



Research Article

## Transmission Dynamics of Aleutian Mink Disease Virus on a Farm Under Test and Removal Scheme

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### Abstract

One-hundred black female mink naturally infected with the Aleutian mink disease virus (AMDV) were monitored between Nov. 2005 and Feb. 2008. Animals were tested for antibodies against the virus by counter-immunoelectrophoresis (CIEP) and for serum globulin level by the iodine agglutination test (IAT) on nine occasions. CIEP and IAT tests were conducted twice each year on kits at 4 and 7 months of age. The prevalence of CIEP positive adult females and kits was 12% and 20.9% (n=411) in 2006, respectively, but one female and none of the kits (n=491) were seropositive in 2007. IAT positive cases ranged between 14.1% and 80.7% in adults and between 17.0% and 57.6% in kids, suggesting infection by pathogens other than AMDV. Three of the seropositive females cleared the virus and were considered to be resistant, although they continued producing antibodies until pelted at 34 months of age. The pattern of viral transmission among individuals which were in contact with one another was complex. The virus was not transmitted from two infected males to five seronegative females to which they were mated. There was a significantly higher incidence of CIEP positive kits from seropositive dams in 2006 (63.4%) compared with kits from seronegative parents (16.8%), showing transplacental transmission of the virus. All eight progeny of the only infected male in 2006 were CIEP- and PCR-positive, implying that the male possibly transmitted susceptible gene(s) to his progeny. Estimates of heritability for the CIEP-positive kits at 4 and 7 months of age were 0.573 and 0.497, respectively, suggesting the strong contribution of the host genetics to this trait. It was concluded that resistance to infection played a more important role than the survival rate of infected individuals on the herd's health status. Measures of reproduction were not affected by the IAT scores of the dams.

### Keywords

American mink; Aleutian mink disease virus; Viral transmission; Resistance; Anti-viral antibody; Iodine agglutination test

### Introduction

The Aleutian mink disease virus (AMDV, *Carnivore amdoparvovirus 1*) typically causes persistent infection, enhanced antiviral antibody production, polyclonal hypergammaglobulinemia, plasmacytosis, and formation and deposition of immune complexes in various organs in adult mink, leading to glomerulonephritis, arteritis and sometimes death [1]. The disease has neither cure nor an effective

vaccine [2] and the conventional strategy for combating the virus has been the elimination of seropositive mink identified by the counter-immunoelectrophoresis (CIEP) test [3], and has been an effective tool for viral eradication from mink farms in some mink producing countries [4-7]. The test-and-cull strategy in combination with disinfection practices and implementation of biosecurity measures have been used in Nova Scotia, the largest mink pelt producing province in Canada, since the mid-1970s, but has not been effective in eradication of the virus from many farms, particularly in the western part of the province where they are in high concentration [8].

Uncertainty of the outcome of virus eradication strategies inspired farmers in some regions of North America and Europe to select for tolerant mink based on low antibody titers or negative iodine agglutination test (IAT). It has been suggested that animals may show two different responses to infection; either they are resistant, preventing viral entry, minimizing viral load or clearing the virus; or they are tolerant, remaining productive while viral infection persists [9]. The degree of variation in mink on naturally infected farms for resistance and tolerance has not clearly been determined. It is known that the severity of Aleutian disease (AD) symptoms depends on mink genetics, the strain of AMDV and environmental factors (reviewed in 1), and some non-Aleutian mink can tolerate the infection, showing persistent infection with the normal level of serum gamma-globulin, low anti-AMDV antibody titer, and mild or no gross or microscopic lesions characteristic of AD [10]. It has been shown that AMDV spreads slowly on infected farms [4], but the extent of genetic control of susceptibility to infection and antibody response has not been reported. The objective of this study was to investigate the manner of spread of AMDV on a naturally infected herd of black mink subjected to the test-and-removal strategy, to monitor anti-AMDV antibody production and serum globulin levels and to estimate heritability of seroconversion.

### Materials and Methods

#### Statement of animal care

This experiment was performed on a commercial farm, where industry standards were followed. Sampling procedures were performed according to the standards of the Canadian Council on Animal Care after approval by the institutional Animal Care and Use Committee.

#### The farm

This commercial mink farm is located in a rather isolated area in eastern Nova Scotia, 160 Km from the nearest mink farm. The farm was established in 1984 with 50 CIEP-negative black females and 10 males. The number of breeder females gradually increased to approximately 4,000 by 2006. Over the years, pregnant females were purchased from three farms in the USA (Utah and Wisconsin), three farms in Nova Scotia and one farm in Ontario, Canada. By 2006, the herd consisted almost entirely of the Wisconsin blood line, because of superior performance of these animals. New entries were always tested by CIEP on the farm of origin prior to importation, and were quarantined in a separate building for eight weeks before retesting and integration into the herd. The farm was surrounded partly by a

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chain link fence and partly by a solid fence, and biosecurity measures were strictly followed. Animals were kept in one row of cages each of which contained a wooden nest box and were separated by a plastic sheet to avoid direct contact. Initially, the CIEP test was performed only on animals which were to be sold as breeders. Two of the 12 mink that were tested prior to being sold in 2001 were CIEP positive. The source of infection was unclear, and could have been from wild animals because a weasel was once spotted in the feed kitchen in 2000. Starting in 2002, all adult breeders and potential replacement kits were tested by CIEP in November and selected animals were tested again in February prior to breeding, and positive individuals were pelted. The virus spread slowly, reached a peak of 34.2% in 2003 and was eliminated by 2008 (Table 1).

In addition to being CIEP negative, selection of replacement animals has been based on vigor, reproductive performance and fur quality traits. Replacement females were selected from litters of 5 or larger, and males were selected from litters of 5 to 8 because males from litters of 9 and larger were believed to have a slower growth rate and lower sperm production. Starting early after birth, all litters were regularly checked and those with any problem were marked for elimination. Any female that lost two or more kits was culled. Special emphasis was put on body size, hair color and short guard hair (nap) when selecting replacements. By 2006, close to 75% of the pelts from this herd had large sizes (00 or larger). Average litter size of the entire herd was 3.8 prior to 2002, but increased to 5.4 after the AMDV outbreak. These unexpected observations were the reasons for taking a closer look at the status of AMDV infection and animal performance of a group of mink on this farm.

### Experimental procedures

One-hundred black females and 30 males which were CIEP negative in Nov. 2005 were housed in individual cages in one shed. The pedigrees of the animals were known. Each female was exposed to one male between March 6 and 9, 2006, and again to the same male 8 to 10 days later. The animals whelped between late April and early May and kits were weaned at the end of June. Kits were either kept in individual cages after weaning or a male and a female sibling were caged together. Blood was collected from breeder females into heparinized capillary tubes by toe-nail clipping for the CIEP test on four occasions in 2006 (Feb. 10, July 17, Oct. 12 and Nov. 27), and from the males in Feb. Kits were CIEP tested twice: on Aug. 25 and Nov. 27. The CIEP tests were performed at the Nova Scotia Department of Agriculture Animal Pathology Laboratory in Truro, Nova Scotia, which is accredited for this test by the Standards Council of Canada. The test was performed using a cell-cultured antigen supplied by United Vaccine, Inc., Madison, Wisconsin, USA. Breeder females were subjected to the Iodine Agglutination Test (IAT) by the farmer on the same days that blood samples were drawn for the CIEP test. The farmer had several years of experience in performing the IAT. The IAT results were scored as clear (0), weak positive as shown by a few precipitates after swirling of the mixture for up to a minute or two (1), moderate as shown by the presence of a cloudy mixture containing many small precipitates (2), positive as shown by heavy precipitates (3), and strong positive as shown by immediate formation of dark clumpy precipitates (4) [11]. As part of routine

farm practices, low producing females were pelted in December and breeder males were pelted in March after breeding.

For 2007 breeding, 46 of the experimental females with satisfactory reproductive performances were retained in December 2006, and the number increased to 100 using 29 CIEP-negative kits from the 2006 experimental herd and 25 CIEP-negative adult females from other groups on this farm. Two females that were seropositive in July 2006 (#2, #46) were retained for breeding because they appeared healthy and each weaned 4 kits. Another female which tested CIEP positive before breeding in Feb. 2007 (#74) was also retained for breeding. These three females were monitored until Feb. 2008, when they were 34 months of age, and were considered to be resistant. Management, breeding and nutrition of these animals were similar to those in the previous year. Females were tested by CIEP on four occasions in 2007 (Feb. 16, July 3, Sep. 14 and Nov. 23) and on Feb. 8, 2008. Kits were CIEP tested on Sep. 14 and Nov. 23, 2007. Breeder males were tested by CIEP before breeding in Feb. 2006 (n=30) and Feb. 2007 (n=30).

### AMDV DNA detection by PCR

Spleen samples were collected aseptically from dead animals and after pelting from breeders and kits in cases where confirmation of AMDV infection was needed. Samples were stored at -80°C, and DNA was extracted by the high-salt method [12]. The presence of AMDV DNA was tested by the polymerase chain reaction (PCR) using primers 60F/60R and four volumes of sample DNA (2.55, 1.5, 0.15 and 0.075 µL) as previously explained [13]. PCR products were bi-directionally sequenced in 11 samples from this farm and presence of AMDV was confirmed.

### Necropsy and histopathology

Within 24 h after killing, carcasses of the eight CIEP-positive females pelted in Feb. 2006, the two CIEP-positive females pelted in Dec. 2006, and the three resistant females pelted in Feb. 2008 were subjected to postmortem examination and histology by an experienced veterinary pathologist at the Nova Scotia Department of Agriculture, Truro, Nova Scotia. Size, color, inflammation and necrosis of the spleen, kidneys, lungs, heart, brain and liver were evaluated at necropsy. Histopathological lesions in these organs, except the spleen, were subjectively scored on a scale of 0 (no lesion) to 4 (very severe lesions of advanced AD) as previously explained [14]. Slides from kidneys of the three resistant mink were further examined by staining with the Masson's Trichrome [15] and Mallory's phosphotungstic acid haematoxylin (PTAH) [16]. Because lesions on the lungs of most mink were suspicious of a bacterial infection, swabs of the lung tissues of the three resistant mink were tested for bacterial growth for 48 h on blood and MacConkey agars incubated at 35°C, and on blood CO<sub>2</sub> and chocolate agars incubated at 37°C in a CO<sub>2</sub> incubator.

### Data analysis

Data were analyzed using the SAS program, V9.4 (SAS Institute Inc., Cary, NC). The likelihood ratio chi-square was used for comparisons involving counts. The associations among individuals for IAT results on different occasions were calculated by the Spearman's rank correlation, and intraclass correlations were calculated using

Table 1: Percentage of CIEP positive mink from 2000 to 2010 on the farm.

Measurement	2000	2001	2002	2003	2004	2005	2006	2007	2008	2010
Number tested	149	12	6025	7861	4737	7725	7354	7496	9917	2693
% CIEP positive	0.0	16.7	14.9	34.2	22.5	20.5	4.1	0.2	0.0	0.0

variance components estimated by the PROC MIXED. Heritability of and genetic correlation between CIEP test results of kits at 4 and 7 months of age in 2006 were estimated using a multi-trait animal model which included the CIEP results of parents and kits, fixed effects of sex and age at the time of testing (month), the random animal additive genetic effect and the random residual effect. Variance components were estimated using REML on the basis of the analytical gradients method with normality assumption, despite the binary nature of the trait, using the VCE5 software [17]. Breeding values were obtained by the PEST software [18]. The number of records in the pedigree file was 527.

## Results

### CIEP and PCR results of adult mink

The number of new CIEP positive adult females was 8, 2, 1 and 1 in Feb., July, Oct. and Nov. 2006, respectively (Table 2). The eight CIEP-positive females were pelted in Feb. before breeding, which is the standard farm practice, and were all PCR positive. The two females which were seropositive in Oct. and Nov. 2006 were pelted in Dec., and both were PCR positive. The CIEP status of the three resistant females fluctuated over time. Animal #2 became seronegative in July 2007, all three were seronegative in Sep. 2007, and all were CIEP-positive again in Nov. 2007 (Table 2). AMDV DNA was not detected by PCR in the spleens of these females after pelting in Feb. 2008. Four and six females died in 2006 and 2007, respectively (Table 2), and all were seronegative.

Only one of the 30 males used in the 2006 breeding was CIEP positive in Feb. and was mated with two seronegative females. This male was the only PCR positive case amongst the 30 males pelted after breeding in March 2006. The two seronegative females which bred to this infected male remained seronegative when tested three times after mating (July, Oct., Nov.), and were PCR negative after pelting in Dec. 2006. Two males were CIEP-positive in Feb. 2007 and were also PCR positive when pelted in March 2007. One of them mated with one of the seropositive (resistant) females, and the other with the

two resistant and three seronegative females. The three seronegative females remained seronegative in July, Sep. and Nov. tests, and were PCR negative after pelting in Feb. 2008.

### CIEP status of kits

The frequency of CIEP-positive kits was 3.8% in Aug. 2006 (16/416), and increased by more than five-fold to 20.9% (86/411) by Nov., whereas all the kits which were tested in Sep. (n=491) or Nov. (n=478) of 2007, even the 28 progenies of the two CIEP-positive males which bred to the three resistant and three CIEP-negative females, remained seronegative. All the kits that were CIEP positive in Aug. 2006 either died (n=5) or remained seropositive until Nov. (n=11). The CIEP status of kits in 2006 was significantly influenced by the CIEP status of their parents (Table 3). In Aug. 2006, 1.3% of the progenies of seronegative parents, 27.3% of the progenies of the four females which became seropositive by Nov. 2006 and mated with seronegative males, and 62.5% of the progenies of the seropositive male mated to two seronegative females, were seropositive (Table 3). The corresponding estimates in the Nov. test were 16.8%, 63.4% and 100.0%, respectively.

The 16 CIEP-positive kits in Aug. 2006 were the progeny of five males, and the 86 CIEP-positive kits in Nov. (including the five seropositive kits that died) were the progeny of 20 males. None of the 115 progenies of nine sires that mated with 24 females were CIEP positive. Of the two females which were seropositive by July 2006, one (#2) had three progenies which were all seropositive by Aug. 2006, whereas none of the four progenies of the other female (#46) were seropositive by Nov. 2006 (Table 4). Estimates of heritability for the CIEP-positive kits at four and seven months of age (Aug. and Nov. 2006) were  $0.573 \pm 0.044$  and  $0.497 \pm 0.049$ , respectively, and the genetic correlation between the two measurements was  $0.748 \pm 0.051$  (Table 5).

There was no statistical difference between male and female kits for the percentage of CIEP test results in Aug. ( $\chi^2_{(1)}=0.53$ ,  $P=0.47$ ) or Nov. ( $\chi^2_{(1)}=1.04$ ,  $P=0.31$ ). Of the 128 pairs of siblings which

**Table 2:** Number of adult females positive on counter-immunoelectrophoresis (CIEP) in 2006 and 2007.

Measurements	2006				2007				2008
	02/10	07/17	10/12	11/27	02/16	07/03	09/14	11/23	02/08
No. of females tested	100	90	88	88	100	95	95	94	99
No. died	0	2	2	0	0	5	0	1	1
New CIEP positive cases	8 <sup>*</sup>	2 <sup>§</sup>	1 <sup>¶</sup>	1 <sup>¶</sup>	1 <sup>§</sup>	0	0	0	0
CIEP status of resistant females <sup>c</sup>									
#2	-	+	+	+	+	-	-	+	+
#46	-	+	+	+	+	+	-	+	+
#74	-	-	-	-	+	+	-	+	+

\* These 8 mink were killed before 2006 breeding

§ These three were used in 2007 breeding

¶ Pelting in December 2006

<sup>c</sup>Positive (+), negative (-)

**Table 3:** CIEP status of kits, their sires and dams in 2006.

Parents' CIEP		August test		November test	
Sire	Dam	Number of kits tested	% CIEP positive	Number of kits tested	% CIEP positive
Negative	Negative	386	1.3	381	16.8
Negative <sup>*</sup>	Positive <sup>*</sup>	22	27.3	22	63.4
Positive <sup>§</sup>	Negative <sup>§</sup>	8	62.5	8	100.0
Chi-square (probability)		45.8 (P<0.001)		47.0 (P<0.001)	

\*Three seronegative males bred to four seropositive females

§One seropositive male bred to two seronegative females

**Table 4:** Litter size of the three resistant females and CIEP test results of their progenies in 2006 and 2007.

Measurement	Animal ID		
	#2	#46	#74
2006			
Number of kits born alive	7	9	8
Number of kits weaned	4	5	7
Number of CIEP positive kits/number tested (Aug. 25)	3/3	0/4	0/5
Number of CIEP positive kits/number tested (Nov. 27)	3/3	0/4	0/5
2007			
Number of kits born alive	7	0	9
Number of kits weaned	7	0	9
Number of CIEP positive kits/number tested (Sep. 14)	0/7	0/0	0/9
Number of CIEP positive kits/number tested (Nov. 23)	0/7	0/0	0/9

**Table 5:** Estimates of additive genetic variance ( $\sigma^2_A$ ), residual variance ( $\sigma^2_e$ ) and heritability ( $h^2$ ) in August and November 2006 tests.

Source	August	November	Covariance <sup>s</sup>
$\sigma^2_A$	0.016985	0.068248	0.02548
$\sigma^2_e$	0.012657	0.069207	-.00155
$h^2$	0.573	0.497	0.748

<sup>s</sup>Genetic covariance between additive genetic effect and residuals of August and November tests, and the genetic correlation between the two measurements.

were housed in the same cage, both were CIEP negative in 98 cages (76.6%), both were positive in 21 cages (16.4%) and one of the pairs was positive in nine cages (7.0%) in the Nov. 2006 test. Seropositive and seronegative siblings from four cages were pelted in Dec., and the virus was present in the spleens of the four seropositive kits, whereas it was not detected in their seronegative siblings.

### Adult IAT

Prevalence of IAT positive females in 2006 ranged between 27.3% in Nov. and 80.7% in Oct. (Table 6). The estimates were lower in 2007, ranging between 16.0% in Nov. and 33.7% in July, but the patterns of changes in both years were almost similar, i.e. an increase from Feb. to July followed by a decline by Nov. The largest proportion of adult females had weak positive IAT (score 1) in most tests and a small proportion showed strong positive (score 4) only in July and Oct. 2006. The IAT status of a considerable proportion of the females changed from one test to the next in both years. In 2006, only 3.8% of the 88 females that completed all the tests remained IAT negative in all tests and 4.5% remained IAT positive throughout the entire period (Table 7). The largest proportion of females (25%) were IAT negative in Feb., positive in July and Aug., and negative again in Nov. In 2007, the largest number of females had negative IAT in the three tests (44.7%), followed by those which were positive in July and negative in Feb. and Nov. (21.3%), and only one female (1.1%) remained IAT positive in all tests (Table 8). The Spearman's rank correlation coefficient between IAT scores of the pairs of tests were small and non-significant within each year, ranging between 0.16 (Feb.-July) and -.04 (Feb.-Nov.) in 2006, and between 0.05 (Feb.-Nov.) and -.01 (Feb.-July) in 2007. The estimates of intraclass correlations between IAT scores were 0.33 and 0.00 in 2006 and 2007, respectively. The three resistant females showed varying degrees of IAT results over time. They showed clear signs of elevated levels of serum globulins in July 2006, including #74 which was seronegative at this time. Females #46 and #74 had negative IAT results from Feb. 2007 to Feb. 2008, but female #2 was IAT positive (score 3) in Feb. and Nov. 2007 (Table 6).

### Kit IAT

Consistent with the adults, higher percentages of kits were IAT positive in both tests in 2006 than in 2007, and the proportion of IAT-

**Table 6:** Distribution of females by the IAT scores in 2006 and 2007.

IAT scores	2006				2007			2008
	Feb. 10	Jul. 17	Oct. 12	Nov. 27	Feb. 16	July 3	Nov. 23	Feb. 8
Number of females	100	88	88	88	100	95	94	99
IAT scores, %								
Clear (0)	65.0	29.6	19.3	72.7	79.0	66.3	84.0	85.9
Faint (1)	35.0	25.0	28.4	21.6	0.0	24.2	3.2	4.0
Cloudy (2)	0.0	25.0	35.2	2.3	8.0	6.3	5.3	4.0
Spots (3)	0.0	13.6	14.8	3.4	13.00	3.2	7.5	6.1
Clump (4)	0.0	6.8	2.3	0.0	.0	0.0	0.0	0.0
Total IAT positive, %	35.0	70.4	80.7	27.3	21.0	33.7	16.0	14.1
IAT status of 3 females								
#2	1	4	2	0	3	0	3	0
#46	0	3	0	1	0	0	0	0
#74	1	4	3	1	0	0	0	0

**Table 7:** Distribution of 88 female mink by the IAT status in different tests in 2006.

Sampling month	IAT status <sup>s</sup>													
February	-	-	-	-	-	-	-	-	+	+	+	+	+	+
July	-	-	-	+	+	+	+	-	-	+	+	+	+	+
August	-	+	+	-	-	+	+	-	+	-	-	+	+	+
November	-	-	+	-	+	-	+	+	-	-	+	-	+	+
Number of mink	3	12	5	5	4	22	8	1	5	2	2	15	4	4

<sup>s</sup>IAT positive(+) and negative (-) results

**Table 8:** Distribution of 94 female mink by the IAT status in different tests in 2007.

Sampling month	IAT status <sup>s</sup>							
February	-	-	-	-	+	+	+	+
July	-	-	+	+	-	-	+	+
November	-	+	-	+	-	+	-	+
Number of mink	42	6	20	5	11	3	6	1

<sup>s</sup>IAT positive (+) and negative (-) results

positive kits declined from the first to the second test in both years (Table 9). Small percentages of kits showed the highest IAT score (4), and the majority of kits had low score in three of the four tests (Table 9). The IAT status of a considerable number of kits changed from one test to the next, and the changes between the two measurements were significant in 2006 ( $\chi^2_{(1)}=5.5, P=0.02$ ) but not in 2007 ( $\chi^2_{(1)}=0.02, P=0.88$ ). Of the 404 kits that had IAT information on Aug. and Nov. 2006, only 18.1% and 24.3% were positive or negative on both tests, respectively. Of the 482 kits that had IAT information in Sep. and Nov. 2007, 4.8% and 60.4% were positive or negative on both tests, respectively. The Spearman's rank correlation coefficient between the two measures of IAT were -0.12 ( $P=0.02$ ) and 0.02 ( $P=0.73$ ) in 2006 and 2007, respectively.

## Reproduction

In addition to the eight CIEP-positive females which were discarded, two died before breeding, and the remaining 90 females were exposed to males in 2006. One did not breed, and six bred but did not whelp, four of them bred to an infertile male. The means of the number of kits born alive and weaned per female exposed to males were 6.37 and 4.71, respectively, in 2006 and 6.70 and 5.29, respectively, in 2008 (Table 10). Measures of reproduction were comparable between seronegative and seropositive (resistant) females in 2007 (Table 10), but were not statistically analyzed because of only three resistant females. The proportion of animals whelped ( $\chi^2_{(1)}=0.59$ ,  $P=0.44$ ), number of kits born alive ( $\chi^2_{(9)}=11.8$ ,  $P=0.23$ ) and weaned ( $\chi^2_{(8)}=11.5$ ,  $P=0.17$ ) were not different between the two years. Chi-square tests showed that the IAT scores did not have a significant effect on the proportion of females which bred, whelped, and the number of kits born and weaned. Spearman's rank correlation coefficients between IAT scores and measures of reproduction were all smaller than 0.07 and non-significant.

## Kit mortality

Pre-weaning mortality of live-born kits was 27.39% and 21.0% in 2006 and 2007, respectively, and kit mortality from weaning to Dec. (pelting) was 2.64% and 1.36% in 2006 and 2007, respectively. Of the 11 kits that died after weaning in 2006, nine were CIEP positive. AMDV DNA was detected in the spleen samples of the nine seropositive dead kits, but not in the spleens of the two dead kits that were seronegative. The mortality rate of CIEP-positive kits (10.4% of 86) was significantly greater than that of CIEP-negative kits (0.62% of 325) in 2006.

## Necropsy and histology

None of the carcasses of the 13 seropositive females showed

**Table 9:** Distribution of kits by the IAT scores in 2006 and 2007.

IAT scores	2006		2007	
	August	November	September	November
Number of kits	416	404	488	483
IAT scores, %				
Clear (0)	42.3	63.9	72.3	83.0
Faint (1)	35.3	17.6	9.0	7.9
Cloudy (2)	18.5	13.4	10.0	5.8
Spots (3)	3.6	4.0	8.2	3.1
Clump (4)	0.2	1.2	0.4	0.2
Total IAT positive, %	57.6	36.2	27.6	17.7

**Table 10:** Descriptive statistics of reproductive performance in 2006 and 2007 breeding seasons.

Year	2006		2007	
	Negative	Negative	Positive	Combined
CIEP status at breeding				
Number of females exposed to males*	90	96	3	99
Did not breed	1	0	0	0
Bred but did not whelp	6	4	1	5
Kits born alive/females exposed				
Mean	6.37	6.74	5.33	6.70
Median	7.0	7.0	7.0	7.0
Range	0-12	0-11	0-9	0-11
Kits weaned/females exposed				
Mean	4.71	5.29	5.33	5.29
Median	5.0	6	7	6
Range	0-10	0-10	0-9	0-10

\*These females were alive at whelping and weaning

**Table 11:** Descriptive statistics of the histopathological lesions on organs of the eight CIEP-positive females killed in February 2016.

	Mean	Median	Minimum	Maximum
Brain	0.13	0	0	0.5
Liver	0.57	0	0	2.0
Kidneys	0.50	0	0	4.0
Lung	1.57	2.00	1.0	2.0
Heart	0.06	0	0	0.5

any gross lesions of AD on any of the organs. There were mild AD symptoms (plasma cells infiltrates) in most of the five organs of the eight seropositive mink pelted in Feb. 2006. The lungs of all animals showed the most severe symptoms and heart and brain showed the lowest severity (Table 11). There was no histopathological symptom of AD in any organ of the two seropositive females which were pelted in Dec. 2006, except in their lungs which showed mild lesions in both individuals (score 1.0). There were some mild abnormalities in the organs of the three resistant mink, including irregular thickening of renal glomerular basement membranes (glomerulonephritis) (Table 12). No bacterial growth was observed in the lung swabs of the resistant mink after pelting.

## Discussion

In agreement with the previous studies [4,19], AMDV spread slowly and reached a peak of 34.2% on the entire farm three years after the initial infection, and the incidence of CIEP positive cases was also low for experimental females (12%) and their kits (20.9%) by Nov. 2006. Slow rate of virus transmission often results in varying proportions of mink remaining seronegative on infected farms [4,14,20,21]. The slow rate of virus spread was also manifested in the pronounced increase in the incidence of seropositive kits from 3.8% in Aug. to 20.1% in Nov., which is in agreement with previous studies [19,22]. One possible explanation for the rapid increase in the prevalence of seroconversion after Sep. is that maternal antibodies delay the establishment of infection in some kits which were exposed to the virus at an early age. Stress caused by a combination of handling animals for pelt evaluation, changing animals' cages, reduced cage capacity relative to body size and cold weather, could have affected the immune system and made animals more susceptible to the establishment of infection in the Fall. Changes in the hormonal secretions which modulate molting of the summer fur and growth of winter pelage [23] may also play a role in the increased incidence of seropositive animals. The pattern of change in the CIEP status of the three resistant females (negative in Aug. and positive in Nov., Table 2) supports the latter hypothesis. Alternatively, because AMDV is very resilient [24], an increase in the rate of infection could have occurred during the Fall because the virus could survive and spread more efficiently in the cold weather.

A complex pattern of virus transmission was observed among individuals that were in contact with each other in this study, such as males and females during breeding, parent-offspring and those kits which were kept in the same cage. There was no virus transmission among breeder males and females, shown by the two and three seronegative females which remained CIEP and PCR negative after breeding with the CIEP- and PCR-positive males in 2006 and 2007, respectively. This observation is in agreement with a previous study where none of the eight AMDV-free females that bred to progressively infected males, which were expected to have high virus burdens, remained seronegative when tested up to four months after mating, and only one of the seven non-infected males mated with infected

**Table 12:** Histopathological lesions observed on the five organs of the three resistant females.

Organ	Animal number		
	#2	#46	#74
Brain	No lesion	No lesion	Few lymphocytes in the choroid plexus
Heart	Rare small interstitial lymphoid aggregates in the interstitium	No lesion	No lesion
Liver	Scattered cells of ITO and small foci of hematopoietic cells	Small to moderate numbers of mixed mononuclear cells in portal triads, and around some larger blood vessels. Scattered foci of cells compatible with hematopoietic cells	No lesion
Kidneys	Slight proteinaceous material in some tubules, mostly in the medulla. Glomeruli had equivocal mild irregular thickening of the mesangial matrix and regular thickening of Bowman's capsule on H & E stain. Masson's Trichrome stains revealed a moderate number of small quite fibrotic glomeruli and suggestion of mild irregular fibrosis in other glomeruli. PTAH stain was negative for fibrin.	Rare tubules contain proteinaceous material and similar equivocal glomerular lesions. Masson's Trichrome stains revealed only one definitely fibrotic glomerulus and was as equivocal on the regular glomeruli as in animal #2. PTAH stain negative for fibrin. Several fibrotic glomeruli were observed on one of the slides.	Similar to animal # 2, with a few small bands of interstitial fibrosis extending from glomeruli on only one slide. Irregular glomerular fibrosis was clearly defined in some glomeruli. PTAH stain was negative for fibrin.
Lungs	Very slight increased cellularity in the interstitium and in alveoli, most were macrophages (may be post mortem change).	Similar to animal # 2. Foreign material evident in one macrophage on one of the slides. Other slide showed some mononuclear cells, mostly lymphocytes with a few macrophages, by a bronchiole.	Small to moderate sized mononuclear cell aggregates around large blood vessels and by bronchioles, and a suggestion of a few of these cells in alveolar septa.

females became seropositive after four months [22]. In another study, there was no difference in the number of CIEP-positive dams which bred to seropositive or seronegative sires [21]. Low incidence of AMDV transmission between mates can be attributed to the irregular and short-lived AMDV in oro-nasal cavities of the mink [25,26], which reduces the risk of viral transmission via saliva excretion or biting during mating. It can be concluded that transmission of the virus between mates during breeding is related to factors other than the presence of the virus in the male's blood circulation.

The significantly higher incidence of CIEP positive kits from seropositive dams in Nov. 2006 (63.4%) compared with kits from seronegative parents (16.8%) was the result of transplacental transmission of the virus, although dams could have transmitted genes that modulate susceptibility to infection as well. Transplacental transmission of AMDV has been documented in experimentally inoculated mink [27,28], and has been shown to play a more important role than animal-to-animal contact for viral transmission under natural conditions [4]. Transplacental transmission of AMDV is not certain, and is less likely to happen from dams with non-progressive infection than from progressively infected ones [22]. This was confirmed in an experiment where AMDV was not detected in 92 progenies of mating between non-progressively infected pastel mink when tested at three months of age [29]. The variable results of published reports along with the significant effect of the CIEP status of the parents on the CIEP status of their kits in the current study are evidence that infection with AMDV depends on the genetics of the host, exposure to the virus as well as other unknown factors.

Interestingly, all the eight progenies of the infected male in the current study were seropositive by Nov. 2006, and were PCR-positive when pelleted in Dec., implying that either AMDV was transmitted by sperm or the male transmitted susceptible gene(s) to its progeny. Although some viruses are carried by sperm [30-32], the latter explanation seems to be more plausible and is supported by the previous report that infected black mink sires had protective effects on their progenies [21]. In that study [21], however, the same protective effect was not observed in brown mink or when both black sires and dams were seropositive. Similarly, the 28 progenies of the two infected males used in the 2007 breeding in the current study remained seronegative, showing the presence of genetic differences among individuals for the control of AMDV infection.

The contribution of exposure to the virus and host genetic differences on AMDV transmission were reiterated by the finding that both siblings which were kept in the same cage were seropositive in 16.4% of the cages and only one of the pairs was seropositive in 7.0% of the cages by Nov. 2006. Differences among littermates in becoming infected with AMDV was reported as early as 1964 [33]. In a previous experiment, only one of the two naturally infected mink in 72.4% of 58 cages were seropositive, and both were seropositive in 27.6% of the cages [4]. The above results, and the finding that AMDV DNA was present in the spleens of four CIEP-positive kits which were tested after pelting in the current experiment, whereas the virus was not detected in their seronegative siblings which were kept in the same cage, demonstrate the complexity of the establishment of infection with AMDV in adult mink.

The exact mechanisms involved in the establishment of infection with AMDV under natural conditions, and the significance of different viral and host factors in determining the outcome of exposure to the virus remain to be determined. Undoubtedly, the intrinsic susceptibility of the host cells, early host shutoff mechanisms [34,35] and pathways that modulate viral gene expression and production of progeny virions [36] influence the outcome of exposure to the virus, viral replication and antibody production. One likely scenario under natural conditions is the ability of the immune system of some mink to prevent the establishment of infection when exposed to low doses of the virus. The effects of viral dose on the establishment of infection has been reported in neonatal mice inoculated with the murine leukemia virus [37], and in sapphire and pastel mink inoculated with low doses of the Pullman strain of AMDV [38]. Although the mode of inheritance of AMDV infection and antibody production is complex, moderate estimates of heritability for the number of CIEP-positive animals confirmed the strong contribution of the host genetics to this trait. It should be noted that estimates of heritability in the current study where a linear model was used for binary data, were lower than those which would be obtained from a threshold model [39,40].

Two of the three resistant females very likely cleared the virus before the 2006 breeding and all three were free of the virus before the 2007 breeding, as supportive evidence came from the observation that all their progenies were seronegative (Table 4), the absence of any histopathological signs of AD and no AMDV DNA in their spleens at pelting. AMDV is present in urine and feces of infected mink [25,33],

and the observation that the three resistant females in the current study did not transmit the virus to any mink in their adjacent cages in 2006 or 2007 is farther evidence that AMDV was not present, at least in high amounts, in their blood, kidneys or intestine. It has been shown that viral replication sharply decreases sometimes after infection [41], and viremia is often transient in non-progressively infected mink [25,29,42], but there is limited published evidence of AMDV clearance. In a previous experiment, the virus was not detected in the spleens and mesenteric lymph nodes of 8 of the 9 pasteurized mink inoculated with the Pullman strain and tested between 78- and 125-weeks post-inoculation using tester sapphire mink, whereas they remained seropositive [42].

The CIEP test results of the three resistant females shows that antibody production persisted long after viral clearance, although titers were marginal and were below the detection threshold of the CIEP at some points. Continued antibody production after cessation of viral replication [25,29,43] or after viral clearance [42] has been reported in mink. Antibody response usually results in aggravation of the disease, but the persistent antibody response in these mink was associated with recovery from the disease. It may be postulated that the persistent non-pathogenic antibody response conferred immunity in some mink as a result of (i) cell mediated immunity which prevents the virus from mounting excessive antibody responses that is harmful to the host, (ii) antibody response to the right antigen(s) of the virus that is not harmful, or (iii) restricted viral replication and antibody production at low levels in response to sequestered AMDV [44]. A less likely hypothesis is that persistent antibody production was the result of cross reactive antigens from other proteins.

Viral clearance and the continued antibody production following viral clearance have important ramifications in viral eradication programs as well as in selection for tolerance. In viral eradication programs, those seropositive animals which have cleared the virus, and thus are resistant, will be culled. The sustained use of a test-and-cull program has two antagonistic effects on the population; selection against seropositive mink which cleared the virus, and selection for those which were exposed to the virus but remained uninfected. The net effect of these two actions on the population likely depends on the virus pathogenicity and virus dose. In selection programs for tolerance based on antibody titer, those individuals which do not become infected after exposure to the virus, and those which are capable of clearing the virus after infection, will be retained. Identifying such individuals requires uniform exposure of animals to the virus, which is difficult to achieve on farms because of the slow rate of AMDV transmission.

The finding that nine of the 11 kits that died after weaning in 2006 were CIEP and PCR positive, and that the mortality rate during this period was significantly greater in seropositive (10.4%) than seronegative kits (0.62%) suggest that the virus isolate was moderately pathogenic. The mild histological lesions in organs of the eight seropositive females which were culled in Feb. 2006 supports this conclusion. The low prevalence of seroconversion in adults and kits, which could have been the result of continuous selection for CIEP negative animals on this farm, indicates that resistance to infection on a farm infected with a moderately pathogenic isolate of AMDV is a significant source of genetic variability. The mortality rates of adults and weaned kits were low (less than 6%) in both years, but estimates of pre-weaning mortality (27.39% and 21.0% in 2006 and 2007) were comparable with 20% in non-infected commercial farms in Canada [45].

Successful eradication of AMDV from this farm in seven years after the initial infection was likely achieved by a combination of the removal of seropositive individuals and no viral introduction from the surroundings. The test-and-cull strategy was effective for AMDV eradication from four farms in Ontario, Canada, during the early years of using CIEP [4], but it has not been effective in virus eradication from many ranches in western Nova Scotia, Canada, where the concentration of mink farms is high [8], and the virus is widespread in wild animal populations [13]. AMDV was eradicated in Iceland by 1984 using a test-and-removal program, and mink farms remained free of the virus for 12 years before the reinfection of one farm, probably by a virus originated from wild mink [6]. AMDV infection persisted in a small district in Denmark despite many years of eradication efforts [7], and the virus sporadically appeared in subsequent years in other regions of the country [5,46]. It can be concluded that AMDV eradication by the test-and-cull strategy has uncertain outcomes, and that an essential criterion for keeping a farm free of AMDV is preventing farm re-infected using adequate biosecurity measures.

IAT is a low-cost on-farm test, which is not specific for AMDV infection [20], but has been successfully used to eradicate AMDV from a herd [47] and establishing a tolerant mink herd [48]. Because serum gamma-globulin level is highly correlated with anti-AMDV antibody titer (10), the high number of IAT-positive and the small number of CIEP-positive adults and kits in the current experiment implies that a large proportion of animals were infected with pathogens other than AMDV. The lowest incidence of IAT-positive adults and kits in Nov. in both years, which was opposite to the trend for CIEP results, suggests that the reduced level of serum globulins could have been the result of a low abundance of bacteria in feedstuffs and the environment during the cold season. The large degree of fluctuation in IAT scores over time, which is in agreement with previous reports on IAT scores [49] and serum globulin levels (10), was manifested in non-significant correlation coefficients between different IAT measurements on each animal, and further confirms the above conclusion. The finding that IAT scores of the three resistant females were zero whereas CIEP results were positive in some occasions (Tables 2 and 6) was because gamma-globulin levels in infected animals were likely low, as previously reported in the case of non-progressively infected mink [22,42].

The average number of kits born alive (6.37 and 6.70) and weaned (4.71 and 5.29) per live female at pelting time in the current study were comparable with that for black mink on an uninfected farm, i.e. 5.0 kits within 48 h after birth [50]. This was expected because of a few seropositive females. The above estimates are particularly interesting because each female was bred to only one male, which is believed to have a negative effect on reproductive success. The number of females which bred but did not whelp (6 and 5 in 2006 and 2007) would have potentially been lower had two different males been used. The unexpected increase in reproductive performance of the entire herd after AMDV contamination is contrary to previous reports that AMDV infection reduces reproductive success and increases kit mortality [27,28,51,52]. In another experiment, however, the number of kits weaned and culled per female was not different between naturally infected and seronegative dams [21], possibly because of differences in virus strain and mink genotype.

The lack of a significant relationship between IAT scores and reproductive measures in the current study are in line with a previous report where the effect of IAT scores on female reproduction was

inconclusive [49], and could be because most IAT positive animals had low scores (1 and 2). Lack of association between IAT scores and reproduction measures was also confirmed by the observation that the IAT score of the 12 females which did not breed or did not whelp in 2006 and 2007 were zero in the Feb. tests, except one female in 2007 which had IAT score 3.

The severity of AD lesions is often lowest in the lungs compared to other organs [53,54], but the opposite was observed in the 10 seropositive females in the current experiment. A similar situation is reported in naturally [14] and experimentally [26] infected adult mink in Nova Scotia, suggesting that lung lesions are specific to AD in this province. Alternatively, plasma cell infiltrates in the lungs could have been caused by microorganisms other than AMDV or caused by IgG4 overproduction [55]. Minor lesions that were observed in the organs of the three resistant mink could have been the remnants of an earlier AMDV infection, or caused by other pathogens with cross reactivity with AMDV, because these three mink previously had IAT scores of 3 and 4 (Table 6), although no bacterial growth was observed in their lung swabs when pelted in Feb. 2018. The absence of any histopathological sign of AD in the three resistant mink is in agreement with previous reports that non-progressively infected pastel mink, with normal levels of serum gamma-globulin and low anti-AMDV antibody titers, had no AD lesions a long time after infection [10,29].

## Conclusion

AMDV transmission among mink on this infected farm was slow and showed a complex pattern among animals that were in contact with each other. Becoming infected in animals which were naturally exposed to the moderately pathogenic AMDV isolate was the major source of variation. Three adult females which were monitored until 34 months of age cleared the virus, and were considered to be resistant. It is logical to hypothesize that becoming infected with AMDV after exposure to the virus, and tolerating the disease following the establishment of infection, are possibly modulated by two different immunological pathways, and distinction between these two traits is important when selecting for tolerance or resistance to AMDV.

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