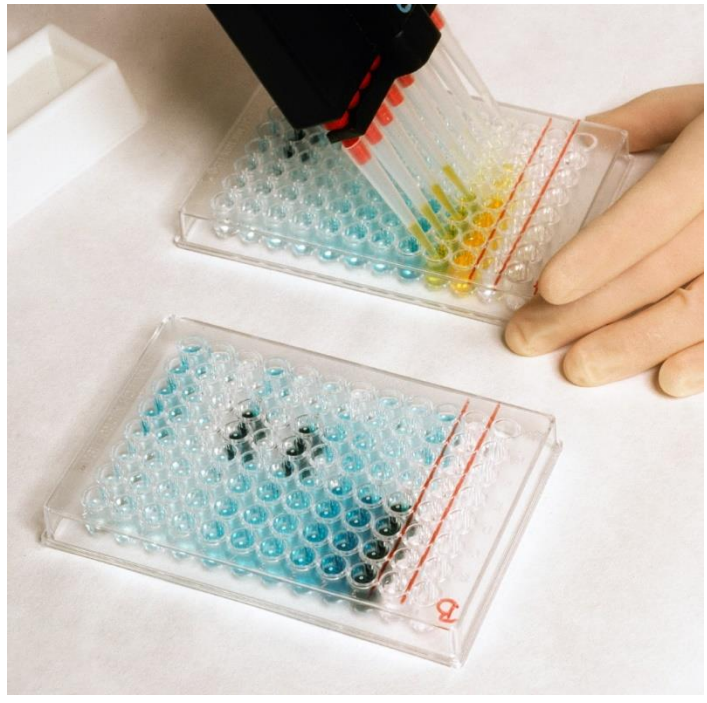

Figure 7. Performance of an ELISA. Photo by SVA.

Several different enzyme-linked immunosorbent assays (ELISA systems; Figure 7) have also been developed and evaluated for diagnosis of ADV infection (Dam-Tuxen *et al.*, 2014; Knuuttila *et al.*, 2014; Knuuttila *et al.*, 2009a; Wright & Wilkie, 1982). The first ELISA for detection of ADV antibodies was based on whole virus antigen (Guelph strain) (Wright & Wilkie, 1982). However, due to false negative results in comparison to CIEP, ELISA was for many years considered not suitable for diagnosis of ADV infection. In 2009, a new ELISA system for ADV based on recombinant VP2 antigen was presented that detected ADV infected mink with high sensitivity and specificity when compared with CIEP (Knuuttila *et al.*, 2009a). In parallel, an ELISA system based on whole virus antigen (ADV-G) was manufactured and used in North America (Aleutian Disease Virus (AMDV) Antibody ELISA Test, Reference ADV3005, Scintilla Development Company LLC, Bath, Pennsylvania, USA). However, until this project started, no scientific validation of the latter had been published. As the ELISA systems became a more reliable diagnostic tool for ADV infection in mink, the possibility to develop an automated ELISA was considered. In 2014, an automated ELISA based on a modified form of the ADV-G antigen, traditionally used in CIEP, was developed and scientifically evaluated (Dam-Tuxen *et al.*, 2014). The same year, an adaptation of the ELISA

based on the recombinant VP2 antigen for an automated system was also published (Knuuttila *et al.*, 2014). The largest advantage of the ELISA compared to CIEP is that it can be used both for qualitative and quantitative detection without any dilution steps. Also, the ELISA is an objective assay which is not dependent upon any subjective interpretation of the visual appearance of the agarose gel as CIEP is.

Both the available ELISA systems and CIEP are dependent upon production of IgG antibodies in the mink, and therefore a positive test result cannot be expected until a couple of weeks after infection (Farid *et al.*, 2015; Jensen *et al.*, 2014; Hadlow *et al.*, 1985; Hadlow *et al.*, 1983). However, the advantage of serological methods are that they detect the immunological response to the infection rather than presence of the infectious agent per se (Maclachlan & Dubovi, 2011) even though a possible limitation is the risk of misclassifications i.e. false positive and false negative results (Murray *et al.*, 2015). Several studies have compared the diagnostic performance of CIEP with other methods such as ELISA, and the position of CIEP as gold standard has been questioned (Dam-Tuxen *et al.*, 2014; Farid & Segervall, 2014; Knuuttila *et al.*, 2014; Knuuttila *et al.*, 2009a; Wright & Wilkie, 1982). For instance, it has been reported that CIEP underestimates the antibody titre and therefore might fail to identify ADV infected mink at an early stage of infection or mink with non-progressive AD i.e. when the antibody levels are low (Farid *et al.*, 2015; Hadlow *et al.*, 1985; Crawford *et al.*, 1977; Bloom *et al.*, 1975).

In addition to detection of specific ADV antibodies, the characteristic hypergammaglobulinemia associated with AD can also be used as a diagnostic criterion. The serum of mink with progressed AD is characterized by an increased total amount of proteins, an increased proportion of gamma globulins and a decreased proportion of albumin (Cepica *et al.*, 2012; An & Ingram, 1977; Porter *et al.*, 1965b; Porter *et al.*, 1965a; Henson *et al.*, 1961). While the proportion of gamma globulins in serum/plasma of healthy mink is around 13% (An & Ingram, 1977; Porter & Dixon, 1966), a proportion of gamma globulins above 21% has been suggested to represent AD associated hypergammaglobulinemia (Cepica *et al.*, 2012; An & Ingram, 1977; Porter *et al.*, 1965b; Porter *et al.*, 1965a; Henson *et al.*, 1961). Alternatively, hypergammaglobulinemia can be expressed in terms of the ratio between albumin and gamma globulin (the A:γG ratio). It has been reported that a ratio greater than 8 represents healthy mink while a ratio lower than 5 represents mink with hypergammaglobulinemia (Cepica *et al.*, 2012).

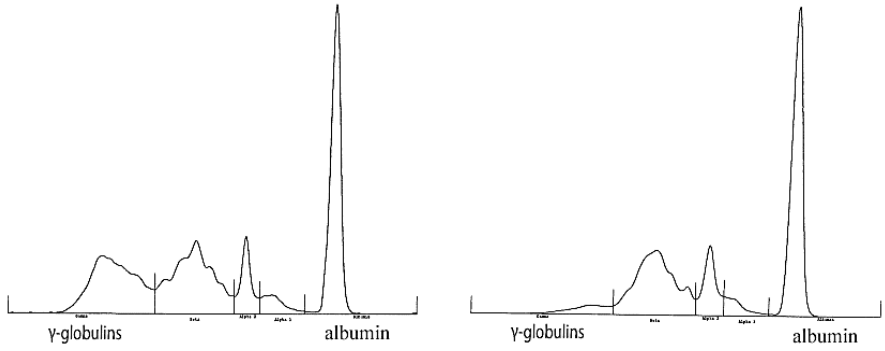

Figure 8. Serum protein profiles in mink with (to the left) and without (to the right) hypergammaglobulinemia.

For the purpose of detecting hypergammaglobulinemia in mink, one alternative is the iodine agglutination test (IAT) (Mallen *et al.*, 1950). In IAT, the test solution reacts with gamma globulins in the serum leading to a precipitation in mink with hypergammaglobulinemia (Porter *et al.*, 1980; Greenfield *et al.*, 1973; Mallen *et al.*, 1950). The IAT is a simple and non-expensive test which has been used at farms since the 1960s when it was first introduced (Greenfield *et al.*, 1973; Mallen *et al.*, 1950). IAT is not a specific test for AD since the test identifies an increased amount of gamma globulins which is a feature typical but not exclusive for AD (Cepica *et al.*, 2012). In areas with endemic AD, however, the power of the test will be sufficient due to the high prevalence of AD compared to other diseases which could cause a corresponding hypergammaglobulinemia (Cepica *et al.*, 2012). Another limitation of IAT is that it is a qualitative test that identifies mink with marked hypergammaglobulinemia i.e. relatively late in the disease process (Cepica *et al.*, 2012; Gorham *et al.*, 1964). Expressed in terms of A:γG ratio, the IAT identifies a A:γG ratio of less than 1 as diseased (Gorham *et al.*, 1965). Consequently, ADV infected mink with an A:γG ratio greater than 1 will remain undetected (Cepica *et al.*, 2012; Cho & Ingram, 1972).

There are other alternative ways of identifying an increased amount of serum gamma globulin which offer better possibilities to quantify the degree of hypergammaglobulinemia and thereby also indicate the degree of AD progression (Bloom *et al.*, 1994). Examples include serum electrophoresis (Porter *et al.*, 1980) and MALDI-ToF (Cepica *et al.*, 2012). However, these methods are either expensive, laborious, or technically demanding and therefore not easily used for mass screening purposes. Consequently, there is a need for a simpler method to separate animals based on their degree of disease progression

and quantitative ELISA systems have been suggested to fit such a purpose. However, before a new diagnostic test can be implemented it needs to be sufficiently evaluated and validated (OIE, 2013).

1.6 Evaluation of diagnostic tests

There is no such thing as a perfect diagnostic test, and any test will as a consequence inevitably lead to misclassifications (Greiner & Gardner, 2000). Knowledge about the probability for misclassifications is therefore essential before applying the test in research or in a clinical setting. Before any evaluation of a new diagnostic assay the purpose of the test must be defined as this will have a large impact on the assessment of the test performance (OIE, 2014a). Demonstrating disease-free status require tests with high sensitivity resulting in low false negative rates and maximum negative predictive values for low-prevalence populations (OIE, 2014a). For a similar reason, eradication of a disease also requires a test with high sensitivity, especially at the late stages of the elimination process when prevalence is low (OIE, 2014a). In both these cases, a confirmatory test with high specificity and low false positive rate can be applied in order to more accurately evaluate the disease status of the individual (OIE, 2014a). For disease prevalence estimates, a test with both moderate to high sensitivity and moderate to high specificity is optimal (OIE, 2014a). For tests based on a continuous scale rather than a binary outcome, the results are often categorized into two to three categories. If there is an overlap in distributions of test values between the different categories, the selected cut-off values must then be aimed to fit the selected purpose (OIE, 2013).

1.6.1 Precision

The precision of a diagnostic test can be described as the ability of the test to produce the same result at different assay occasions and compromises both the repeatability and the reproducibility (Greiner & Gardner, 2000):

- Repeatability: This is an estimation of the internal consistency *i.e.* the variation at the laboratory(European Medical Agency, 1995) when things such as instruments, procedures, materials *etc.* are being held constant (United Nations Office on Drugs and Crime, 2009). Repeatability is often expressed as *intra-assay variability.* Factors affecting this variability could be poor washing efficiency, variations in the plate coating, un-stable antigens, pipetting errors and non-optimized incubation times (Shan, 2010).

- Reproducibility: This is an estimation of both the within laboratory variations as well as the external consistency i.e. the variation between different laboratories (United Nations Office on Drugs and Crime, 2009; European Medical Agency, 1995). Reproducibility can be expressed as the *inter-assay variability* compromising for instance intra-observer variation, inter-observer variation and factors such as the day of the analysis (United Nations Office on Drugs and Crime, 2009; Kanchanaraksa, 2008).

Precision is commonly measured as the coefficient of variation (CV) which is the standard deviation (SD) of the repeated measurements divided by the mean of the measurements (Dohoo *et al.*, 2009). Estimations of the CV are concentration-dependent and should therefore always be evaluated at different concentrations throughout the working range of the diagnostic tool (United Nations Office on Drugs and Crime, 2009). As a guideline, less than 15% intra-assay variation and less than 20% inter-assay in the CV have been regarded as acceptable (Shan, 2010; United Nations Office on Drugs and Crime, 2009; OIE, 2008).

1.6.2 Accuracy

The accuracy is a measurement of the ability of the test to reflect reality i.e. the test performance (Greiner & Gardner, 2000). In diagnostic assay evaluations, it is often measured in terms of sensitivity and specificity in relation to another test such as a gold standard (Figure 9; (Greiner & Gardner, 2000)). Sensitivity is defined as the proportion (a/(a+c)) of diseased individuals (a+c) who are identified by the diagnostic test as diseased/ true positives (a) and not as false negatives (c). Specificity is defined as the proportion (d/(b+d)) of non-diseased individuals (b+d) who are identified as non-diseased/ true negatives (d) and not as false positives (b)(Dohoo *et al.*, 2009).

	Disease positive	**Disease negative**
Test positive	a	b
Test negative	c	d

Figure 9. Two-by-two table of the test result vs the disease status of the animal as decided with the reference test. Sensitivity is calculated as a/(a+c) and specificity is calculated as d/(b+d).

There are several factors that may affect the evaluation of the test performance and therefore estimations of test performance should be regarded to be valid only for the given reference population under the defined conditions (Gardner *et al.*, 2000). The influencing factors can be divided into biological factors and test related factors e.g. choice of reference test and selected cut-off (OIE, 2008).

Biological factors

Sensitivity and specificity of a test can vary both within and among animal populations and sub-populations (Greiner & Gardner, 2000). Disease prevalence, composition of the population (e.g. age, gender, colour type, gestation) as well as the sample size may influence the test performance (OIE, 2008). Hence, it is crucial to evaluate the diagnostic test in the population where it is going to be used. Also, the sample used during the evaluation must be representative for the overall population. If the population later changes in e.g. terms of infection load it is recommendable that the accuracy of the test is re-evaluated (OIE, 2008).

In addition, there are biological factors within the individual animals which may influence the test performance (Greiner & Gardner, 2000). One example is intra-subject variability where short-term variations in the results of the same individual (*e.g.* as the result of physiological changes) will introduce error into estimations of the test performance (Kanchanaraksa, 2008). Other examples of biological factors at the individual level which may affect the estimations of sensitivity and specificity are the stage of infection, the immune status, and other coexistent diseases (Greiner & Gardner, 2000).

The reference test (the gold standard)

Ideally, the gold standard should be a perfect reference standard in order to be representative of the true disease status of the animal population (OIE, 2013; Thrusfield, 2007). However, apart from direct isolation of the organism or pathognomonic histopathological criteria, other tests for disease status risk to suffer from errors (OIE, 2014b; OIE, 2008). When using such tests as reference tests, there is a risk for misclassifications and the estimated sensitivity and specificity of the new assay risk to be flawed (OIE, 2014b; OIE, 2014a). One way to improve the gold standard classification is to use multiple testing (Dohoo *et al.*, 2009), either by re-testing the test positives/ test negatives from the first test (sequential multiple testing) or testing all individuals with both tests (simultaneous multiple testing). Another way to improve the assessment of disease status is to use a population with known disease status (Thrusfield,

2007). For ADV, this can be translated into using ADV seronegative herds to find ADV seronegative animals, and inoculating these ADV seronegative mink with ADV with the aim of achieving ADV seropositive animals. A third alternative to handle imperfectness of the reference test is to statistically adjust the estimated sensitivity and specificity for the new test using Bayesian or Maximum likelihood latent class analysis (LCA) (OIE, 2014c; Georgiadis *et al.*, 2003; Staquet *et al.*, 1981; Hui & Walter, 1980).

If possible, the gold standard should be conditionally independent from the test validated, i.e. measuring a different biological process (Georgiadis *et al.*, 2003; Gardner *et al.*, 2000). Two tests measuring similar analytes such as antibodies, however, are likely to be conditionally dependent given the animal's true disease status (Georgiadis *et al.*, 2003; Gardner *et al.*, 2000). This introduces another source of error when estimating the sensitivity and specificity of the new test in which a new test which is more similar to the gold standard will appear superior compared to a test which is less similar irrespective of true superiority. Any correlation between the tests needs to be addressed during the statistical analysis or otherwise the estimations can be biased (Dohoo *et al.*, 2009; Georgiadis *et al.*, 2003; Gardner *et al.*, 2000).

Selection of cut-off value

For tests that measure on a continuous scale, such as ELISA, a cut-off needs to be created in order to enable categorization of individuals as diseased or non-diseased. Different cut-off points will lead to different estimations for sensitivity and specificity, and the evaluation of one test compared to another test will dependent upon the cut-off values chosen (Dohoo *et al.*, 2009). For instance, a cut-off that identifies more true negatives (high specificity) will also lead to more false negative results. In contrast, a cut-off that identifies more true positives (high sensitivity) will lead to more false positive results (Giesecke, 2001). Sensitivity and specificity are inversely related to each other so that when one of them increases, the other one will decrease (Figure 10). The choice of cut-off is therefore a balance act depending on the purpose of the diagnostic test. (Giesecke, 2001).

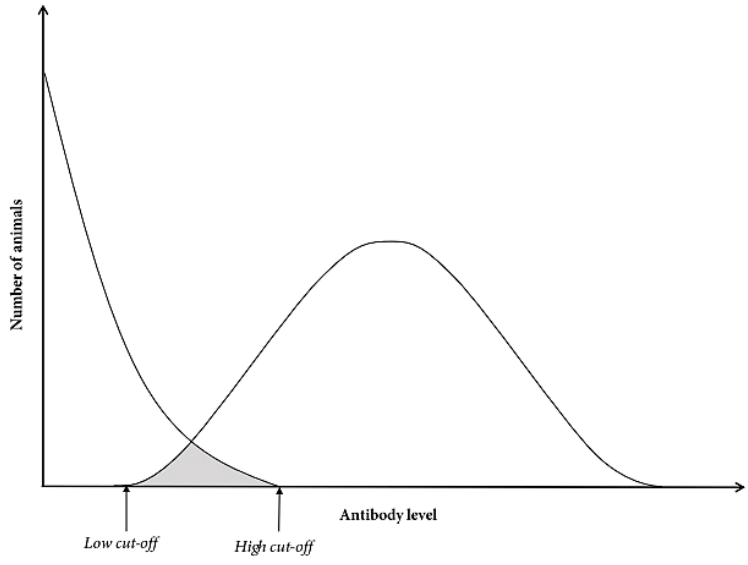

Figure 10. Example of distributions of antibody levels for non-infected animals (left) and infected animals (right) tested with a test measuring on a continuous scale. The overlap between the test values (grey area) include animals which are at the risk of misclassification depending on the selected cut-off. A low cut-off will classify all infected animals as test positive as well as some of the non-infected (grey area) i.e. high sensitivity but lower specificity. A high cut-off will classify all non-infected animals as test negative as well as some of the infected (grey area) i.e. high specificity but lower sensitivity.

One commonly used way to decide the cut-off is to take the mean result of a healthy population/sub-population and add two or three standard deviations (Jacobson, 1998). Statistically, this ensures a specificity of 97.5 – 99.5% i.e. 97.5 – 99.5% of the healthy population will be test negative and the remainder will be test positive. An alternative method to determine the cut-off is using a receiver-operating characteristic (ROC) analysis (Zweig & Campbell, 1993; Hilden, 1991). This is a graphical tool where the area under the curve (AUC) represents the probability that a randomly chosen diseased individual has a greater test value compared to a randomly selected healthy individual (Fawcett, 2006). Through maximization of AUC, the optimal cut-off can be estimated. It is possible to optimize both sensitivity and specificity or only one of them. Depending on the test in question, this can lead to different estimations for the cut-off value (Fawcett, 2006; Zweig & Campbell, 1993; Hilden, 1991).

2 Aims and objectives of the thesis

The overall aim of this thesis was to obtain knowledge about serodiagnosis of ADV infection and Aleutian disease in Swedish mink, and to develop strategies to reduce the impact on welfare and reproduction in mink in ADV endemic areas using estimation of ADV antibodies as a tool. The specific objectives were:

- To evaluate two different available ELISA systems for serological diagnosis of ADV infection and select the most suitable system for serodiagnosis in Swedish mink (**I**).
- To adapt and validate the serum VP2 ELISA selected in **I** for ADV antibody detection in dried blood spots (DBS) with the aim to facilitate sampling and preparations of samples for analysis (**II**)
- To evaluate the short-term stability of the estimated antibody levels of mink (**III**).
- To investigate the relationship between ADV antibody levels, estimated with the DBS VP2 ELISA, and amount of gamma globulins in serum of mink (**II**).
- To evaluate long term consistency of the estimated antibody level and disease status of mink (**III**).
- To evaluate the association between the AD status of female mink, estimated with the DBS VP2 ELISA, and their reproductive performance, expressed as being barren or not and litter size per non-barren female mink (**IV**).

3 Materials and methods

A brief description of materials and methods used in this thesis are presented below. More detailed information is given in papers **I-IV**. In addition, details on extra data analyses performed in the thesis are given in the section *Addendum*.

3.1 Study populations and study designs

All herds studied in **I – IV** were affiliated to the Swedish national health program for mink and situated in the southern part of Sweden where most Swedish mink herds were situated. Participation in all studies was voluntary.

I was designed as an observational cross-sectional study. All ADV positive herds in Sweden (n=58) were offered the opportunity to join and a convenient sample of voluntary farmers, stratified over the three different mink-producing areas was made (n=4). Blood samples from a random sample of 50-100 female mink older than 6 months of age were collected from each herd during December 2010. Negative control samples were collected from 25 randomly selected female mink originating from an ADV seronegative herd which was, at the time, the only herd in Sweden which had been sampled for ADV yearly and had not had any ADV positive test samples over the last ten years.

II was also designed as an observational cross-sectional study including mink from two ADV positive herds. The herds were selected as a convenient sample based on the criteria that they both had a high prevalence of seropositive animals during previous screening but differed in herd size as well as reproductive performance. Moreover, to enable sampling within the stipulated time frame, a relative proximity of the herds but not directly adjacent to each other (approximately 10 km apart) was a decisive criterion. A random sample of 90 female mink from three different non-Aleutian colour types (brown, silver blue and Hedlund white) were sampled from each herd. In addition, data from routine screening of 300 silver blue mink from a low-prevalence ADV positive herd as

well as 1,008 mink (brown, silver blue and Hedlund white as well as sapphire colour type) from an ADV seronegative herd sampled within the framework of the Swedish national health program for mink were included.

III was a descriptive case-serial study divided into two parts. For the investigation of the short-term stability, 120 of the 180 mink scrutinized in **II** were further sampled and included as the study population in **III** $^{\text{part I}}$. For the long-term consistency, data from a high-prevalence ADV positive herd with data from two consecutive routine screenings of 350 Hedlund white mink in October 2011 and February 2012 were used as study population in **III** $^{\text{part II}}$.

IV was a retrospective observational cohort study. Data from routine ADV antibody analysis of DBS samples as well as registered reproductive performance data from four ADV infected herds collected within the framework of the Swedish national health program for mink in 2012 and 2013 (Svensk Mink, 2017) were used in the study. First, a pilot study was performed (**IV** $^{\text{pilot}}$) including 1,001 primiparous and 1,075 older female mink of three different non-Aleutian colour types (brown, silver blue and Hedlund white) from one herd bred with the same colour and with a complete record of the reproductive performance. In order to confirm the findings in **IV** $^{\text{pilot}}$, a follow-up study (**IV** $^{\text{follow-up}}$) was performed and a total of 10,638 female mink from of the same three non-Aleutian colour types from four different mink herds were scrutinized.

3.2 Collection of samples for serological analysis

3.2.1 Serum/plasma samples

Serum and plasma samples were collected into glass capillary tubes from toe-nail. Tubes with Natrium-heparin were used for plasma, and tubes without additive were used for serum. The capillary tubes were directly transported to the lab, centrifuged at 850 gs and stored in -20°C until analysis. After thawing, plasma and serum, respectively, were harvested through breakage of the tubes at the interface between the cell fraction and the plasma/serum fraction.

3.2.2 DBS samples

In **II, III** and **IV**, capillary blood samples from individual mink were or had been collected as DBS by toe nail clips onto filter papers. To avoid cross-contamination of samples, each filter paper was air-dried and stored individually until analysis. The analysis was initiated by manually punching out 25 mm^2 of each DBS, which corresponded to approximately 5 µL of serum (Aulerich *et al.*,

1999). Each punch of filter paper was placed in a separate well of a flat bottomed 96-well plate containing 200 µL elution buffer over night in order to elute the dried blood. The next day, the eluates were transferred to another microtiter plate.

3.3 Laboratory diagnostics

3.3.1 CIEP

CIEP was used as the reference gold standard test in **I** and **II**. In **I**, CIEP was performed according to a modified protocol previously described (Cho & Ingram, 1972). Glass slides were coated with 0.8% agarose in barbital buffer. Undiluted plasma samples were placed in anodal wells. In the cathodal wells, a commercial CIEP antigen was applied. Positive controls were included on each slide. Following electrophoresis, the plates were viewed under indirect illumination and evaluated by two observers. Weak positive reactions were re-evaluated after the gel had been soaked for 60 min in 0.9% saline and dried. Only samples with an immunoprecipitation line remaining after soaking were recorded as positive. In **II**, data from routine CIEP analysis of plasma samples performed at Kopenhagen Diagnostics, Glostrup, Denmark were used.

3.3.2 Plasma ADV-G ELISA

Blood plasma samples were analysed with the ADV-G ELISA kit in **I** according to the instructions of the manufacturer. Plasma samples were diluted 1:100 in phosphate-buffered saline (PBS) and added to the microtiter plates. Positive and negative controls were included on each plate. All incubations were 30 minutes and protein A-HRP was used as conjugate. The optical density was measured at 450 nm (OD_{450}) and the mean OD_{450} value of the blank wells was subtracted from each result. In order to evaluate if the mink were seropositive to ADV, two different cut-offs were calculated and used (i) the mean OD_{450} value + 10 SD of the negative control samples (as suggested by the manufacturer), and (ii) the mean OD_{450} value + 3 SD of the CIEP negative samples (Jacobson, 1998) in order to be comparable to the cut-off used for the serum VP2 ELISA (see below).

3.3.3 Serum VP2 ELISA

In **I**, **II** and **III**, a VP2 ELISA was used to analyse antibodies in mink serum according to a previously published protocol (Knuuttila *et al.*, 2009a). Microtiter plates were coated overnight with antigen diluted in coating buffer and then

blocked with blocking buffer. Serum samples were diluted 1:200 in dilution buffer and added to the wells of the microtiter plates. All incubations were 60 minutes and peroxidase-conjugated goat anti-cat IgG was employed as reagent solution. The optical density was measured at 450 nm (OD_{450}) and the mean OD_{450} value of the blank wells was subtracted from each result. Positive and negative controls were included on each plate, and in **II** and **III** the OD_{450} values of each plate were adjusted to a value of OD_{450} = 1.0 for the positive control. The assay cut-off value was calculated as the mean OD_{450} value + 3 SD of the CIEP negative samples according to the published protocol (Knuuttila *et al.*, 2009a).

3.3.4 Serum electrophoresis

In **II**, capillary serum samples were collected and sent to the Department of Clinical Sciences at the Swedish University of Agricultural Sciences, Uppsala where the proportions of the different serum proteins were determined by serum electrophoresis. The degree of hypergammaglobulinemia for each mink was then estimated as the ratio between the amount of albumin and gamma globulins (the A:γG ratio) (Cepica *et al.*, 2012).

3.3.5 DBS VP2 ELISA

In **II**, **III** and **IV**, antibodies to ADV in DBS were estimated by adapting the VP2 ELISA previously used for serum. When serum and DBS were compared, the analysis protocols were the same except for the dilutions of serum and DBS samples. The optimal dilution of the DBS was found by stepwise dilution of the samples from the ADV seronegative herd in **II**. The DBS eluate dilution when all samples had an OD_{450} value of less than the mean OD_{450} value + 2 SD of the original dilution (1:5) was decided to be optimal *i.e.* an eluate dilution of 1:20 corresponding to a serum dilution of 1:800. Through ROC analysis, the assay cut-off values for ADV infection and different levels of AD progression based on the classifications by Cepica et al. 2012, were defined (Figure 11).

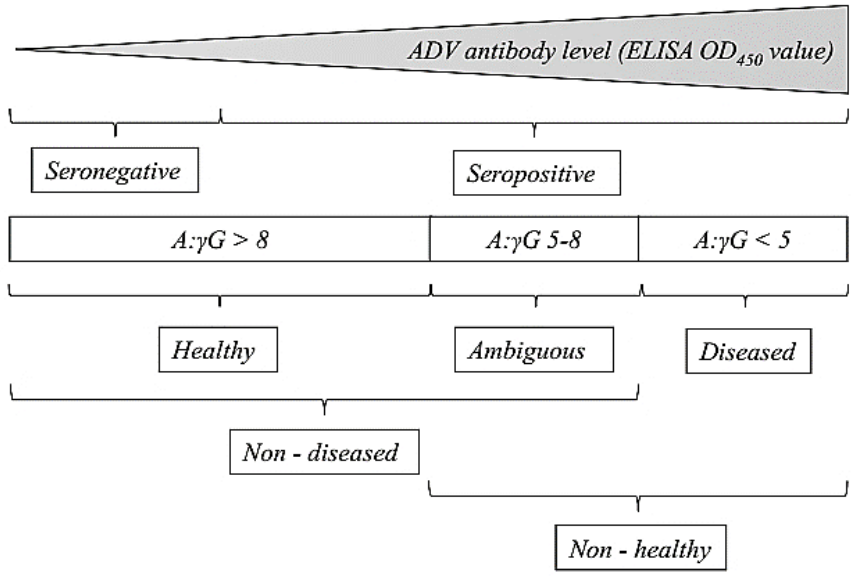

Figure 11. The definition of ADV infection and different levels of AD progression based on the classifications by Cepica et al., 2012.

3.4 Registration of reproductive performance

In farms associated to the Swedish national health program for mink (Svensk Mink, 2016), the staff count the number of kits during the first eight weeks and both the percentage of barren females and the number of kits born per female mink are annually reported to the program by the farmer. Data on the reproductive performance used in **IV** were obtained from a control program for AD within the framework of the health program (Svensk Mink, 2017; Svensk Mink, 2016). For the herds scrutinized in **IV** $^{\text{pilot}}$ and **IV** $^{\text{follow-up}}$, the number of kits had been counted by the farm personnel when the kits were two to four weeks of age during May 2012 and 2013, respectively (Svensk Mink, 2017).

3.5 Statistical analyses

Statistical analysis was used in all four papers to investigate test performance, evaluate significant differences between different test groups or to assess the association between various factors. All statistical analyses and graphical

presentations were made in Stata (Stata/IC 13.1, StataCorp LP, College Station, Texas 77845, USA). A *p*-value of less than 0.05 was considered to be statistically significant.

3.5.1 Evaluation of the different serum/plasma ELISA systems

In **I**, the antibody levels (estimated as OD_{450} values) for CIEP negative and CIEP positive mink were compared using a t-test. Sensitivities and specificities for the different ELISA systems were calculated with the CIEP as reference gold standard test. The cut-off used was calculated either by the mean OD_{450} value + 3 SD of the CIEP negative samples (Jacobson, 1998) , or for the ADV-G antibody ELISA, the mean OD_{450} value + 10 SD of the negative control samples provided in the kit (according to the instructions from the manufacturer).

3.5.2 Evaluation of the DBS ELISA

In **II**, the precision of the DBS VP2 ELISA was evaluated through intra-assay as well as inter-assay variability:

- The intra-assay variability was estimated for 11 randomly selected DBS samples tested in six replicates in a single assay. This was repeated during two consecutive days and the CV for each sample each day was calculated by dividing the SD of the six replicates for each sample with the mean OD_{450} value for all the replicates. The overall intra-assay variability was then computed as the mean CV for all samples.
- The inter-assay variability was evaluated by comparing the mean OD_{450} value for two duplicate wells for each DBS sample analysed during three consecutive days. The CV was calculated as for the intra-assay variability, both for the individual DBS samples as well as the overall inter-assay variability.

The accuracy of the DBS VP2 ELISA to categorize mink from the low-prevalence herd as seropositive or seronegative was first evaluated with CIEP as the reference gold standard test. The mean DBS antibody levels (again estimated as OD_{450} values) for CIEP positive and CIEP negative mink were then compared using a paired t-test assuming unequal variance. The sensitivity and specificity for the DBS ELISA compared to CIEP were calculated using the assay cut-off defined by ROC analysis earlier in **II**.

3.5.3 Evaluation of ELISA as a tool for determining AD progression

A major focus of this project was to evaluate the capacity of the DBS VP2 ELISA to quantitatively estimate the amount of ADV antibodies as an indirect measurement of disease progression. Therefore, in a first step, the correlation between the quantitatively estimated OD_{450} values for DBS and serum samples in **II** were compared by calculating the Spearman rank correlation for the paired samples from the same mink. Thereafter, the Spearman rank correlations between the A:γG ratio and the serum/DBS VP2 ELISA OD_{450} values were calculated and the linear associations between serum/DBS VP2 ELISA OD_{450} values and A:γG ratio were investigated using linear regression models. Prior to the analysis, OD_{450} values for DBS were transformed using the natural logarithm (ln). By stratifying the A:γG ratio into three categories representing diseased, ambiguous and healthy mink respectively (Figure 11), the mean OD_{450} values for DBS and serum samples for each category were statistically assessed using a paired t-test assuming unequal variances. The sensitivity and specificity for the DBS ELISA to predict diseased mink using the assay cut-off defined in by ROC analysis earlier in **II**.

3.5.4 Evaluation of the stability of antibody levels estimated with the VP2 ELISA

For the short-term stability in **III** **part I**, the OD_{450} values were normally distributed and statistical calculations comparing the consecutive sampling occasions were made with a two one-sided test for equivalence (TOST) testing the null hypothesis that there was a difference between the consecutive samplings (Shtaynberger & Bar, 2013; Schuirmann, 1987). The margin of equivalence was set to a level compensating for the methodological variability reported in **II**, ensuring that any difference in antibody levels would be the result of a true difference and not the methodological variance. In other words, equivalence between the two samplings was decided when the 95% confidence interval (CI) for the observed difference between the two measurements was within ±0.1 OD_{450} units (Shtaynberger & Bar, 2013).

As the distribution of the OD_{450} values used for the evaluation of the long-term consistency in **III** **part II** was bimodal the statistical comparison of the two sampling occasions were made with a non-parametric paired-samples sign test testing the null hypothesis that there was no difference between the two samplings. In addition, the Spearman rank correlation between the two sampling occasions was calculated. In order to evaluate whether the infection and disease status of the individual mink were preserved from the first to the second sampling, the data were tabulated and visually assessed.

3.5.5 Association between AD status and reproductive performance

A multivariable zero-inflated Poisson (ZIP) regression model was used to investigate the associations between the reproductive performance and the explanatory variables AD status, colour type and age in **IV** $^{\text{pilot}}$. The aim was to investigate the effects of the process that results in having kits or not (binary outcome) and the process that results in the actual size of the litter (count outcome). Moreover, to facilitate comparisons between the results from **IV** $^{\textbf{pilot}}$ with those from **IV** $^{\textbf{follow-up}}$, we also built two separate models for the binary and the count outcome including only the primiparous female mink. A univariable mixed-effects logistic regression model was used to investigate the association between being barren or not (binary outcome) and AD status. A univariable mixed-effects Poisson regression model was then used to investigate the association between litter size and AD status. In the latter model, all barren females were excluded. In both models, colour was included as a random effect using an independent covariance structure.

Initially, all potential explanatory variables were included and the final model was accomplished with a manual backward selection approach. A p-value ≥ 0.05 was used to eliminate variables in a step-wise manner. In order to reveal any confounder, a limit was set for a maximum change in the coefficient of ±10%. All possible interactions between significant main effects were also investigated and included in the model when the interaction term was significant ($p<0.05$). Collinearity between the explanatory variables was assessed by pairwise Spearman rank correlations. A level of $r \geq 0.70$ was used as a proof of collinearity and if present, the variable with lowest p-value was selected. Finally, the Vuong test was used to evaluate the ZIP model compared to an ordinary Poisson model.

In **IV** $^{\textbf{follow-up}}$, when data from primiparous mink in four different herds were included, the direct effect of colour type or herd was not of interest and a model which could include repeated measurements within herd and colour type as random effects was preferred over the zero-inflated model. Inclusion of repeated measurements within herd and colour type takes into account that mink within a herd and colour type are more similar than mink between different herds and colour types. Therefore, two separate models were applied for the binary and the count outcome. A univariable mixed-effects logistic regression model was used to investigate the association between being barren or not and the AD status of the female mink, and a univariable mixed-effects Poisson regression model was used to investigate the association between litter size and AD status. In the latter model, all barren females were excluded. For both models, an independent covariance structure was used allowing a distinct variance for each random effect.

3.5.6 Addendum

In this thesis, the data in **I** were re-analysed using Bayesian LCA allowing CIEP and the two ELISA systems to be compared more equally. In a LCA, none of the tests is considered a gold standard. Instead, the sensitivity and specificity, as well as the prevalence of the investigated disease in each of the populations, are simultaneously estimated for all tests. Neither of the three tests used in **I** could be assumed completely conditionally independent on the AD status since they all measure antibodies. Hence, a model (1) allowing for conditional covariance between the two ELISA systems was first ran while assuming conditional independence of CIEP. In addition, models (2) allowing for conditional covariance between CIEP and each of the ELISA systems, or (3) assuming conditional independence between all tests, i.e. the Bayesian formulation of the LCA for three test in k-populations (Branscum *et al.*, 2005), were also run. One of the advantages of Bayesian analysis is that all parameters can be modelled using distributions based on previous knowledge (so called priors). Informative priors for the sensitivity and specificity of the CIEP and the two ELISA systems were obtained from previously published studies (Dam-Tuxen *et al.*, 2014; Knuuttila *et al.*, 2014) while uniform priors were used for the prevalences and covariances. Based on these priors, alpha and beta parameters of the beta distributions were calculated using the probability calculator tool on the EpiTools website (Sergeant, 2016), and then used in the models. In addition, a model with uniform priors for all parameters were also run for comparison as the priors obtained were from only two studies.

The LCA were performed using OpenBUGS, version 3. 2. 2 rev 1063 (OpenBUGS, 2010–2011 Members of Open BUGS Project Management Group) which uses a Markov Chain Monte Carlo (MCMC) sampling algorithm (Gibbs sampling algorithm) to obtain a random sample from the joint posterior distribution of all model parameters. Three MCMC chains, with different starting values, were run for 20,000 iterations of the model, with a thinning of 1 in 10, and the first 10,000 were discarded as the burn-in phase. Convergence of the MCMC chains after the initial burn-in was assessed by visual inspection of the time-series plots of selected variables as well as by inspecting Gelman-Rubin diagnostic plots and autocorrelation plots. The median of the posterior distribution was used as an estimate for the sensitivity and specificity of the three tests, the possible covariance of between the tests, and the prevalence in each population; the 2.5 and 97.5 points were used as estimates of the 95% posterior credibility intervals (PCI; the Bayesian analogue of CI).

Complementary to the strategy for disease control developed in **IV**, the potential to use the quantitatively estimated antibody level as a tool to discriminate diseased mink with different levels of AD progression was also

investigated in this thesis. First, the litter size of each mated female mink was plotted against the OD_{450} value in February using the *lowess* command in Stata. To further investigate the association between the reproductive performance and the OD_{450} value of the female mink, two separate models for the percentage of barren females and the mean litter size of the non-barren female mink were then made. Unlike in **IV**, the mink were not categorized into diseased and non-diseased but instead the OD_{450} value for each mink was initially used in the models.

For the risk of being barren, a logistic regression model was made using the data from the 10,638 mink originally included in **IV** $^{\textbf{follow-up}}$ followed by estimation of the average predicted percentage of barren females for each OD_{450} value using the margins command in Stata. In the next step, the female mink were subdivided into three categories based on the OD_{450} value: non-diseased ($OD_{450} \leq 0.8$), diseased^{+} (OD_{450} 0.9-1.1), and diseased^{++} ($OD_{450} \geq 1.2$). A new logistic regression model was then made using these three categories and the average predicted percentage of barren females was estimated for each category.

For the litter size of the non-barren female mink, a Poisson regression model was first made followed by estimation of the average predicted litter size per non-barren female mink for each OD_{450} value using the margins command in Stata. In a second step, a new Poisson regression model was made using the three new disease categories and the average litter size per non-barren female mink was then estimated for each disease category.

3.6 Ethical consent

The studies were approved by the Swedish Ethical Committee in Uppsala (Dnr C306/10) & the Swedish Ethical Committee in Gothenburg (Dnr 117-2012). Additional data were obtained from routine ADV antibody analyses of clinical screening samples. These samples were collected by the farmers and submitted for diagnosing ADV in their attempts to control the disease (i.e. not collected for scientific purpose). Further, the reproductive performance data were obtained within the framework of the Swedish national health program for mink in 2012 and 2013, respectively (Svensk Mink, 2017).

4 Results and Discussion

4.1 Evaluation of different ELISA systems for detection of ADV antibodies

4.1.1 Serum/plasma ELISA

Several biological factors are known to affect the evaluation of the accuracy of a test and therefore the performance of a test should always be evaluated in the population where it is intended to be used (OIE, 2008; Gardner *et al.*, 2000). In this thesis, the performance of two different available ELISA systems for detection of ADV antibodies were evaluated in Swedish mink (**I**).

The mink were tested for ADV antibodies with CIEP, the ADV-G ELISA and the VP2 ELISA. The mean antibody level (defined as the OD_{450} value) for the groups of CIEP negative and the CIEP positive mink differed significantly ($p<0.05$) for both ELISA systems, respectively. Moreover, the accuracy for each ELISA system was expressed in terms of sensitivity and specificity using CIEP as the reference gold standard test (**I)** and also by LCA, in the extended data analysis in this thesis, allowing a more equal comparison between all three tests.

For the ADV-G ELISA, the cut-off value for categorizing plasma samples as negative or positive was defined as $OD_{450} = 0.19$ when the cut-off value was calculated based on the negative control samples (as suggested by the manufacturer). The estimated sensitivity and specificity using this cut-off were 54.3% and 93.2%, respectively. The second cut-off investigated was defined as $OD_{450} = 0.28$ (based on the mean OD_{450} value +3 SD of the CIEP negative samples). The estimated sensitivity and the specificity with this cut-off was 37.6% and 98.3% instead, which illustrates the trade-off between sensitivity and specificity occurring whenever a cut-off level is used for categorization of continuous data. In this case, the OD_{450} values for the CIEP negative mink and

CIEP positive mink overlapped in the ADV-G ELISA to a large extent (Figure 12) which inevitably lead to a large number of false positive and false negative results. Consequently, the choice of cut-off will have a major impact on the estimations for sensitivity and specificity.

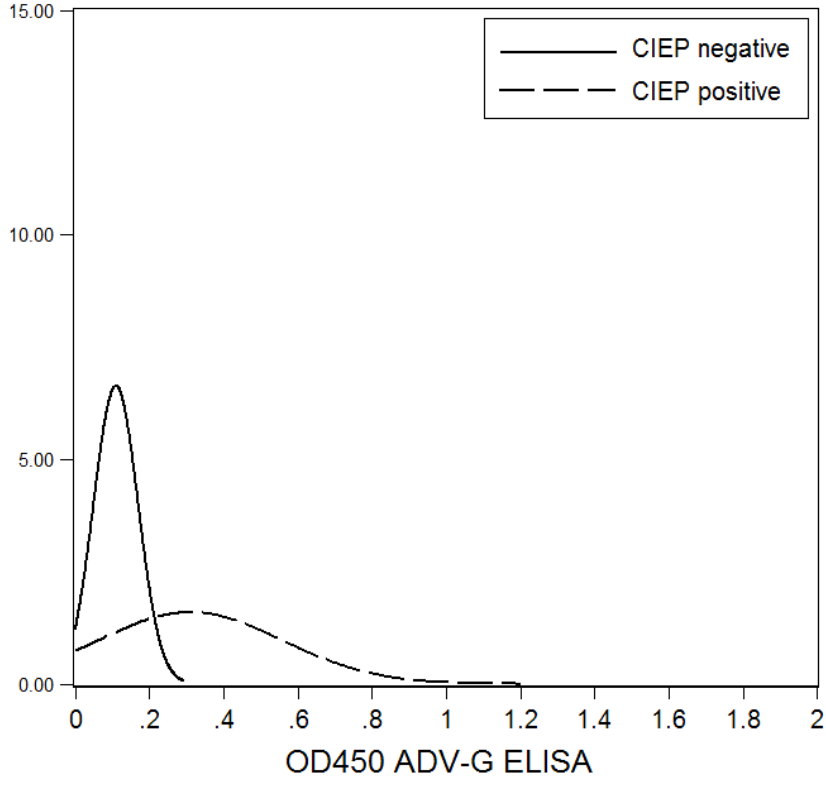

Figure 12. The distribution of OD_{450} values in the ADV-G ELISA for CIEP negative and CIEP positive mink. The mean OD_{450} was 0.11 for the CIEP negative mink (SD=0.06), and 0.31 for the CIEP positive mink (SD=0.25).

No details about the development of the ADV-G ELISA used in **I** (ADV-G $ELISA_{USA}$) have been published which makes further interpretations of the poor performance difficult. Another ADV-G ELISA has been developed in Denmark (ADV-G $ELISA_{DK}$) and reported a higher sensitivity but a lower specificity compared to CIEP for diagnosing ADV infection in serum and blood (Farid & Rupasinghe, 2016; Dam-Tuxen *et al.*, 2014). The only scientific evaluation published of the performance of ADV-G $ELISA_{USA}$ has been concerning the accuracy of ranking mink based on their estimated antibody level where the ADV-G $ELISA_{USA}$ had the lowest accuracy compared to VP2 and the ADV-G

$ELISA_{DK}$ (Farid & Rupasinghe, 2016). The poor performance of the ADV-G $ELISA_{USA}$ could either be caused by the antigen per se or the optimization of the ELISA. In a previous study, the ADV-G antigen has been reported to be inferior to antigens based on ADV structural proteins (VP1 and VP2) for identifying mink with low as well as higher ADV antibody levels (Clemens *et al.*, 1992). However, there are also differences in the operation procedure between the ADV-G ELISA and the VP2 ELISA in **I** which could have contributed to the different accuracies of the two tests. For instance, plasma samples diluted 1:100 were used in the ADV-G ELISA compared to serum samples diluted 1:200 used in the VP2 ELISA. There are many possible ways to optimize the performance of an ELISA (Shan, 2010) . However, in **I**, we merely used previously developed systems and therefore we performed the ADV-G ELISA according to the instructions from the manufacturer. In the end, based on the poor performance of the test irrespective of the selected cut-off, the ADV-G ELISA was concluded to be substandard for detection of antibodies to ADV in the scrutinized population.

For the VP2 ELISA, on the other hand, a single assay cut-off value was employed in **I** based on the mean OD_{450} value + 3 SD in accordance with a previous study of the VP2 ELISA (Knuuttila *et al.*, 2009a), and was defined as OD_{450} = 0.14. Using this cut-off value, the estimated sensitivity of the VP2 ELISA was 99.7% and the specificity was 98.3% which was comparable to the previous evaluation of the VP2 ELISA in Finnish mink where the sensitivity was established to 99% and the specificity was 97% (Knuuttila *et al.*, 2009a). The overlap between CIEP positive and CIEP negative mink was considerably reduced for the VP2 ELISA (Figure 13) compared to the overlap for the ADV-G ELISA, and therefore the sensitivity and specificity of the VP2 ELISA were less dependent upon the cut-off. Based on the superior sensitivity and specificity, the VP2 ELISA was concluded to be the preferred ELISA system for ADV diagnosis in the scrutinized mink population.

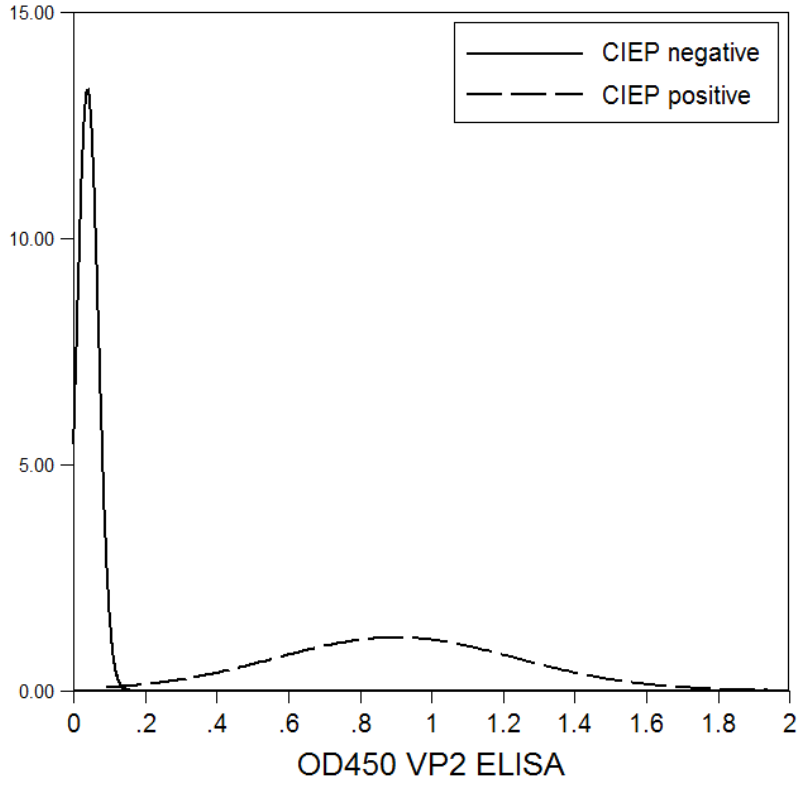

Figure 13. The distribution of OD_{450} values in the VP2 ELISA for CIEP negative (mean=0.04; SD=0.03) and CIEP positive mink (mean=0.90; SD=0.34)

CIEP was chosen as the reference test since it has been the primary choice for routine diagnostics in many countries and therefore has been informally established as the gold standard test for ADV infection (Knuuttila, 2015; Uttenthal, 1992; Cho & Greenfield, 1978). However, CIEP cannot be regarded as a perfect reference test since CIEP itself have estimated to have a sensitivity of 79% - 99% and a specificity of 90% - 100% (Dam-Tuxen *et al.*, 2014; Knuuttila *et al.*, 2014; Aasted *et al.*, 1986; Wright & Wilkie, 1982) and it has not been properly validated according to the OIE standards (Knuuttila, 2015). In addition, CIEP cannot be assumed to be conditionally independent from the two ELISA systems since they all measure antibodies (Georgiadis *et al.*, 2003; Gardner *et al.*, 2000). Therefore, estimations of the accuracy of the ELISA systems risk to be both flawed due to misclassifications and biased due to the dependence of the tests (OIE, 2014b; OIE, 2014a; Dohoo *et al.*, 2009; Georgiadis *et al.*, 2003; Gardner *et al.*, 2000). One alternative to handle this statistically is through LCA (OIE, 2014a; Georgiadis *et al.*, 2003; Staquet *et al.*, 1981; Hui &

Walter, 1980), but unfortunately this was not performed in **I**. However, in this thesis, the data in **I** were re-analysed using Bayesian LCA. The results have not been compiled into a scientific paper but are presented below (Table 1). As the covariances between the three tests were low (<0.001), only the model assuming conditional independence using informative or uniform priors is presented below. In conclusion, estimations for sensitivity and specificity for the two ELISA systems were very similar to the sensitivities and specificities presented in **I** - especially the results from the model using uniform priors (which could be expected).

Table 1. *Posterior median and 95% posterior credibility interval (95% PCI) of populations specific prevalence, sensitivity and specificity obtained from latent class analysis, assuming conditional independence between the two ELISA systems the CIEP test, using informative or uniform priors. Results from the comparison between the two ELISAs and CIEP using CIEP as golden standard (results from I) is also presented.*

	Informative priors		Uniform priors		Results in **I**
	Median	95% PCI	Median	95% PCI	
Prevalence					
Herd A	0.94	[0.88; 0.98]	0.92	[0.86; 0.96]	0.92
Herd B	0.92	[0.86; 0.96]	0.92	[0.86; 0.96]	0.93
Herd C	0.89	[0.81; 0.94]	0.86	[0.78; 0.92]	0.86
Herd D	0.89	[0.78; 0.95]	0.86	[0.73; 0.94]	0.84
Herd E	0.03	[0.0009; 0.14]	0.03	[0.001; 0.13]	0.00
Sensitivity					
CIEP	0.87	[0.84; 0.89]	0.99	[0.97; 1.00]	Ref.
VP2 ELISA	0.98	[0.96; 0.99]	0.99	[0.98; 1.00]	0.99
ADV-G ELISA	0.76	[0.73; 0.79]	0.56	[0.51; 0.61]	0.54
Specificity					
CIEP	0.95	[0.91; 0.98]	0.99	[0.93; 1.00]	Ref.
VP2 ELISA	0.98	[0.96; 1.00]	0.95	[0.85; 1.00]	0.98
ADV-G ELISA	0.99	[0.99; 1.00]	0.88	[0.79; 0.95]	0.93

4.1.2 DBS ELISA

In **II**, the VP2 ELISA system was further evaluated for detection of ADV antibodies in DBS. Initially, a DBS eluate dilution of 1:5 (corresponding to a serum dilution of 1:200) was used. Employing this dilution, 1,008 mink originating from an ADV seronegative farm had a mean OD_{450} = 0.15 (SD=0.07) with values ranging from 0.03 to 1.04. In total, 28 mink had OD_{450} values exceeding 0.28 (corresponding to the mean OD_{450} value +2 SD) and all these mink were re-evaluated with another test to confirm their disease status. The confirmatory test was PCR of the spleen and mesenteric lymph nodes or plasma CIEP and all the 28 mink were confirmed ADV negative. The increased OD_{450} values were consequently regarded as background noise due to the use of whole blood eluted from DBS instead of serum. Through stepwise dilution, the DBS eluate dilution when all samples had an OD_{450} value < 0.28 was established to be 1:20 (corresponding to a serum dilution of 1:800). In a second step, using an eluate dilution of 1:20, mink from two ADV seronegative herds analysed for ADV antibodies had no suspected seropositive mink and a mean OD_{450} value of 0.21 (SD=0.06) and 0.11 (SD=0.04), respectively.

When evaluating the precision of the DBS VP2 ELISA, the repeatability in terms of mean intra-assay CV for the two consecutive days was 5.5% and 5.8%, respectively, and the reproducibility in terms of mean inter-assay CV was 12.0%. Although the ELISA worked well, the variability could have been reduced further. One thing that could reduce the variability is a more homogenous antigen solution for the plate coating. Indeed, following this study, the risk of persistent precipitations in VP2 ADV antigen solution has been further decreased by an extra centrifugation both prior to delivery of the antigen[3] as well as in the lab prior to dilution of the antigen. In the end, our evaluation confirmed that antibodies to ADV can be detected in DBS eluates with a precision within the acceptable maximum CVs of 15% and 20%, respectively (Shan, 2010; United Nations Office on Drugs and Crime, 2009; OIE, 2008). The only sample with an inter-assay variability outside this range (CV=26.1%) had an OD_{450} value of 1.9 which is outside the optimal ELISA OD reading range and the sample would therefore have benefitted from further dilution in order to improve the estimation. However, seen from a clinical perspective this was not necessary as it does not make any practical difference for mink with such high OD_{450} values.

The accuracy of the DBS VP2 ELISA in categorizing mink from a low-prevalence herd as seropositive or seronegative was evaluated with CIEP as the reference gold standard test. The mean OD_{450} value was significantly (p<0.05)

3. Kirsi Aaltonen, personal communication

higher in the group with CIEP positive mink than in the group with CIEP negative mink. Through ROC analysis, the assay cut-off for seropositive mink was defined as OD_{450}=0.15 which was comparable to the cut-off established for the serum VP2 ELISA in **I**. A cut-off based on the mean OD_{450} value + 2 SD of the mink from the ADV seronegative herds was also defined (OD_{450}=0.30). The estimated sensitivities of the DBS VP2 ELISA for these cut-offs, were 97.3% and 89.2%, respectively, and the specificities were 93.2% and 97.7%, respectively, which were comparable to what had been reported from other evaluations of filter paper VP2 ELISA systems (Farid & Segervall, 2014; Knuuttila *et al.*, 2014). Compared to the serum VP2 ELISA, the OD_{450} values for the CIEP negative mink and CIEP positive mink overlapped more for the DBS VP2 ELISA (Figure 14) and the trade between sensitivity and specificity was again more prominent. However, irrespective of the cut-off chosen the sensitivity and specificity of the DBS VP2 ELISA were lower than the performance of the serum VP2 ELISA demonstrated in **I** (sensitivity 99.7%; specificity 98.3%). This is most likely an effect of using blood instead of serum. One factor which might have improved the DBS VP2 ELISA could be to use another elution buffer for the extraction of the DBS; now only one elution buffer (PBS/Tween) was tested in **II**. In the end, the choice of cut-off depends on the aim with the test and whether high sensitivity or high specificity is most important to fulfil the purpose e.g. demonstrating disease-free status, eradication of disease, or estimating disease prevalence.

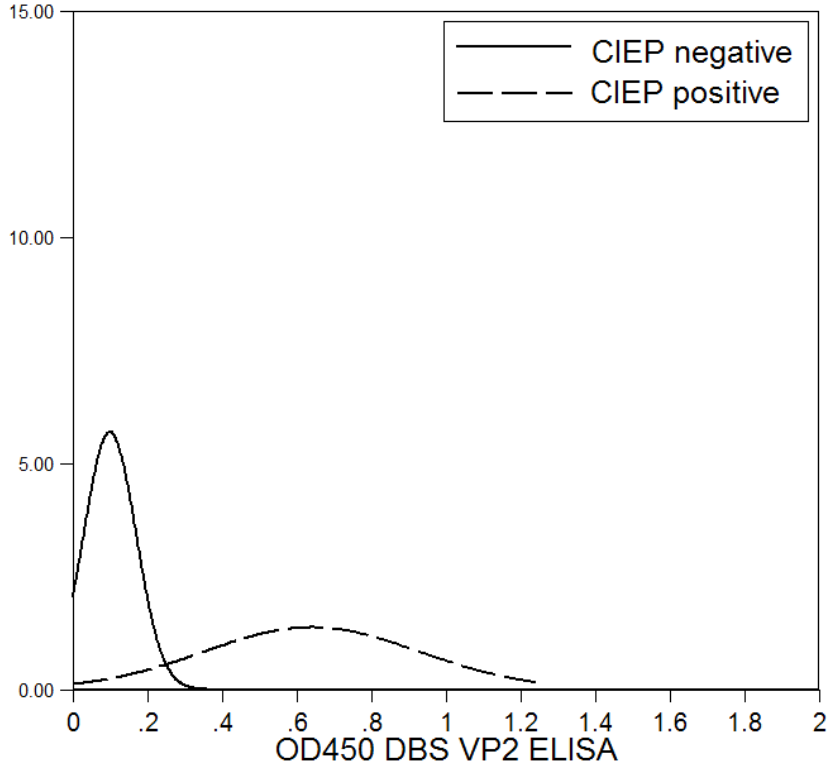

Figure 14. The distribution of OD_{450} values in the VP2 ELISA for CIEP negative (mean=0.10; SD=0.07) and CIEP positive mink (mean=0.64; SD=0.29).

4.2 Evaluation of ELISA as a tool for determining AD progression

A major focus in this project, was the capacity of the ELISA to quantitatively estimate the amount of ADV antibodies as an indirect measurement of AD progression. Categorization of mink into infected and non-infected was of subordinate importance. Using ELISA as a tool for quantitative determination, antibody stability over time is an important criterion since the sampling for serological screening of many thousand mink for AD typically extends over some time and in larger herds can be stretched over a couple of weeks. For mink with progressive AD, IgG antibodies can typically be detected a couple of weeks after infection (Farid *et al.*, 2015; Bloom *et al.*, 1994; Hadlow *et al.*, 1985; Aasted *et al.*, 1984a; Hadlow *et al.*, 1983). The IgG levels gradually increase and level out after a couple of months (Bloom *et al.*, 1994; Hadlow *et al.*, 1983). For

mink with non-progressive AD, the IgG antibody levels will instead remain low or decrease with time following an initial increase. When using the estimated antibody level as a diagnostic tool to determine the AD status in mink based on their result at a single sampling occasion, it is therefore essential to secure that the estimated antibody level will not depend on the sampling order of the mink in the herd. In **III part I**, comparisons between the OD_{450} values in serum from mink sampled either on consecutive days or one week apart showed that the difference between different measurements were all within the predefined interval of equivalence based on the methodological variance in **II** ($\delta=\pm0.1$ OD_{450}). It was therefore concluded that the measurements were equal and that antibody levels can be considered stable over a couple of weeks and thereby useful as a diagnostic tool.

With the introduction of ELISA for measuring ADV antibodies, the possibility to improve the sampling method arose. The use of DBS instead of serum could facilitate both sampling as well as the preparations for analysis at the laboratory. Prior to the initiation of **II**, DBS had been introduced at some laboratories but the method had not yet been scientifically published. In **II**, the quantitative comparison of OD_{450} values for paired samples of serum and DBS revealed an overall good agreement ($r= 0.90$). The mean OD_{450} value in DBS was slightly higher than the mean OD_{450} value in serum (1.02 compared to 0.91). Considering these results, DBS was concluded to be a fully applicable alternative to serum for quantitative analysis of ADV antibodies.

In the next step, the capacity of the DBS VP2 ELISA to work as an indirect quantitative measurement of AD progression was investigated through evaluation of the correlation between the DBS antibody level (expressed as the OD_{450} value) to the degree of hypergammaglobulinemia (expressed in terms of the A:γG ratio). Since the concentrations of antibodies and gamma globulins have been reported to depend on many factors such as the host, the virus strain, the total pathogen load and time after infection (McKenna *et al.*, 1999; Aasted & Hauch, 1988; Porter *et al.*, 1984a; Hadlow *et al.*, 1983; Eklund *et al.*, 1968), it has previously been argued that quantitative ELISA analysis for antibodies cannot replace a direct test for hypergammaglobulinemia (Cepica *et al.*, 2012). However, in **II**, a moderate - strong correlation between the level of antibodies and the degree of hypergammaglobulinemia ($r_{serum}=-0.66$ and $r_{DBS}=-0.81$) as well as a moderate - high coefficient of determination ($R^2_{serum}=0.47$ and $R^2_{lnDBS} = 0.66$) for the regression models were demonstrated. Both the correlation as well as the association between the OD_{450} value and the A:γG ratio were generally superior for DBS than for serum samples from the same mink. The superiority of DBS compared to serum can reflect the significantly higher OD_{450} values in DBS than serum for diseased mink (A:γG <5) which were not found for non-diseased mink

(A:γG >5). This indicated that there were components in the blood of diseased mink that created a background in the ELISA that increased the OD_{450} values and improved the association with AD progression estimated as hypergammaglobulinemia. Consequently, the DBS VP2 ELISA was concluded to be preferable over serum VP2 ELISA as a quantitative tool for estimating the degree of hypergammaglobulinemia and identify diseased mink.

Through ROC analysis, an OD_{450} value of 0.50 in the DBS VP2 ELISA was found to correspond to healthy mink (defined as having an A:γG >8) and a cut-off at $OD_{450} \geq 0.83$ in the DBS VP2 ELISA was found optimal to predict diseased mink (Figure 15). The sensitivity and specificity of the DBS VP2 ELISA to identify mink with hypergammaglobulinemia, using $OD_{450} \geq 0.83$ as cut-off, were determined to 87.6% and 76.0%, respectively. From this, it was concluded that the DBS VP2 ELISA can be a fully applicable diagnostic tool for estimating AD progression of individual mink in the absence of any available direct diagnostic method for the A:γG ratio.

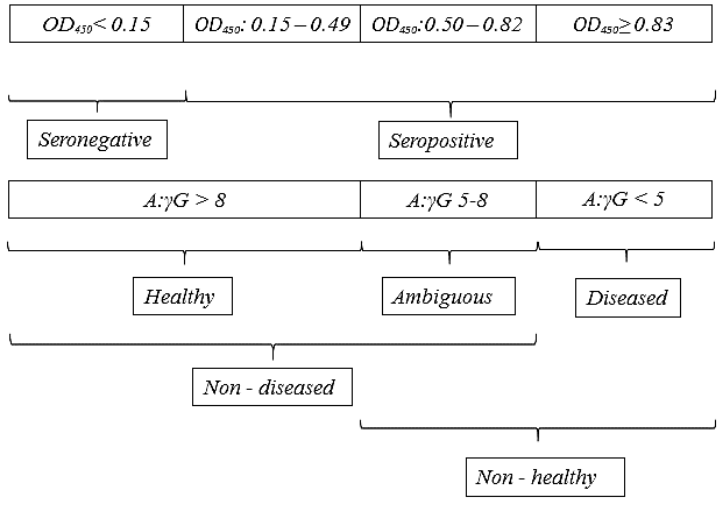

Figure 15. The OD_{450} cut-off values for ADV infection and different stages of AD progression originally published in **III**.

The idea behind estimations of AD progression is to control AD through selection of non-diseased mink as breeding mink (Thomsen, 2016; Aronen, 2011; Greenfield *et al.*, 1973), and thereby minimize the number of mink which will develop clinical symptoms in the herd the following year. Traditionally, this selection has depended on the result from IAT, and the sampling strategy throughout the year have varied between different herds; while some farmers sample both in October prior to pelting and in February prior to mating, many mink farmers still only select their future breeders prior to pelting (Svensk Mink, 2017). Due to this, it is also important to investigate whether the same mink will have a similar OD_{450} value irrespective of the sampling period. In **III part II**, comparisons between the OD_{450} values in DBS from mink sampled in October and February revealed a significantly higher median OD_{450} value in February than in October (0.72 compared to 0.58). Consequently, the proportion of mink categorized as seropositive ($OD_{450} \geq 0.15$) was also higher in February than in October (90% compared to 83%). More than half of the mink (55%) that were seronegative in October had seroconverted until February while a small proportion (3%) of the mink that were seropositive in October were categorized as seronegative in February. In herds in areas where AD is endemic, estimating AD progression in terms of ELISA OD_{450} value has been suggested as a tool for grading mink during the selection of future breeding animals. In that process, variations in OD_{450} value with time would consequently lead to different estimations of the AD progression in the mink. In addition, in **III part II**, the correlation between the October and February samplings was only moderate (r=0.53) indicating that there is a risk that the grading order of the animals will vary between the two sampling occasions. Within a control program, this means that the time point of sampling mink will influence the selection of future breeders.

To investigate how selection at the two different time points would affect which mink were selected, the AD progression was categorized and the AD status of each mink was compared between the two sampling occasions. The results (**III part II**) showed that 64% of the mink categorized as healthy ($OD_{450} < 0.50$) during the October testing were categorized as healthy in February as well. Similarly, a majority of the mink (75%) that were categorized as non-diseased ($OD_{450} < 0.83$) during the October testing remained non-diseased until February. Of the mink categorized as non-healthy ($OD_{450} \geq 0.50$) during the October testing, 92% were categorized non-healthy in February as well while 8% were categorized as healthy in February. Similarly, 66% of the mink categorized as diseased ($OD_{450} \geq 0.83$) during the October testing, were categorized diseased in February as well while 31% were categorized as ambiguous and 4% were categorized as healthy. One reason for the general increase in antibody level

between the two sampling occasions could be true disease progression, either in mink not infected in October or in infected mink experiencing a gradual increase of immunoglobulins typical of AD (An *et al.*, 1978; Kenyon *et al.*, 1963). Another possible explanation for the difference could be that the ADV antibody levels are showing similar seasonal changes as those that has been reported for gamma globulin concentrations in ADV infected mink (Bazeley, 1976). However, the correlation between the two sampling occasions was only moderate, indicating that the antibody levels in the individual mink were not affected in a similar way and that there may be other factors that affect the long-term consistency in ADV antibody levels. For instance, a decreased antibody level between the two samplings could mirror "resistant" animals or animals with non-persistent infection (An & Ingram, 1978). Thus, mink with low to decreasing concentrations of antibodies to ADV could thereby be of interest to select for breeding. It could also be argued that the observed difference in antibody levels between October and February was caused by a methodological variance. However, the median absolute difference between the two samplings in **III** $^{\text{part II}}$ exceeded the predefined equivalence interval based on the methodological variance found in **II** (0.18 and 0.10, respectively) and the difference was concluded to be a real difference and not a consequence of methodological variation.

4.3 Association between AD status and reproductive performance

AD has previously been reported to have a negative impact on the reproductive performance (Reichert & Kostro, 2014; Segervall, 2011; Kangas & Smeds, 1983; Kangas, 1971). To investigate the association between AD status estimated with the DBS VP2 ELISA and the reproductive performance, the reproductive performance of the mink in **IV** was measured and expressed separately in terms of percentage of barren females and the mean litter size of the non-barren female mink. For the single herd scrutinized in **IV** $^{\text{pilot}}$, the percentage of barren females (both primiparous and older female mink) was 31% and the mean litter size of the non-barren female mink was 5.2 (SD=2.0). In **IV** $^{\text{follow-up}}$, the mean percentage of primiparous barren females for the four herds scrutinized was 14% (range: 13-19%) and the mean litter size of the non-barren female mink was 5.9 (range: 5.6 – 6.1). In all herds in **IV** $^{\text{pilot}}$ and **IV** $^{\text{follow-up}}$, the percentage of barren females was higher than the recorded mean percentage of barren females for Swedish ADV infected herds (11.5% in 2012 (n=68) and 8.8% in 2013 (n=64) (Svensk Mink, 2017)). A selection bias can be one possible explanation for the generally increased percentage of barren females in the

scrutinized herds since farmers experiencing a more progressive ADV infection in their herd can be more likely to seek advice and enrol to the AD control program. However, the large percentage (20 %) of non-diseased barren females in **IV** $^{\textbf{pilot}}$ is noteworthy, and other un-diagnosed factors could have contributed to an overall increased percentage of barren females. Such factors can for instance be nutrition in terms of the amount of energy and nutrients, the feed quality, as well as the feeding strategy (Børsting, 1998; Tauson, 1992).

In **IV**, both AD status and the age of the female mink were significantly associated with both being barren and litter size. Diseased mink, defined as having an OD_{450} value > 0.8 before mating, had a higher risk of being barren compared to non-diseased female mink in an interaction with the age in of the female mink in **IV** $^{\textbf{pilot}}$. The risk of being barren was 2.6 times higher among primiparous diseased female mink compared to the primiparous non-diseased female mink while for the older female mink, no significant difference in percentage of barren females due to the AD status was found. For both the diseased as well as non-diseased female mink, the percentage of barren females and the risk for being barren was significantly higher for primiparous compared to older female mink. In **IV** $^{\textbf{follow-up}}$, when only primiparous mink were scrutinized, being barren was significantly associated with the AD status although the risk of being barren for diseased mink (OR=1.6) was lower than in **IV** $^{\textbf{pilot}}$. One possible reason for this difference in risk could be that mink selected as breeders in the **IV** $^{\textbf{follow-up}}$ had a lower mean antibody level ($OD_{450=}$ 0.5 in **IV** $^{\textbf{follow-up}}$ compared to $OD_{450=}$1.0 in **IV** $^{\textbf{pilot}}$) indicating that mink with the highest antibody levels had been de-selected as breeders by the farmer prior to mating to a larger extent than in **IV** $^{\textbf{pilot}}$. Due to this, the mean antibody level of the diseased mink was lower and consequently, a more limited negative effect on reproductive performance could be expected.

Regarding litter size of the non-barren female mink, no significant association between the litter size of the non-barren female mink and the AD status was found in **IV** $^{\textbf{pilot}}$. However**,** in **IV** $^{\textbf{follow-up}}$ when more herds were scrutinized, the effect of being diseased compared to non-diseased was a reduction in litter size by 5%. Thus, the results in **IV** agreed with earlier studies reporting smaller litter sizes as well as increased early kit mortality in diseased compared to non-diseased/healthy female mink (Reichert & Kostro, 2014; Kangas & Smeds, 1983; Kangas, 1971). Age was also associated with litter size of the non-barren female mink where older female mink had approximately 5% larger litter sizes compared to the primiparous female mink (**IV** $^{\textbf{pilot}}$), which agreed with previous reports (Dziadosz, 2010; Lagerkvist, 1992). However, a bias in the selection of breeding female mink could also have contributed to the results in **IV**. Older female mink are more likely to perform better since they

have been selected due to a high reproductive performance during the previous year, and they may have had a superior resistance to AD which also could have influenced the reproductive performance.

In **IV**, the colour type of the mink was also associated with the reproductive performance, which previously has been reported by others (Seremak, 2013; Brzozowski *et al.*, 2012; Demontis *et al.*, 2011; Felska & Sulik, 2000). A higher percentage of barren females and a lower mean litter size of the non-barren female mink for the Hedlund white female mink than for the brown female mink was observed in **IV** $^{\text{pilot}}$. In **IV** $^{\text{follow-up}}$, significant differences between colour types within the same herd could also be observed even though the superior colour types varied between different herds. In addition, in **IV** $^{\text{follow-up}}$ when four different herds were scrutinized, the observed mean percentage of barren females for the four herds ranged from 13% to 19% and the observed mean litter size of the non-barren female mink ranged from 5.6 to 6.1 with statistically significant differences between some of the herds. The model in **IV** $^{\text{follow-up}}$ was therefore adjusted for both herd and colour type of the mink. However, the influence of colour type and herd effects were not further evaluated since the main aim of this project was to evaluate the influence of the AD status on the reproductive performance.

In conclusion, disease status of the female mink had an impact on the reproductive performance in **IV** and can therefore be a subject for a control program. However, it is important to be consistent when registering the reproductive performance in order to facilitate comparisons between different countries as well as between different herds. Previously, the mean litter size per mated female mink (including both barren and non-barren female mink) has commonly been reported (Segervall, 2011; Lagerkvist, 1992; Joergensen, 1985b; Kangas & Smeds, 1983; Kangas, 1971), and has been traditionally used among farmers (Svensk Mink, 2017). The mean litter size per mated female mink is a joint measure combining two different processes, i.e. being barren and litter size of the non-barren female mink, that were differently associated with the estimated A:γG ratio in **IV**. Therefore, in order improve an AD control program, there are advantages of reporting both the percentage of barren females as well as the litter size of the non-barren female mink separately. In **IV**, the effect of disease on the reproductive performance was mainly an effect on the percentage of barren females although a slightly negative effect on the litter size was observed in **IV** $^{\text{follow-up}}$. Further, the time at which the number of kits are counted can influence the reported reproductive performance. In the present project, litter size was defined as the number of kits alive at three weeks *post-partum* which has previously been used in fur animal breeding programs and in research (Kempe & Strandén, 2016; Hansen *et al.*, 2010; Lagerkvist, 1992).

However, this measure is also a joint measure combining the litter size at parturition with the pre-weaning survival rate. Due to this, the true percentage of barren females and the pre-weaning mortality are often unknown. Certainly, litter size at birth and pre-weaning mortality can be affected separately by ADV and the reported effect in **IV** on the reproductive performance could have been different if we had counted the number of kits earlier.

4.4 Suggested strategies to improve the reproductive performance

The overall aim of this thesis was to develop strategies to reduce the impact on welfare and reproduction in mink in ADV endemic areas using estimation of ADV antibodies as a tool. Knowledge that that the OD_{450} value in the DBS VP2 ELISA was accurate for diagnosing ADV infection in the scrutinized population (**II**), stable in the short-term (**III**), highly correlated to the level of hypergammaglobulinemia (**II**), significantly associated to the reproductive performance (**IV**), quick to perform and can be offered at a reasonable price all make the DBS VP2 ELISA a suitable tool to use for disease control. Based on this, a suggested strategy to improve the reproductive performance is presented below.

4.4.1 Categorizing mink based on AD progression

When selecting future breeders, it is important to preserve the magnitude of the breeding stock and at the same time create space for genetic selection for desirable traits such as temperament, pelt quality, body size, and litter size (Joergensen, 1985b). In AD endemic areas, one way to control the disease is through selection of suitable breeding mink and this selection has traditionally depended on the result from IAT. In **II**, the alternative to use of the DBS VP2 ELISA instead of IAT to categorize mink based on AD progression was presented (Figure 15). Depending on the selected cut-off value, the mink could either be categorized into seropositive/seronegative, healthy/non-healthy, or diseased/non-diseased and at each sampling time, any of these categorizations could possibly be used. However, controlling AD and preserving the size of the breeding stock at the same time can be challenging in herds with a high prevalence of ADV infected mink. In this project, the proportions of ADV infected mink of the herds in **I** were between 85% - 93%, according to the results obtained in the serum VP2 ELISA and the herds in **IV** between 78% - 95% according to the results obtained in the DBS VP2 ELISA. As will be illustrated below, this high prevalence of infected mink makes it impossible to select only

non-infected mink for breeding. Focusing on AD progression instead, the proportion of diseased mink (OD_{450} value > 0.8), was 60% in the single herd in **IV** $^{\textbf{pilot}}$ with a significant difference between primiparous mink (73%) and older mink (48%). In **IV** $^{\textbf{follow-up}}$, including primiparous mink from four herds, in average 19% of the mink were categorized as diseased with a significant difference in proportion of diseased mink between different herds (8% - 51%).

To illustrate the effect of the selected cut-off on the number of potential future breeding female mink in a herd, a hypothetical example was created in **IV** and further modified here (Table 2). The example is based on two samplings per year, *i.e.* in October and February, where the same cut-off is used for the two samplings. Using 14% barren females and 5.9 kits per non-barren female mink (as was reported in **IV**) in the calculations, a breeding result at three weeks *post-partum* of 5.1 kits per mated female mink can be expected. Hence, a farm with 1,000 female breeders, will deliver approximately 2,550 new potential female breeders (when the calculated number of female kits in a litter is 50%). Together with the initial 1,000 female breeders, the total number of future potential breeders is then approximately 3,550. However, due to normal mortality in the herd (Svensk Mink, 2017; De Rond. & Kleyn Van Willigen, 2012; Møller, 2011; Clausen, 2006), a loss of approximately 5% of the mink can be expected from three weeks *post-partum* until pelting. In this example, 177 female mink can therefore be expected to die throughout the production year and 3,373 female mink remaining for grading and ADV sampling at the end of October.

Although Table 2 is based on only one publication (**III**), it indicates that not only culling all seropositive female mink but also all non-healthy female mink may lead to too few female mink left to preserve the size of the breeding stock in herds with a high prevalence of AD positive mink. By culling only mink categorized as diseased, around 40% of the female mink would still be potential breeding animals at three weeks *post-partum*, which is approximately 1.5 times the number of female mink needed for breeding and leaves freedom to select for other traits.

Table 2. *Hypothetical example of how the number of potential future breeding female mink is affected by the choice of cut-off in the DBS VP2 ELISA. The figures are extrapolated and modified from the results originally published in **III** and **IV**.*

		Selection threshold		
		Seronegative (OD_{450} <0.15)	**Healthy** (OD_{450} < 0.50)	**Non-diseased** (OD_{450} <0.83)
Size of female breeding stock	(n)	1000	1000	1000
New female mink born	(n)	2550	2550	2550
Total number of female mink at 3 w *p.p*	(n)	3550	3550	3550
Remaining female mink at the end of October [1]	(n)	3373	3373	3373
October				
Percentage of female mink above cut-off	(%)	83[2]	58[3]	39[3]
Female mink potentially culled due to high OD_{450} value	(n)	2800	1956	1315
Potential breeding female mink remaining	(n)	573	1417	2058
February				
Percentage of female mink above cut-off	(%)	55[3]	36[3]	25[3]
Female mink potentially culled due to high OD_{450} value	(n)	315	510	514
Potential breeding female mink remaining	(n)	258	907	1544
Female breeders required for the next season	(n)	1000	1000	1000
Balance (basis for selection)	(n)	-742	-93	+544

1 Expected mortality from three weeks post-partum to pelting of up to 5% (Svensk Mink, 2017; De Rond. & Kleyn Van Willigen, 2012; Møller, 2011; Clausen, 2006)

2 Based on the results in **III** and **IV**

3 Based on the results in **IV**

Besides preserving the size of the breeding stock and create space for genetic selection for desirable traits, it is essential to minimize the risk of poor reproductive performance in ADV positive herds. A quantitative test for ADV antibodies creates a possibility to control AD. Based on the results in **IV**, removal of primiparous diseased female mink from the breeding stock before mating in February was concluded to be beneficial for the reproductive performance of the herd by minimizing the risk for barren females. There is a potential advantage of choosing a higher cut-off for the first sampling in October since this will create more space for selection for other desirable traits, and at the same time decreases the risk of culling mink that will go from a higher to a lower antibody level between the two sampling occasions *i.e.* mink that most probably had an immune system handling ADV without developing hypergammaglobulinemia. At farm level, this can either be done by an arbitrary chosen higher cut-off in the ELISA adjusted to the farm-specific distribution of OD_{450} values. Alternatively, IAT can be chosen for the first screening even though it is likely to result in a more unspecific selection (Porter *et al.*, 1980; Gorham *et al.*, 1976; Greenfield *et al.*, 1973). Employing a higher cut-off value and keeping more mink will be a balance act with an increased risk of viral spread from seropositive mink. It should, however, be mentioned that irrespective of the test in October, it was the disease status before mating that was associated with the reproductive performance in **IV**.

4.4.2 Further discrimination between more or less diseased mink

In **IV** the mean OD_{450} value varied between the different herds, and there is a risk that not all mink with an OD_{450} value > 0.8 can be removed from the breeding stock in herds with a higher mean value without decreasing the size of the breeding stock and impede selection for other economically important traits. It has therefore been discussed whether the OD_{450} value can be used as a continuous variable instead of categorical as was presented in **IV**. To illustrate the epidemiological rationale behind such a strategy, an extended investigation of the reproductive performance of the diseased mink (OD_{450} value > 0.8) was performed for the four herds originally included in **IV** $^{\textbf{follow-up}}$**.** The results of this extended investigation have not been compiled into a scientific paper but are presented below.

In order to visibly assess the linearity between the OD_{450} and the reproductive performance, the OD_{450} value in February and the litter size for all 10,638 female mink (both barren and non-barren) were plotted using the" lowess" command in Stata (Figure 16). As is illustrated in the graph, the net reproductive performance per mated female mink was relatively constant for mink with an OD_{450} value ≤

0.8. Based on this data, there is no evidence of an advantage of using a lower DBS VP2 ELISA cut-off value nor of sorting non-diseased mink based on their OD_{450} value. In contrast, there seems to be a possible negative association between the OD_{450} value and the reproductive performance in mink with OD_{450} values > 0.8.

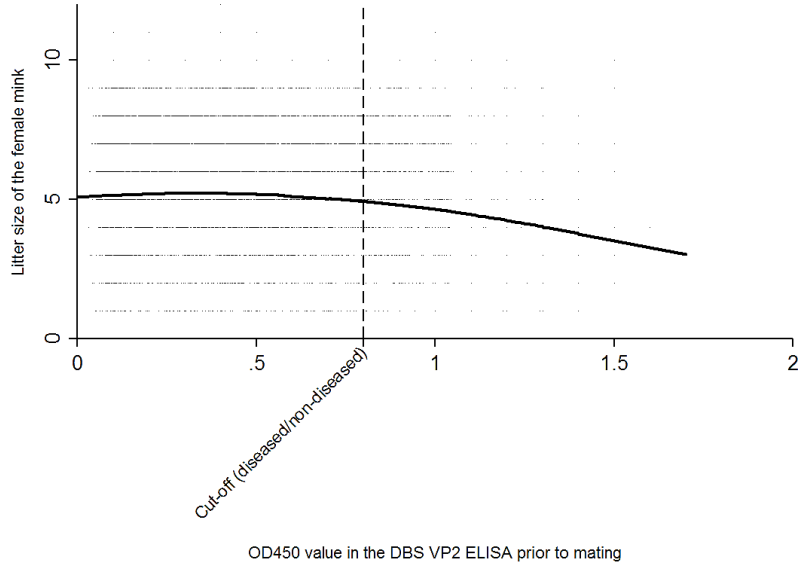

Figure 16. The litter size and the OD_{450} value in February for the 10,638 female mink originally included in **IV** $^{\text{follow-up}}$. The litter size of each mink is marked as a dot in the graph and the lowess curve represents the mean number of kits per mated female mink for each OD_{450} value.

When further investigating the association between the reproductive performance and different OD_{450} values of the diseased mink, there seemed to be two different risk classes: (i) OD_{450} values 0.9 – 1.1, and (ii) OD_{450} values ≥ 1.2 which both were significantly associated with an increased risk of being barren. (Table 3). For practical reasons, the female mink may be subdivided into three categories: non-diseased ($OD_{450} \leq 0.8$), diseased^{+} (OD_{450}: 0.9 – 1.1), and diseased^{++} ($OD_{450} \geq 1.2$). To validate this categorization, another logistic regression model was made using these three categories and the average predicted percentage of barren females was estimated for each category (Table 4). For the litter size of the non-barren female mink, an OD_{450} value ≥ 1.2 was significantly associated with a decreased litter size (Table 3). Using the same three categories for the OD_{450} values as for the risk of being barren above, only the diseased^{++}- category was significantly associated with a decreased litter size of the non-barren female mink (Table 4).

Table 3. *The odds ratio (OR) for being barren, the predicted percentage of barren females, the incidence risk ratio (IRR) and predicted litter size per non-barren female mink for different DBS VP2 OD_{450} values for the models in addendum using the data from the 10,638 primiparous mink originally investigated in* ***IV.***

	Logistic model			**Poisson regression model**		
	OR	*p*-value	Predicted percentage of barren females (95% CI)	IRR	*p*-value	Predicted litter size per non-barren female mink (95% CI))
OD_{450} value						
≤ 0.8	Ref.	Ref.	13 (12;13)	Ref.	Ref.	5.9 (5.9-6.0)
0.9	1.3	0.05	16 (13; 19)	0.97	0.21	5.8 (5.6-6.0)
1.0	1.6	<0.001	19 (15; 22)	0.99	0.74	5.9 (5.7-6.1)
1.1	1.4	0.04	17 (13; 21)	0.96	0.12	5.7 (5.4-6.0)
1.2	2.1	<0.001	24 (18; 29)	0.90	0.001	5.3 (5.0-5.7)
1.3	2.4	<0.001	26 (20; 31)	0.88	<0.001	5.2 (4.9-5.5)
≥ 1.4	2.2	0.004	24 (14; 33)	0.87	0.013	5.2 (4.6-5.7)
Herd						
1	Ref.	Ref.	15 (13;17)	Ref.	Ref.	6.0
2	0.9	0.19	14 (12;15)	0.99	0.69	6.0
3	1.1	0.30	17 (15;19)	0.93	<0.001	5.6
4	0.8	0.03	13 (12;14)	0.98	0.17	5.9
Colour						
812	Ref.	Ref.	15 (14;16)	Ref.	Ref.	5.8
813	0.9	0.08	13 (12;15)	1.07	<0.001	6.2
814	0.7	<0.001	11 (9;12)	1.03	0.03	6.0
Intercept	0.2			5.98		

Table 4. *The odds ratio (OR) for being barren, the predicted percentage of barren females, the incidence risk ratio (IRR) and predicted litter size per non-barren female mink for three categories of diseased mink in addendum using the data from the 10,638 primiparous mink originally investigated in **IV**.*

	Logistic model			**Poisson regression model**		
	OR	*p*-value	Predicted percentage of barren females (95% CI)	IRR	*p*-value	Predicted litter size per non-barren female mink (95% CI))
Disease status						
Non-diseased	Ref.	Ref.	13 (12;13)	Ref.	Ref.	5.9 (5.9; 6.0)
Diseased^{+}	1.4	<0.001	17 (15;19)	0.98	0.11	5.8 (5.7;6.0)
Diseased^{++}	2.3	<0.001	25 (21; 28)	0.89	<0.001	5.3 (5.1;5.5)
Herd						
1	Ref.	Ref.	15 (13;17)	Ref.	Ref.	6.0 (5.9;6.2)
2	0.9	0.21	14 (12;15)	0.99	0.67	6.0 (5.8;6.1)
3	1.1	0.26	17 (15;19)	0.93	<0.001	5.6 (5.4;5.7)
4	0.8	0.03	13 (12;14)	0.98	0.19	5.9 (5.8;6.0)
Colour						
812	Ref.	Ref.	15 (14;16)	Ref.	Ref.	5.8 (5.7;5.9)
813	0.9	0.08	13 (12;15)	1.07	<0.001	6.2 (6.1;6.4)
814	0.7	<0.001	11 (9;12)	1.03	0.03	6.0 (5.8;6.1)
Intercept	0.2			5.98		

In conclusion, mink with an OD_{450} value in the DBS VP2 ELISA between 0.9 and 1.1 had a slightly increased risk of being barren (OR=1.4) but there was no evidence of any effect on the litter size of the non-barren female mink. For mink with an OD_{450} value ≥ 1.2, however, there was a more prominent risk of being barren (OR=2.3) in addition to an expected decreased litter size of the non-barren female mink of approximately 11% compared to non-diseased female mink. This implies that diseased mink can further be discriminated categorically if needed to facilitate selection of future breeders in herds with a high mean DBS VP2

OD_{450} value. The results from this limited material shows no evidence of a true linear relationship between the reproductive performance and the OD_{450} value. Therefore, the use of the OD_{450} value as a continuous variable in AD control programs still needs to be validated.

4.4.3 Concluding remarks

In conclusion, disease status of the female mink had an impact on the reproductive performance in **IV** and for herds with a low percentage of diseased mink, it can be possible to select for desirable traits and at the same time cull all diseased mink. In herds with a higher percentage of diseased mink, however, culling all diseased mink can have a negative impact on the genetic selection for other traits due to the impeded size of the breeding stock by selection for AD status. In such herds, there are indications that the negative impact on the reproductive performance can be limited by culling mink based on the three disease categories presented above.

Today, the VP2 ELISA has been implemented as a tool for selection of breeders in several Swedish herds but due to lack of available large-scale national diagnostic possibilities, many mink farmers still use IAT as the preferred tool for AD screening. Screening mink with for ADV antibodies with the VP2 ELISA can be expected to identify mink with hypergammaglobulinemia at an earlier stage and have the potential to improve both welfare as well as reproductive performance when employed at strategic time points throughout the production year. Therefore, it would be desirable with a future coordinated national strategy for the control of AD in Sweden based on the current knowledge presented in this thesis.

5 Conclusions

This work has generated increased knowledge about serodiagnosis of ADV infection and AD in Swedish mink including suggested strategies to reduce the impact of AD on reproduction in mink in endemic areas. Specific conclusions of the projects were:

- The ELISA system based on VP2 antigen detected ADV antibodies with high sensitivity and high specificity when compared to CIEP. In contrast, the ELISA system based on ADV-G antigen did not perform as well and had a low sensitivity compared to CIEP.
- The adaptation of the VP2 ELISA to detect antibodies to ADV in dried whole blood eluted from filter paper (DBS) had a preserved high sensitivity and specificity when compared to CIEP.
- ADV antibody levels in mink were stable over a period of two weeks which correspond to the time that can be needed to sample all mink in large herds.
- ADV antibody levels estimated with the VP2 ELISA in DBS correlated well to the antibody levels and to the gamma globulins levels in serum. The correlation and association between the antibody level and the level of gamma globulins were generally superior for DBS compared to serum samples from the same mink, and the use of DBS VP2 ELISA was therefore concluded to be the preferable indirect method for estimating AD progression in mink.
- ADV antibody levels in samples collected from the same mink four months apart differed significantly, indicating non-consistency over time, and further studies are needed to follow the alterations in antibody levels throughout the production year.
- In primiparous mink, the risk of being barren was associated with the AD status prior to mating with a higher risk in diseased mink than in non-diseased mink. In addition, there was an association between the litter size of primiparous non-barren female mink and the AD status prior to mating. In

diseased mink, the litter size was reduced by approximately 5 % compared to non-diseased mink. Selection of primiparous female mink with low OD_{450} values for breeding can therefore reduce the risk of reduced reproductive performance.

- In older mink, no association between the risk of being barren and AD status prior to mating was found. The litter size of non-barren female mink was approximately 5 % larger than for primiparous mink irrespective of disease status. However, it should be kept in mind that these mink had been selected due to good reproductive performance during the preceding production year.
- Herd and colour type of the female mink influenced both the percentage of barren females and the litter size of the non-barren female mink.
- By categorizing mink into three different categories based on the ADV antibody level, the control of AD in herds with high mean antibody levels can be facilitated.

6 Future perspectives

For many years, the only way to control AD was by eradication of either CIEP positive mink or IAT positive mink, which reduces the flexibility in control programs. During the past decade, quantitative measurement of AD progression has grown in popularity as a method to select future breeding animals. However, control of AD in endemic areas has not previously been the focus of research, and scientific evaluations of quantitative ELISA as a method to control AD have therefore been sparse. The information provided in this thesis can be used to improve control programs based on quantitative ELISA systems which would contribute to increased welfare, reproductive performance and production economy in areas where AD is endemic. However, there are still many challenges ahead.

The strategy presented in this thesis is only based on one study (**IV**) and in the future, it would be interesting to verify the results in a larger number of randomly selected herds. Similar strategies have been introduced and implemented in other countries with endemic AD, such as the Netherlands and Canada. However, these works have not yet been properly statistically evaluated and published despite convincing clinical experience. Consequently, it would be valuable to confirm the findings in **IV** by including herds in different AD endemic countries into future statistical modulations.

Based on this thesis, several research gaps can be identified and new questions have been generated:

Host factors

- More knowledge about the variations in ADV antibody levels throughout the production year is needed in order to optimize the strategic time points when mink should be sampled in order best predict the future reproductive performance.

- The number of kits as well as kit related parameters (birth weight, growth, and early mortality) ought to be monitored and recorded at different times *post-partum* e.g. at birth, at 3 w and at weaning with the aim to evaluate the effect of the female antibody level on several different aspects of reproductive performance.
- Simulations of the reproductive performance of mink have been presented for ADV seronegative mink including parameters such as body size (Gautason *et al.*, 2016). In the future, it would be interesting to mimic this simulation for ADV positive mink to further improve the models presented in this thesis.

Viral factors

- There is a large variety of ADV strains circulating in the mink populations and it has previously been shown that the virus strain affect the progression of AD (McKenna *et al.*, 1999; Hadlow *et al.*, 1983; Eklund *et al.*, 1968). Therefore, it would be interesting to include viral strains into the statistical models with the aim to evaluate whether the association with reproductive performance differs between different viral strains.

Breeding factors

- In this project, the focus was on the antibody level of the female mink. In the future, it would also be interesting to evaluate the association between the levels of ADV antibodies in male mink during the months prior to mating, and reproductive performance.
- In this project, the follow-up period for the evaluations of the effect of high and low ADV antibody levels was short, and it would be interesting to follow the reactions to ADV infection through future generations both in relation to the female as well as the male AD status.
- As the reproductive performance of older female mink was superior to that of primiparous female mink, it is tempting to increase the number of older female mink in the breeding stock. With an improved reproductive performance of juvenile mink, a larger percentage of these primiparous mink can be considered as breeding animals the next season (*i.e.* be older female mink the following year) and less female breeders will need to be recruited from the juvenile mink (*i.e*. less primiparous female mink in the breeding stock). Consequently, the mean age of the female breeding stock will increase.
- An improved reproductive performance of primiparous female mink will also increase the total number of potential female breeders which in turn creates

space to select for other desirable traits than AD status, which in turn will improve the overall productivity of the herd. To further explore the benefits of ELISA for improving productivity in terms of other parameters, studies on the relationship between the number of available female mink to select from and selection for other desirable traits are of great importance.

Technical factors

- Increased knowledge and awareness of the value of validation of diagnostic tests for AD are desirable. Today, there exist several different tests which are available to mink farmers in different countries. It is essential to ensure that every method used, for research purposes as well as commercial purposes, have been validated for the purpose it is intended for *i.e.* for qualitative or quantitative ADV antibody estimation. Ideally, each method should be accompanied with an easily accessible research-based report on the accuracy as well as the precision of the test.
- Registration of farm related data for Swedish mink such as mortality, reproductive performance and disease incidence is relatively novel to the Swedish mink farmers. The quality of the registered data as well as the usability of the data would benefit from more automated computer based systems.

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Populärvetenskaplig sammanfattning

Plasmacytos kallas även aleutiansjukan från engelskans Aleutian disease (AD). AD är en sjukdom som drabbar mårddjur och är en sjukdom av stor betydelse för minkar världen över. Sjukdomen orsakas av infektion med plasmacytosvirus (på engelska Aleutian disease virus; ADV). ADV har en lång överlevnadstid utanför sitt värddjur och anses vara ett mycket smittsamt virus som lätt överförs direkt från mink till mink eller indirekt via redskap, foder m.m. Viruset smittar även från moderdjur till avkommorna via moderkakan.

Det finns två olika former av sjukdomen. Hos unga minkar utan medfött antikroppsskydd leder infektionen till en akut lunginflammation med andnöd och hög dödlighet. Hos valpar med medfött antikroppsskydd och hos äldre minkar orsakar viruset självt endast en begränsad skada på kroppens celler och det som orsakar sjukdomen är istället minkens eget immunförsvar. Genom en komplicerad mekanism lyckas viruset undslippa immunförsvaret och infektionen blir istället kronisk. Den kroniska närvaron av virus i kroppen leder till en fortsatt stimulering av immunförsvaret vilket skapar en ond cirkel. Successivt bildas mer och mer antikroppar (hypergammaglobulinemi) av en ökande mängd antikroppsbildande plasmaceller (plasmacytos). Antikropparna binder till viruspartiklar i kroppen och tillsammans bildar de komplex som ansamlas i de mindre blodkärlen och fastnar i olika organ i kroppen t.ex. njurarna och levern. Detta kommer med tiden att störa organens funktion vilket ger symtom som nedsatt aptit, viktminskning, blodbrist, nedsatt fortplantningsförmåga, njursvikt och förhöjd dödlighet. Inkubationstiden från smitta till synliga symtom är lång och kan vara upp till ett par år. På gårdsnivå har sjukdomen betydelse både för djurens välfärd och för produktionsekonomin.

Eftersom det är minkens eget immunförsvar som orsakar sjukdomssymptomen så finns det inget vaccin som kan skydda mot sjukdomen. Än så länge finns det inte heller någon fungerande, prisvärd, effektiv behandling och sjukdomen hanteras istället med hjälp av olika bekämpningsprogram. Traditionellt har bekämpningsprogram i områden där plasmacytosviruset är

mindre vanligt bestått i att försöka utrota sjukdomen med hjälp av s.k. ”stamping out” där alla minkarna på gården avlivas och ersätts med friska djur efter en omfattande sanering av gården. I och med att viruset är så motståndskraftigt och smittsamt så är dock ”stamping out” förenat med risker för återinfektion. I områden med endemisk plasmacytos (ständig närvaro av plasmacytos) har ”stamping out” visat sig vara svårare och istället söks det kontinuerligt efter nya alternativ för bekämpning av sjukdomen.

För att påvisa plasmacytos hos levande djur tas blodprov. Traditionellt har blodproverna undersökts med motströmslektrofores (CIEP) men de senaste åren har en nyare metod blivit en alltmer populär metod för att påvisa antikroppar mot plasmacytos, Metoden kallas ELISA och har fördelen att den inte bara kan påvisa om minken är infekterad eller inte utan även kan mäta mängd antikroppar hos de infekterade minkarna. Detta är viktigt eftersom mängden antikroppar ökar ju längre sjukdomen fortskrider. Därför har bestämning av mängden antikroppar föreslagits som ett indirekt mått på hur långt sjukdomen fortskridit hos minken. För gårdar där smittan inte kan utrotas så kan bestämning av djurens antikroppsmängd vara grunden till en alternativ bekämpning av AD. Genom en riktad provtagning innan avelssäsongen kan man identifiera djur som trots att de är smittade inte har utvecklat AD och därför är lämpliga att använda som avelsdjur. Denna metod för att välja ut lämpliga avelsdjur har inspirerat till frivilliga kontrollprogram i exempelvis Kanada och Nederländerna och är ett tilltalande alternativ för plasmacytosbekämpning även i Sverige.

Innan det här doktorandarbetet påbörjades saknades vetenskapliga studier som visade att mängden antikroppar som uppmättes med ELISA också gav ett mått på graden av sjukdom hos mink och hur detta mått på graden av sjukdom påverkar viktiga egenskaper såsom fortplantningsförmågan. Det övergripande målet med det här doktorandarbetet var därför att öka kunskapen om diagnostik av plasmacytos hos svenska minkar för att ge ett redskap som kan användas i en strategi för att kontrollera plasmacytos i områden där sjukdomen förekommer endemiskt såsom i Sverige.

Den här avhandlingen baseras på fyra vetenskapliga arbeten (**I** – **IV**). Den första studien, **I**, genomfördes på fem svenska minkgårdar med målet att jämföra två olika ELISA-system för analys av plasmacytosantikroppar i minkblod (plasma eller serum) med CIEP. Vi valde att jämföra ELISA-systemen med CIEP då CIEP anses vara det test som är bäst (den s.k. gyllene standarden för att påvisa plasmacytosantikroppar). Det visade sig att det ELISA-system som baserades på VP2 protein påvisade antikroppar mot ADV hos svenska minkar med hög tillförlitlighet.

I den andra studien, **II**, låg fokus på att förenkla provtagningen och analysförfarandet genom att använda intorkat blod filterpapper (dried blood spots; DBS) istället för blod i kapillärrör. Genom en viss anpassning av VP2 ELISA-systemets utförande så kunde plasmacytosinfekterade djur särskiljas från plasmacytosfria djur med hög tillförlitlighet på gårdar med låg förekomst av infekterade djur. Vid jämförelse mellan den uppskattade antikroppsmängden i serum och den i DBS så var överensstämmelsen hög även om de uppmätta värdena i genomsnitt var högre i DBS än i serum. Vid vidare analys visade det sig att statistiskt säkerställda skillnader av betydelse mellan DBS och serum endast fanns hos de djur som hade förhöjd nivå av gammaglobuliner i blodet (uttryckt som kvot mellan albumin och gammaglobuliner i blodet). För minkar med normala nivåer av gammaglobuliner kunde inga skillnader påvisas. När den uppskattade mängden av plasmacytosantikroppar jämfördes med mängden gammaglobuliner i blodet så var överenstämmelsen högre för DBS än för serum.

I den tredje studien, **III**, undersöktes stabiliteten av den uppmätta antikroppsmängden över tid. I den första delen av studien konstaterades att antikroppsmängden i blodprov som tagits upp till två veckors mellanrum stämde väl överens med varandra. Resultaten visade att antikroppsmängden uppmätt med VP2 ELISA därför var tillförlitligt för att rangordna minkar på en gård även om provtagningen sträcker sig över ett par veckor. I den andra delen av studien jämfördes antikroppsmängden vid två olika provtagningstillfällen med fyra månaders mellanrum; den första i samband med det stora urvalet av avelsdjur strax innan pälsningen på hösten (i oktober) och den andra strax före parningen (i februari). Här var skillnaderna i antikroppsmängd mellan de två olika provtagningarna större och antikroppsmängden var signifikant högre i februari än i oktober. Överenstämmelsen mellan höst- och vårprovtagningen var låg och vidare studier behövs därför för att utreda betydelsen av när antikroppsmängden bäst bör mätas i ett kontrollprogram för AD.

Plasmacytosens påverkan på avelsresultatet kan visa sig både som en ökad andel gallhonor (d.v.s. honor som inte får några valpar) och som en minskad kullstorlek bland de honor som får valpar. På gårdsnivå uttrycks detta tillsammans som kullstorleken per parad hona d.v.s. antalet valpar utslaget på alla honor inklusive gallhonorna. I den sista studien, **IV**, studerades sambandet mellan avelsresultatet och antikroppsmängden i DBS. I den första delen av studien analyserades avelsresultatet och antikroppsmängden för 2067 förstaårshonor och äldre avelshonor från en gård. Den studerade gården hade 31 % gallhonor och en genomsnittlig kullstorlek på 5,2 valpar per valpad hona (motsvarande 3,6 valpar per parad hona). Det var stora skillnader i antalet minkhonor hade en hög antikroppsmängd och därmed klassades som plasmacytossjuka. Totalt klassades 73 % av förstaårshonorna och 48 % av de

äldre honorna som sjuka. Denna skillnad var även tydlig i avelsresultatet – förstaårshonorna hade en genomsnittlig kullstorlek på 5,0 valpar per valpad hona med en gallprocent på 45 % (motsvarande 2,3 valpar per parad hona) medan de äldre honorna hade en genomsnittlig kullstorlek på 5,2 per valpad hona och en gallprocent på 17 % (motsvarande 4,3 valpar per parad hona). Risken för att *bli gallhona* var generellt högre för förstaårshonor än äldre honor oavsett antikroppsmängd men det var en större risk för förstaårshonorna som klassats som plasmacytossjuka att bli gallhona jämfört med förstaårshonor som inte klassats som plasmacytossjuka. För de äldre honorna kunde däremot ingen statistiskt säkerställd skillnad ses i avelsresultat mellan plasmacytossjuka och friska honor. Även minkens färg spelade roll för risken att bli gallhona med en något högre risk för Hedlund-vita minkhonor än bruna minkhonor oavsett antikroppsmängd. För *kullstorleken per valpad hona* så kunde inget samband med antikroppsmängden statistiskt säkerställas i den här delen av studien. Åldern hos minkhonan hade en signifikant betydelse för kullstorleken även om den effekten var liten; medelkullstorleken hos äldre honor var ungefär 5 % större än medelkullstorleken hos förstaårshonorna.

I den andra delen av **IV** analyserades avelsresultatet och antikroppsmängden för 10 368 förstaårshonor från fyra gårdar. De fyra gårdarna hade i genomsnitt 14 % gallhonor och en genomsnittlig kullstorlek på 5,9 valpar per valpad hona (motsvarande 5,1 valpar per parad hona). Det var stora skillnader i antalet honor som kategoriserades som plasmacytossjuka på de olika gårdarna, med en variation från 8% till 51 %. Denna skillnad sågs till viss del även i avelsresultatet där gallprocenten varierade mellan 14 – 19 % och den genomsnittliga kullstorleken varierade mellan 5,6 och 6,1 valpar per valpad hona. Sammanfattningsvis hade plasmacytossjuka honor högre risk för att gå gall och hade i genomsnitt färre valpar per valpad hona jämfört med friska honor. Även minkhonans färg och i från vilken gård hon kom från hade betydelse för det slutliga avelsresultatet.

I den här avhandlingen illustreras sambandet mellan minkhonans antikroppsmängd och avelsresultatet vidare. Istället för att dela in minkhonorna i *sjuk* och *frisk* delades minkhonorna in i tre olika kategorier beroende på deras antikroppsmängd: *frisk*, *sjuk*$^{+}$ och *sjuk*$^{++}$. Minkhonorna som klassificerades som *sjuk*$^{+}$ hade en 1,4 gånger större risk att bli gallhonor men ingen påvisbar påverkan på kullstorleken per valpad hona; minkhonor som klassificerades som *sjuka*$^{++}$ hade 2,3 gånger större risk att bli gallhonor och fick i genomsnitt 11 % färre valpar per valpad hona jämfört med de friska honorna. Genom att kategorisera minkhonorna i tre olika kategorier så är ett kontrollprogram för AD möjligt även på gårdar med hög förekomst av plasmacytos.

Utvärdering av ELISA-systemet baserat på VP2 antigen visade att testet kan användas för att mäta antikroppar i både serum och DBS med hög tillförlitlighet och antikroppsmängden i DBS kan även användas för att hitta minkar med förhöjd nivå av gammaglobuliner i blodet. Detta test kan därför användas för att välja ut minkonor för avel som kan förväntas att hålla sig friska. Att kunna avla på individer som förväntas hålla sig friska säkrar bibehållen välfärd hos minkarna, minskar risken för negativ påverkan på avelsresultatet och kan förväntas leda till en förbättrad produktionsekonomi i områden med endemisk AD. Rent praktiskt kan en strategi för bekämpning av AD baseras på att identifiera plasmacytossjuka djur på gården med hjälp av djurens antikroppsmängd i februari. På gårdar där en hög andel djur har en hög antikroppsmängd är det inte realistiskt att välja bort alla djur med höga antikroppsnivåer i en snabb process. På dessa gårdar finns möjligheten att dela upp djuren i ytterligare kategorier för att underlätta urvalet av avelsdjur och på så vis arbeta långsiktigt för en gradvis förbättrad plasmacytosstatus på gården i samklang med andra kriterier för urval av avelsdjur.

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