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Factors affecting seroprevalence of *Toxoplasma gondii* in the endangered Iberian lynx (*Lynx pardinus*)

I. García-Bocanegra [a], J.P. Dubey [b,*], F. Martínez [c], A. Vargas [c], O. Cabezón [d], I. Zorrilla [e], A. Arenas [a], S. Almería [f,g]

[a] *Departamento de Sanidad Animal, Facultad de Veterinaria, UCO, Campus Universitarios de Rabanales, 14071 Córdoba, Spain*
[b] *Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, Agricultural Research Service, United States Department of Agriculture, Building 1001, Beltsville, MD 20705, USA*
[c] *Centro de Cría de Lince Ibérico el Acebuche, Matalascañas, Huelva, Spain*
[d] *Servei d'Ecopatologia de Fauna Salvatge (SEFaS), Departament de Medicina i Cirurgia Animals, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain*
[e] *Centro de Análisis y Diagnóstico de la Fauna Silvestre (CAD), Conserjería de Medio Ambiente (EGMASA), Junta de Andalucía, Spain*
[f] *Departament de Sanitat i Anatomia Animals, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain*
[g] *Centre de Recerca en Sanitat Animal (CReSA), UAB-IRTA, Campus de la Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain*

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ABSTRACT

Wild felids are considered important in maintaining the sylvatic cycle of *Toxoplasma gondii*. Although, *T. gondii* antibodies have been reported in several species of wild felids, little is known of the epidemiology and risk factors associated with *T. gondii* infection in wild cats. The Iberian lynx (*Lynx pardinus*) is the most endangered felid species in the world. In the present study, seroprevalence and associated risk factors for *T. gondii* infection in a large population of Iberian lynx in Spain were determined. Serum samples from 129 Iberian lynx collected from 2005 to 2009 and 85 wild rabbits (*Oryctolagus cuniculus*), sharing the habitat with the Iberian lynx, were tested for antibodies to *T. gondii* by the modified agglutination test (MAT) using a cut-off value of 1:25. Antibodies to *T. gondii* were found in 81 of 129 (62.8%) Iberian lynx. Seroprevalence to *T. gondii* in Iberian lynx significantly increased with age ($P < 0.001$). *T. gondii* seroprevalences were similar in free-ranging (66.7% of 93) and wild-caught captive lynx (69% of 84) but significantly lower in captive-born lynx (22.5% of 40). Seroprevalence was higher in lynx with concurrent *Cytauxzoon felis* (88% of 25) but not with concurrent Feline Leukemia Virus (FeLV) infection (53.8% of 13). There were no significant differences in seroprevalence between sexes, geographic region and year of sample collection (2005–2009). Oocysts of *T. gondii* were not detected microscopically in fecal samples from 58 lynx. Wild rabbits are considered the most important food for the lynx. Antibodies to *T. gondii* were found in 14 (11.9%) of 85 rabbits tested. The present results indicate that *T. gondii* infection is widespread in the two areas where Iberian lynx survive in Spain. The fact that four captive-born lynx seroconverted was indication of contact with *T. gondii* also in the Captive Breeding Centers, hence, control measures to prevent *T. gondii* infection would be necessary in these centers.

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1. Introduction

Felids are important in the epidemiology of *Toxoplasma gondii* infection because they are the only hosts that can excrete environmentally resistant oocysts in nature (Dubey, 2009). A recent review by Jones and Dubey

* Corresponding author. Tel.: +1 301 504 8128; fax: +1 301 504 9222.
E-mail address: jitender.dubey@ars.usda.gov (J.P. Dubey).

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(in press) summarized worldwide serological surveys in all felid species. Most of these surveys were from domestic cats in semi-urban or urban areas.

The Iberian lynx (*Lynx pardinus*) is the most endangered felid species in the world (Nowell and Jackson, 1996) and the most endangered carnivore in Europe (IUCN, 2009). In the past few decades, the progressive disappearance of the Iberian lynx populations has been mainly attributed to the drastic reduction in the numbers of its staple prey, the European wild rabbit (*Oryctolagus cuniculus*). Habitat destruction and fragmentation, illegal trapping, road kills, illegal hunting and diseases have also been important factors in the decrease of their populations (Ferreras et al., 1992, 2001; Rodríguez and Delibes, 2004). Presently, the Iberian lynx is considered as critically endangered (IUCN, 2009), and its distribution has been reduced by 99% in the last 50 years, with approximately 250 individuals currently inhabiting only two isolated metapopulations in southern Spain (Sierra Morena and Doñana) (Palomares et al., 2002). To save this species from extinction an EU LIFE nature project is underway that includes habitat preservation, lynx population monitoring, and rabbit management. In addition, the Central Government of Spain in conjunction with the Regional Government of Andalusia has initiated an *ex situ* conservation program comprising Captive Breeding Centers to maintain the genetic variability present in the free-ranging populations and produce individuals for future reintroduction efforts (Vargas et al., 2009).

There is limited information on *T. gondii* infection in the Iberian lynx. In previous studies, the number of lynx analyzed was comparatively small (Sobrino et al., 2007; Roelke et al., 2008; Millán et al., 2009a) with little information on the risk factors.

The aims of the present study were (1) to study *T. gondii* seroprevalence in a large number of Iberian lynx, (2) to provide information on possible risk factors associated with seroprevalence levels, such as age, sex, habitat, metapopulation, year of sample collection, presence of active infection of *Cytauxzoon felis* and infection by Feline Leukemia Virus (FeLV) infection and (3) to assess the dynamics of seropositivity in the Iberian lynx sampled several times along the 5-year study period to further understand the possible routes of transmission of *T. gondii*.

2. Materials and methods

2.1. Source of animals

Serum samples ($n = 217$) from a total of 129 Iberian lynx were collected from 2005 to 2009; 93 samples were from free-ranging lynx, 84 from wild-caught captive lynx (free-ranging lynx captured and kept in captivity) and 40 samples from captive-born lynx. Most ($n = 205$) samples were from live lynx. Twelve samples were from dead animals; 6 from lynx dead due to FeLV outbreak (López et al., in press; Meli et al., 2009), 4 from road kills, and 2 samples from stillborn lynx (captive-born). Sixty-nine lynx were from Sierra Morena (Jaen province, 38°13′N, 4°11′W), 51 from Doñana (Huelva province, 37°9′N, 6°26′W) and 9 were animals of mixed origin (lynx born to parents from both areas). Thirty-three, 14, and 9 animals were sampled twice, three and four times, respectively, at 1–2-year intervals.

In most of the animals the date of birth was registered from previous field studies and medical records. In animals in which the date of birth was unknown, age was estimated by weight, tooth-wear and facial, body and coat features. Animals were classified as cub-juvenile lynx (1–9 months), sub-adults (9–24 months) and adults (>24 months). Samples from lynx born between 1989 and 2009 were collected. At the time of sample collection clinical signs of disease from live animals were evaluated.

Most of the samples were collected from lynx captured by mean of box-traps, 44 samples were collected from animals captured by nets, blow-dart or manual immobilization. Animals were handled according to regulations of the LIFE project and to collect samples the appropriate permits from Regional Ministry of Environment of the Government of Andalusia were obtained. Lynx were chemically immobilized with a combination of ketamine hydrochloride (5 mg/kg, Imalgene 1000®, Merial, Barcelona, Spain) and medetomidine (50 μg/kg; Domtor®, Pfizer, Madrid, Spain) administered intramuscularly. Blood (2 ml) samples were obtained from each animal by venipuncture. Blood or peritoneal fluid was collected from respectively the heart or the thoracic cavity of dead lynx. Samples were collected in serum separator tubes (Becton-Dickinson, Rutherford, New Jersey, USA), centrifuged at $400 \times g$ for 15 min and stored at −20 °C until analyzed. One milliliter of whole blood was also placed in lithium heparin-coated tubes for *C. felis* examination. Fecal samples (3–5 g) were collected from the individual cages just after deposition from 34 wild-caught captive and from 24 captive-born lynx.

Serum samples from 85 translocated wild rabbits (*O. cuniculus*) sharing areas with the Iberian lynx were also tested for *T. gondii* antibodies. Two milliliter of whole blood were obtained by an incision in the auricular marginal vein from live rabbits captured for translocations and restocking purposes. Samples were collected in serum separator tubes (Becton-Dickinson, Rutherford, New Jersey, USA), centrifuged at $400 \times g$ for 15 min and sera stored at −20 °C until analyzed.

2.2. Laboratory methods

The presence of antibodies to *T. gondii* was determined by the modified agglutination test (MAT) as described previously (Dubey and Desmonts, 1987). Serum from each lynx was tested at 1:25, 1:50 and 1:500 dilutions. Positive or doubtful samples were re-tested. Positive and negative controls were included in each test. Sera with a titer of ≥1:25 were considered positive as has been previously described for felid species (Dubey and Thulliez, 1989; Gauss et al., 2003; Ryser-Degiorgis et al., 2006; Sobrino et al., 2007; Millán et al., 2009a).

Fecal samples were examined for the presence of *T. gondii* oocysts by a combined sedimentation and flotation procedure using concentrated sucrose solution as previously described by Schares et al. (2005). Floated material was transferred to a slide and examined by light microscopy using a magnification of at least 200×.

The presence of the intraerythrocytic piroplasm *C. felis* was determined in 112 animals by PCR from blood samples as previously described (Millán et al., 2007; Meli et al., 2009). Active infection of Feline Leukemia Virus was evaluated from 44 individuals using antigen ELISA and real-time PCR (López et al., in press; Meli et al., 2009).

2.2.1. Statistical analysis

The prevalence of antibodies against *T. gondii* was estimated from the ratio of positive results to the total number of lynx examined, with the exact binomial confidence intervals of 95% (Martin et al., 1987). For lynx sampled multiple times, only results from the last sampling date were used to calculate prevalence.

The associations between serological results and independent variables such as sex, age, habitat, geographic region, year of sample collection and presence of concurrent *C. felis* and FeLV infections were analyzed by means of a Pearson's chi-square test or, when there were less than six observations per category, by the Fisher's exact test. The differences between variables were analyzed by Bonferroni or Tukey tests. For multiple comparison Dunn's test was performed. Values with $P < 0.05$ were considered as statistically significant. Statistical analyses were performed using SPSS 14.0 (Statistical Package for Social Sciences (SPSS) Inc., Chicago, IL, USA).

3. Results

Antibodies (MAT ≥1:25) to *T. gondii* were found in 81 (62.8%; 95% CI: 57.0–68.6) of 129 lynx with titers of 1:25 in 7 lynx (3.2%), 1:50 in 31 lynx (25.2%), and ≥1:500 in 91 lynx (68.7%).

Prevalence among sexes, ages, regions, habitats, years and concurrent infections with *C. felis* and FeLV are shown in Table 1. Seroprevalence to *T. gondii* significantly increased with age ($P < 0.001$). Antibodies to *T. gondii* were found in 19 of 62 (30.6%) cub-juvenile lynx, in 25 of 47 (53.2%) sub-adult lynx, and in 85 of 108 (78.8%) adult lynx (Table 1).

Captive-born lynx had significantly ($P < 0.001$) lower seroprevalence compared to wild-caught captive and free-ranging lynx. No statistical differences between prevalence in wild-caught captive and free-ranging lynx were observed. Most of the wild-caught captive lynx were seropositive (94%, of 38) before captivity.

C. felis was found in 26.9% of 112 animals (95% CI: 20.6–33.2) and all positive animals were from Sierra Morena. Significantly higher seroprevalence of *T. gondii* was observed in lynx with presence of *C. felis* in blood ($P = 0.03$).

No statistically significant differences were observed in the prevalence of antibodies against *T. gondii* and year of sample collection.

No statistically significant differences were observed in the seroprevalence to *T. gondii* between sexes, regions, year of sample collection and presence of active infection of FeLV. The seroprevalence of female lynx was not statis-

Table 1
Prevalence against *T. gondii* antibodies in Iberian lynx.

Category	No. examined	No. positive (%)
Sex		
Female	66	43 (65.2)
Male	63	38 (60.3)
Age		
Cub-juveniles	62	19 (30.6)
Sub-adults	47	25 (53.2)
Adults	108	85 (78.7)
Geographic region		
Doñana	51	32 (62.7)
Sierra Morena	69	44 (63.8)
Mixed origin	9	5 (55.6)
Habitat		
Free-ranging	93	62 (66.7)
Wild-caught captive	84	58 (69.0)
Captive-born	40	9 (22.5)
Year sampled		
2005	28	19 (67.9)
2006	48	30 (62.5)
2007	56	29 (51.8)
2008	58	38 (65.5)
2009	27	13 (48.4)
Cytauxzoon felis		
Negative	87	50 (57.5)
Positive	25	22 (88.0)
FeLV		
Negative	31	20 (64.5)
Positive	13	7 (53.8)

Table 2
Toxoplasma gondii antibody titers in 12 Iberian lynx that seroconverted in the present study.

Name	Habitat	2005	2006	2007	2008	2009
Almoradoux	Captive-raised	–[a]	NEG	–	50	–
Arrayán	Free-ranging	NEG	500	–	–	–
Castañuela	Captive-born	–	–	NEG	25	–
Centáurea	Free-ranging	–	NEG	NEG	500	–
Coca	Captive-raised	–	NEG	NEG	NEG	500
Cromo	Captive-raised	NEG	500	50	–	–
Cynara	Captive-born	–	NEG	500	–	–
Dama	Captive-born	–	–	NEG	50	50
Dardo	Free-ranging	–	–	NEG	500	–
Datil	Captive-born	–	–	NEG	50	–
Dulce	Free-ranging	–	–	NEG	–	500
Durillo	Free-ranging	–	–	NEG	500	–

[a] Not done.

tically associated to the serostatus of their progeny ($P = 0.34$).

Of the 56 lynx sampled more than once, 15 were seronegative in all the samplings, 29 were seropositive in all the samplings and 12 seroconverted along the study period. Four of the animals that seroconverted were captive-born lynx, 3 were wild-caught captive lynx and 5 were free-ranging lynx (Table 2).

T. gondii—like oocysts were not observed in any of the fecal samples analyzed; bioassays were not performed.

Antibodies against *T. gondii* were detected in 14 (11.9%) of 85 wild rabbits.

4. Discussion

Seroprevalence of *T. gondii* infection in the endangered Iberian lynx in Spain has been previously reported (Table 3). However, in previous studies the number of lynx analyzed was comparatively small and only data on free-ranging lynx were reported. In the present study, over half of the estimated remaining population of Iberian lynx was analyzed to establish seroprevalence to *T. gondii* in both free-ranging and captive Iberian lynx populations. Wide variations in prevalence of antibodies to *T. gondii* have been reported in other lynx species (Table 3). The differences in seroprevalences in these species could be due to the serological methods, biological particularities, diet, climate conditions, size or origin of the samples. The high seroprevalence of *T. gondii* observed in the Iberian lynx indicated widespread *T. gondii* infection in the areas where the Iberian lynx survives. Seroprevalence of *T. gondii* in domestic cat in the same areas has also been observed to be high. Seroprevalence higher than 50% was reported from 25 domestic cats in the same areas inhabited by Iberian lynx with detection of *T. gondii* oocysts in the faeces of some adult domestic cats (Millán et al., 2009a) and 71.4% of 14 serum samples from domestic cats were positive in the most recent survey in the area (unpublished data). High differences in seroprevalence of other wild felid species have been found (reviewed by Jones and Dubey, in press). Strictly wild living cats could be considered the most similar felids to free-living Iberian lynx. Although, there is little information from these cats because they are difficult to catch, in one study, *T. gondii* antibodies were found in 84.2% of 19 feral cats that had minimum human contact from Mona Island, Puerto Rico (Dubey et al., 2007), and recently a very high seroprevalence of *T. gondii* antibodies (84.7%) was observed in 59 wild living cats in Mallorca, Balearic Islands, Spain (Millán et al., 2009b), suggesting that free-ranging felids in Spain have a high rate of *T. gondii* infection with important implications for public health.

Seroprevalence of *T. gondii* infection in the Iberian lynx was age-related which is not unexpected, because cumulative likelihood for exposure to *T. gondii* has been reported to increase during the life of the animals (Roelke et al., 2008). In addition, 12 animals seroconverted.

The higher seroprevalence observed in adult and subadult lynx and the lack of association between the seropositivity of female lynx and their progeny could indicate horizontal transmission as the main route of transmission of *T. gondii*. *Toxoplasma gondii* can be

Table 3
Seroprevalence of *T. gondii* antibodies in lynx species.

Species	Location	No.	Seropositive (%)	Type of test	Reference
Iberian lynx (*Lynx pardinus*)	Spain	27	81.5	MAT	Sobrino et al. (2007)
	Spain	26	80.7	MAT	Millán et al. (2009a)
	Spain	48	44.0	LAT, IHA	Roelke et al. (2008)
	Spain	129	62.8	MAT	Present study
Eurasian lynx (*Lynx lynx*)	Sweden	207	75.4	MAT	Ryser-Degiorgis et al. (2006)
	Czech and Slovak	2	100	IFA	Sedlák and Bártová (2006)
	Finland	70	73.0	–	Oksanen and Lindgren (1995)
	Brazil	1	100	MAT	Silva et al. (2001)
	Canada	5	20.0	ELISA	Philippa et al. (2004)
Canada lynx (*Lynx canadensis*)	Canada	106	44.0	MAT	Labelle et al. (2001)
	USA	1	100	IFA	Spencer et al. (2003)
Bobcat (*Lynx rufus*)	Canada	10	40.0	MAT	Labelle et al. (2001)
	USA	131	83.0	MAT	Mucker et al. (2006)
	USA	58	51.7	LAT	Kikuchi et al. (2004)
	USA	21	71.4	IHA	Riemann et al. (1974)
	USA	86	69.0	IHA	Franti et al. (1976)
	USA	25	88.0	LAT	Riley et al. (2004)
	USA	103	61.0	IHA	Riemann et al. (1978)
	USA	6	83.3	MAT	Dubey et al. (2004)
	USA	2	50.0	DT	Smith and Frenkel (1995)
	USA	3	100	IFA	Miller et al. (2008)
	USA	3	33.3	IFA	Spencer et al. (2003)
	USA	150	18.0	IHA	Oertley and Walls (1980)
	Mexico	27	44.0	Sabin–Feldman test	Marchiondo et al. (1976)
Caracal (*Caracal caracal*)	USA	1	100	IFA	Spencer et al. (2003)
	USA	4	50.0	MAT	De Camps et al. (2008)

MAT, modified agglutination test; LAT, latex agglutination test; IHA, indirect hemagglutination; IFA, indirect fluorescent antibody; ELISA, enzyme-linked immunosorbent assay; DT, dye test.

transmitted via carnivorism, fecal-orally, and transplacentally. In wild felids, the ingestion of infected tissue is the most efficient means of transmission of *T. gondii*, although transplacental or lactational infections of kittens have also been reported (Dubey, 2009). The diet of free-ranging lynx mainly includes wild rabbit (90.8%) with small proportions of red-legged partridge (4.5%) and wild ruminants (2.8%) (Gil-Sánchez et al., 2006). Captive lynx are mostly fed live farmed rabbits or quail and occasionally live wild rabbits. Wild rabbits may be an important source of *T. gondii* infection for Iberian lynx as previously suggested (Almería et al., 2004). The seroprevalence observed in wild rabbits sharing the habitat with Iberian lynx, although low, was similar to those previously reported in southern Spain by Almería et al. (2004). In addition to hunting wild rabbits, free-ranging Iberian lynx could have access to carcasses from wild ruminant species, in which *T. gondii* antibodies have been reported in Sierra Morena (Gauss et al., 2006). We are not aware of *T. gondii* prevalence in natural infections of free-ranging avian species in Spain, including the red-legged partridge.

Interestingly, in the study by Roelke et al. (2008) there were significant differences on prevalence of *T. gondii* between Doñana and Sierra Morena, while no differences in seroprevalence were observed in the present study. These findings may be related to the number of samples analyzed in both studies.

The similar seroprevalence observed among years in the present study would indicate endemic *T. gondii* infection in the Iberian lynx populations. Seropositive lynx sampled several times along the study period maintained their seropositivity which may indicate repeated exposure to infection or long lasting humoral immune responses. It is noteworthy that more than 68% of lynx had high antibody titers (1:500 or higher). In a recent study of *T. gondii* antibodies in feral cats in Mallorca (Balearic Islands, Spain), a similar finding was observed with 38 of 59 (64.4%) cats having titers of 1:1000 or higher (Millán et al., 2009b). Whether this high titer positivity is related to the strains of *T. gondii* circulating in wild felid species in Spain is unknown and merits further investigation.

Seroprevalence in wild-caught captive lynx was similar to that in free-ranging lynx, probably due to the fact that the majority of seropositive wild-caught captive lynx were already seropositive before capture. On the other hand, captive-born lynx seroprevalence was significantly lower compared with wild-caught captive and free-ranging lynx, indicating a decreased contact with the parasite in captivity. However, the fact that four captive-born lynx seroconverted showed that *T. gondii* infection by Iberian lynx can be acquired in captivity under strict biosecurity. The biosecurity measures at the Iberian Lynx Captive Breeding Centers are very strict and therefore, direct contact with other wildlife species is unlikely. Ingestion of food infected with tissue cysts or water contaminated with oocysts are the most likely sources of infection in captive-born lynx.

Although no oocysts were detected in the Iberian lynx fecal samples analyzed in the present study and to date, *T. gondii* oocyst excretion has not been found in the Iberian lynx, it seems a fair assumption that they excrete *T. gondii* oocysts as all other felids tested do (Jones and Dubey, in press) and that seropositive lynx might have shed oocysts. Even in cats, coprological surveys are unrewarding because at any given time less than 1% of domestic cats were found to have shed oocysts (reviewed by Jones and Dubey, in press). Domestic cats excrete oocysts for only 1–2 weeks during the initial phase of infection by *T. gondii* when become infected for the first time and after re-infection they shed only reduced numbers of oocysts on limited occasions. On the other hand, the lack of oocysts in lynx feces was probably due to the high *T. gondii* seropositivity. In domestic cats seropositivity indicates that cats have already shed oocysts (Dubey, 2009).

Concurrent microbial infections can modify the course of *T. gondii* infection. Two good examples are the Canine Distemper Virus (CDV) infection in dogs and related canids and, the Human Immunodeficiency Virus (HIV) in humans (Dubey and Beattie, 1988; Dubey, 2009). Most cases of clinical toxoplasmosis in canids are in animals with concurrent CDV infections and hundreds of persons concurrently infected with active HIV have died of clinical toxoplasmosis. There are several immunosuppressive microbial infections of felids (FIV, FeLV, *Bartonella* spp.) that are thought to modify the course of *T. gondii* infection in felids. However, a recent review of all data in naturally infected domestic cats concluded that there is no evidence that any of these immunosuppressive agents modify *T. gondii* infection in naturally infected cats (Dubey, 2009; Dubey et al., 2009). Only limited data are available on concurrent *T. gondii* and other microbial infections in the Iberian lynx. To our knowledge, there has not been any report to date of clinical toxoplasmosis in the Iberian lynx.

A high prevalence of *C. felis* in lynx from Sierra Morena was previously reported (Millán et al., 2007; Meli et al., 2009) and also found in the present study, in which the presence of *C. felis* was significantly associated to the seroprevalence of *T. gondii*. Whether there is any clinical relevance between these two infections has yet to be established.

In conclusion, the high seroprevalence of *T. gondii* observed in the Iberian lynx indicated widespread *T. gondii* infection in the areas where the Iberian lynx survives. Horizontal transmission may be the main route of transmission of *T. gondii* in this species. The high seroprevalence observed in the Iberian lynx suggests an important role in the sylvatic cycle of *T. gondii* in the areas where they survive. The seroconversion of some captive-born lynx in the Captive Breeding Centers is a matter of concern and requires further investigation. Because freezing kills encysted *T. gondii* (Kotula et al., 1991; Silva et al., 2007), feeding meat previously frozen at $-12\,^{\circ}C$ for a period >7 days is one of the most practical method to reduce exposure to *T. gondii* in zoo carnivores.

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