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Risks of SARS-CoV-2 transmission between free-ranging animals and captive mink in the Netherlands

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Abstract

In the Netherlands, 69 of the 126 (55%) mink farms in total became infected with SARS-CoV-2 in 2020. Despite strict biosecurity measures and extensive epidemiological investigations, the main transmission route remained unclear. A better understanding of SARS-CoV-2 transmission between mink farms is of relevance for countries where mink farming is still common practice and can be used as a case study to improve future emerging disease preparedness. We assessed whether SARS-CoV-2 spilled over from mink to free-ranging animals, and whether free-ranging animals may have played a role in farm-to-farm transmission in the Netherlands. The study encompassed farm visits, farm questionnaires, expert workshops and SARS-CoV-2 RNA and antibody testing of samples from target animal species (bats, birds and free-ranging carnivores). In this study, we show that the open housing system of mink allowed access to birds, bats and most free-ranging carnivores, and that direct and indirect contact with mink was likely after entry, especially for free-ranging carnivores and birds. This allowed SARS-CoV-2 exposure to animals entering the mink farm, and subsequent infection or mechanical carriage by the target animal species. Moreover, mink can escape farms in some cases, and two SARS-CoV-2-positive mink were found outside farm premises. No other SARS-CoV-2-RNA-positive free-ranging animals were detected, suggesting there was no abundant circulation in the species tested during the study period. To investigate previous SARS-CoV-2 infections, SARS-CoV-2 antibody detection using lung extracts of carcasses was set up and validated. One tested beech marten did have SARS-CoV-2 antibodies, but the closest SARS-CoV-2-infected mink farm was outside of its home range, making infection at a mink farm unlikely. Knowing that virus exchange between different species and the formation of animal reservoirs affects SARS-CoV-2 evolution, continued vigilance and monitoring of mink farms and surrounding wildlife remains vital.

KEYWORDS
animal husbandry, transmission, mustelids, One Health, SARS-CoV-2, wildlife

1 | INTRODUCTION

SARS-CoV-2 has the ability to infect a range of mammalian species, both in laboratory and field settings. Natural infections have been reported worldwide in many different farmed, pet, zoo and wildlife species (Aguiló-Gisbert et al., 2021; Hobbs & Reid, 2021). Animals from the order of carnivores (cats, mustelids, raccoon dogs, amongst others) seem to be especially susceptible (Freuling et al., 2020; Oude Munnink et al., 2021; van Aart et al., 2022). This was also evidenced from large-scale SARS-CoV-2 outbreaks on mink farms around the world (European Food Safety Authority et al., 2021; Lu et al., 2021). To date, evidence of large-scale transmission in wildlife populations is absent, with the exception of a high number of infections and transmission between white-tailed deer in the United States and Canada (Kotwa et al., 2022; Kuchipudi et al., 2022).

In the Netherlands, 69 of the 126 (55%) mink farms in total became infected with SARS-CoV-2 between 24 April 2020 and 4 November 2020. In addition, employees and their family members at 61% of the infected mink farms tested SARS-CoV-2 positive. In all human cases that could be sequenced, a mink SARS-CoV-2 strain was detected (Lu et al., 2021). However, these mink SARS-CoV-2 strains only rarely spilled over to and spread among the general Dutch population (Lu et al., 2021). From 8 January 2021 onwards, a ban was imposed on commercial mink farming, eliminating the risk of new infections with SARS-CoV-2 on mink farms in the Netherlands. Some farm-to-farm transmissions could be explained by shared employees, but for the majority of cases it remained unclear how the virus was able to spread between mink farms despite strict biosecurity measures and extensive epidemiological investigations (Lu et al., 2021). A better understanding of transmission of SARS-CoV-2 between mink farms is of relevance for preventive measures in other countries where mink farming is still common practice and can be used as a case study to improve future emerging disease preparedness (European Food Safety Authority et al., 2021; Fenollar et al., 2021; Koopmans, 2021). In addition to the need to protect farmed mink and mink workers from infection, high infection rates of farmed mink may also increase the risk of spillover of SARS-CoV-2 into free-ranging animals, with subsequent development of an animal SARS-CoV-2 reservoir. This might have devastating effects on free-ranging animal populations, as well as increase the risk of development of novel SARS-CoV-2 strains that can spill back into farmed animals or humans (Kuchipudi et al., 2022).

Here, we tried to describe the possibility of SARS-CoV-2 spillover from mink to free-ranging animals, and the possibility of SARS-CoV-2 transmission between mink farms via free-ranging animals, in the Netherlands. Therefore, we executed a qualitative analysis of the possibility of free-ranging species to enter and leave mink farms, the possibility of direct contact with mink and the possibility of SARS-CoV-2 infections in free-ranging animals and subsequent transmission to neighbouring farms. The study included farm visits, farm questionnaires, SARS-CoV-2 RNA and antibody testing of samples from free-ranging animals and expert workshops.

2 | MATERIALS AND METHODS

2.1 | Species selection for risk assessment

We assumed that animal species that can become infected and subsequently shed virus play a potentially larger role in SARS-CoV-2 transmission compared to animals that can only transmit virus mechanically. The species selection was based on existing literature on animal species SARS-CoV-2 susceptibility, animal species abundance in the geographical area with SARS-CoV-2 infected mink in the Netherlands, likelihood of entering mink farms (halls or sheds; Tables S1 and S3) and animal species home ranges (related to the chance that these animals were involved in farm-to-farm SARS-CoV-2 transmission; Tables S2 and S3). Criteria were discussed amongst researchers (veterinarians) with expertise in virology, public health, epidemiology, serology, infectious diseases in the farmed animal–wildlife interface, as well as mammal biologists and ornithologists with knowledge of the local situation.

The following carnivore species were selected: domestic cat (*Felis catus*), fox (*Vulpes vulpes*), badger (*Meles meles*), beech marten (*Martes foina*), polecat (*Mustela putorius*), weasel (*Mustela nivalis*) and American mink (*Neovison vison*). In addition, the selected bat species were : brown long-eared bat (*Plecotus auritus*), grey long-eared bat (*Plecotus austriacus*), serotine bat (*Eptesicus serotinus*), Natterer's bat (*Myotis nattereri*) and common pipistrelle (*Pipistrellus pipistrellus*). All selected bat species are Vespertilionid bats (members of the Vespertilionidae family).

Mice and rats were not included in the analysis, since there was no evidence for SARS-CoV-2 infections in those species at the time of the study and their home range was estimated to be too small (*Rattus norvegicus* and *Rattus rattus* [Lambert et al., 2008; Velkers et al., 2017]: up to 500 m; *Mus musculus*: up to 100 m [Howell, 1954; Mikesic & Drickamer, 1992]; *Apodemus sylvaticus* and *Clethrionomys glareolus*: up to 260 m [Korn, 1986]) to play a significant role in farm-to-farm transmission. There is no evidence, to date, of SARS-CoV-2 infections in birds. Still, because of the ability of birds to move over long distances per day, and because of their expected high abundance on farms, birds were included to assess their possible role in mechanical transmission of SARS-CoV-2 between mink farms (Frederiksen et al., 2020).

2.2 | Mink farming in the Netherlands

In 2019, 4.5 million American mink (*Neovison vison*) were bred on 126 Dutch farms, making the Netherlands the fourth mink-producing country in the world (Fenollar et al., 2021). In total around 1200 full-time and 400 part-time employees worked in the mink farming industry in the Netherlands.

Mink are commonly fed a mixture of slaughterhouse or fish offal with other by-products such as oil and grain products, that is offered to the animals by putting the feed on the wire mesh cages (Lyhs et al., 2019). Whelping takes place in April and May. Adult females give birth to around four to six kits per litter. The kits are vaccinated against botulism, *Pseudomonas* and mink enteritis virus in June and weaning takes place in late June and July (Lu et al., 2021). After whelping, each cage houses one adult female with their offspring. After weaning, young mink are kept in small groups of on average three mink per cage until pelting at the end of the year. In the same period, the breeding females for the next year are selected. Additional photos of mink farms in the Netherlands can be found in the Supporting Information.

2.3 | Farm visits

In the period October–November 2020, a total of 33 mink farms in the region where most SARS-CoV-2-infected farms were located were visited and assessed for accessibility for free-ranging animal species (including domestic cats). At the time this study started, most mink farms in the region had already been infected with SARS-CoV-2 and their mink subsequently had been culled. The remaining active farms had incorporated strict infection prevention measures, including a visitor ban and biosecurity protocols to prevent the introduction of SARS-CoV-2 infections into their farm, hampering a detailed analysis on site. Three of six SARS-CoV-2-uninfected mink farms gave permission to inspect the farm from outside the farm premises by a mammal biologist (R.J.). The other 30 farms were selected from 69 infected farms (where the animals had already been culled) based on the period of infection, location and virus cluster (Oude Munnink et al., 2021). The virus cluster refers to the five sequence clusters described previously (Oude Munnink et al., 2021) that were formed following five seperate introductions into mink from the human population, and subsequent farm-to-farm transmission (three out of five clusters). The goal was to obtain a representative selection of farms based on these criteria. Ten out of 30 farms were inspected from the inside and outside. The remaining 20 farms were assessed from the public road. All inspections were performed by the same mammal biologist (R.J.). Two farmers provided additional information by telephone.

During the visits, several risk factors were scored: (1) the possibility for each selected animal species (birds, foxes, mustelids, cats, bats and [escaped] mink) to enter the farms, (2) the possibilities of direct and indirect contact with the farmed mink, and (3) the possibility for the selected animal species to leave the farm after entering (Supporting Information S2). Accessibility was scored as 'accessible' (multiple opportunities to access farm premises, well within the limits of climbing and jumping capacities of the selected animal species), 'accessible with difficulty' (only one possible weak spot identified, access opportunities at the limit of jumping and climbing capacities) or 'not accessible'. Table S1 shows the climbing and digging capabilities of selected carnivore species used for this score. In addition, all traces of selected species and the presence of birds were recorded, and if possible the owner was asked about previous observations of free-ranging carnivores, birds and bats. Further, the expected effect of electric fences placed for keeping out foxes, mustelids (including mink) and cats was discussed with importers of electrical fences and assessed taking into account previous research on the use of electric fencing for predator exclusion (Day & MacGibbon, 2007; White & Hirons, 2019).

2.4 | Mink farm information collection

All SARS-CoV-2-positive mink farms were visited by the Netherlands Food and Consumer Product Safety Authority (NVWA) for clinical inspections, official sampling and epidemiological investigations for contact tracing purposes. During these visits, and during a follow-up epidemiological investigation of each of these farms, photos of the farm premises and farm layouts were made and outbreak data and farm characteristics (type of housing [sheds/halls], number of housed mink, applied biosecurity measures, etc.), observations of free-ranging animals and details of pest control strategies were collected.

Reports of the presence of free-ranging carnivores, bats by mink farmers were combined with sightings of animals or indications of their presence (faeces, tracks, nests) on SARS-CoV-2-infected farms ($n = 30$) by the visiting mammal biologist (R.J.) and other visiting professionals (information collected during expert consultations) to obtain data on presence of wildlife on mink farms.

2.5 | Expert consultations

Three expert consultations were organized in which employees of the NVWA and veterinarians involved in the monitoring and control of SARS-CoV-2 as well as local farm veterinarians, who had visited the selected farms previously, were interviewed (Supporting Information S3). At these meetings, all selected farms were jointly reviewed to assess the accessibility of the farms by the selected free-ranging animals. Observations of free-ranging animals (including domestic cats) as well as farm set-up and intactness of the fencing were inventoried and discussed.

2.6 | Free-ranging animal sample collection

2.6.1 | Free-ranging carnivore and bat carcasses

Carcasses of bats (April–September 2020) and wild carnivores (July–November 2020) in the region with infected mink farms (~30 km around infected mink farms, in the Netherlands) were actively collected following citizen and volunteer reports (Figure 1b,c). In addition,

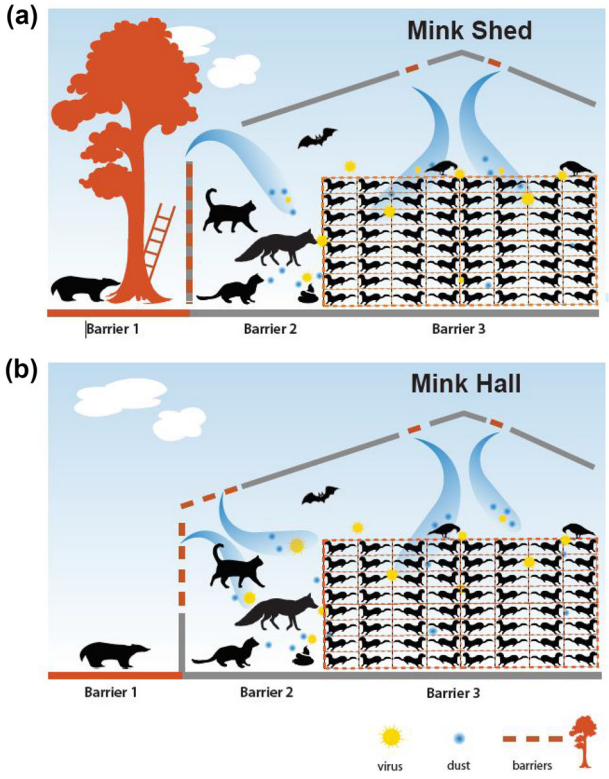

FIGURE 1 **Mink farm access and possible SARS-CoV-2 exposure of wild carnivores, bats and wild birds.**
Barrier 1 is the perimeter fencing, which is high enough to prevent carnivores to cross it. However, breaches such as trees and structures that allow carnivores (as well as birds and bats) to cross it were present in all mink sheds. Barrier 1 is generally absent in farms with mink halls. Barrier 2 is the barrier that prevents animals from entering the buildings (walls, windbreak netting). These were insufficient for the majority of carnivores, birds and bats. Barrier 3 is the mink cage wire mesh that prevents mink from escaping but allows for direct contact between mink and free-ranging animals that accessed the mink sheds/halls

bat carcasses that had been submitted for lyssavirus monitoring, originating from the region with SARS-CoV-2 infections in mink and submitted between January–September 2020 were included in our study (Figure 1c). Bat carcasses were stored at −20°C or −70°C until autopsy and sampling.

For SARS-CoV-2 RNA detection in bat carcasses, the following samples were collected: faeces from the rectum stored in virus transport medium (VTM; if faeces were absent, a rectal swab instead) and an oral swab in VTM. In addition, the nose was sampled by a wash from the nose (by inserting a 200-μl pipet filled with VTM into the choanae and collecting the fluid from the nose in a 2-ml tube) and the oral swab was then stored in the product of the nasal wash. Of the carcasses originating from the lyssavirus monitoring, only faeces samples (or alternatively rectal swabs, if no faeces were available) were collected. From carnivore carcasses, lung tissue samples (no medium) and nasopharyngeal swabs (in VTM) were collected. Samples for RNA detection were stored at −70°C until further analyses.

2.6.2 | Free-ranging carnivore and bat faecal sample

Fresh faeces were collected based on published evidence that SARS-CoV-2 RNA can be found in faeces of infected animals and humans, although SARS-CoV-2 RNA cannot be detected in faeces of all infected cases (Kim et al., 2020; Zhang et al., 2021). Therefore, using faeces of free-ranging carnivores and bats may lead to under-detection of SARS-CoV-2. Bat coronaviruses have been detected in bat faecal samples previously (Hernández-Aguilar et al., 2021; Wong et al., 2019). The advantage of faeces is that it can be collected without having to handle or disturb (free-ranging) animals.

Faeces of badgers and foxes from the regions of infected mink farms were collected close to badger setts (badger setts are also regularly used by foxes) between September and November 2020 (Figure 1b,c). Badgers use latrines that can be detected near known setts by careful inspection of the area. Faeces of other carnivores that were found during badger latrine searching and sampling were also included in the analysis. Several grams of faeces per stool sample were collected in stool sampling containers, transported to the laboratory within a day and subsampled and stored in RNAlater (Thermo Fisher Scientific, USA), fixed for 24 h at 4°C and then frozen at −20°C until further analyses.

Faeces from bats in the region with SARS-CoV-2-infected mink farms were collected from known bat roosts in use, mainly attics, but also behind house shutters and from bat boxes, between October and November 2020. Faecal pellets were collected in 2-ml tubes containing RNAlater (Thermo Fisher Scientific, USA). Generally, three faecal pellets were stored in one tube, when they originated from one location and when they were from the same bat species. It was not possible to collect faeces from the Natterer's bat (*Myotis nattereri*) due to its lifestyle as a mainly tree-roosting species and the lack of known roosts in the vicinity of the mink farms. In addition, two longitudinal faeces collections (collected for other purposes and under different sampling and storage protocols) were included. One set was from brown long-eared bats (*Plecotus auritus*) foraging in the risk area, collected on 14 different days between June and July 2020. These faecal pellets were stored in 70% ethanol, and stored frozen at −70°C. A second set was from a colony of serotine bats sampled weekly from May to September 2020, a total of 19 weeks. These samples originated from their permanent summer roost attic, in the risk region. In September, when our study started and this set of samples was identified, nine faecal pellets of these weekly samples were transferred to RNAlater (Thermo Fisher Scientific, USA), three pellets per tube, transported to the lab and stored at −20°C until further analyses.

2.7 | Sample processing and PCR analysis

All samples were processed under BSL2 conditions. Faeces (bats and carnivores) in VTM were thoroughly mixed by stirring and subsequently vortexed for 15 s. Next, approximately 200 μl of the mixture was added to 900 μl S.T.A.R. buffer (Roche, Switzerland) and 120 μl chloroform. This mixture was vortexed and then centrifuged for 5 min

at 10,000 × *g*, and 200 μl of the supernatant was added to 300 μl MagNA Pure lysis buffer (Roche, Switzerland).

Bat rectal swabs and oral swabs in VTM were vortexed. Then 200 μl of the VTM was added to 300 μl MagNA Pure lysis buffer (Roche, Switzerland). The combined bat oral swabs in nose washes were vortexed, then 200 μl was added to 300 μl MagNa Pure lysis buffer (Roche, Switzerland). The carnivore nasopharyngeal swabs in VTM were vortexed and 600 μl of VTM was added to a MagNApure 96 (MP96) compatible vial, containing 600 μl external lysis buffer (Roche, Switzerland). All RNA from samples in lysis buffer was extracted using the MP96 with total nucleic acid kit large volume (Roche, Switzerland).

From lung material, about a quarter size of a pea was sliced off and homogenized in a vial containing 300 μl tissue lysis buffer (Roche, Switzerland) and a 1/4″ ceramic sphere. Homogenization was performed at a speed of 5 m/s during 60 s. After spinning down the sample at maximum speed during 5 min, 60 μl was added to an MP96 compatible vial, containing 600 μl external lysis buffer (Roche, Switzerland) and 540 μl VTM. Total nucleic acid from nasopharyngeal swabs and lung tissue of carnivores was extracted using the MP96 with the total nucleic acid kit large volume (Roche, Switzerland).

Faecal samples of bats and wild carnivores as well as faeces (or rectal swabs) and nasal washes combined with oral swabs of bat carcasses were tested by RT-PCR for the SARS-CoV-2 E gene (Corman et al., 2020) and by a pan-coronavirus PCR (de Souza Luna et al., 2007). All lung tissue samples and throat swabs from mustelids were tested by RT-PCR for the E gene (Corman et al., 2020). Some samples gave an inconclusive result. For these samples, the SARS-CoV-2-specific PCR was repeated, with two instead of one target (E-gene and RdRp) (Corman et al., 2020). Samples were considered positive if they tested positive (Ct < 40) on two different targets, or with two different assays.

2.8 | Lung extracts and SARS-CoV-2 serology

When available, one lung lobe of the bat carcasses or a lung fragment of variable size (0.1–3 cm^3) taken from mustelid carcasses was cut into two pieces, and put in a 15-ml tube with 2 ml PBS on ice. After 20 min on ice, tubes were rotated at 4°C for 20 min, put back on ice and spun down (15 min at 1000 × *g*). The supernatant was processed further for serology.

The protein microarray technique, using SARS-CoV-2 spike ectodomain (bats and carnivores) and S1 (carnivores), was used as a screening assay to detect binding IgG antibodies, as described before (Sikkema et al., 2022). Briefly, the recombinant protein was printed on nitrocellulose-coated glass slides (Sartorius, Germany) with a non-contact printer (Scienion, Germany). After drying and blocking the slides (Blocker Blotto, Thermo Fisher Scientific, USA), lung extract was incubated in a 1:2 dilution at 37°C for 1 h. For the mustelid lung extracts, slides were incubated for 1 h at 37°C using goat anti-ferret IgG-Biotin (antibodies-online.com; ABIN117100) in a 1:100 dilution, followed by secondary mouse-anti-biotin (Jackson Immuno Research; 200-602-211) IgG conjugated to AlexaFluor647 (Jackson Immuno Research, 200-602-211) in a 1:500 dilution. For the bat lung extracts, slides were incubated with 1:500 goat-anti-bat antibody (Bethyl Laboratories; A140-118A) labelled with Alexa fluor 647 Conjugation Kit (Fast)—Lightning-Link® (Abcam; ab269823), according to manufacturer instructions. After washing, slides were dried, scanned (Powerscanner, Tecan group Ltd, Switzerland) and fluorescence values were analysed (Imagene 8.0 software, Biodiscovery, USA). More information on the validation of the use of lung extracts for SARS-CoV-2 antibody detection using the protein microarray can be found in the Supporting Information.

2.9 | Plaque reduction neutralization test

Lung extracts with a fluorescence value above the determined cut-off, in the protein microarray, were subjected to a plaque reduction neutralization test (PRNT) as final confirmation, as described previously (Okba et al., 2020; Sikkema et al., 2022). PRNT assays are considered the gold standard in coronavirus serology (GeurtsvanKessel et al., 2020).

3 | RESULTS

3.1 | Possibility of focus species entering and/or leaving mink farms

Free-ranging animals have to cross up to three barriers before direct contact with farmed mink is possible (Figure 2). First, many farms have a barrier (fencing or hedge) around their farm premises (barrier 1), followed by walls of the buildings and/or windbreak netting (barrier 2) and thirdly, inside the buildings farmed mink are housed in cages (barrier 3). Mink in the Netherlands are either housed in open sheds or halls (including glasshouses). Of the 33 selected farms in this study, 11 farms housed their mink in halls, 19 farms had sheds and three farms had both. All sheds had long roofs on poles without walls (absence of barrier 2), but the farm premises with such sheds are surrounded by fencing of corrugated iron or other material. Halls also have long roofs on poles, but with walls approximately 1 m in height, and windbreak netting in the open space between wall and roof. The majority of sheds and halls (30/33) have open roof ridges (Figure 1).

Although all selected farms had fencing or a wall either around their farm premises or as part of the halls, 30 of 33 selected farms had at least one weak spot in barriers 1 and 2 where free-ranging carnivores could enter and leave the mink farms. The most common weak spots of farms with sheds were holes in the fencing or nearby trees or other objects that could be used to climb the fencing (Figure 2). Although most halls had installed windbreak netting, they were often kept open for optimal ventilation. Most free-ranging carnivores can therefore easily enter and exit halls, since the height of the wall is well below their jumping capacities (Figure 2; Table S1). Twenty out of 33 farms (no differences between halls or sheds) had installed electric fences, either around farm premises or in the open space between wall and roof of the buildings. However, electric fencing is not suitable to keep out

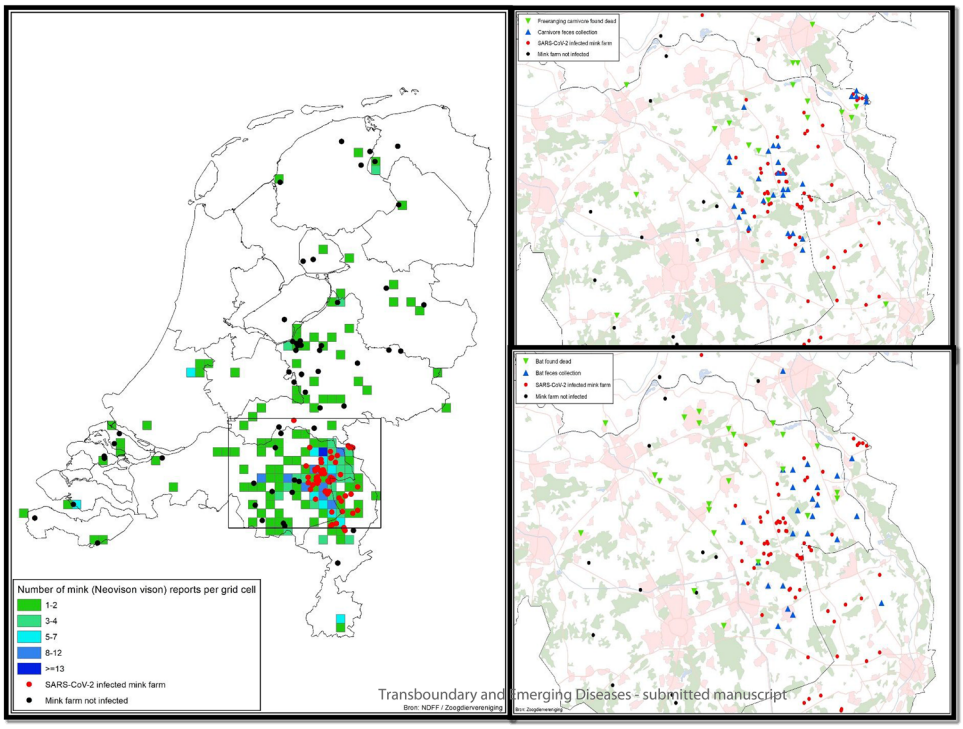

FIGURE 2 **Locations of mink sightings and free-ranging animal sampling.**
(a) Mink (*Neovison vison*) observations in the period January 2015 to January 2021; data extracted from National Databank Flora and Fauna (NDFF; English—Nationale Databank Flora en Fauna [ndff.nl]). (b) Locations of carnivore sample collection (faeces and carcasses). (c) Locations of bat sample collection (faeces and carcasses)

free-ranging carnivores because they do not touch the ground when they jump (Day & MacGibbon, 2007; White & Hirons, 2019). This was supported by the reported presence of stray and/or unknown cats on the majority of farm premises (22 of 30 selected positive farms).

Based on data on wild carnivore climbing and jumping abilities (Table S1), farm layouts and farm visits, it was estimated that 91% (30/33) of farms were accessible to beech marten, fox and domestic cat, and one farm (1/33) was rated accessible with difficulty for these species. For the polecat and (escaped) American mink. 27% (9/33) of farms were rated accessible and 58% (19/33) accessible with difficulty. Five out of 33 farms (15%) were rated accessible to badgers, when only assessing climbing and jumping abilities. Twenty-one farms had fences that went <20 cm below ground level. This means that most farm could be accessed by means of digging, although no traces of badger presence (such as tunnels) were seen during farm visits. All 33 selected farms were accessible to birds and bats. The majority (31/33) of inspected farms had open sides that allowed birds and bats to enter easily. In two farms, both with halls, the open sides of the halls were covered by a combination of windbreak nets and additional mesh that was specifically made to prevent mink from escaping. This made access by birds and bats harder, but these halls were still accessible through the open roof ridges.

On 80% (24/30) of the included farms, cats were reported. Also, foxes (6/30; 20%), martens (6/30; 20%), badgers (4/30; 13%) and mink (3/30; 10%) were reported nearby the farms. Fifty-four out of 69 infected mink farms participated in an extensive questionnaire that included questions on observations of free-ranging animals at mink farms. Bats were reported at 13 out of 54 infected farms, although they were not often seen inside sheds or halls. Insects such as flies, wasps and beetles that may attract insect-eating birds and bats were reported at 20 out of 54 infected farms. Despite pest control, mice and rats were reported at 18 out of 54 infected mink farms. These may also play a role in attracting cats or other carnivores, as well as birds of prey. Bird species most often reported by farmers were corvids (28/50), house sparrows (24/50) and starlings (13/50). Other commonly reported bird species are pigeons, gulls and swallows and also, to a lesser extent, blackbirds, tits, finches, wagtails, owls and buzzards (Hissel, 2021). These were also reported inside halls and sheds.

It is also possible for mink to escape from mink farms, at some occasions. Each year approximately 100 observations of free-ranging mink in the Netherlands are registered in the National Databank Flora and Fauna. After banning mink farming in the Netherlands in January 2021, the number of sightings decreased sharply to almost zero in October 2021 indicating observations prior to the ban were most likely recently escaped mink. This is confirmed by the correlation between the former distribution of mink observations and the presence of mink farms (Figure 1a). Previous research also indicated that the Netherlands did not have a wild self-sustaining population of American mink (Dekker & Hofmeester, 2014).

3.2 | Possibility of contact between focus species and farmed mink

All mink are kept in adjoining cages consisting of a nest box filled with bedding and a wire mesh section [10]. After entering the sheds or halls, it is possible for free-ranging animals to have direct contact with farmed mink through the mesh (Figure 2; barrier 3). Selected mustelids, domestic cats and foxes in the vicinity of mink farms are likely attracted to the smell of mink and their feed (especially the residual products from the fish and poultry slaughterhouses) which is placed on top of the wire mesh cages. Free-ranging carnivores and some bird species can be expected to eat from that food and get into direct contact or close proximity to farmed mink. Free-ranging carnivores can also come into contact with litter and dust that fell from the cages onto the floor of the sheds and halls.

All selected bats were insectivorous species, and are likely to be attracted due to the many flies (*Brachycera* spp.) in the sheds and halls. Serotines and common pipistrelles hunt for flies above the cages in the rows and outside the buildings, while the brown long-eared bat and grey long-eared bat probably also take flies and other insects from the cages, ceilings or other structures (Janssen & Dekeukeleire, 2014; Siemers et al., 2012). Bats probably have no or very little direct contact with the bedding, mink faeces or mink themselves, even if flies are present (Figure 2) (Siemers et al., 2012). The flies, including pupae and larvae, present also make the sheds and halls attractive for various insectivorous bird species to enter. Other bird species are more attracted to mink food, making direct contact more likely. Three farmers indicated that they found bird carcasses or bird legs in mink cages and 22 farmers (out of 69) indicated that there was direct contact between birds and mink feed and bedding. Only two farmers indicated that there were no sightings of birds near to their mink. This shows that there is ample opportunity for close contact between birds and mink (Frederiksen et al., 2020; Hissel, 2021).

3.3 | Possibility of focus species SARS-CoV-2 infections and their role in the spread to neighbouring farms

Previously, it was described that there had been five separate SARS-CoV-2 introductions into mink farms, followed by farm-to-farm spread following three of five introductions, resulting in three virus clusters (Oude Munnink et al., 2021). The distances between successively infected farms, within the virus clusters, ranged between 0.1 and 54.9 km (median 10.0) for cluster A, 0.49 and 31.9 km (median 7.5) for cluster C and 0.2 and 4.4 km (median 0.7) for cluster D. In 24% (cluster A, 42 farms), 33% (cluster C, 15 farms) and 100% (cluster D, 7 farms) of consecutive infections within a known cluster, the distance between farms was less than 5 km.

The home range of most mustelids and foxes is around 5 km, meaning that farms located within 5 km from each other could have been visited by the same animal (Table S2). Any visits to mink farms are likely to be limited to one or a few nearby farms because most species are territorial and will not tolerate 'foreign' individuals in their own habitat. It is therefore unlikely that one individual wild mustelid or fox visits a large number of farms. Cats and (escaped) American mink are less territorial and therefore it is more likely that they visit multiple farms per night. The selected bat species are much more mobile. Bats can fly several kilometres per night to forage, and visit several farms per night. The mink farms in the risk areas are mainly embedded in a landscape with rows of trees and hedgerows, which makes it easy for bats to find mink farms to forage. Similarly, some bird species can visit several mink farms per day. Especially starlings, corvids and gulls have larger foraging distances (Hissel, 2021).

Between April and November 2020, 1036 bat faecal samples, 76 badger faecal samples and 22 fox faecal samples were collected and tested for SARS-CoV-2 RNA, as well as 32 bat carcasses and 21 wild carnivore carcasses (Table 1; Figure 1b,c). All samples were collected from the region with SARS-CoV-2 infected mink farms. SARS-CoV-2 RNA was not detected in any of the collected samples.

Lung extracts were made using carcasses found dead in teh risk region and tested for SARS-CoV-2 antibodies (supporting information). In total, 21 lung extracts of wild carnivores and 29 lung extracts of bats were obtained. One lung extract of one beech marten in the south of the Netherlands was positive for SARS-CoV-2-binding antibodies both in protein microarray (SARS-CoV-2 S1 and S-ectodomain) and PRNT (titre 80). The carcass was collected >30 km from the nearest SARS-CoV-2-infected mink farm. None of the bat lung extracts contained SARS-CoV-2 (S-ectodomain) binding antibodies.

4 | DISCUSSION

In this study, we show that the open housing system of mink allowed access to birds, bats and most free-ranging carnivores into the farms, and that direct contact with mink and their faeces, feed or bedding is possible after entry, especially for carnivores and birds. This allows for SARS-CoV-2 exposure to free-ranging animals entering the mink farm, and subsequent infection of susceptible species as well as mechanical carriage by non-susceptible species. No SARS-CoV-2-RNA-positive free-ranging animals were detected in our study, suggesting there was no abundant circulation in the species tested during the study period. One beech marten in the south of the Netherlands did have SARS-CoV-2 antibodies, although this animal was likely not exposed at an infected mink farm, because the closest infected mink farm was well outside its expected home range.

To date, the routes of the majority of mink farm-to-farm SARS-CoV-2 transmission events are unknown (Lu et al., 2021). In approximately one third of the farm-to-farm transmission events, wild carnivores, escaped mink and domestic cats theoretically could have played a role, when only looking at the distance between subsequent infected farms within one virus cluster. Previously, it has been shown that free-ranging carnivores can enter mink farms and exchange pathogens with mink. For example, regular outbreaks with canine distemper virus on Dutch mink farms are an indication that mink exchange viruses with free-ranging carnivores (Deem et al., 2000; Molenaar & Buter,

TABLE 1 Sample collection and SARS-CoV-2 detection in selected animal species from the region with SARS-CoV-infected mink farms

	SARS-CoV-2 RNA detection (number positive/number available for testing)			SARS-CoV-2 antibody detection
Species	Carcass	Environmental (faeces)	Total	Lung extract
Cross sectional study (multiple locations and dates)				
Badger (*Meles meles*)	0/12	0/76	0/87	0/12
Marten (*Martes foina* and *M. martes*)	0/6	0/0	0/7	1/6
Weasel (*Mustela nivalis*)	0/3	0/0	0/2	0/3
Fox (*Vulpes vulpes*)	0/0	0/22	0/22	0/0
Long-eared bat (*Plecotus* spp.)	0/2	0/370	0/372	0/1
Pipistrelle bat (*Pipistrellus* spp.)	0/23	0/144	0/167	0/21
Serotine bat (*Eptesicus serotinus*)	0/7	0/168	0/175	0/7
Total	0/53	0/780	0/828	1/50
Longitudinal sampling design (each single location, for 2–5 months)				
Brown long-eared bat (*Plecotus auritus*)	–	0/184	0/184	–
Serotine bat (*Eptesicus serotinus*)	–	0/170	0/170	–
Total	–	0/384	0/384	–

2018; Tavernier et al., 2012). Moreover, SARS-CoV-2-infected cats and SARSCoV-2 positive escaped mink have been found in the proximity of infected mink farms in 2020 (Lu et al., 2021; van Aart et al., 2022). Their contribution to SARS-CoV-2 transmission between mink farms could not be excluded. Since there were no indications of SARS-CoV-2 infections in wild carnivores near mink farms, SARS-CoV-2 transmission due to infected wildlife seems less likely.

Although it is considered more likely that infected animals are involved in virus spread as compared to animals that can only act as mechanical vector, mechanical transmission via free-ranging animals cannot be excluded. This could mainly be the case for certain bird species (e.g., starlings, corvids, gulls), which were sighted on mink farms (on 26%, 56% and 6% of mink farms that participated in the questionnaire, respectively), can bridge large distances and are often seen on mink cages. SARS-CoV-2 RNA, and likely also infectious virus, was present in high amounts in infected mink farms (faeces, surfaces and airborne dust) (de Rooij et al., 2021) and thus there is a possibility that infectious virus was mechanically transferred via birds, after accessing an infected mink farm. The possibility of virus introductions via birds was exemplified by previous avian influenza outbreaks in mink farms, although in this case wild birds were also infected (Englund, 2000; Sun et al., 2021). The number of bats per farm was much lower and close contact with mink is unlikely, making them less likely candidates for virus transfer.

We were unable to detect virus in the collected animal samples, strongly suggesting there was no continued transmission and establishment of the virus in the selected free-ranging animal species in the risk region. Unfortunately, much of the sample collection was performed after the peak of mink infections. As a result, the sampling period was not optimal for determining possible spillover from the mink into a free-ranging animal population, except when it would have led to continued transmission and establishment of the virus in host populations. To investigate previous exposure or infections, SARS-CoV-2 antibody detection was performed in lung extracts of target animal species that were found dead. The choice for lung extracts of dead animals, instead of serum collected from live birds, to prevent disturbance and discomfort caused by capture and handling of free-ranging animals, limited the number of samples as well as the sensitivity of the antibody detection. However, we have shown that it is possible to use lung extracts to detect SARS-CoV-2 antibodies in wild carnivores, making it a very valuable addition to the molecular methods used in this study as well as previous studies looking at SARS-CoV-2 in wildlife around mink farms. The serology results con that SARS-CoV-2 likely has not established itself in free-ranging animals selected in our study.

Our study shows that mink farms are accessible to free-ranging animals, both mammals and birds, and that mink escaped from farms on a regular basis. Therefore, infected mink formed a potential source of infection for susceptible free-ranging animals that had access to the mink farm. In addition, infected mink that escaped from farms could have been a source of SARS-CoV-2 infection for free-ranging animals outside farms. While this scenario of farmed mink as a stepping stone species for the spread of SARS-CoV-2 from humans to free-ranging animals is no longer possible in the Netherlands, where mink farming is no longer allowed, it remains a possible scenario elsewhere in the world where SARS-CoV-2 is still circulating in the human population, and mink are being farmed in the above-described way. Examples of countries with a sizable mink farming industry are China, Finland, Poland, Lithuania and Greece. Moreover, with the extended host ranges of recent SARS-CoV-2 variants, which can also infect mice and rats, the risks of interspecies transmission at mink farms have even increased [36]. Taken together, this is a situation of great concern, knowing

that virus exchange between different species and the formation of animal reservoirs affect SARS-CoV-2 evolution (Koopmans, 2021; Telenti et al., 2021). This can pose significant public health risks when novel variants spill back to humans (Koopmans, 2021; Telenti et al., 2021).

AUTHOR CONTRIBUTIONS

RS, LB, TK and MK conceived and designed the study. RJ, WW, RHH, PE, WP, JB, RS, MH and FV were involved in data collection. CGK, EB, WP, RS and LB were involved in laboratory analyses. RS, LB, RJ, RS, MH, MK, FV and TK were involved in data interpretation. RS wrote the first version of the manuscript. All authors read and critically assessed the final version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as there was no live animal handling or sampling included in the set-up of this study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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