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SEROLOGIC SURVEY FOR SELECTED VIRAL PATHOGENS IN FREE-RANGING ENDANGERED EUROPEAN MINK (*MUSTELA LUTREOLA*) AND OTHER MUSTELIDS FROM SOUTH-WESTERN FRANCE

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ABSTRACT: To investigate the possible role of selected pathogens in the decline of endangered European mink (*Mustela lutreola*) populations and the potential for these pathogens to affect mink survival, a serologic survey was conducted using serum samples collected from March 1996 to March 2003 in eight departments of south-western France. In total, 481 free-ranging individuals of five mustelid species (including the European mink) were tested. Sympatric mustelids can serve as sentinels to determine the presence of antibodies to viruses in the study area that could potentially infect mink. Antibodies to *Canine distemper virus* (CDV) were detected in all species; 9% of 127 European mink, 20% of 210 polecats (*Mustela putorius*), 5% of 112 American mink (*Mustela vison*), 33% of 21 stone marten (*Martes foina*) and 5% of 20 pine marten (*Martes martes*). Antibody prevalence was significantly higher in stone marten and polecats, possibly because their ranges overlap more closely with that of domestic species than that of the other species tested. Antibodies to *Canine adenovirus* were detected in all species but the pine marten; antibody prevalence estimates ranging from 2% to 10%. Antibodies to canine parainfluenza virus were detected in 1% of European mink, 1% of American mink and 5% of tested polecats but were not detected in *Martes* species. Antibodies to *Rabies virus* (RV) were detected in three animals, possibly because of interspecies transmission of bat lyssaviruses as the sampling area is considered to be free of RV, or to a lack of test specificity, as antibody titers were low. The high antibody prevalence to potentially lethal CDV suggests that this pathogen could have significant effects on the free-ranging populations and has implications for the conservation efforts for the endangered European mink.

Key words: *Canine adenovirus*, *Canine distemper virus*, canine parainfluenza virus, *Martes*, *Mustela*, *Rabies virus*, serologic survey.

INTRODUCTION

The European mink (*Mustela lutreola*), a small, semiaquatic mustelid, has retracted dramatically from its former territory during the last century (Youngman, 1982; Rozhnov, 1993; Maizeret et al., 2002; Maran and Henttonen, 2005) and is currently listed as endangered by the International Union for the Conservation of Nature and Natural Resources (IUCN, 2007). Presently, the remaining population is spread out over two distinct areas: a relatively larger fragmented eastern population (in Russia, Belarus, and Romania) and a very small western population located in south-western France and northern Spain (IUCN, 2007). In France, there are probably no more than a few hundred individuals, and population density seems to be low. Possible reasons for the decline include excessive trapping; change or loss of habitat; competition with the larger,

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introduced American mink (*Mustela vison*); and infectious diseases (Fournier and Maizeret, 2003; Maran and Henttonen, 2005). Recent studies in the western population of European mink have shown the presence of *Aleutian disease virus* (ADV), which could contribute to the decline (Mañas et al., 2001; Fournier-Chambrillon et al., 2004). So far, the incidence of other infectious diseases has not been investigated in free-ranging European mink. A number of other viruses have been reported in captive or free-ranging mustelids, which could potentially damage free-ranging European mink populations: *Canine distemper virus* (CDV; Appel, 1987; Williams et al., 1988; Appel and Summers, 1995; Van Moll et al., 1995; Froelich et al., 2000), *Rabies virus* (RV) (Ulbrich, 1969), *Canine adenovirus* (CAV; Karstad et al., 1975; Sumner et al., 1988; Woods, 2001; Philippa et al., 2004), canine parainfluenza virus (CPIV; Klingeborn et al., 1985; Baumgaertner et al., 1989; Durchfeld et al., 1991; Philippa et al., 2004), parvoviruses, including *Feline panleukopenia virus*, *Mink enteritis virus*, and *Canine parvovirus* (Truyen et al., 1995; Steinel et al., 2001), coronavirus-associated epizootic catarrhal enteritis (Williams et al., 2000), *Severe acute respiratory syndrome* (SARS) *coronavirus* (Martina et al., 2003), *Feline leukemia virus*, *Rotavirus*, *Powassan virus*, and herpes viruses, including Aujeszky's disease virus and an α-herpes virus (herpes necrotizing encephalitis) (Fernandez-Moran, 2003).

To investigate the potential threat of viruses to the western range of European mink, a serologic survey was conducted in several mustelid species, including European mink, feral American mink, polecat (*Mustela putorius*), stone marten (*Martes foina*), and pine marten (*Martes martes*). All of these mustelids have much larger ranges than the European mink and coinhabit certain habitats with European mink, thereby providing opportunities for interspecies transmission of pathogens. American mink were introduced in Europe for the fur trade, but subsequent escapes from fur farms and successful colonization of habitats have led to the establishment of populations in large parts of Europe (Dunstone, 1993). Polecats are found throughout most of Europe; pine martens are found throughout central/northern Europe and as far east as Siberia, whereas stone martens are found throughout central and southern Europe (Nowak, 1999). There are no current studies on population sizes and densities of these four mustelid species in the sample area.

Serologic surveys can be used to determine prevalence of antibodies to different pathogens and to gain knowledge on whether these pathogens are endemic, whether repeated infections occur from an external source, and whether an epidemic has occurred. Differences in prevalence can also be attributed to differences in population density or differences in host-virus interaction. However, prevalence of antibodies should be interpreted with caution because these estimates to not necessary equate to the prevalence of infection. Infected animals that die, that have not yet seroconverted, or that no longer have detectable antibody titers will not be detected in such ad hoc serologic surveys. Furthermore, serum antibody tests are usually produced for use in domestic species and have not been validated for use in nondomestic species.

To our knowledge, free-ranging mustelids in Europe have previously only been tested for the presence of antibodies to RV (Potzsch, 2004), ADV (Mañas et al., 2001; Yamaguchi and MacDonald, 2001; Fournier-Chambrillon et al., 2004), and CDV (Kolbl et al., 1990; Alldinger et al., 1993; Lopez-Pena et al., 1994; Van Moll et al., 1995; Froelich et al., 2000). Apart from recent data on prevalence of ADV (Fournier-Chambrillon et al., 2004), there is no knowledge of the infection status of the mustelid populations in south-western France.

We tested for antibodies against four viruses that are common in domestic

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animals (CDV, CAV, CPIV, and RV) and for which serologic tests are readily available. Although ADV is seen as a potential threat to the European mink, this virus was not included in this survey because the data on ADV prevalence in this population have been published recently (Fournier-Chambrillon et al., 2004).

Mustelids are known to be very susceptible to CDV infection (Pearson and Gorham, 1987; Williams, 2001). In the highly endangered black-footed ferret (*Mustela nigripes*) of Wyoming, USA, CDV has contributed to the decline of free-ranging and captive populations (Williams et al., 1988). Effects of CDV on European mink is poorly documented, but fatal vaccine-induced distemper has been published (Sutherland-Smith et al., 1997; Ek-Kommonen et al., 2003), and the endangered European mink is, therefore, expected to be very susceptible. Members of the Canidae, Ursidae, and Mustelidae (including striped skunks [*Mephitis mephitis*], American mink, and ferrets) are susceptible to CAV-1 infection, and transmission among domestic and wildlife species is well documented (Cabasso, 1981), but reports of clinical disease in free-ranging species associated with natural infection are limited (Woods, 2001). Experimental intranasal infections with CPIV in ferrets usually cause mild respiratory symptoms (Durchfeld et al., 1991), but its prevalence and significance in free-ranging mustelids is largely unknown. The zoonotic potential of RV has initiated effective vaccination programmes of domestic dogs and free-ranging vector species, which have eradicated it in many areas, including our study area.

The objectives of this study were 1) to determine the prevalence of antibodies to CDV, CAV, CPIV, and RV in free-ranging European mink from south-western France as a measure of exposure to these major pathogens; and 2) to determine antibodies in sympatric mustelids, which coinhabit home ranges of the European mink and which can serve as sentinels to determine the presence of these four viruses in the study area or which could potentially pass virus to them.

MATERIALS AND METHODS

For this study, we used banked serum samples, collected from 127 European mink, 112 American mink, 201 polecats, 20 pine martens, and 21 stone martens trapped during several studies (Fournier-Chambrillon et al., 2004) in eight departments of south-western France (42°47′ to 46°22′N, 0°54′ to 4°7′W) between March 1996 and March 2003 (Fig. 1). Most animals ($n=327$) were caught in live traps, between September and April, to avoid birth and nursing periods. Some animals ($n=154$) were also accidentally captured in live traps during pest-control campaigns. Individuals were sometimes caught several times.

Animals were anaesthetised with an intramuscular injection of 150 μg/kg medetomidine (Domitor®, 1 mg/ml, Pfizer Sante Animale, Paris, France) and 7.5 mg/kg ketamine (Ketamine UVA 500®, 50 mg/ml, Laboratories UVA, Ivry-sur-Seine, France), and a detailed clinical exam was performed (Fournier-Chambrillon et al., 2004). All animals were marked by a cut on the ear and received a subcutaneous transponder (Injectable Trovan®, Eid Aalten B.V., Aalten, The Netherlands) between the shoulders. Blood was taken from the jugular vein using a disposable syringe with a 0.6 × 25-mm disposable needle (Terumo®, Terumo Europe N.V., Leuven, Belgium), and transferred into a plain, silicone-coated glass tube (Venoject, Terumo). When the procedures were completed, anaesthesia was reversed with 750 μg/kg Atipamezole (Antisedan®, 1 mg/ml, Pfizer Santé Animale), and the animal was placed back in the trap to recover and was released at the capture site 2–3 hr after recovery. Blood was centrifuged at 3,000 × G for 5 min on the same, or the next, day, and serum was stored at −20 C.

Serum was centrifuged for 5 min at 10,000 × G, heat-inactivated at 56 C for 30 min, and screened for antibodies against CDV, CAV, CPIV, and RV using an indirect enzyme-linked immunosorbent assay (ELISA), as described by Orvell et al. (1985). In short, horseradish-peroxidase–conjugated protein A was used to detect the pathogen-specific immunoglobulins bound to the antigen-coated wells (European Veterinary Laboratory, Woerden, The Netherlands). An optical

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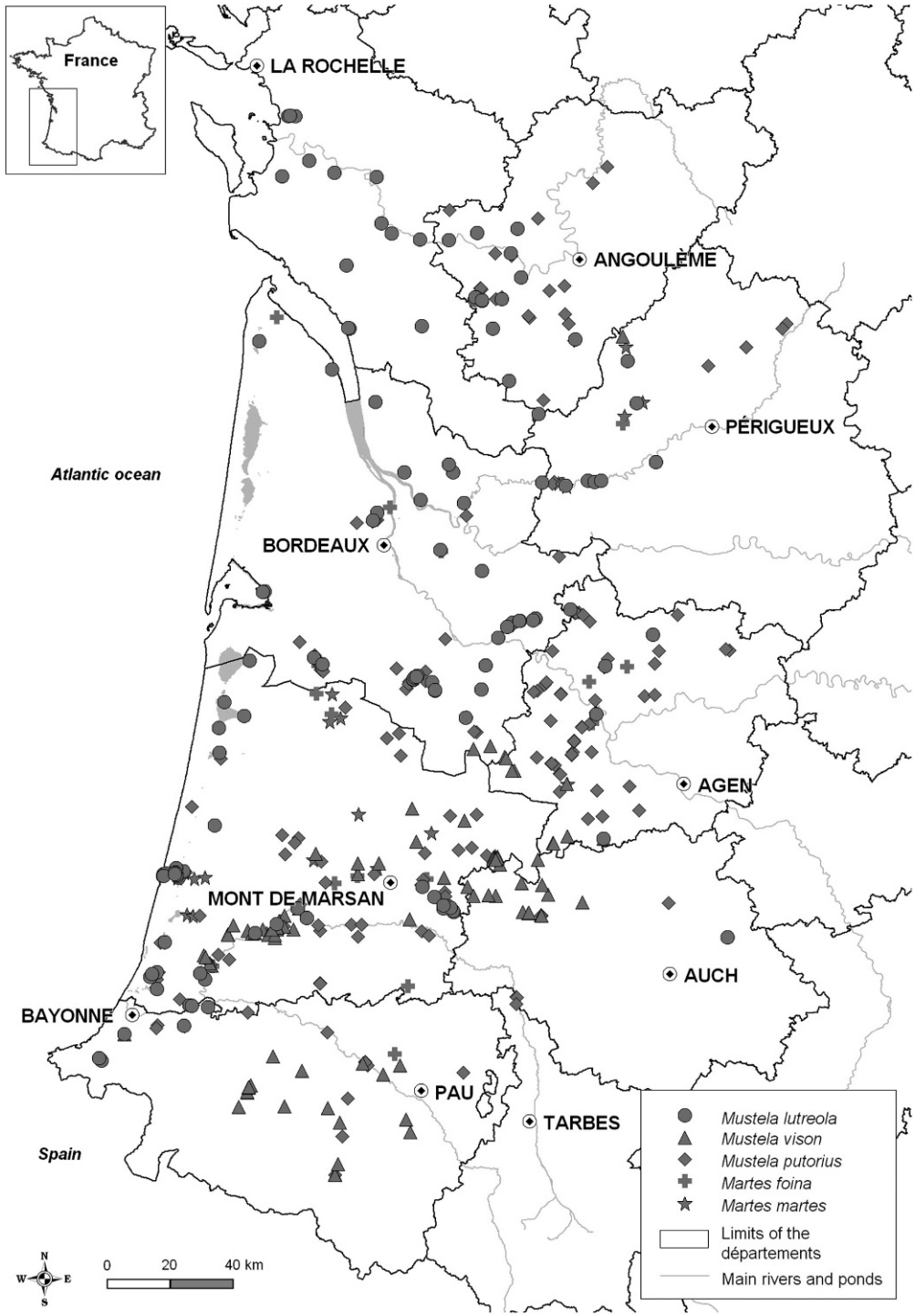

FIGURE 1. Geographic distribution of 480 free-ranging mustelids tested for antibodies to *Canine distemper virus* (CDV), *Canine adenovirus* (CAV), canine parainfluenza virus (CPIV), and *Rabies virus* (RV) in southwestern France.

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density (OD) read at 450 nm of three times the background OD was considered positive. Dilutions of serum were made in a buffer consisting of phosphate-buffered saline solution, 0.2% bovine serum albumin, 0.1% milk powder, and 5% sodium chloride. Positive and negative control sera were included in the tests. Positive samples in the screening dilution of 1:50 were then retested using a 2-log dilution series (1:10 to 1:1,280) to determine the titer. *Canine adenovirus* type 1 and CAV-2 are closely related viruses (CAV-2 causes milder, predominantly respiratory, disease in domestic species), and antibodies against these viruses cannot be distinguished with the methodology used. Results are, therefore, given for CAV (without specification of the subtype).

The CDV-specific serum antibody titers of samples considered positive by ELISA were subsequently determined by means of a virus neutralization (VN) test, as previously described (Visser et al., 1990) using a 2-log dilution series of the prediluted samples (1:10 to 1:1,280). The endpoint titer of each serum was expressed as the reciprocal of the highest dilution that completely inhibited the cytopathic effect (CPE) in Vero cells after 5 days of incubation.

Twenty European mink, four polecats and two American mink were sampled repeatedly, two to four times (one European mink, three times, and one, four times), with a mean interval of 48 wk (6 to 123 wk). For determination of antibody prevalence and for all statistical tests, resampled animals were represented once (the first sample that tested positive). Cytotoxic sera in the VN test ($n=11$) were excluded from calculations of prevalence.

For each disease, we used the chi-square test to compare the prevalence of antibodies between sex, within species, or we used a Fisher's exact test when the contingency table contained an expected frequency of less than 1.0 in any cell (Scherrer, 1984). For CDV, the same tests were used to compare, within species, the difference between prevalences measured by the ELISA and VN tests. Difference of prevalence of antibodies between species was tested using a chi-square test, followed by a multiple comparisons test (Scherrer, 1984; Sokal and Rohlf, 1995). For all statistical tests, $P \leq 0.05$ was considered significant.

RESULTS

None of the animals sampled showed clinical signs of disease upon capture and sampling. Antibody titers to CDV were detected in all species (Table 1 and Fig. 2), without significant differences in prevalence between sexes tested per species. For each species, the difference of prevalence between the ELISA and the VN test was not significant. Prevalence tested by ELISA was significantly different between species ($\chi^{2-}=26.8$, $P<0.005$), and the multiple-comparison test (with $\alpha'=0.0051$) revealed that for both polecat and stone marten prevalence was significantly higher than in European mink ($\chi^{2-}=8.0$ and $\chi^{2-}=10.3$, respectively) than in American mink ($\chi^{2-}=14.6$, and $\chi^{2-}=17.9$, respectively). Prevalence tested by VN was also significantly different between species ($\chi^{2-}=18.8$, $P<0.005$), and multiple-comparisons test only revealed significantly higher prevalence for

TABLE 1. Antibody prevalence to *Canine distemper virus* (CDV) in free-ranging small mustelids from southwestern France using indirect enzyme-linked immunosorbent assay (ELISA) and virus neutralization tests.

	CDV by ELISA			CDV by virus neutralization		
	Positive/ tested	Prevalence[a]	(95% CI[b])	Positive/ tested	Prevalence[a]	(95% CI[b])
Mustela lutreola	11/127	8.7 ab	(3.4–14.0)	8/126	6.3 fg	(1.7–11.0)
Mustela putorius	41/201	20.4 ac	(14.6–26.2)	30/192	15.6 fh	(10.2–21.0)
Mustela vison	5/112	4.5 cd	(0.2–8.8)	4/111	3.6 hi	(0.0–7.5)
Martes foina	7/21	33.3 bde	(14.6–57.0)	5/20	25.0 gi	(8.7–49.1)
Martes martes	1/20	5.0 e	(0.1–24.9)	1/20	5.0	(0.1–24.9)

[a] Prevalence (in %), means values with the same letters are significantly different between species ($P \leq 0.05$).

[b] CI = confidence interval.

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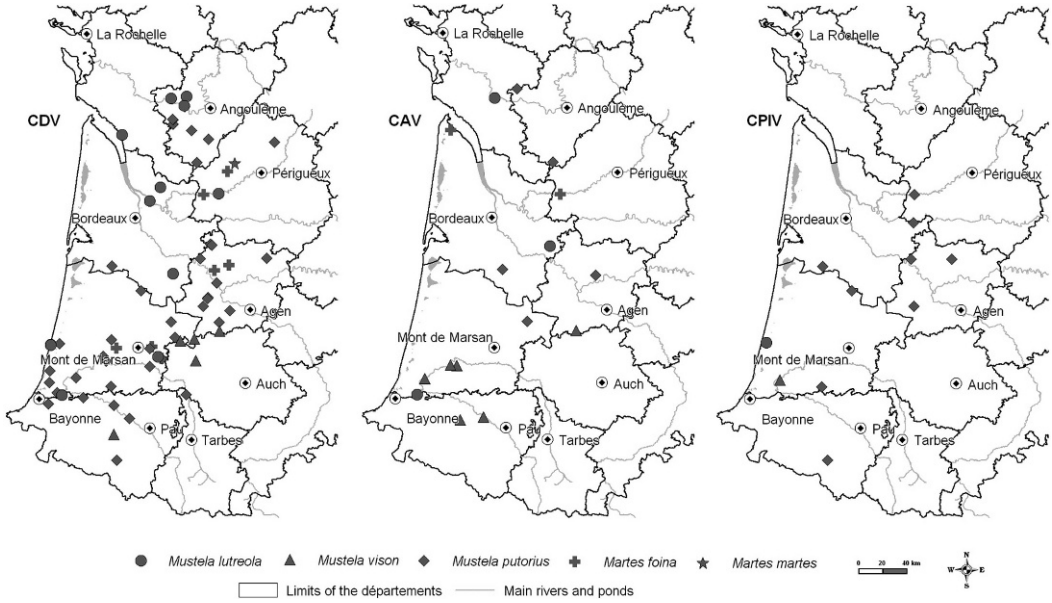

FIGURE 2. Geographic distribution of the free-ranging mustelids seropositive for antibodies to *Canine distemper virus* (CDV), *Canine adenovirus* (CAV), and canine parainfluenza virus (CPIV) in southwestern France.

both polecat and stone marten versus American mink ($\chi^{2-}=10.2$ and $\chi^{2-}=12.1$, respectively).

Antibody (VN) titers ranged from 40 to 640 in European mink and polecat, from 20 to 160 in American mink, from 80 to 160 in stone martens, and was 320 in the positive pine marten. Seroconversion was not observed in 21 negative, resampled individuals. One European mink had an increased titer when recaptured 48 wk later (320 to 640). One other (positive in ELISA test only) was negative in both tests 13 mo later. Three polecats with an antibody titer of 80 were negative ($\leq$20) 12, 42, and 44 wk later, respectively.

Antibody titers to CAV were detected in all species except pine marten (Table 2 and Fig. 2), without any significant difference in prevalence between sexes. No significant difference was observed between species ($\chi^{2-}=5.4$). All resampled individuals were negative, without any serologic conversion.

Antibody titers to CPIV were only detected in one European mink, nine polecats, and two American mink, without any significant difference between these three species ($\chi^{2-}=5.9$) (Table 2 and Fig. 2). One negative polecat was positive 12 wk later. All other resampled animals were negative, without any serologic conversion.

Low, borderline antibody titers to RV ($\leq$50) were only detected in one European mink and two polecats (Table 2). All resampled individuals were negative, without any serologic conversion.

One stone marten and two polecats were positive to both CDV and CAV, three polecats were positive to both CDV and CPIV, and one European mink was positive to CDV and had a low, possibly nonspecific, titer to RV.

DISCUSSION

In the present study, we showed the presence of antibodies to CDV in all species investigated, to CAV in all species but the pine marten, and to CPIV and RV in all *Mustela* spp. Serologic evidence of exposure to CDV occurred in all five mustelid species tested and throughout the sample area. The significantly higher

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TABLE 2. Antibody prevalence to *Canine adenovirus* (CAV), canine parainfluenza virus (CPIV), and *Rabies virus* (RV) in free-ranging small mustelids from southwestern France using an indirect enzyme-linked immunosorbent assay (ELISA) test.

	CAV by ELISA			CPIV by ELISA			RV by ELISA		
	Positive/ tested	Prevalence[a] (95% CI[b])		Positive/ tested	Prevalence[a] (95% CI[b])		Positive/ tested	Prevalence[a] (95% CI[b])	
Mustela lutreola	3/126	2.4	(0.0–5.5)	1/126	0.8	(0.0–2.7)	1/126	0.8	(0.0–2.7)
Mustela putorius	5/201	2.5	(0.1–4.9)	9/201	4.5	(1.4–7.6)	2/201	1.0	(0.0–2.6)
Mustela vison	6/112	5.4	(0.7–10.0)	1/112	0.9	(0.0–3.1)	0/112	0.0	(0.0–0.4)
Martes foina	2/21	9.5	(1.2–30.4)	0/21	0.0	(0.0–16.1)	0/21	0.0	(0.0–16.1)
Martes martes	0/20	0.0	(0.0–16.8)	0/20	0.0	(0.0–16.8)	0/20	0.0	(0.0–16.8)

[a] Prevalence (in %), means values with the same letters are significantly different between species ($P \leq 0.05$).

[b] CI = confidence interval.

prevalence observed in polecats and stone martens (33% and 20%, respectively, versus 9% and 5% in European mink and American mink, respectively) correlates with previous prevalence rates found in stone martens from Germany (Hentschke, 1995; Froelich et al., 2000). The high prevalence of CDV antibody is possibly related to the natural habitat of these species. They live in close proximity to humans (Delibes, 1983; Baghli et al., 2005), making it more likely that they come into direct or indirect contact with CDV-infected, domestic dogs, which can act as an external source of virus for free-ranging populations. Studies have shown that CDV strains in dogs and free-ranging carnivores in Germany are identical, suggesting transmission of the virus between these populations (Hentschke, 1995; Froelich et al., 2000). In the study area, hunting with hounds is widespread in rural regions, and CDV infection probably occurs regularly in these hounds. To our knowledge, no published data are available on the vaccination status of the domestic dog population in the study area. Although the European mink is strongly specialized to aquatic habitats, generally remote from human activity, they have very large home ranges (Fournier et al., 2003), occasionally resting near rural human habitation (Fournier et al., 2007). American mink are known to cause damage to hen houses and poultry farms. Therefore, interspecies contact with domestic species is also likely to occur, but less frequently than for polecats or stone martens. Although a high CDV antibody prevalence was observed in free-ranging polecats, a CDV epidemic could not be demonstrated because the numbers of animals sampled were too small to perform statistical tests between years. It is possible that CDV is endemic in this species or in these species collectively or that CDV is repeated introduced from external sources. Whatever the source, polecats are known to have close contact with European mink, as hybrids have been found in the wild (Lode et al., 2005). The high antibody prevalence observed in this species and the very high mortality rate reported for CDV in naïve ferrets (Pearson and Gorham, 1987) suggests that CDV could pose a serious threat to the European mink.

Although specific data on longevity of detectable CDV antibodies are unknown for these species, three polecats that were positive for CDV antibodies at the time of their first capture were negative when recaptured, illustrating that serologic studies may underestimate the true prevalence of previous infection. The CDV neutralization titers (20 to 640) observed in our study are higher than those previously reported (Froelich et al., 2000), although differences in methodology impede direct comparisons.

With the exception of pine marten,

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antibodies to CAV were detected in all species, throughout the sampled area. In our study, prevalence ranged from 2% to 10%. Previous serologic surveys of Canadian mustelids have shown antibody prevalences of 4% in 28% fishers (*Martes pennanti*) and 0% in 15% American badgers (*Taxidea taxus*; Philippa et al., 2004), but a 62% antibody prevalence was observed in striped skunks, and two cases of fatal disease have been described in this species (Karstad et al., 1975). Although CAV-1 infection causes more severe disease than CAV-2 in domestic dogs, clinical disease caused by CAV infection in nondomestic carnivores is seen only sporadically (Woods, 2001). Our results probably reflect a relatively low exposure to canine adenoviruses.

Antibodies to CPIV were found mainly in polecats (5% of 201 individuals). Of all other species, only one positive American mink and one positive European mink were detected. Our results probably reflect a low exposure to the virus, particularly in minks, and suggest that CPIV infection is a lesser threat for free-ranging mustelids.

The detection of antibodies against RV is surprising because the sampling areas are considered rabies-free. There are two possible explanations. First, it could be attributed to a lack of specificity in the test method used, as the titers detected were low ($\leq$50). Usually a higher cut off (=100) is used for positivity in this ELISA. Second, the antibodies detected may be directed against *European bat lyssavirus* 2 (EBLV-2) because there is a high level of cross-reaction between the closely related rabies and bat lyssaviruses, and it is difficult to distinguish the antibodies to either of these viruses by the serologic method used. Spillover of bat-origin lyssavirus type-1 has been documented in stone martens in Germany (Muller et al., 2004); however, such spillover infections do not occur frequently and are usually fatal (Bourhy et al., 1999). It is interesting to note that experimental infection of ferrets with EBLV-2 has induced high neutralizing antibody titers, and all ferrets survived (Vos et al., 2004). Insufficient volumes of serum from these animals did not allow for additional testing to confirm EBLV-2 exposure.

We have shown that free-ranging mustelids of south-western France are exposed to all the viruses investigated (with the possible exception of RV). The high prevalence of antibodies against the, potentially lethal, CDV suggests that this pathogen could have significant effects on the free-ranging European mink populations, and its contribution to the decline of this endangered population cannot be excluded. This has several implications for the conservation of the species. Strict sanitary protocols should be implemented during trapping programs to exclude live-traps as sources of infection; European mink are occasionally accidentally captured in live-traps used for pest control. Recently a breeding program was set up in Spain, with the intention of releasing European mink. A similar program is under consideration in France. Virus burdens in future release areas may be reduced by vaccination campaigns of domestic dogs in the region, as is done to protect endangered free-ranging carnivores in Africa (Randall et al., 2006; Cleaveland et al., 2007), or by restrictive dog-hunting measures. Vaccination of immunologically naïve European mink (especially against CDV) before release into endemic or epidemic areas may be required for these programs to be maximally successful. Vaccines against the viruses reported in this study are commercially available for domestic dogs, but unfortunately, these may contain a modified-live CDV component. Only inactivated vaccines (or other vaccines that have proven to be safe and effective in the targeted species) should be used in nondomestic animals because fatal, vaccine-induced diseases have occurred in several nondomestic species, including vaccine-induced CDV infections in European mink. Currently, there is no safe and

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effective CDV vaccine commercially available for nondomestic species in the European Union, and the safety, efficacy, and the extralabel use of vaccines against other pathogens have not been described in European mink.

In conclusion, this study has shown that free-ranging mustelids in south-western France have been exposed to CDV, CAV, and CPIV. Future studies should focus on isolation and identification of these viruses through collection of whole blood/buffy coats and swabs from pharynx, conjunctiva, and rectum to improve our understanding of their epidemiology and impact on these species and on the development and evaluation of preventive measures, such as the development of safe and effective vaccines.

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