A distemper outbreak in beech martens *(Martes foina)* in Flanders

Een uitbraak van hondenziekte bij steenmarters (Martes foina) *in Vlaanderen*

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ABSTRACT

An outbreak of distemper was observed in beech martens (*Martes foina)* in the eastern part of Flanders (Belgium) for the first time. The clinical and pathological findings were consistent with other outbreaks described in mustelids in Europe. Using reverse transcriptase polymerase chain reaction, morbillivirus RNA was detected in an organ homogenate inoculated on Vero.dogSLAM cells. The virus was sequenced and was identified as a canine distemper virus (CDV) strain, hundred percent identical to an earlier isolate from a marten from Germany. After a period of nearly complete absence of beech martens in Flanders due to intensive prosecution, their population density has increased substantially in the last decennia. Although the exact mechanisms behind the observed population changes stay unclear, spread of CDV from central Europe following dispersion of beech martens is indicated by our findings. Further CDV spread could negatively impact on the highly threatened pine marten *(Martes martes)* and the decreasing polecat (*Mustela putorius)* populations in Flanders.

SAMENVATTING

Voor het eerst werd een uitbraak van hondenziekte waargenomen bij steenmarters (*Martes foina*) in het oostelijk deel van Vlaanderen. De klinische en pathologische bevindingen waren gelijklopend met andere uitbraken beschreven bij marterachtigen in Europa. Door middel van *reverse transcriptase polymerase chain reaction* werd morbillivirus RNA gedetecteerd in een orgaanhomogenaat geïnoculeerd op Vero.DogSLAM-cellen. Het virus werd gesequeneerd en geïdentificeerd als een hondenziektevirusstam, honderd procent identiek aan een eerder geïsoleerd virus uit een marter uit Duitsland. Na een periode van quasi afwezigheid van steenmarters in Vlaanderen door intense vervolging is hun populatiedichtheid in de laatste decennia beduidend toegenomen. Hoewel de onderliggende mechanismen van de waargenomen populatieveranderingen onduidelijk zijn, wijzen onze bevindingen op een spreiding van hondenziektevirus vanuit centraal Europa volgend op dispersie van steenmarters. Verdere spreiding zou een negatieve impact kunnen hebben voor de sterk bedreigde boommarter (*Martes martes*) en de teruglopende bunzing (*Mustela putorius*) populatie in Vlaanderen.

INTRODUCTION

Canine distemper virus is an enveloped, single-stranded negative sense RNA virus, classified in the genus morbillivirus, family paramyxoviridae, order mononegavirales. It is closely related to the viruses of *rinderpest*, *peste des petits ruminants*, measles and the morbilliviruses of marine mammals (Harder and Osterhaus, 1997). The distemper virus envelope contains two immunogenic glycoproteins, named H (hemagglutinin) and F (fusion) glycoprotein. Genetic diversity is known for both glycoproteins but is highest for the H-gene and is responsible for the existence of seven geographically separate lineages of the virus, called America-1, America-2, Arctic-like, Asia-1, Asia-2, Europe and European wildlife (Mochizuki *et al.*, 1999; McLachlan and Dubovi, 2011; McCarthy et al, 2007). For phylogenetic analysis, the H gene and the P gene are used. Canine and mustelid strains can be distinguished in Europe and an expansion of the host range of CDV has been observed in the last decades (Haas *et al.*, 1997; Harder and Osterhaus, 1997). There

is a close relationship of CDV strains in new hosts with known CDV strains (Frölich *et al.*, 2000; Philippa *et al.*, 2008). Genetic drift in wild type CDV strains has been demonstrated and could explain cross-species transmissions and resurgence of distemper in highly vaccinated dog populations (Harder and Osterhaus, 1997).

Distemper outbreaks in Europe in wild carnivores have been documented in Germany (Geisel, 1992; van Moll *et al.*, 1995; Sekulin *et al.*, 2009), Switzerland (Palmer *et al.* 1983), the Czech Republic (Pavlacik *et al.*, 2007), Austria (Benetka *et al.*, 2011), Spain (López-Peña *et al.*, 1994; 2001) and Italy (Monne *et al.*, 2011). In southern Belgium (Wallonia), distemper has been diagnosed in foxes (*Vulpes vulpes*), but also in a wolf (*Canis lupus*) (personal communication A. Linden, 2011) and an Asian clawless otter (*Aonyx cinereus*), both kept in captivity (De Bosschere *et al.*, 2005). The seroprevalence reported in wild canids (foxes and wolves) in Europe ranges between 5 and 20% (Santos *et al.*, 2009; Sobrino *et al.*, 2008; Åkerstedt *et al.*, 2010; Frölich *et al.*, 2000; Truyen *et al.*, 1998; Damien *et al.*, 2002) and in mustelids between 20 and 33 % (Frölich *et al.*, 2000; Philippa *et al.*, 2008) illustrating a wide exposure and an endemic situation in many countries. In Germany, CDV prevalences between 5 and 33 % were detected on tissue samples of wild carnivores using reverse transcriptase polymerase chain reaction (RT-PCR) (Frölich *et al.*, 2000) and up to 37 % using immunohistochemistry on brains of mustelids (van Moll *et al.*, 1995).

Mustelids are known to be very susceptible to distemper (Philippa *et al.*, 2008). Next to the signs reported in dogs (conjunctivitis, rhinitis, tracheitis, pneumonia, vomiting, diarrhea, progressive central nervous impairment including ataxia, tremor and seizures; Harder and Osterhaus, 1997; Williams, 2001; Declercq *et al.*, 2011), the signs reported in martens are abnormal behavior, loss of fear for humans, circling gait, jumping, screaming fits, exhaustion, salivation, thickening of the eyelids, lips and anus, and pruritus (Geisel, 1992; Williams, 2001; van Moll *et al.*,1995).

This paper describes an outbreak of distemper in beech martens (*Martes foina)* in Flanders (Belgium) and discusses the ecological context of this outbreak. To our knowledge it is the first report of distemper in wild carnivores in Belgium.

HISTORY

In the spring of 2009, beech martens with neurological symptoms were observed at different locations in the eastern part of Flanders (Figure 1). About 30 of them were admitted to a wildlife rescue centre. The clinical picture was similar in all the martens examined. It included conjunctivitis (80%), rhinitis (100%), dyspnoea with forced abdominal respiration (75%), generalized muscular tremors (75%), convulsions with lateral decubitus (100%), and sometimes screaming. Fever was seen in some animals only. Eventually, most of them died and some of them were euthanized after one to three days.

METHODS

Postmortem examination following standard procedures was carried out on five submitted beech martens. Tissue samples of four animals were preserved in 4% phosphate buffered formalin, embedded in paraffin, sectioned at 5 µm thickness and stained with hematoxylin-eosin for histopathological examination.

Sections of brain, lung, heart and urinary bladder were immunolabelled for CDV antigen with a mono-

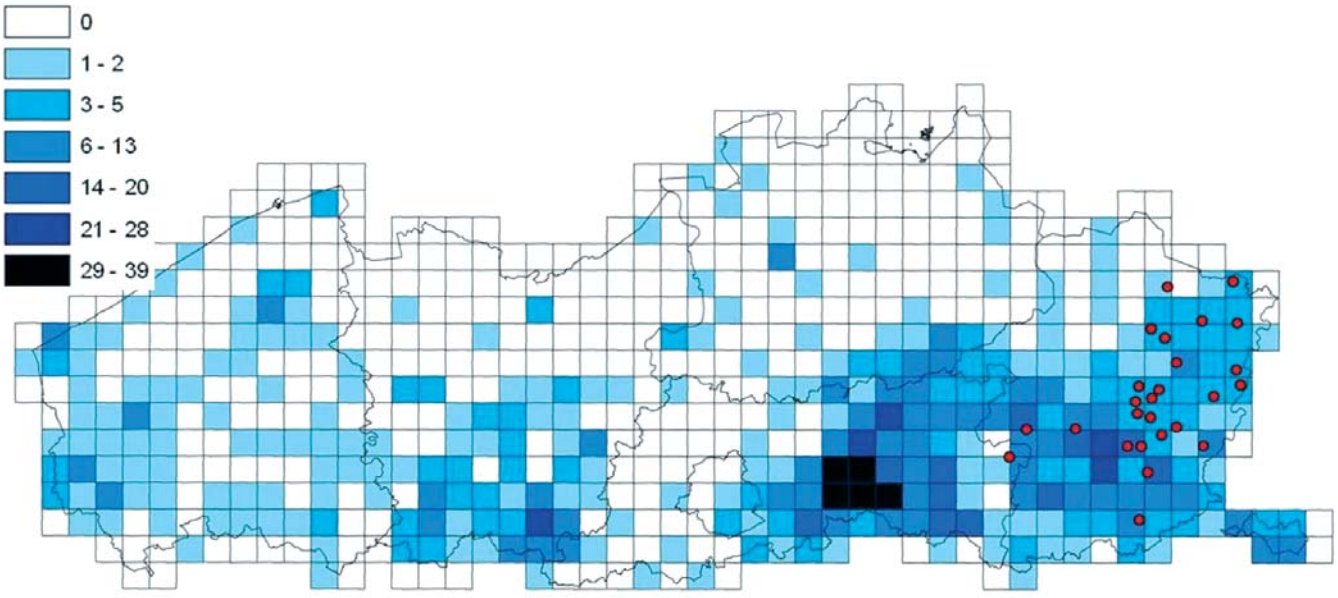

Figure 1. Beech marten population density estimates in Flanders based on observations from 1995 till 2009 on a 5km² UTM-grid (Universal Transverse Mercator). Sick martens were found at several locations in the province of Limburg (dots). Carnivore database of the INBO (Institute for Nature- and Forestry).

clonal mouse antibody against CDV (VHRD, ref. CDV-NP, Pullman, USA) using a commercial kit (Dako Denmark A/S, envision system HRP Mouse (DAB+), ref. K 4007 11, Glostrup, Denmark).

For virus isolation, a 10% organ homogenate (mixture of liver, lung, stomach, intestine, spleen and brain of one animal) in Dubelcco's Minimum Essential Medium (DMEM) supplemented with antibiotics was made using a tissue homogenizer (Kinematica Polytron, Lucerne, Switzerland). The homogenate was inoculated on Vero.DogSLAM cells for 1 hour at 37°C (Seki *et al.*, 2003). Cell cultures were washed twice with culture medium, DMEM supplemented with antibiotics and 10% fetal calf serum, and incubated at 37°C humid atmosphere 5% CO_2. Cultures were checked routinely for cytopathic effects (CPE).

For RT-PCR, 300 µl of the 10% organ homogenate or culture supernatant was mixed with 300 µl of lysis buffer for RNA isolation. The High Pure Viral Nucleic Acid Kit (Roche diagnostic GmbH, Mannheim, Germany) was used following the protocol provided by the manufacturer. RT-PCR was performed to detect morbilliviral RNA after first strand synthesis with specific morbilliviral primers P1: 5'ATGTTTATGATCACAGCGGT3' and P2: 5'ATTGGGTTGCACCACTTGTC3'. RT-PCR reactions were checked for fragments of the correct size on agarose gel.

Automated sequencing of RT-PCR fragments was performed on an ABI 3130XL genetic analyzer with the Big Dye Terminator Cycle Sequencing Kit (ABI, Applied Biosystems, Foster City California, USA) using the RT-PCR primers P1 and P2. For identification of sequenced fragments, the Basic Local Alignment Search Tool (BLAST) option of the NCBI (National Center for Biotechnology Information) website was used (http://www.ncbi.nlm.nih.gov/).

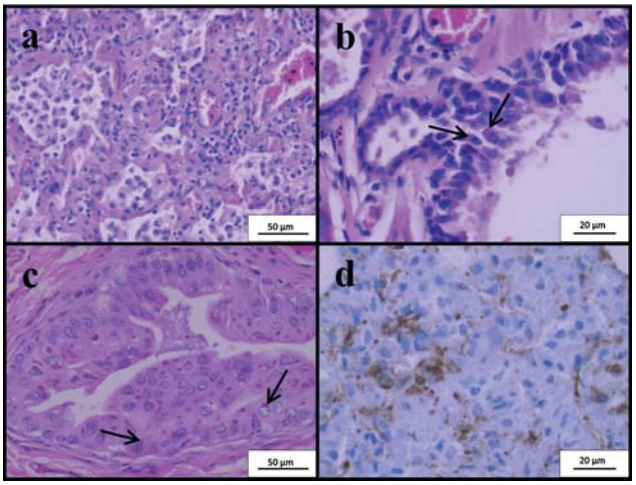

Figure 2. a. Histopathological changes in the lungs (Hematoxylin Eosin staining), bronchointerstitial pneumonia with scant mixed (neutrophilic and lymphocytic) infiltration and patchy necrosis b and c. Multiple acidophilic intracytoplasmatic viral inclusions in bronchiolar (b) and bladder (c) epithelial cells (arrows) d. Positive immunohistochemistry result in the lungs; the brown color shows the presence of the CDV antigens.

RESULTS

The most obvious gross lesions in all the beech martens were cachexia and focal pneumonia. Less frequent lesions included anemia (1/5), hemorrhages in the pyloric and duodenal mucosa with melena (1/5) and the absence of nidation in the uterus of a sexually mature female, although in spring female martens are usually pregnant through delayed nidation (Libois and Waechter, 1991). Histopathologic examination of the brain showed non-purulent meningo-encephalitis (2/4) and focal gliosis with demyelination (1/4). Broncho-interstitial pneumonia was observed in the lungs, characterized by thickened alveolar septa (with congestion, infiltration of macrophages and type 2 pneumocyte hyperplasia) (2/4) and necrotic epithelial cells in the bronchio-alveolar lumina (1/4) (Figure 2a). A mild infiltration of lymphocytes was seen in the lamina propria of the stomach (1/4). Lymphocytic and plasmacellular infiltrates were present in the portal areas of the liver (2/4). Large eosinophilic intracytoplasmatic inclusion bodies were observed in epithelial cells of the conjunctiva (1/4), the lungs (1/4), the urinary bladder (1/4) and the stomach (1/4) (Figures 2b,c). Immunohistochemical staining confirmed the presence of CDV in the brain, lungs, hearth and urinary bladder tissue (each 1/4) (Figure 2d).

Cytopathogenic effect (CPE) was observed in the inoculated cell culture on day 4 of the second passage. The RT-PCR for morbillivirus was positive for the original homogenate and for the Vero-dogSLAM culture supernatants from both passages. All three PCR fragments were sequenced and were shown to be identical when compared. The use of BLAST allowed to identify the RT-PCR fragment of 388 nucleotides as canine distemper virus. The nucleotide sequence was submitted to Genbank (accession number JN873341). The most closely related CDV sequence identified with BLAST (100% identity) originated from a marten in Germany (Genbank accession no.: AJ582389) (Figure 3).

DISCUSSION

The clinical image of the distemper outbreak in beech martens here described was dominated by respiratory (dyspnoea) and central nervous symptoms (tremor, seizures, ataxia), consistent with other reports of distemper in mustelids. Gastrointestinal signs were absent. Also the histopathological findings with presence of inclusion bodies in epithelial cells of different organs and the immunohistochemical findings were consistent with earlier descriptions.

The morbillivirus identified as CDV by sequencing was identical to an isolate included in Genbank from a marten in Germany. Phylogenetic analysis of CDV is usually based on the analysis of both a 388 base pairs fragment of the P gene and the entire encoding region of the H gene (Harder and Osterhaus, 1997). As the clusters of CDV strains obtained in a phylogenetic tree prove to be similar by both methods,

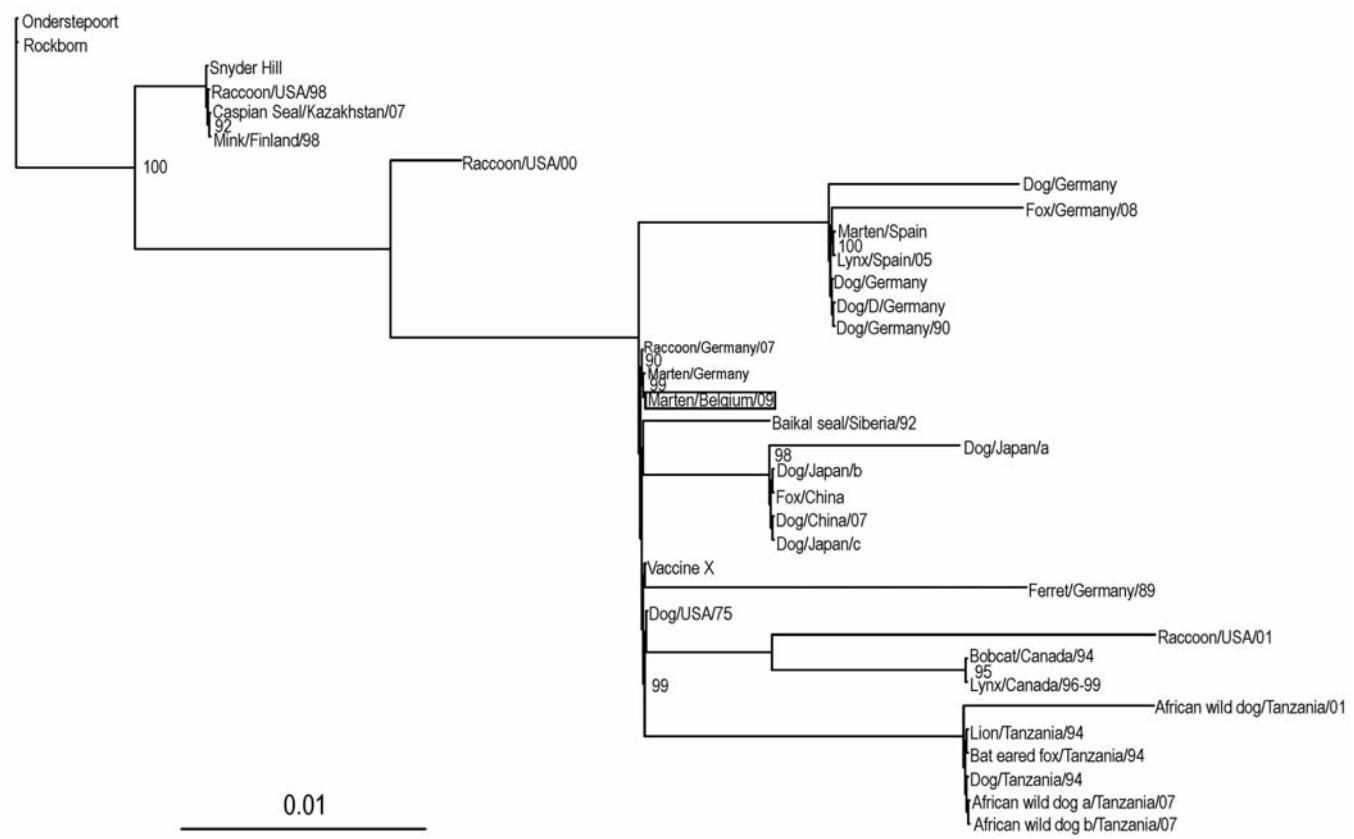

Figure 3. Phylogenetic tree of representative CDV sequences based on a 388 nucleotide fragment of the phosphoprotein (P) gene. The maximum likelihood tree was generated with the SEQBOOT and DNAML program of the Phylip package (Felsenstein 2004) with 500 bootstraps.
Genbank accession numbers are given in parenthesis: Onderstepoort: Onderstepoort strain (AF305419); Rockborn: Rockborn strain (AF181446); Snyder Hill: Snyder Hill strain (AY286481); Raccoon/USA/98: raccoon isolate 98-2645, USA (AY445077); Caspian Seal/Kazakhstan/07: strain phoca/Caspian/2007, Kazakhstan (EU594261); Mink/Finland/98: European Mink Finland, 1998, vaccine origin (AY130856); Raccoon/USA/00: raccoon brain 00-2601, USA, 2000 (AY443350); Dog Germany: Dog isolate 1259/95, Germany (AJ582388); Fox/Germany/08: red fox, isolate 458/08, Germany, 2008 (JN153031); Marten/Spain: Stone marten, Spain (GU001866); Lynx/Spain/05; Iberian Lynx, Spain, 2005 (GU001865); Dog/Germany: dog, Germany (AY386316); Dog/D/Germany: German dog (AF259549); Dog/Germany/90: dog 5804, Gemany, 1990 (AY386315); Raccoon/Germany/07: Raccoon strain 236/07, Germany, 2007 (JN153029); Marten/Germany: Marten, isolate 207/97, Germany (AJ582389); Marten/Belgium/09: Beech marten (*Martes foina*) , Belgium, 2009 (this study, JN873341); Baikal seal/Siberia/92: Baikal seal (*Phoca sibirica*), Siberia, 1992 (AF259551); Dog/Japan/a: Dog strain Hamamatsu, Japan (AB028915); Dog/Japan/b: Dog strain Yanaka, Japan (AB028914); Dog/Japan/c: Dog strain Jujo, Japan (AB028916); Fox/China: Fox strain HLJ1-06, China (EU545143); Dog/China/07: Dog strain HLJ2-07, China, 2007 (HQ540292); Vaccine X: vaccine X, Hungary, 2006 (EU072201); Dog/USA/75: domestic dog A75/15, USA, 1975 (AF259550); Ferret/Germany/89: Ferret, Germany, 1989 (AF295550); Raccoon/USA/01: raccoon brain 01-2689, USA, 2001 (AY649446); Bobcat/Canada/94: bobcat 94-X5538, Canada, 1994 (FJ240228); Lynx/Canada/96-99: lynx 97-X6984, Canada, 1996-99 (FJ240229); African wild dog/Tanzania/01: African wild dog (*Lycaon pictus*) isolate LP/Tan/01, Mkomazi Tanzania, 2001 (FJ011000); Lion/Tanzania/01: lion 94-52.10, Tanzania, 1994 (U53712); Bat eared fox/Tanzania/94: bat-eared fox (*Otocyon megalotis*) 94-200, Tanzania, 1994 (U53714); Dog/Tanzania/94: dog isolate A9411/15, Tanzania, 1994 (U53715): African wild dog a/Tanzania/07: African wild dog isolate X6534, Tanzania, 2007 (EU184828); African wild dog b/Tanzania/07: African wild dog isolate X6511, Tanzania, 2007 (EU481827).

our isolate was identified by the analysis of the P gene solely.

The hundred percent identity of our isolate with an earlier isolate from a marten in Germany is an interesting finding when compared to the population biology of beech martens in this part of Europe. The life history of beech martens in Flanders shows a particular evolution (Van Den Berge *et al.*, 2003; Van Den Berge and De Pauw, 2003). From the end of the 19th century on, large scale extermination caused a dramatic population decline, and the species disappeared almost completely in large parts of Western Europe. However, after the Second World War, a small bulwark localized in the south-eastern part of Flanders was able to develop, emanating from Walloon relic populations. From the 1960's onwards, a general population revival could be noticed, starting in Central Europe and reaching the eastern part of The Netherlands in the seventies

and eighties (Broekhuizen and Müskens, 1984). Within this context, a remarkable and spontaneous recolonization – be it only from the 1990's onwards – has been observed in Flanders too. Currently, the population density is still the highest in the south-eastern part of Flanders (Figure 1).

Canine distemper has not been documented in wildlife in Belgium before and the observed cases in Flanders and Wallonia date from the last few years. This could be consistent with spread from endemic areas in the central part of Europe towards naive West European populations, according to the preceding density and area expansion of beech martens in the outbreak area. It could also explain the hundred percent identity of our strain with an earlier isolate from Germany. Furthermore, this hypothesis is consistent with the survival strategy of CDV. Despite known high mutation rates in RNA viruses, morbilliviruses show a limited genetic diversity and induce long-term immunity. This means that, for their survival, they have to rely on a continued access to susceptible host populations with periodic epidemic spread (McLachlan and Dubovi, 2011).

Geisel (1992) found young beech martens to be affected more frequently than adults, suggesting a higher degree of immunity in older animals. If this is the case, a population with a large proportion of young animals is more at risk. As an expanding population is usually characterized by a large relative number of young animals, this population structure could be an additional factor favoring the spread of CDV.

The transmission of CDV through direct contact depends on host population density. Host density dependent infectious agents with one single host don't drive their host species to extinction, but reduce their numbers to the level where transmission is stopped (infection reproduction rate $R_0 < 1$) and the agent disappears locally in the host population. However, the reservoir of CDV is a metapopulation of multiple host species (Riordan *et al.*, 2006; Craft *et al.*, 2008). This means that CDV can be maintained in one of the host species that is at high density, while driving another host species at low density to extinction. For example, African wild dogs (*Lycaon pictus)* are threatened by distemper epizootics in domestic dogs (Alexander and Appel, 1994). A European example of a species at risk is the endangered European mink (*Mustela lutreola*) with a distribution restricted to two distinct areas, and living in close contact with sympathric polecats (*Mustela putorius*) carrying high CDV antibody prevalences (Philippa *et al.*, 2008). We couldn't find information about a possible role of CDV in the ongoing decrease of European mink populations since the introduction of American mink (*Mustela vison*). In Flanders, beech martens are not threatened but pine martens *(Martes martes)* are (Van Den Berge *et al.*, 2000; Van Den Berge and De Pauw, 2003) and it is likely that the spread of the CDV infection to this species would have a devastating effect. As for polecats, concern is also warranted as their population density in Flanders has been steadily decreasing in the last decades (Van Den Berge and De Pauw, 2003; Pertoldi *et al.*, 2005).

Taking into account reports of simultaneous distemper outbreaks in domestic dogs and wild mustelids, and the close genetic relationship between the isolated virus strains, transmission between domestic dogs and mustelids in such events is probable (Frölich *et al.*, 2000; Philippa *et al.*, 2008 ; Steinhagen and Nebel, 1985; Harder and Osterhaus, 1997). The beech martens preference for human dwellings largely facilitates this scenario (Libois and Waechter, 1991). However, the majority of domestic dogs are vaccinated in Belgium and substantial distemper outbreaks in domestic dogs have not been reported in the area concerned, as confirmed by veterinary practicians we have inquired**.** Vaccine virus strains are able to cause disease in susceptible species (Ek-Kommonen *et al.*, 2003), but do not circulate in the field and are not considered responsible for outbreaks in wildlife or vaccinated dogs (Harder *et al.*, 1996; Lan *et al.*, 2006; Mochizuki *et al.*, 1999; Kapil *et al.*, 2008). Therefore beech martens are more likely to be a reservoir in Flanders than domestic dogs.

Our observations indicate that number and density increase of beech martens as a consequence of population expansions following earlier large scale extirpations, could possibly play an important role in the spread of distemper and could constitute a threat to low density carnivore species. For comparison, it is unclear whether the recolonization of Western Europe with foxes had any effect on the epidemiology of distemper (Chautan *et al.*, 2000). Geospatial analysis of wild carnivore movements compared to the phylogenetic analysis of CDV strains from distemper outbreaks is necessary to clarify the role of population dynamics in the epidemiology of distemper. Next, the role of CDV in population number shifts between different mustelid species merits to be further examined. Our future research will focus on the analysis of conserved organ samples of wild carnivores collected in Flanders during the last two decennia.

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Persbericht

De E.U. voorziet 3 miljoen euro voor onderzoek naar verbeterde controle van worminfecties bij herkauwers. Het project met de acroniem "GLOWORM" (GLObal changes in parasitic WORMs) ging recentelijk (22-24 februari) officiëel van start met een stuurgroepmeeting in Berlijn. GLOWORM verzamelt de expertise van een multidisciplinair team van 14 partners (academici, onderzoeksinstellingen en KMO's) uit heel Europa met als doelstelling een beter inzicht te krijgen in de gevolgen van veranderingen in het klimaat, landgebruik, veehouderijmethoden en resistentie tegen de courant gebruikte geneesmiddelen op het voorkomen en het economisch belang van parasitaire wormen bij herkauwers. Het project loopt over 3 jaar en wordt gecoördineerd door prof. Jozef Vercruysse (Universiteit Gent). De drie peilers van het project zijn:

1) de ontwikkeling van nieuwe diagnostische testen en hun interpretatie om het monitoren van parasitaire infectieniveaus in Europa te verbeteren.

2) het modelleren van de infectierisico's in tijd en ruimte om de invloed van management- en klimaateffecten te onderzoeken en onze controlestrategieën hieraan aan te passen.

3) het uittesten van nieuwe controlestrategieën onder veldomstandigheden.

Dit ambitieuze project heeft een uitgebreid trainingsprogramma voor jonge onderzoekers en ondersteunt verscheidene KMO's voor het omzetten van de wetenschappelijke resultaten naar praktijkgerichte dienstverlening en producten. Meer informatie is te vinden op http://www.gloworm.eu of Dr. Johannes Charlier: Laboratorium voor Parasitologie, Faculteit Diergeneeskunde, Universiteit Gent, Salisburylaan 133, 9820 Merelbeke, johannes.charlier@ugent.be, Tel: +32 9 264 74 04.