

Supplemental Information accompanying manuscript

Occupational and environmental exposure to SARS-CoV-2 in and around infected mink farms

Authors

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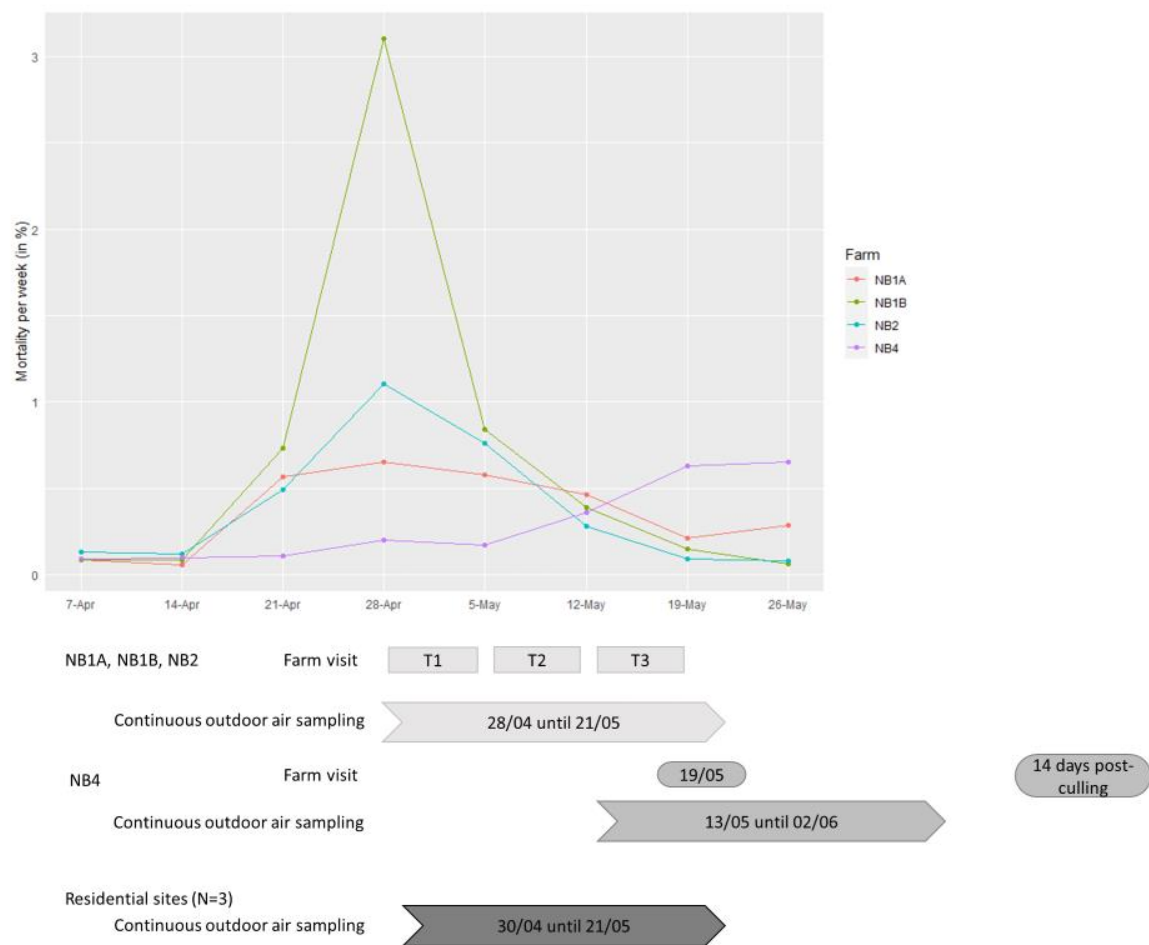
Supplemental Methods

Supplemental Methods A. Background information on investigated farms

The first SARS-CoV-2 infected mink farms were investigated thoroughly including sampling of animals, humans and the environment. These farms were all situated in the region of the Netherlands with the highest mink farm density, the eastern part of the province of Noord-Brabant (NB). On April 23rd, SARS-CoV-2 infection was established in the first farm, NB1, which has two separate locations, NB1A and NB1B, which are 115m apart. Two days later another farm was diagnosed positive, NB2, situated at 14 km distance from NB1. For NB1 as well as NB2, the source of the outbreak amongst minks was traced back to an SARS-CoV-2 infected farmer/worker at the specific farm. Results of whole-genome sequencing research indicated the virus strain at NB2 to be different from NB1, thus underlining the occurrence of two separate antropozoonotic transmission events^{2,9}. Both NB1 and NB2 experienced increased mortality amongst the minks coinciding with respiratory signs starting in the first half of April but, as it was unprecedented, initially SARS-CoV-2 infection was not suspected. Serological findings suggested, based on a random subset of minks tested, many minks to already have seroconverted by the time of diagnosis, indicating the outbreak to be indeed circulating for a while. This was confirmed by sequencing research showing considerable viral genomic diversity at the time of diagnosis. Animal investigations performed from the moment of diagnosis onwards, indicated the number of infected minks to be decreasing over time thus substantiating that both NB1 and NB2 were in a later phase of the outbreak when detected. From 28th of April onwards, environmental sampling started at and around NB1 and NB2. On May 6th, SARS-CoV-2 infection was detected at two more farms, NB3 and NB4. NB4 was, in contrast to the other farms, at the time of diagnosis in a more early phase of the outbreak based on disease history, serology and sequence diversity. Therefore, NB4 was included for environmental sampling from May 13th onwards, while NB3 was not included. For a detailed description on the course of the outbreak in the farmed minks, clinical/pathological findings, and genomic epidemiology see Oreshkova et al², Molenaar et al¹⁰, and Oude Munnink et al⁹.

Minks kept at NB1, NB2 and NB4 were housed in wire netting cages placed in halls. Halls are naturally ventilated via both large openings in the roof as well as walls which are only (partially) closed in the winter months. Cages are arranged in long single rows, on one side separated by a narrow manure conveyer belt and on the other side by a feeding alley (see Figure S2 and S3 for pictures). In the front of each cage is a sleep/nest box which contains bedding material. Solid boards are attached onto the sides of the cage to prevent direct contact between minks in bordering cages. Per cage, one adult mink is housed and if present kits are kept with their mothers. Whelping takes place once a year in the period end of April/beginning of May. The population before whelping consisted of: 8971 females and 24 males at NB1A, 2923 females and 1699 males at NB1B, 7500 females and 90 males at NB2, and 10300 females and 242 males at NB4.

52 Timeline of the outbreaks at the mink farms and environmental sampling



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54 Note. T1: NB1A 28/04, NB2 30/04, NB1B 02/05

55 T2: NB1A 05/05, NB2 07/05, NB1B 09/05

56 T3: NB1A 12/05, NB2 14/05, NB1B 16/05

57 Mortality is expressed in percentage of naturally deceased minks per week.

58 GD Animal Health is acknowledged for providing the data on mortality

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Supplemental Methods B. Additional information on sampling

Technical details on air sampling (see figure S2 for pictures)

Air sampling was performed by means of filter-based techniques using teflon filters (Pall Corporation, Ann Arbor, USA). Air sampling was performed in parallel to measure both Particulate Matter 10 (PM₁₀) and inhalable dust based on the by European norm (EN) defined size fractions^{12,13}. PM₁₀ is defined by particles of a nominal aerodynamic diameter of 10 µm or less, particles of this size or smaller can penetrate the tracheobronchial regions of the airways. Inhalable dust covers all particles that may enter the nose and mouth thus including small particles (PM₁₀) but also larger particles (inhalable criterion: 100% penetration for particles <10µm, dropping to 50% for 100µm particles, no median cut-off aerodynamic diameter).

Outdoor air sampling

Outdoor air sampling involved continuous multi-day sampling in order to measure high volume of air per sample as levels in the outdoor environment were not expected to be high. Multiple-day sampling of PM₁₀ was performed by means of Harvard impactors (Air Diagnostics and Engineering Inc., Naples, ME, USA) which selectively sample PM₁₀ by trapping larger particles on an impaction plate. Harvard impactors were connected to self-designed air pumps with critical orifices calibrated at a flow of 10.0 l/min (optimal flow rate for this sampling head type). The air flow was checked before and after sampling using a calibrated rotameter. Sampling duration was typically 4 days (hence 57.6 m³ air sampled), minimally 3 days (43.2 m³ air) and maximally 5 days (72 m³ air). To enable sampling of total suspended particles the impaction plate was removed from the Harvard impactor for a series of measurements at farm NB4. For inhalable dust sampling, GSP (Gesamtstaubprobenahme; JS Holdings, Stevenage, UK) sampling heads were used connected to a Gilian GilAir 5 pump (Sensidyne, St. Petersburg, USA) calibrated at a flow of 3.5 l/min (optimal flow rate for this sampling head type). The air flow was checked before and after sampling using a calibrated rotameter. Sampling duration was typically 4 days (hence 20.16 m³ air sampled), minimally 3 days (15.12 m³ air) and maximally 5 days (25.2 m³ air). Sampling heads were attached side-by-side onto a pole at 1.50m height (average breathing height of humans); see Figure S2 for pictures. Field blank controls were collected each measurement period, a Harvard impactor field blank and a GSP sampling head field blank. These field blanks underwent the same procedure except that no air was drawn through the sampling device.

Sampling inside farm and downwind/upwind

Six-hour stationary air sampling was performed and 8-hour personal air sampling by using Gilian GilAir 5 pumps with battery pack. Inhalable dust sampling was performed by means of GSP sampling heads at a flow of 3.5 l/min. PM₁₀ sampling was performed by means of PEM (Personal Environmental Monitor) sampling heads (MSP Corporation, Minnesota, USA) at a flow of 4.0 l/min (optimal flow rate for this sampling head type) which selectively samples PM₁₀ by trapping larger particles on an impaction plate. The air flow was checked before and after sampling using a calibrated rotameter. For inhalable dust samples, 6-hour sampling resulted in 1.26 m³ air sampled and 8-hour sampling in 1.68 m³. For PM₁₀ samples, 6-hour sampling resulted in 1.44 m³ air sampled and 8-hour sampling in 1.92 m³. Stationary air sampling was performed by attachment of the sampling heads side-by-side onto a pole at 1.50m height (average breathing height). Personal air samples were collected by attachment of the sampling heads within the breathing zone of the fieldworker and the pumps clipped on a belt; see Figure S2 for pictures of the measurement set-up. Inside the farm, stationary air sampling was performed at three spots distributed within the farm which remained the same per farm. The spots of the six-hour air sampling outside the farm were based on the wind direction of the measurement day. Upwind sampling was performed at 50 meter distance from the farm, and downwind sampling at 10-20 meter and 100 meter distance. Field blank controls were collected each measurement day, a GSP sampling head field blank and a PEM sampling head field blank. These field blanks underwent the same procedure except that no air was drawn through the sampling device. EDC samplers were placed in cardboard boxes on top of the minks' housing units, on hanging PVC plates and on stands (see Figure S2 for pictures). EDC field blank control was collected each EDC measurement period. These

EDC field blanks underwent the same procedure including opening and closing of the folder except for being opened for 7 days inside the farm.

Technical details on sampling minks' housing units (see figure S3 for pictures)

Swipe samples were collected by sterilized electrostatic cloths (polyester electrostatic cloth; Albert Heijn, Zaandam, the Netherlands) to collect material settled on hardboard rim. The swiped surface was 135mm in length and 65mm in width. Before every sampling, clean gloves were worn. Cloths were not-wetted. Swabbing of the drinker cup was performed by rolling the swab (Cultiplast (dry sterile swab rayon/plastic); LP Italiana, Milano, Italy) on the rim. Swabs were not-wetted. Bedding material was collected from the night/nest box by scooping. Pre-culling a pooled sample of both the top layer and bottom layer was collected. Post-culling, the top layer and bottom layer were sampled separately. Food residues were collected by scraping off the wire with remaining residues. Faecal material was collected by scooping either from within the cage when present or from the floor beneath the cage. These collected materials (bedding, faeces, food) were stored in containers (Cellstar tubes; Greiner bio-one; Kremsmünster, Austria).

Details on fieldworkers

All fieldworkers wore PPE including full face mask (silicon mask 6800 with filters 6035; 3M, St. Paul, U.S.A.) and coverall (Tyvek 500 Xpert Blue; Dupont, Wilmington, U.S.A.); fieldworkers remained SARS-CoV-2 negative.

Supplemental Methods C. Details on laboratory analyses

Sample processing for storage at the end of fieldwork day

For the sampling heads used for air measurements, this involved disassembly to collect the exposed filter and transfer it to an enclosed tube (Greiner 15ml, DNA/RNA-ase free). For the EDCs, this involved collection of the exposed electrostatic cloth out of the holder and place it in an enclosed tube (Greiner 50ml, DNA/RNA-ase free). The other samples were stored directly in their collection container at -80°C.

Further processing for RNA extraction

Per sample type, an adequate processing procedure was performed.

The exposed Teflon air filters, stored at -80°C in 15 ml Greiner tubes with the dust-surface facing the inner of the tube were thawed in the safety-cabinet of the BSL-2 lab. Using a 5 ml sterile pipet 4 ml of PBS-Dulbecco's was added. To submerge the complete filter of 37 mm in diameter in the fluid, the filter was pushed carefully, without damaging the Teflon surface, to the bottom of the tube using the tip of this 5 ml pipet. Tubes were placed on an end-over-end roller and after 90 minutes of rolling at room temperature the tubes were placed vertical for a few minutes allowing the fluid to drip to the bottom of the tube. Using a 2 ml pipet the fluid and large dust particles that sank to the bottom, were homogenised and 1 ml of the suspension was transferred to a clean Eppendorf tube. After vortexing the Eppendorf, 200 µl of suspension was mixed with 200 µl lysis buffer of the ID Gene™ Mag Fast Extraction Kit (ID-VET) and RNA extraction was performed on the KingFisher (ThermoFisher). The remaining suspensions were stored at -80°C for potential virus isolation.

EDC cloths stored at -80°C in 50 ml Falcon tubes with the swiped surface facing the inner of the tube were thawed in the safety-cabinet of the BSL-2 lab. Using a sterile pipet 10ml PBS-PEFABLOC buffer was added (PBS-PEFABLOC buffer: 1% v/v of stock solution in PBS-Dulbecco's. Stock solution: 1 PEFABLOC tablet [Sigma Aldrich, Zwijndrecht, the Netherlands] dissolved in 1ml PBS-Dulbecco's and stored in aliquots at -20°C) and rolled for 1 hour on an end-over-end roller. Two hundred µl of the suspension with dust particles was collected from each tube and mixed with lysis buffer in a similar manner as described above for Teflon air filters.

Approximately 0.5 ml bedding material was transferred to a 15ml Falcon tube to which 3ml PBS-PEFABLOC buffer was added. After vortexing, tubes were incubated one hour at room temperature and centrifugated 10 minutes at 2500xg. Two hundred µl of intermediary fluid was collected and mixed with 200 µl of lysis buffer for RNA extraction.

For food residue samples as faecal material, approximately 0.5 ml was transferred to a 15ml Falcon tube. To each tube 2 ml PBS-PEFABLOC buffer was added and tubes were vortexed vigorously until the material was properly suspended. After incubation of one hour at room temperature, tubes were centrifuged for 10 minutes at 2500xg rpm and 200 µl of the supernatant was collected and mixed with 200 µl of lysis buffer for RNA extraction.

Sampled swabs from drinker cups) were transferred to a 15ml Falcon tube with 2 ml of medium used for maintaining Vero-E6 cells (Minimum Essential Medium with 5% v/v foetal bovine serum (Gibco, Thermo Fisher Scientific, Bleiswijk, The Netherlands), 1% v/v Antibiotic-Antimycotic mixture (anti-anti, Gibco), 1% L-Glutamine (Gibco), 1% nonessential amino acids (Gibco). Tubes were vortexed and incubated for one hour at room temperature. After centrifugation for 10 minutes at 2500 g, 200 µl of the supernatant was collected and mixed with 200 µl of lysis buffer for RNA extraction. See Supplemental Table S1 for a schematic overview of processed volume per sample type.

PCR

Subsequently, samples were tested for SARS-CoV-2 using the accredited E gene PCR as described by Corman et al.²⁶ using the TaqMan Fast virus 1-Step Master Mix (Applied Biosystems), with minor modification in the reverse transcription conditions (52°C for 10 min). The calibration curve ($-3 * \log_{10}(\text{copies}) + 39.249$) was established based on serial dilutions of inactivated cell culture produced SARS-CoV-2 with known titer (SARS-CoV-

2/human/NL/Lelystad/2020). Samples of the same type were tested in one or maximally two runs. Negative controls (no template) and positive controls were included in each PCR run. To each sample, an internal control (intype IC-RNA, Qiagen) was added to check for inhibition of reverse transcription and amplification. Samples with Ct values below the threshold Ct, were defined positive. The threshold Ct was set at 36 based on optimization of sensitivity and specificity analysed by means of the ROC curve using the function spEqualSe from R package OptimalCutpoints.

Attempted virus isolation from air filters

After establishing the presence of SARS-CoV-2 RNA by E gene qPCR in air samples, samples with a Ct value below 32 were subjected to virus isolation using Vero-E6 cells. After thawing, 400 hundred µl of suspension was used to infect monolayers containing of 1×10^6 Vero-E6 cells (ATCC) grown in T25 cm² flask. Cells were cultured in MEM supplemented with 5% FCS, 1% antibiotic/antimycotic, 1%, glutamine and 1% non-essential amino acids at 37°C and 5% CO₂. Monolayers were inspected regular with a microscope to identify the typical cytopathogenic effect induced by SARS-CoV-2. After five days of growth 200 µl of the medium was analysed by E-gene qPCR to detect replication of SARS-CoV-2.

199 **Supplemental Tables**

200 **Supplemental Table S1.** Schematic overview of quantitative Polymerase Chain Reaction analysed proportion per sample type

Sample type	Original sample	Volume of medium added (ml)	Volume aliquot of sample for qPCR (ul)	Volume lysisbuffer added to aliquot (ul)	Volume eluens (ul)	Volume in qPCR (ul)	Proportion volume qPCR/volume eluens	Proportion volume aliquot/sample volume medium	Proportion subsample of original sample
Teflon filter (filter used in active air sampling)	1 filter ~ cubic meter of air sampled dependent on flow rate and sampling duration	4.00	200.00	200	60	5	0.083	0.05	0.004167
EDC	1 EDC cloth ~ 0.2035 m^2	10.00	200.00	200	60	5	0.083	0.02	0.001667
Bedding material	1 patch ~ 0.5 ml	3.00	200.00	200	60	5	0.083	0.067	0.005556
Food residue	1 scoop ~ 0.5 ml	3.00	200.00	200	60	5	0.083	0.067	0.005556
Swab drinker cup	1 swab ~ swabbed rim drinker cup: 17 cm diameter	3.00	200.00	200	60	5	0.083	0.067	0.005556
Faecal material	1 scoop ~ 0.5 ml	3.00	200.00	200	60	5	0.083	0.067	0.005556
Swipe normal size	1 normal sized swipe ~ swiped hardboard: 60cm long and 2cm width	5.00	200.00	200	60	5	0.083	0.04	0.003333
Swipe large size	1 large sized swipe ~ swiped hardboard: 60cm long and 2cm width	10.00	200.00	200	60	5	0.083	0.02	0.001667

Supplemental Table S2. Results of multivariable modelling on SARS-CoV-2 RNA load in settling dust sampled by means of EDCs

	Virus load (copies/m ² per day)
Variable	Ratio (95% confidence interval)
Farm NB1A (indicator)	.
Farm NB1B	0.43* (0.21-0.90)
Farm NB2	15* (6.8-32)
Timepoint 1 (indicator)	.
Timepoint 2	0.37* (0.19-0.72)
Timepoint 3	0.075* (0.038-0.15)
Positioned in close proximity to minks	2.3*(1.1-4.9)

Note. Censored regression was applied. Associations expressed in ratio's = estimate of associations to the power 10 to represent ratio in viral load.
Marked * then P-value < 0.05

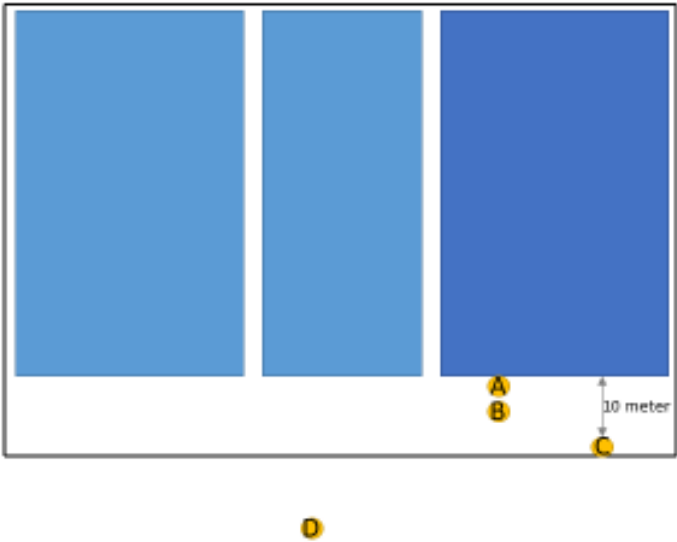
210 **Supplemental Table S3.** Comparisons of SARS-CoV-2 RNA detection in 14 housing units at NB4 sampled pre-
211 culling and post-culling

Sample type	% positive samples pre-culling	% positive samples post-culling
Swipe	100%	14%
Bedding material	Mix of layers top and bottom: 100%	Bottom layer: 85% Top layer: 57%
Faeces material	71%	21%
Swab drinker cup	50%	0%
Food residue	14%	0%

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Supplemental Figures

Supplemental Figure S1. Map showing the sampling locations at farm NB4. The blue blocks are mink stables.



219 **Supplemental Figure S2.** Pictures showing sampling of air and settling dust

220 Picture showing personal air sampling (1), stationary air sampling (2) and sampling of settling dust in close
221 proximity of minks (3).



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223 Close-up of EDC placed in cardboard box



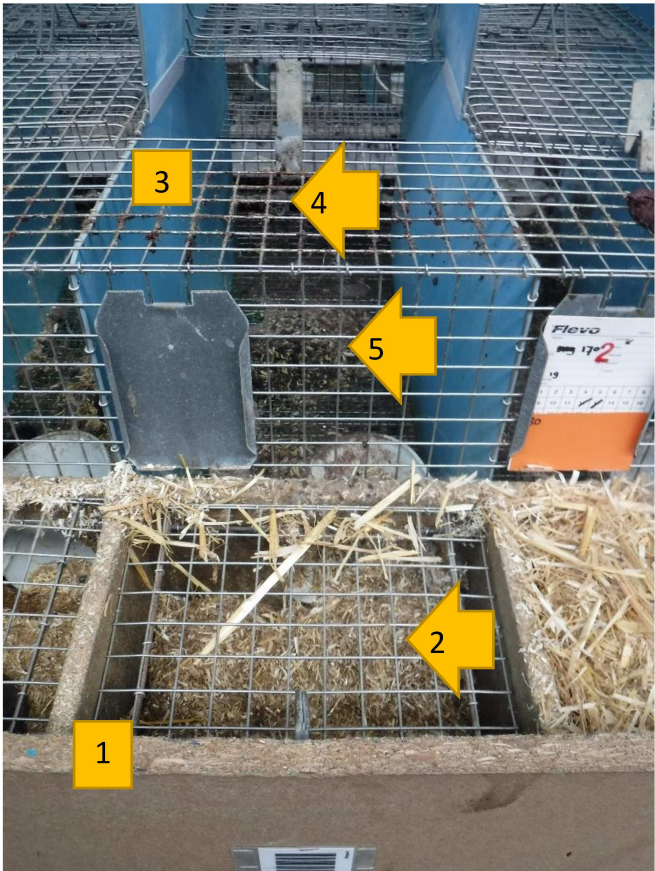
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- 225 Pictures showing sampling of settling dust at further distances from minks; left by placement of EDC on hanging
226 PVC plate (red circle), right by placement of EDC on stand in empty row



228 **Supplemental Figure S3. Pictures showing sampling of mink’s housing units**

229 Picture of mink’s housing unit



Number	Sample type
1	Swipe
2	Bedding material
3	Food residue
4	Swab of drinker cup
5	Faeces material

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231 Picture showing typical way of feeding minks by placing suspension of raw meat on top of the cage



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1. Collection of swipe



2. Collection of bedding material



242 **3. Collection of food residue**



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245 **4. Collection of swab of drinker cup**



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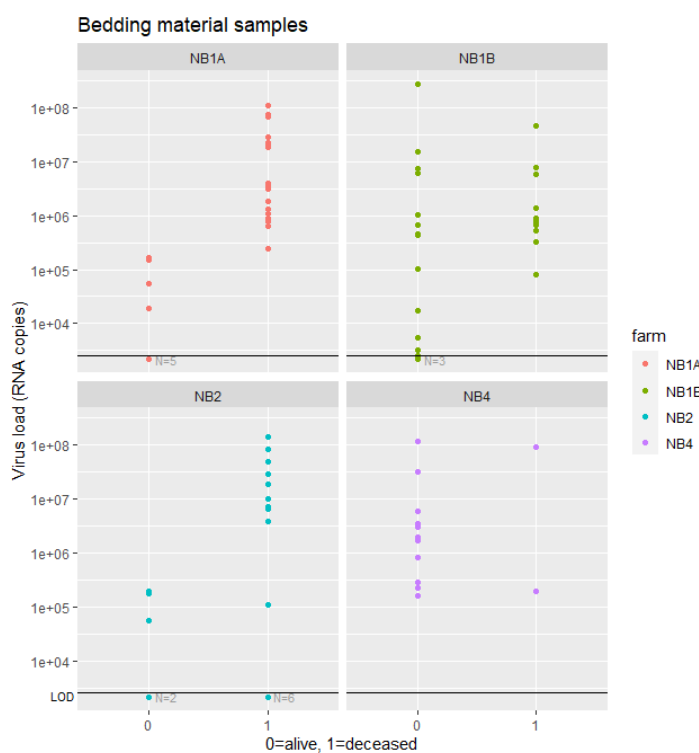
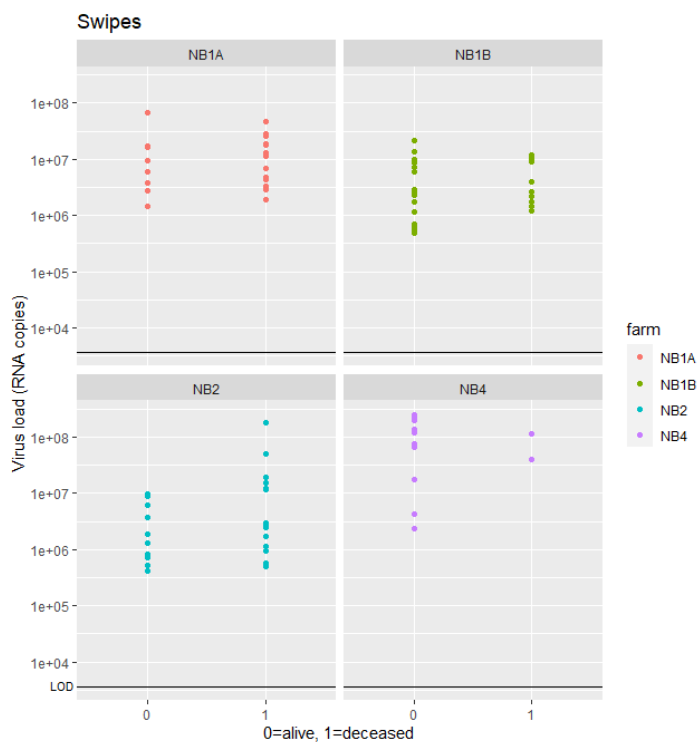
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248 **5. Collection of faeces material**

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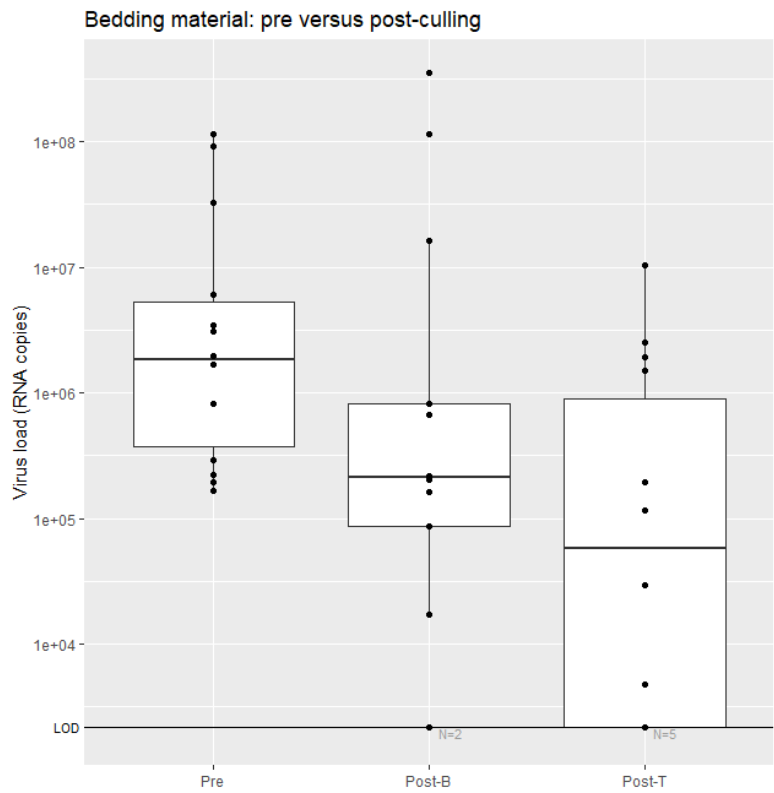
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Supplemental figure S4. Overview of viral load in swipes and bedding material samples per farm in housing units belonging to recently deceased minks versus minks still alive



Note. Percentiles: 25th, 50th and 75th of virus load detected in swipes of 2.06E+06, 6.03E+06, 1.60E+07; respectively. Percentiles: 25th, 50th and 75th of virus load detected in bedding material of 6.36E+04, 8.16E+05, 7.41E+06; respectively.

Supplemental Figure S5. Overview of viral loads in bedding material collected pre-culling versus post-culling (divided in bottom layer sampling and top layer sampling)



Note. Pre = bedding material samples collected pre-culling
Post-B = bedding material samples collected post-culling of bottom layer
Post-T = bedding material samples collected post-culling of top layer