Dirofilaria immitis: A New Potential Pathogen for the Endangered Iberian Lynx (*Lynx pardinus*)

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ABSTRACT

Heartworm disease caused by *Dirofilaria immitis* is a vector-borne disease that affects many species of both domestic and wild carnivores. We investigated the seroprevalence of *D. immitis* infections in one of the most endangered felids in the world, the Iberian Lynx (*Lynx pardinus*), which currently occupy natural restricted and protected areas in southern of Spain. Eight out of 191 animals were seropositive to the analyses of IgG antibodies anti-*D. immitis* and anti WSPr (*Wolbachia* surface protein recombinant) (4.2%). These data suggest that both free and captive animals could be at risk of infection, which could be important on his conservation.

INTRODUCTION

The Iberian lynx (*Lynx pardinus*) is one of the most endangered felids in the world, listed as Endangered by The IUCN Red List of Threatened Species, Version 2017.[3] (www.iucnredlist.org, 2017). In 2002, only

Figure 1. *Location of the studied areas in Spain where the study areas are located: the Doñana area (1) and Sierra Morena (2) in Andalusia, Spain.*

around 100 individuals remained, distributed in two populations: one in Andújar-Cardeña, Eastern Sierra Morena, and the other one in Doñana, southern Spain (data from M.A. Simón).

Since then, the Iberian lynx has undergone a significant increase in population size due to integrated conservation projects for the species.[1] As a result, in 2015 the IUCN downgraded the Iberian lynx from "critically endangered" to "endangered." The in-situ conservation program is reinforced by means of five breeding centers, three of them located in Doñana, Jaén and Jerez de la Frontera, Andalusia (Spain), another one in Zarza de Granadilla, Cáceres (Spain), and the last in Vale Fuzeiros, Silves (Portugal) (https://www.lynxexsitu.es/). Endangered populations are markedly sensitive to diseases, but unfortunately, due to the particular situation of this species little is known about parasitic diseases.

Heartworm diseases caused by *Dirofilaria immitis* is a vector-borne parasitic zoonosis with cosmopolitan distribution. *D. immitis*, which shows little specificity of vertebrate host, have been described infections in many species of carnivores, both domestic and wild. Of these, canids and mainly domestic dogs are the most important biological reservoirs, so the existence of canine dirofilariasis is a risk for both human populations living in endemic areas and their other pets (cats and ferrets).[2]

The cat is host resistant to *D. immitis*, which translates, with respect to the pattern of canine infection, into a very low parasitic load, lower life expectancy of adult worms, and absence of microfilariae in blood.[3,4] The laboratory diagnosis of feline dirofilariasis is uncertain. Unlike the dog, in which the one-step tests for the detection of circulating antigens reach great sensitivity and specificity, the low parasite load determines that these tests are inefficient in the cat. However, *Wolbachia*, an intracellular symbionts of *D. immitis* stimulates a strong antibody response in the cat, observable at least for several months after the destruction of immature or fully developed vermes.[5]

Heartworm disease is endemic in many areas of peninsular and insular Spain,[2] and favorable geoclimatic conditions for its transmission occur in geographic areas that are more extensive than those revealed by known epidemiological data.[6] The existence of canine dirofilariasis and extensive areas with favorable conditions for transmission pose a risk to other hosts. As regards the feline population, the existing data are very limited, but clearly reveal the existence of dirofilariasis in domestic cats from rural and urban areas of Barcelona, Canary Islands, and Madrid,[3,4,7] with seroprevalence values ranging between 7.3% and 18%. Several studies have demonstrated the existence of *D. immitis* in numerous species of wild carnivores (fox, jackal wolf, wild cat, and raccoon dog) in Europe. In Spain, *D. immitis* has been detected exclusively in foxes, where it can reach high prevalences, as well as an isolated case in a wolf.[2]

In the present work, the possible existence of *D. immitis* in the endangered Iberian lynx was studied through the analysis of the presence of anti-*D. immitis* and anti-*Wolbachia* surface protein recombinant (WSPr) antibodies in serum samples from free-living specimens and kept in captivity in Spain.

MATERIAL AND METHODS

The study was carried out in two areas:

1) The Doñana area (37°0′N, 6°30′W) covers 2,000 km2 of land, mostly within Doñana National Park and Doñana Natural Park in the provinces of Cadiz and Huelva, with altitudes ranging from 0 to 47 m above sea level, and

2) The Sierra Morena (38°13′N, 4°10′W), which includes 1,125 km2 in two bordering protected natural parks and several private hunting estates at 500–1,300 m above sea level.

The climate of both areas is sub-humid Mediterranean with marked seasons (Figure 1). Serum samples from 191 Iberian lynx specimens (90 females and 101 males) were analyzed: One hundred seven came from animals kept in captivity in the three breeding centers of Andalusia, and 84 from free animals in the wild (42 from the Sierra Morena area and 42 from the Doñana National Park), whose ages were between 2 months and 12 years. The samples were taken during the routine controls that are practiced within the monitoring and recovery programs, both in free-living lynxes and in captivity, or immediately after the natural death of some individuals.[8,9] Several biological parameters such as age, sex, and geographical origin were considered. The samples used in this study were from the Iberian lynx Biological Resource Bank (CONSEMEDAMAND1.05X), which has been performed under agreement with the Environmental Council of the Regional Government of Andalusia.

The seroprevalence of *D. immitis* infections in lynx was assessed by ELISA techniques for anti-*D. immitis* and anti-*Wolbachia* antibody detection, as described by Montoya-Alonso et al. with some modifications.[3,5,7] In brief, the plates were coated with 0.8 µg of adult *D. immitis* somatic antigen (DiSA) or the *Wolbachia* surface protein recombinant (WSPr). Serum samples were analyzed at 1/100 for anti-DiSA serum antibodies and 1/40 for anti-WSPr antibody detection. Anti-feline IgG secondary antibody, horseradish peroxidase-labelled (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA), was used at 1/4000 dilution. The optical densities were measured in an Easy-Reader (Bio-Rad Laboratories, Hercules, CA, USA) at 492 nm.

Table 1. *Number of samples and positive cases of dirofilariasis distributed by age and sex.*

	Frequency n (%)	**SEROPOS n (%)**
Sex		
Male	101 (52.9)	4 (50.0)
Female	90 (47.1)	4 (50.0)
Age group		
<1 year	60 (31.4)	0 (0.0)
1-5 years	92 (48.2)	6 (75.0)
>5 years	39 (20.4)	2 (25.0)
Origin		
In situ	84 (44.0)	5 (62.5)
Ex situ	107 (56.0)	3 (37.5)
Total n (%)	191 (100)	8 (100)

**Foodnote: In situ: living in wild; Ex situ: captivity; SEROPOS: Positive simultaneously to anti-D. immitis and anti-WSPr of Wolbachia antibodies; n: number; %: Percentage.*

The cut-off points for both ELISAs (0.8 and 0.6, respectively) were obtained as arithmetic mean optical density ± 3 standard deviations of 20 serum samples from clinically healthy lynx. Lynx were considered seropositive when positive results of anti-DiSA and anti-WSPr antibodies presented jointly.[3,4,5,7,10] Statistical analysis was carried out using Prism 7 software (GraphPad Software, California, USA). Descriptive analysis of the considered variables was carried out considering the proportions of the qualitative variables. $\chi 2$ to compare proportions was performed. In all cases, a p value below 0.05 was considered statistically significant.

RESULTS

Eight of the 191 animals studied (4.2%) presented positive serology simultaneously with DiSA and WSPr (see Table 1). Three of them were animals kept in captivity, and the remaining five animals were free, three from Sierra Morena and two from Doñana. By

age, two females were 11 and 12 years old, while the rest were between 1 and 4 years old. No seropositive lynxes less than 1 year were detected. Considering the sexes, four seropositive animals were males (3.9%) and four females (4.4%). We did not observe any association between seropositivity and sex, age group, and location. Two of the seropositive samples came from dead lynxes due to chronic kidney disease, an 11-year-old female and a 4-year-old male, both kept in captivity.

DISCUSSION

Several epidemiological studies have established the existence of *D. immitis* infections in wild canids such as the fox, jackal, raccoon dog, and wolf in numerous areas of Europe, where canine dirofilariasis is endemic.[2] However, the data on feline dirofilariasis, both in domestic cats and in wild cats are much scarcer.[2,4,11,12,13,14] In the present study, the presence of antibodies against antigens of *D. immitis* and WSPr of *Wolbachia*, suggests the existence of antibodies against *D. immitis* in the Iberian lynx, an endemic species of the Iberian fauna, which contributes to the expansion of the list of hosts of this parasite. In several epidemiological studies, experimental ELISAs have been used simultaneously to detect anti-*D. immitis* and anti-WSPr of *Wolbachia* antibody modifications,[3,5,7] with results according to the seroepidemiological situation of the studied areas.

Although the existence of canine dirofilariasis is considered a fundamental risk factor for the occurrence of heartworm in humans and in other susceptible hosts, both domestic and wild,[2] it is very difficult to establish to what extent they interact the domestic and wild cycles. Our study shows the existence of infections both in lynxes kept in captivity and free in the wild. Assuming that, because it is a feline, the infections will be mostly amicrofilaremic. The cases that occur in animals kept in captivity may have their origin in the existence of infected canine reservoirs in urban areas near the maintenance centers. However, the cases detected in lynx living in the wild, given their way of life and customs away from the anthropogenic environment, should be more related to wild reservoirs, probably foxes, suggesting the existence of a wild cycle of *D. immitis*.

On the other hand, the existence of dirofilariasis, independent of the life form of the lynxes, is consistent with the fact that both the natural distribution zones (Sierra Morena and Doñana), as well as the maintenance centers, are located in areas of high transmission risk, according to the predictive results of the geoclimatic model generated for the Spanish territory.[6] Given that in many cases the cause of the death was not determined and taking into account the glomerulonephritis that can develop as a result of the immune complex fixation of *D. immitis*, which faces the heartworm, this pathology could be one of the among the possible causes of the death of these animals. Nowadays is performed investigations in this sense.

CONCLUSION

These data demonstrate the existence of antibodies against *D. immitis* in the Iberian lynx, showing that both free and captive animals could be at risk of infection. Current protection (o endangerment) of lynx makes the necropsies or direct study of tissue helminth infections difficult, highligting the importance of serological analyses. Complementary studies are necessary to obtain more data that allow us to determine if *D. immitis* infections constitutes a risk for the health and/or life expectancy of the endargered Iberian lynx. Control measures to prevent hearthworm disease could have a positive impact on the Iberian lynx conservation.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

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