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Detection of anti-*Leishmania infantum* antibodies in wild minks (*Mustela lutreola* and *Neovison vison*) from Northern Spain

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Word count (main text + Lit Cite): 1923

ABSTRACT:

The European mink (*Mustela lutreola*) is listed as a critically endangered species because of ongoing population reduction due to habitat degradation and the effects of introduced species such as American Mink (*Neovison vison*). This small and fragmented population becomes vulnerable to many other threats including diseases. Leishmaniosis is a zoonotic disease caused by the protozoan parasite *Leishmania infantum* (*L. infantum*) found in the Mediterranean area that affects a wide range of mammals, including wild small mammals. Furthermore, clinical disease caused by *L. infantum* has recently been described in other mustelids. To assess the exposure to *Leishmania* infection in mink species in Northern Spain, blood samples from 139 feral American mink, and 42 native European mink from the north Spain were evaluated for *Leishmania* infection using enzyme-linked immunosorbent assays against *Leishmania* antibodies, obtaining 45.3% of seropositivity against *L. infantum* in European minks and 52.4% in American minks. This finding raises questions on how the disease may affect these species and the repercussions that may follow on conservation efforts. Despite that a high seroprevalence was observed in wild minks in this study, association with clinical or pathological signs of disease is yet to be elucidated.

Key words: L. infantum, serological survey, wild minks

Leishmaniosis caused by *Leishmania infantum* is a vector-borne zoonotic disease endemic in southern Europe, which is spreading to northern regions (Pennisi et al. 2015). This parasite is transmitted under natural conditions by female phlebotomine sand flies during blood-feeding. In Spain, dogs (*Canis familiaris*) are considered to be the main reservoir for *L. infantum*. However, the role of other potential reservoirs for this parasite, such as wild small mammals, is being investigated (Alcover et al. 2020). The detection of the parasite infection in wild carnivores in Spain have been shown suggesting the existence of a sylvatic cycle of the *L. infantum* independent of dogs (Sobrino et al., 2008). A recent study detected a seroprevalence of 20% among 200 farmed minks (*Neovison vison*) with absence of skin and visceral lesions. Nevertheless, seropositivity was associated with poor body condition (Tsakmakidis et al. 2019). Recently, the first clinical cases of leishmaniosis in mustelids have been published in a domestic ferret (*Mustela putorius furo),* and a captive Eurasian otter (*Lutra lutra*). The ferret had a papular lesion in the right pinna (Giner et al. 2020) and the otter had bilateral epistaxis, and signs of anorexia, apathy, and weight loss (Cantos-Barreda et al. 2020).

The European mink (*Mustela lutreola*) belongs to the Mustelidae family (Carnivora), and is classified as a critically endangered species according to the IUCN Red List (Maran et al. 2016). During the 20th century, the numbers of European mink declined and the range of distribution has been reduced to a few fragmented populations; today this species faces extinction (Amstislavsky et al. 2008). Several causes have been put forward to explain the disappearance of the species in different time periods. Over-hunting was the most critical cause during the first half of the 20th century and nowadays, climate change, destruction of habitat or the presence of the introduced

American mink in the same region where European mink exists aggravates the situation and often makes it irreversible (Frankham, 2003).

The aim of this study was to determine the prevalence of natural infection with *L. infantum* in wild minks (*Mustela lutreola* and *Neovison vison*) using an in-house enzyme-linked immunosorbent assay (ELISA). The information provided would help ascertain the degree of exposure to the parasite in both mink species (native and introduced) in their two distribution areas in northern Spain, the Ebro basin with a semiarid climate with dry, hot summers and cold winters, and the Cantabrian basin characterized by mild winters and warm summers.

From 2014 to 2020, a total of 181 animals (139 American minks, and 42 European minks) were examined. For each animal, information including geographical coordinates and (river basin, sex and body scoring). Blood samples from native European mink were obtained from various sources: population surveys of the European mink in the Spanish distribution areas; periodic mink population controls in river drainages; campaigns to capture founders for the European mink breeding program in Spain; and accidental trapping during culling campaigns of feral American mink. Samples from feral American minks were collected during the population control operations, which were conducted by several governmental authorities and performed by rangers and biologists acting as trappers. This survey was included under LIFE project approved by the European Commission that opts for the conservation of the European mink (00NAT/E/7299; 00NAT/E/7335;00NAT/E/7331). The care and use of animals were performed according with the Spanish Policy for Animal Protection RD 53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

A total of 139 American minks were included (72 females and 67 males), whilst 42 European minks were evaluated (24 females and 18 males). These animals came from different riverbanks from northern Spain. The total number of samples processed in the sampling period (2014 to 2020) ranged from 1 to 67 in each year. All animals in this study were apparently healthy and presented an ideal condition (3/5) using a body scoring based on a five-point scale (Rouvinen-Wat and Amstrong, 2002). In this sense, animals with ideal condition have the following characteristics: the mink has a slender neck and a straight body shape, there is a slight amount of subcutaneous body fat and finally, the shoulder and hip bones and the ribs can be easily felt.

Both species were captured in single entry 15x15x 60 cm wire cage traps. Captured European mink were anesthetized intramuscularly with a combination of 5 mg/kg ketamine hydrochloride (Imalgene 1000, Merial, Lyon, France) and 0.10 mg/kg medetomidine hydrochloride (Domtor, Orion Corporation, Espoo, Finland).

Atipamezole (Antisedans, Orion Corporation, Espoo, Finland) was used for a reversal at five times the medetomidine dose. All European mink were clinically examined and bled by a jugular puncture; sex, weight and body condition score were recorded, and they were marked with subcutaneous passive transponder tags for identification. After recovery from anesthesia, they were released at their capture locations.

American minks were also anesthetized, and blood samples were collected from the jugular vein or by cardiac puncture. Routine laboratory tests such as a complete blood count and biochemistry profile were not performed. After data collection and while still under anesthesia, animals were sacrificed following the welfare legal standards.

An ELISA was performed on all sera as described previously, with some modifications using 100 µL of mink sera diluted 1:50 (Giner et al. 2020). As a positive control, each plate included serum from a ferret (*Mustela putorius furo*) (Giner et al. 2020) from Spain diagnosed with leishmaniosis, and as a negative control, serum from a healthy, non-infected ferret. The cutoff was set to 0.200 Optical Density units (OD units) (mean + 3 standard deviations of values from 40 healthy indoor ferrets). Sera with an OD unit ≥ 1.00 were classified as high positive, with an OD unit ≥ 0.60 and < 1.00 as moderate positive, and with an EU > 0.20 and < 0.60 as low positive.

Data were analyszed using SPSS vs. 22 software (SPSS Inc., Chicago, USA).

Descriptive analysis of the variables (sex, Ebro basin or Cantabrian basin, species) was carried out considering the proportion of the qualitative variables. Fisher´s exact test and 95% confidence interval (CI) were used to compare proportions. In all analyses, the significance level was established at P <0.05.

Among the 139 American minks, 63 were seropositive for *L. infantum* with variable antibody levels including low positive (n=137), moderate positive (0.610 OD value, n=1) and high positive levels (1.59 OD value, n=1) (Figure 1). Regarding sex, 44.4% of females (32/72) and 46.3% of males (31/67) were seropositive. By contrast, 22 European minks were seropositive for *L. infantum* with low antibody levels (Figure 2). Considering sex, 50.0% of females (12/24) and 55.6% of males (10/18) were seropositive. Real seroprevalence values of 45.3% [CI 34-52.4] and 52.4% [CI 36.4-66.6] of *L. infantum* infection in American minks and European minks respectively were obtained (Table 1 and Table 2). No significant association (P>0.05) was found between seropositivity for anti-*Leishmania* antibodies and the variables studied (Table 3).

In Spain, the seroprevalence of canine leishmaniosis differs from one area to another, and varies from 3.7% to 34.6%, with the highest prevalence cited for southern and eastern Spain and substantially low prevalence in the northern provinces of the Iberian Peninsula (3.7-4.4%) (Miró et al. 2012; Montoya et al. 2020,).

During the last two decades, many wild mammals have been diagnosed with *Leishmania* infection by serological and/or molecular methods (Oleaga et al. 2018). In the same way, studies evidence the widespread of *L. infantum* infection among wild carnivores in *L. infantum* periendemic northern Spain with the presence of *Leishmania* in 28% (44/156) of animals in the Basque Country. Specifically, in 26% of Eurasian badgers (*Meles meles*) (n = 53), 29% of foxes (*Vulpes vulpes*) (n = 48), 29% of beech martens (*Martes foina*) (n = 21) and in 25–50% of less abundant species including genets (*Genetta genetta*), wild cats (*Felis silvestris*), pole cats (*Mustela putorius*), European mink and weasels (*Mustela nivalis*) (del Rio et al. 2014). Oleaga et al. (2018) reported a prevalence of 33% for wolves (*Canis lupus)* and an overall prevalence of 40% for all the wild carnivores studied in North-Western Spain, including a prevalence of 70% for the Eurasian otter (*Lutra lutra*), 62% of European pine marten (*Martes martes*) and 67 % of beech marten (Oleaga et al. 2018). In Catalonia, it has been detected a 29.5% prevalence in wild mammals by *Leishmania* DNA and specific anti-*Leishmania* antibodies were detected (Alcover, 2020).

The high occurrence of *L. infantum* in American mink in this study suggests that further studies are needed to have a deeper knowledge in order to avoid an added potential risk for European mink, including animal monitoring using PCR analyses, xenodiagnostic experiments to confirm that sandflies take blood meals from minks and traps for the capture of adults *Phlebotomus*.

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Figure 1. Location of seropositive American minks detected.

Figure 2. Location of seropositive European minks detected.

Supplementary material: European mink

Table 1. Summary of positivity based on ELISA from all animals.

River	River basin	Number of	Year (n)	Positive minks	Serology classification (n)	Sex seropositive	Year seropositive
			American minks				
Alegria	Ebro	1	2014	0			
Aramayona	Cantabrian	2	2019 (2)	1	Low (1)	Female	2019 (1)
Ayuda	Ebro	5	2014 (1) 2015(4)	1	Low (1)	Male	2015 (1)
Barrundia	Ebro	13	2014 (10) 2015 (2) 2016 (1)	7	Low (7)	Male (3) Female (4)	2014 (6) 2016 (1)
Bayas	Ebro	1	2014	0			
Berron	Ebro	6	2014 (6)	3	Low (3)	Female (3)	2014 (3)
Ebro	Ebro	28	2014 (11) 2015 (15) 2016 (2) 2018 (1)	14	Low (13) High (1)	Female (7) Male (7)	2014 (6) 2015(7) 2018 (1)
Ega	Ebro	2	2014 (1) 2015 (1)	1	Low (1)	Female (1)	2014 (1)
Errekabarri	Ebro	1	2015 (1)	1	Low (1)	Female (1)	2015 (1)
Izoria	Cantabrian	7	2015 (7)	0			
Najerilla	Ebro	16	2014 (16)	11	Low (11)	Female (5) Male (6)	2014 (11)
Nervion	Cantabrian	16	2014 (3) 2015 (6) 2016 (4) 2017 (1) 2018 (1) 2019 (1)	7	Low (7) Moderate (1)	Female (2) Male (5)	2015 (4), 2016 (2), 2019 (1)
Salburua	Ebro	3	2014 (3)	2	Low (2)	Female (1) Male (1)	2014 (2)
Urbion	Ebro	1	2014 (1)	1	Low (1)	Female (1)	2014 (1)
Yalde	Ebro	1	2014 (1)	1	Low (1)	Male (1)	2014 (1)
Zadorra	Ebro	34	2014 (12) 2015 (19) 2016 (3)	12	Low (12)	Female (6) Male (12)	2014 (5), 2015 (7)
Zirautza	Ebro	1	2018 (1)	1	Low (1)	Male (1)	2019 (1)
			European minks				
Alegría	Ebro	2	2014 (1) 2019 (1)	1	Low (1)	Female (1)	2019 (1)

Alhama	Ebro	1	2017 (1)				
Arroy	Ebro	2	2020 (2)	2	Low (2)	Female (2)	2020 (2)
Bayas	Ebro	2	2016 (2)	2	Low (2)	Male (2)	2016 (2)
Cidacos	Ebro	3	2017 (1) 2019 (2)	0			
Ea	Ebro	1	2019 (1)	0			
Ebro	Ebro	7	2015 (1) 2016 (1) 2017 (2) 2020 (3)	6	Low (6)	Female (1) Male (5)	2015 (1) 2017 (2) 2020 (3)
Ega	Ebro	5	2015 (1) 2016 (1) 2017 (2) 2020 (1)	3	Low (3)	Female (2) Male (1)	2015 (1) 2017 (1) 2020 (1)
Iregua	Ebro	1	2017 (1)	1	Low (1)	Female (1)	2017 (1)
Laguna de los dos Reinos	Ebro	1	2019 (1)	1	Low (1)	Male (1)	2019 (1)
Leza	Ebro	2	2017 (2)	2	Low (2)	Female (2)	2017 (2)
Najerilla	Ebro	3	2018 (1) 2019 (1) 2020 (1)	2	Low (2)	Female (1) Male (1)	2018 (1) 2020 (1)
Oja	Ebro	2	2014 (2)	0			
Salburúa	Ebro	1	2014 (1)	0			
Tiron	Ebro	2	2014 (2)	1	Low (1)	Female (1)	2014 (1)
Zadorra	Ebro	3	2014 (1) 2017 (1) 2018 (1)	0			
Zirauntza	Ebro	4	2016 (2) 2018 (1) 2020 (1)	1	Low (1)	Female (1)	2016 (1)

Table 2. Seroprevalence of *L. infantum* studied by gender, species, and habitat

	European minks			American minks		
Animals (Seropositive animals/ total)	22/42			63 /137		
	n	%	95% CI	n	%	95% CI
Gender						
Male	10	23.8	[13.5 – 38.5]	31	22.6	[16.4 – 30.3]
Female	12	28.6	[17.2 – 43.6]	32	23.4	[17.1 – 31.1]
River basin						
Ebro	22	52.4	[37.7 – 66.6]	55	40.1	[32.3 -48.5]
Cantabrian	0	0	N.A	8	5.8	[3.0 -11.1]

N.A: not available

Table 3. Factors evaluated with the presence of anti-*Leishmania* antibodies

	All minks			**American Mink**		**European Mink**	
	Sex	River Basin	Species	Sex	River Basin	Sex	River Basin
Leishmania seropositivity	0.767	0.132	0.482	0.867	0.183	0.763	Not Available

Fisher´s exact test. Associations with a P value of < 0.05 were to be considered statistically significant