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ORIGINAL PAPER

Exposure to disease agents in the endangered Iberian lynx (*Lynx pardinus*)

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Abstract The Iberian lynx (*Lynx pardinus*) is the most endangered felid species in the world. Lynx populations have decreased dramatically in size and distribution in the last four decades, thus becoming increasingly vulnerable to catastrophic events such as epizooties. From 1989 to 2000, serum samples were obtained from 48 free-ranging lynx captured in the Doñana National Park (DNP, n=31) and mountains of Sierra Morena (SM, n=17) in southern Spain. Samples were tested for antibodies against *Toxoplasma gondii*, feline herpesvirus 1 (FHV-1), feline calicivirus (FCV), feline/canine parvovirus (FPV/CPV), feline coronavirus, feline immunodeficiency virus (FIV), feline leukaemia virus and canine distemper virus (CDV) and for FeLV p27 antigen, to document baseline exposure levels. Antibodies against *T. gondii* were detected in 44% of lynx, with a significantly greater prevalence in DNP (61%) than in SM (12%). In DNP, prevalence was significantly higher in adult (81%) than in juvenile and sub-adult (41%) lynx, but no such difference was observed in SM. Low prevalences (≤11%) of minimally positive titres were found for FHV-1, FCV and FPV/CPV. This, combined with the lack of evidence for exposure to CDV, FIV and FeLV, suggests that these lynx populations are naïve and might be vulnerable to a disease outbreak in the future. Because of the reduced size of lynx populations, the documented low level of genetic variation (particularly in the DNP population) coupled with the recently documented state of immune depletion in a majority of necropsied lynx, it is important to better understand the threat and potential impact that disease agents might pose for the conservation of this endangered species. Future surveillance programs must include possible disease reservoir hosts such as domestic cats and dogs and other wild carnivores.

Keywords Andalusia · Conservation · Disease risk · Serosurvey · Spain

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Introduction

The Iberian lynx (*Lynx pardinus*) is the most endangered felid species in the world (Nowell and Jackson 1996), with

less than 200 individuals currently inhabiting only two localities 230 km apart in Andalusia, southern Spain, namely, Doñana and Sierra Morena (SM; Guzmán et al. 2004). The drastic reduction in numbers of its staple prey, the wild rabbit (*Oryctolagus cuniculus*), because of two viral diseases outbreaks (myxomatosis and rabbit haemorrhagic disease), have resulted in the reduction in lynx numbers and range contraction, and local extinction of the species throughout most of Spain (Villafuerte et al. 1994; Rodríguez and Delibes 2002). An increasing concern exists about the role of diseases as a threat for conservation of endangered species (e.g. Smith et al. 2006). It has been shown that infectious diseases can cause significant mortality in wild carnivore populations (e.g. the Ethiopian wolf *Canis simiensis* [Sillero-Zubiri et al. 1996] and the African lion *Panthera leo* [Roelke-Parker et al. 1996]) and can result in the species extinction (the black-footed ferret *Mustela nigripes*, Thorne and Williams 1988), especially when the population size is small and reservoir hosts are present in the area (de Castro and Bolker 2005). Diseases not only reduce survival but also reduce fitness and/or alter movement patterns of infected individuals (Scott 1988). The Iberian lynx apparently has experienced one or several Pleistocene demographic bottlenecks that reduced levels of mitochondrial sequence variation and levels of microsatellite size variation compared to most other wild felid species (Johnson et al. 2004). More recently, the Doñana population has suffered further loss of genetic variation because of inbreeding (Johnson et al. 2004). Inbreeding was recently proposed as a cause of the loss of effectiveness of the immune system observed in lynx (Peña et al. 2006). Thus, remnant populations of the Iberian lynx might be particularly vulnerable to a fatal epidemic.

Apart from descriptions of the lynx helminth and ectoparasite fauna (e.g. Rodríguez and Carbonell 1998; Pérez and Palma 2001; Vicente et al. 2003), there is limited published information concerning disease agents of importance to Iberian lynx populations. Lynx are known to suffer from tuberculosis caused by *Mycobacterium bovis* (bTB, Pérez et al. 2001; Martín-Atance et al. 2006). Peña et al. 2006 found direct evidence of active infection with *M. bovis*, *Toxoplasma gondii*, feline coronavirus (FCoV), feline calicivirus (FCV) and *Chlamydophila* sp. in 6 of 17 necropsied lynx (positive samples detected by histology, immunohistochemistry, immunofluorescense and/or polymerase chain reaction deoxyribonucleic acid techniques). The Iberian lynx has been recently found to host piroplasmids belonging to the genus *Cytauxzoon* (Luaces et al. 2005; Millán et al. 2007). However, it has not been established to what extent diseases are threats to the conservation of the Iberian lynx. In Switzerland, an analysis of causes of mortality in reintroduced Eurasian lynx (*Lynx lynx*) suggested that disease may have a serious impact on population dynamics as 18% of all deaths (13 of 72 lynx), but 40% of the radio-collared sub-set (6 of 15 deaths) were attributable to disease processes such as sarcoptic mange and feline panleukopaenia (FPL; Schmidt-Posthaus et al. 2002). In Spain, feline retrovirosis, parvovirosis, canine distemper and other viral diseases are fairly common in populations of domestic (e.g. Arjona et al. 2000; Decaro et al. 2006) and wild carnivores (e.g. Nieto et al. 1992; Gortazar 1999). In light of the potential importance of infectious diseases for the Iberian lynx, the goal of this study was to compare two surviving populations of Iberian lynx for evidence of exposure of lynx to seven feline viral agents and *T. gondii*, all known to be pathogenic in other species of felids.

Materials and methods

From 1989 through 2000, 57 blood samples were collected from 48 free-living Iberian lynx that were captured as part of ongoing ecological studies. Thirty-one lynx were sampled in Doñana National Park (DNP, Huelva province, 37°9′N 6°26′W) and 17 in SM (Jaén province, 38°13′N 4°11′W). Seven lynx were sampled twice and one lynx three times at 1- to 2-year intervals (all them in DNP). Lynx were captured with box traps or padded foot traps (Victor soft catch no. 1.5 and 2, Woodstream, Lititz, PA) and immobilised with a combination of ketamine (4.5 mg/kg; Imalgène, Merial, France) and xylazine hydrochloride (4.0 mg/kg; Rompun, Bayer, Germany; Ferreras et al 1994) or with tiletamine–zolazepam (5.0 mg/kg; Zoletil, Virbac, Spain). Age was estimated by tooth wear and an evaluation of facial, body and pelt features. Lynx were classified as juvenile (6 to 12 months), sub-adult (13 to 23 months) and adult (≥2 years) according to Ferreras et al. 2004. Whole blood was obtained from each by venipuncture of either cephalic, saphenous or jugular veins. Samples were collected in serum separator tubes and allowed to clot and then centrifuged at 2,000 rpm for 10 to 15 min; the serum was removed and kept frozen at −20°C until analysed. Serum samples were tested for antibodies against the following disease agents: *T. gondii*, FCV, FCoV, feline immunodeficiency virus (FIV), feline/canine parvovirus (FPV/CPV), feline leukaemia virus (FeLV), canine distemper virus (CDV), and feline herpesvirus 1 (FHV-1). Active infection with FeLV was determined by the enzyme-linked immunosorbent assay (ELISA) for antigen (p27) detection. For lynx sampled multiple times, only results from the last bleed date was used to calculate prevalences. Serologic test methods, numbers of lynx tested by each method, positive antibody thresholds and references for standard test procedures or manufacturer and catalogue number of the test kit used are

summarised in Table 1 with the following modifications and clarifications. Indirect immunofluorescence assay (IFA) tests for feline enteric coronaviruses (FECV)/feline peritonitis virus (FIPV) were performed as described by Evermann et al. 1986. The IFA is a group-specific test that detects cross-reacting antibodies to FIPV, FECV, canine coronavirus and transmissible gastroenteritis virus of swine. The lowest serum dilution used was 1:25. Sera that consistently reacted at 1:25 or greater dilutions on repeat evaluations were considered positive. The presence of FIV antibodies was detected by immunoblot (Western blot) assays as described by Troyer et al. 2005. The serum (diluted 1:200) was tested against the viral proteins derived from isolates of the domestic cat, puma (*Puma concolor*) and African lion FIV using a chemiluminescence Western blot. Test results (developed on X-ray film) were scored manually as positive, indeterminate or negative based on the presence and intensity of antibody binding to the p24 *gag* capsid protein. Sera were tested for CDV-neutralizing antibodies against the Rock-borne strain of CDV. Other than the FIV Western blot, the majority of assays (47 samples) were performed by the Washington Animal Disease Diagnostic Laboratory (WADDL) in Pullman, WA. A smaller set of ten lynx sera collected in SM from 1992 to 1995 were analysed in the Infectious Diseases Area, Faculty of Veterinary Medicine, University of Murcia, Spain.

Results

Twenty-one lynx were found to present antibodies against *T. gondii* (44%, Table 2). Antibody titres ranged from 1:64 to 1:2048. Antibodies were also found against FHV-1, FCV and FPV/CPV, with prevalences less than or equal to 11%. Antibodies against CDV, FCoV, FeLV and FIV and the FeLV p27 antigen were not detected (Table 2). The ten lynx captured in SM between 1992 and 1995 and tested in Spain resulted in two seropositive tests (*T. gondii* and FPV/CPV). FHV, with minimal titres (1:4), was only sporadically evident in the populations in 1994–1995 and 1998–1999, mostly in younger lynx. Prevalence of antibodies against *T. gondii* was higher in DNP (61%) than in SM (12%; Fisher exact $p=0.03$), even if only adult individuals were included into the analysis (Fisher exact $p=0.001$). Prevalence was greater in the adult (81%) than in the young lynx in DNP (juveniles+sub-adults, 41%; $\chi^2=8.1$, $p<0.01$). No such age-related differences were found in SM. Among the eight individuals that were sampled more than once over time in

Table 1 Disease agents tested in free-ranging Iberian lynx sera, test method, threshold, and reference or manufacturer and kit number

Serologic tests run	Number tested	Lab[a]	Assay[b]	Detects	Criterion and titre	References or manufacturer[c], name and number of kit
Toxoplasma gondii	47	W	IHA test	IgG	Positive ≥1:64	Lappin and Powell (1991)
	10	S	LA	IgG	Positive ≥1:25	Mazumder et al. (1988)
Feline herpesvirus-1	35	W	VN test	IgG	Positive ≥1:4	Scott (1977)
	10	S	ELISA	IgG	Qualitative	Eurovet: F107-AB02[d]
Feline calicivirus	34	W	VN test	IgG	Positive ≥1:4	Scott (1977)
	10	S	ELISA	IgG	Qualitative	Eurovet: F1008-AB02[d]
Feline/canine parvovirus	27	W	IFA test	IgG	Positive ≥1:25	Scott et al. 1970
	10	S	ELISA	IgG	Qualitative	Ingezim CPV© 15.CPV.K1[e]
Feline coronavirus	27	W	IFA test	IgG	Positive ≥1:25	Evermann et al. 1986
	10	S	ELISA	IgG	Qualitative	Ingezim FCoV© 16.FCV.K1[e]
Feline leukaemia virus	28	W	ELISA	Antigen p27	Qualitative	Mia et al. 1981
	10	S	ELISA	Antigen p27	Qualitative	Ingezim FeLV-Das© 16.FLV.K2[e]
	10	S	ELISA	IgG	Qualitative	Ingezim FeLV gp-70© 16.FLV.K1[e]
Feline immunodeficiency virus	28	N	WB	IgG	Qualitative	Troyer et al. 2005
	28	W	ELISA	IgG	Qualitative	Barr et al. 1989
	10	S	ELISA	IgG	Qualitative	Ingezim FIV© 16.FIV.K1[e]
Canine distemper virus	27	W	VN test	IgG	Positive ≥1:64	OIE 2004
	10	S	ELISA	IgG	Qualitative	Ingezim Moquillo IgG® 15.CDG.K1[e]

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[b] *ELISA* Enzyme-linked immunosorbent assay, *IFA* indirect immunofluorescence assay; *IHA* indirect haemagglutination, *LA* latex agglutination, *VN* virus neutralisation, *WB* Western blot

[c] Eurovet Veterinaria ([d]), Daganzo-Madrid, Spain; Ingenasa ([e]), Madrid, Spain.

Table 2 Sero-evidence of exposure to infectious disease agents in free-ranging Iberian lynx

	Sierra Morena (%)	Doñana NP (%)	Total (%)
Toxoplasma gondii	11.7 (2/17)	61.0 (19/31)	44.0 (21/48)
Feline herpesvirus	6.2 (1/16)	13.8 (4/29)	11.1 (5/45)
Feline calicivirus	6.2 (1/16)	3.6 (1/28)	4.5 (2/44)
Feline parvovirus	6.2 (1/16)	0 (0/21)	2.6 (1/37)
Feline coronavirus	0 (0/16)	0 (0/21)	0 (0/37)
Feline leukaemia virus	0 (0/17)	0 (0/21)	0 (0/38)
Feline immunodeficiency virus	0 (0/17)	0 (0/21)	0 (0/38)
Canine distemper virus	0 (0/16)	0 (0/21)	0 (0/37)

Prevalence in percent (positive/sampled)

DNP, we documented three cases of sero-conversion from negative to positive, and one with a low titre became seronegative 2 years later (Table 3).

Discussion

With the exception of *T. gondii*, seroprevalence of antibodies against infectious disease agents found in the present study was very low. This agrees with studies in other species of lynx, e.g. Eurasian lynx (Ryser-Degiorgis et al. 2005), Canada lynx (*L. canadensis*; Biek et al. 2002) or bobcat (*L. rufus*; Riley et al. 2004), as well in other species of solitary felids as the European wildcat (*Felis silvestris*; Daniels et al. 1999; Leutenegger et al. 1999). As observed by Ryser-Degiorgis et al. 2005 and Biek et al. 2002, we believe that the solitary social system of wild Iberian lynx limits the frequency of intra-specific contacts and thus keeps the possibility of disease transmission at a low level.

Almost half of the lynx were seropositive for *T. gondii*. Actual exposure rates to this parasite may be slightly underestimated as the indirect haemagglutination method employed in this study detects only IgG antibodies in feline serum, thus would have missed early infections with only IgM antibodies present (Lappin and Powell 1991). High prevalence of antibodies against *T. gondii* are commonly observed in free-living individuals of other lynx species (Ryser-Degiorgis et al. 2006 and review therein). As in other lynx species, it is likely that Iberian lynx are infected after exposure to infected prey. As aforementioned, wild rabbits constitute more than 85% of the Iberian lynx food base (Delibes 1980; Gil-Sánchez et al. 2006). In Huelva province (where DNP is found), antibodies against *T. gondii* were detected in 17% of wild rabbits (Almería et al. 2004). No data are available for SM, but the seroprevalence in the nearby province of Toledo was 12% (Almería et al. 2004). Thus, rabbit is the most probable source of exposure to *T. gondii*, although transplacental or lactational infection of kittens can also occur (Omata et al. 1994; Powell et al. 2001). An alternative source may be food or water contaminated by oocysts from cat faeces (Dubey and Odening 2001). We found that older lynx presented higher seroprevalence than young ones in DNP. In addition, three seronegative individuals were found to seroconvert when later re-sampled. Age-related differences in *T. gondii* infection rates as those observed in this study are common in wild *Lynx* species (e.g. Labelle et al. 2001; Zarnke et al. 2001; Kikuchi et al. 2004; Ryser-Degiorgis et al. 2006). Such age-related differences were not observed in the SM subpopulation, however, and the overall prevalence in this area was markedly lower than in DNP. Reasons for these differences are not apparent but may have been the result of insufficient sampling. Domestic cats are widespread in both areas. On the other hand, although exposure to *T. gondii* from rabbits might be lower in SM, there have been no studies of toxoplasmosis in rabbits in SM. Although *T. gondii* infection is common in felids, clinical toxoplasmosis is believed to be rare (Dubey et al. 1987). Peña et al. 2006 did not detect histopathological lesions associated to toxoplasmosis in

Table 3 Levels of antibody titers to *Toxoplasma gondii* in eight Iberian lynx that were sampled multiple times at different ages during their life

Name	Juvenile	Sub-adult	Sub-adult, sampled later	Adult	Adult, sampled ≥1 year later
Ajoli	–	1:128 (1991)	–	1:1024 (1992)	–
Algaida	–	–	–	1:2048 (1991)	1:256 (1996)
Barro	–	–	–	Neg (1996)	1:128 (1997)
Borja	–	–	–	1:512 (1993)	1:256 (1995)
Escarlata	–	–	–	1:128 (1994)	Neg (1996)
Ganga	Neg (1989)	–	–	1:256 (1991)	1:256 (1992)
Pepe	1:128 (1993)	1:128 (1994)	–	–	–
Uda	–	Neg (1996)	1:64 (1997)	–	–

The sampling year is shown in parentheses.

two Iberian lynx found to be positive for *T. gondii* by means of the polymerase chain reaction. One of them was an adult male who was seropositive when live captured in 1991 and that died in 2002 with signs of bTB, suggesting that lynx are persistently infected with this species of Protozoa. However, toxoplasmosis has been proven fatal in neonatal bobcat (Dubey et al. 1987), and therefore a potential impact of *T. gondii* infection on Iberian lynx populations has to be considered.

Only one lynx from SM was found to be seropositive for cross-reacting antibodies to FPV/CPV. FPL, caused by FPV, is a highly contagious disease causing high mortality chiefly in young kittens, especially in case of stress or co-infection with other agents or parasites (Scott 1990). FPL has been reported in free-ranging bobcats (Wassmer et al. 1988) and in Eurasian lynx in Switzerland (Schmidt-Posthaus et al. 2002), and low prevalence of antibodies against FPV have been found in Eurasian lynx in Sweden (Ryser-Degiorgis et al. 2005), bobcats (Fox 1983) and Canada lynx (Biek et al. 2002). This low seroprevalence may indicate that contacts with the agent are rare or that mortality because of the infection is very high. Remarkably, in Florida panthers (*P. concolor*), where the prevalence of FPV antibodies is particularly high (78%) and 32% presented titres more than 1:5,000, no disease has ever been documented (Roelke et al. 1993). Infection with FPV is thought to occur by ingestion of the virus in faeces, vomitus or urine from infected individuals (Cotter 1980). Feral cats are the more likely reservoir host of parvovirus (Sleeman et al. 2001). However, some canine parvovirus antigenic variants are able to replicate and cause disease in cats (Truyen et al. 1996) and can be very common in certain cat populations (Ikeda et al. 2000). Thus, the domestic dog or the red fox (*Vulpes vulpes*) could be also considered a potential source of infection for the lynx (Barker et al. 1985). One concern is that FPV/CPV might be having a large, undocumented effect on Iberian lynx kittens, for which parvovirus is particularly harmful (Barker and Parrish 2001), which is not detected from the current process of sampling.

FHV and FCV are the etiological agents of most of the cases of respiratory disease in cats (Gaskell and Dawson 1998). Antibodies against FHV were detected in a low number of lynx in both study populations (DNP 14%, SM 6%). It is possible that we have under-reported the true number of antibody-positive animals in this study, as the viral neutralisation method used for the majority of the samples (by WADDL) has been shown to detect fewer positives than the ELISA method (66 vs 97%, respectively; Stiles 2000; Maggs et al. 1999). However, at least for the SM population, where an ELISA method was used for 60% of those sampled, the virus neutralisation results are comparable to the ELISA (one of seven positive vs zero of ten). In other *Lynx* species, FHV prevalences of 1% in Canada lynx (Biek et al. 2002) and no detection in bobcats (Riley et al. 2004) and Eurasian lynx (Ryser-Degiorgis et al. 2005) have been reported. Exposure to FHV has been also recorded in wildcats in different European locations, ranging from 0 to 16% (Artois and Remond 1994; Daniels et al. 1999; Leutenegger et al. 1999). Antibodies against FCV were detected in only two lynx, one from each location. Although this low prevalence is similar to that reported in Canada (Biek et al. 2002) or in Eurasian lynx (Ryser-Degiorgis et al. 2005), this virus was found to be quite common in bobcats as reported by Riley et al. 2004. In European wildcat, seroprevalence of FCV ranges from 16 to 37% (Artois and Remond 1994; Daniels et al. 1999; Leutenegger et al. 1999). Transmission of FHV and FCV occurs via close contact and transmission of body fluids, particularly respiratory secretions, and the virus remains viable in the environment for only a few hours or days (Gaskell and Dawson 1998). About half of the FHV-infected cats and almost all the FCV-infected cats are carriers that undergo dormant periods without shedding but renew shedding virus after stress situations (Gaskell and Dawson 1998; Williams and Barker 2001). Thus, the most likely source of FHV and FCV infection for the Iberian lynx may be domestic or feral cats. In agreement with this, Riley et al. 2004 observed that bobcats sampled in areas where cats were frequent presented a markedly higher seroprevalence of antibodies against FCV. As observed for *T. gondii* and FPV, the effect of FHV and FCV on Iberian lynx is unknown. However, these agents deserve attention because high mortality can be observed in kitten and immunosuppressed cats (Gaskell and Dawson 1998). There was no evidence of lynx exposure to FIV, FeLV, FCoV or CDV. FIV and FeLV require direct or intimate contact for transmission, and both can be lethal to the domestic cat (Courchamp et al. 1995). FCoV infection has had devastating effects on cheetahs (*Acinonyx jubatus*) in a number of captive facilities (Evermann et al. 1986; O'Brien et al. 1985). This higher susceptibility to this and other viral infections (i.e. FHV-1; Munson et al. 2004) is believed to be related to the species' lack of genetic diversity compared to other species of cats (Evermann et al. 1986; O'Brien et al. 1985). The extant Iberian lynx population has even less genetic variability than the cheetah (Johnson et al. 2004), so may be similarly at risk if they were to encounter this virus. CDV, which has emerged as a felid pathogen, is especially problematic in species of the *Panthera* genus and caused significant mortality in lions in Serengeti National Park in Tanzania (Roelke-Parker et al. 1996). CDV was also a leading factor in the extinction in the wild of the black-footed ferret (Thorne and Williams 1988). Lack of evidence of exposure of lynx to these agents suggests that these populations might be vulnerable to an outbreak in the future. Moreover, additional factors, such as malnutrition or

stress can exacerbate the effect of infectious agents (Murray et al. 1999).

Given the small number of Iberian lynx remaining in the wild, it is imperative that measures be taken to protect all individual lynx from potential pathogens wherever possible. As lynx are removed from the wild for rehabilitation or captive breeding, care must be taken to prevent transmission of disease agents from these individuals to the captive felid population or from captive animals to those which will be released and could potentially contaminate the free-ranging population (Roelke et al. 1991). Furthermore, it is important to understand the role of these pathogens in the environment. Surveillance for pathogens should be carried out in other sympatric species that may act as reservoir, especially the widespread cat, dog and fox. Consideration should also be given to the reduction in or vaccination of cat and dog populations in and around the remaining core populations of the Iberian lynx.

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