University of Évora

Master's dissertation on

Conservation Biology

# "Contribution to the knowledge of selected parasitic infections in red-foxes (*Vulpes vulpes*) of the Alentejo area"

**Antónia da Conceição dos Reis Pão Mole**

Supervisor: Professor Helder Cortes

Co-supervisor: Professor Bruno Gottstein

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This Master´s dissertation includes critics and suggestions from the jury.

Évora 2010

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## Abbreviations list

| | |
|---|---|
| CFT | Complement fixation test |
| DAT | Direct agglutination test |
| DMEM | Dubelco minimal essential medium |
| ELISA | Enzyme-linked immunosorbent assay |
| FCSI | Fetal calf serum inactivated |
| IFAT | Immunofluorescence antibody test |
| MAT | Modified agglutination test |
| PCR | Polymerase chain reaction |
| PBS | Phosphate buffer saline solution |

## Acknowledgments

The author thanks Professors Helder Cortes, Bruno Gottstein, Luís Cardoso and Saul Semião Santos for supervising; Dr. Ana Patrícia Lopes for technical expertise; Maria João Vila-Viçosa for technical expertise and help on necropsy; Maria Luísa for help on necropsy; Doctor Miguel Fernandes, hunters association from S. Cristovão, Jorge Gonçalves and Rafael Carvalho, for collaboration on hunting days; and Terry Benson for reading the manuscript. Personal thanks to Maria Godinho (my mother), Luís Oliveira, Professor José Potes, and Marie & Olly Holloway.

## Abstract

The present project was developed as an investigation for a master's dissertation in Biology Conservation, a study that links Biology and health surveillance. Twenty-five foxes were obtained during hunting season and serological tests were used to assess the presence of *Leishmania infantum*, *Toxoplasma gondii* and *Neospora caninum* in the red-fox from the Alentejo area; this descriptive surveillance study provides a first insight into the health of the ecosystem and reservoirs of diseases both to endangered sympatric wild species, such as the Iberian lynx, and to domestic mammals and even humans. It's important to better understand the threat and potential impact that disease agents might pose for the conservation of endangered species.

Anti-*L. infantum* antibodies were detected by ELISA in 5.6% of the animals, anti-*T. gondii* antibodies were identified by IFAT and MAT in 50% of the animals; finally anti-*N. caninum* antibodies were detected by IFAT in 16.7% of the foxes.

Keywords: *Leishmania infantum*; *Neospora caninum*; *Toxoplasma gondii*; health surveillance; red-fox; reservoir

Contributo para o conhecimento de infecção parasitária em raposa-vermelha (*Vulpes vulpes*) no Alentejo.

## Resumo

Esta dissertação foi desenvolvida no âmbito do mestrado em Biologia da Conservação e resultou da ligação entre a Biologia e a vigilância sanitária. Durante a época de caça foram capturadas vinte e cinco raposas-vermelhas na região do Alentejo, tendo sido submetidas a provas serológicas para detecção de presença de *Leishmania infantum*, *Toxoplasma gondii* e *Neospora caninum*. Este estudo de vigilância permitirá inferir sobre a saúde do ecossistema e sobre reservatórios de doença tanto para animais silvestres ameaçados, como o lince ibérico, assim como para mamíferos domésticos e para o próprio Homem. Foram detectados, com ELISA, anticorpos anti-*L. infantum* em 5.6% dos animais, anticorpos anti-*T. gondii* em 50% dos animais, com IFAT e MAT e finalmente anticorpos anti-*N. caninum* em 16.7% das raposas, com IFAT.

Palavras-chave: *Leishmania infantum*; *Neospora caninum; Toxoplasma gondii*; vigilância sanitária; raposa-vermelhas; reservatório

# Introduction

Wildlife has long been recognized as a potential source of emerging infectious diseases in humans and domestic animals, and wildlife diseases have historically gained attention primarily when they were considered a threat to agricultural systems and the economic, social or physical health of humans. In fact, the potential impact of infectious diseases on wildlife populations has often been overshadowed by the apparently more pressing anthropocentric issues. Lately, our attention has been diverted towards the impact of human and domestic animals on wildlife, either by environmental changes, overhunting and species extinction or by disease exchange (Thompson *et al.*, 2009). Although this spill-over of parasites to naive species of wildlife requires more profound investigations, there are data confirming diseases exchange, such as tuberculosis in wild animals in South Africa's Kruger Park game reserve, as well as leishmaniasis, toxoplasmosis and neosporosis in wild animals on the Iberian Peninsula. However today, there is a rapidly evolving understanding of the ecology of infectious diseases in wildlife, including a new appreciation of the impact that infectious diseases can have on the dynamics and sustainability of wildlife populations; detection of infectious organisms in wild-life animals reflects autochthony of transmission (Thompson *et al.*, 2009). In particular, the serious threat that disease can impose on genetically impoverished endangered wildlife species is increasingly recognized, as is the importance of preserving biodiversity in wildlife ecosystems to prevent and control the emergence or re-emergence of diseases. Multihost situations are also of concern for wildlife management and conservation, as diseases can affect the productivity and density of wildlife populations. Our current understanding of many parasitic zoonoses is inadequate, in part because it lacks reliable information on parasite identification which is essential for making epidemiological determinations (Thompson *et al.*, 2009). Thus, more experimental approaches are needed to produce substantial knowledge. In the last two decades, ecologists, forest engineers and other professionals have joined veterinarians to form multidisciplinary research teams (Gortázar *et al.*, 2007). Among the most intriguing aspects of several studies is the link between wildlife pathogens, the

environment and human activities. This net of factors creates a dynamic situation where new pathogens or new hosts emerge, changes in population density or host behavior affects disease prevalence and disease agents can suddenly boost their virulence and widen their host range. Many parasites, especially if environmental changes occur, can infect multiple host species and these parasites are primarily responsible for emerging infectious disease outbreaks in humans, livestock and wildlife. Moreover, the ecological and evolutionary factors that constrain or facilitate such emergences are poorly understood. In addition to surveillance, three basic forms of disease management strategies for wildlife are known: prevention of introduction of disease, control of existing disease or, almost impossible, eradication. Wildlife disease control begins with surveillance, knowing which diseases are present, their past and current distribution and the trends in their prevalence (Gortázar *et al.*, 2007).

The objective of this study is to investigate the presence of selected parasitic infections with protozoan parasites, namely *Leishmania infantum*, *Toxoplasma gondii* and *Neospora caninum* in red-foxes from the Alentejo area. A prevalence study in nonspecific predators, as red-foxes are, may attempt to gauge the threat of this same parasitosis on to endangered wild carnivores. On the Portuguese mainland, where attempts are being made to reestablish the Iberian lynx, there are few reports on this issue. Prevalences of the three protozoan of this study in this endangered species are not well known. More studies are required on the prevalence of lynx infection with *Leishmania infantum*; it is reported by Sobrino *et al.* (2008) and CFSPH (2009) that *L. infantum* has been detected in the Iberian lynx; DNA of the parasite was detected by polymerase chain reaction (PCR) in 1 of 4 animals in Spain (Sobrino *et al.*, 2008). There is also limited information on *Toxoplasma gondii* infection in the Iberian lynx (García-Bocanegra *et al.*, 2010). Although *T. gondii* infection is common in felids, clinical toxoplasmosis is believed to be rare (Roelke *et al.*, 2008). García-Bocanegra *et al.* (2010) has analyzed over half of the estimated remaining population of Iberian lynx, to establish seroprevalence to *T. gondii* in both free-ranging and captive aimals. The high seroprevalence of *T. gondii* observed in the Iberian lynx (62.8%, using MAT – modified agglutination test) indicated widespread of *T. gondii* infection in the areas where

this endangered species survives. 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 (García-Bocanegra *et al.*, 2010, Millán *et al.*, 2008, Roelke *et al.*, 2008). In addition, 12 animals seroconverted during the study period of García-Bocanegra *et al.* (2010). The higher seroprevalence observed in adult and sub-adult 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* (García-Bocanegra *et al.*, 2010). *Toxoplasma gondii* can be 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 lactogenic infections of kittens have also been reported (García-Bocanegra *et al.*, 2010, Millán *et al.*, 2008, Roelke *et al.*, 2008, Sobrino *et al.,* 2007). 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%) (García-Bocanegra *et al.*, 2010). The seroprevalence observed by Almería *et al.* (2004) in wild rabbits sharing the habitat with Iberian lynx in some areas of Spain was up to 53.8% (Catalonia). 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 (García-Bocanegra *et al.*, 2010). 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) and for other wild carnivores like the red-fox. Ingestion of food infected with tissue cysts or water contaminated with oocysts are the most likely sources of infection in captive-born lynx (García-Bocanegra *et al.*, 2010). Although *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 and that seropositive lynx might have shed oocysts (García-Bocanegra *et al.*, 2010, Millán *et al.*, 2008). Even in cats, coprological surveys are unrewarding because at any given time less than 1% of domestic cats were found to have shed oocysts (García-Bocanegra *et al.*, 2010). Domestic cats excrete oocysts for only 1–2 weeks during the initial phase of infection by *T. gondii* when they become infected for the first time;

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. 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 (García-Bocanegra *et al.*, 2010). Millán *et al.* (2008) reported the presence of antibodies against *Neospora caninum* in the Iberian lynx, for the first time, with a seroprevalence of 21%, using ELISA and IFAT.

The European wild rabbit (*Oryctolagus cuniculus*) is native to the Iberian Peninsula and plays a key ecological role in Mediterranean ecosystems and their biodiversity (Delibes-Mateos *et al.,* 2008). Rabbits are common prey for many carnivorous species with individuals debilitated by any cause being easy prey. They are mainly preyed upon by red foxes (*Vulpes vulpes*) and Iberian lynx (*Lynx pardinus*) (Almería *et al.,* 2004). Because diet is the main route for parasite ingestion it is important to know the infection seroprevalences in this most hunted prey. More than 25 years ago, Kapperud (1978) reported *T. gondii* antibodies in 21% of 34 wild rabbits in Norway and Sweden, using the Sabine-Feldman dye-test. In Victoria, Australia, Cox (Cox *et al.*, 1981) found a 7.5% prevalence in 1697 wild rabbits, by indirect immunofluorescence testing (IFAT). In the Czech Republic, Hejlícek (Hejlícek *et al.*, 1997) using CFT (complement fixation test) found *T. gondii* antibodies in 8% of 79 wild rabbits. Almería (Almería *et al.,* 2004) detected a 14.2% seroprevalence, using MAT, in wild rabbits in Spain, while in north Yorkshire in the UK Hughes (Hughes *et al.*, 2008) identified a 68.4% seroprevalence in wild rabbits, by PCR.

In 2008 *N. caninum* was, for the first time, isolated from brain samples of wild rabbits in Yorkshire, UK; 10.5% prevalence from 6 out of 57 rabbits was identified by PCR (Hughes *et al.,* 2008). Indeed, rabbits can play an important role as infection reservoir for wild species that prey upon them.

The knowledge of the prevalence of a given infection in red-foxes may be an important step for the evaluation of such pathological agents, either in their prey or other predators and ultimately as ecosystem contaminants.

## *Leishmania infantum*

Visceral leishmaniasis is a major potentially fatal zoonotic infection caused by *Leishmania infantum* in the Old World. The domestic dog is the main reservoir of human infection, and phlebotomine sand flies are the biological vectors of this protozoal disease. The parasite life cycle is presented in Figure 1.

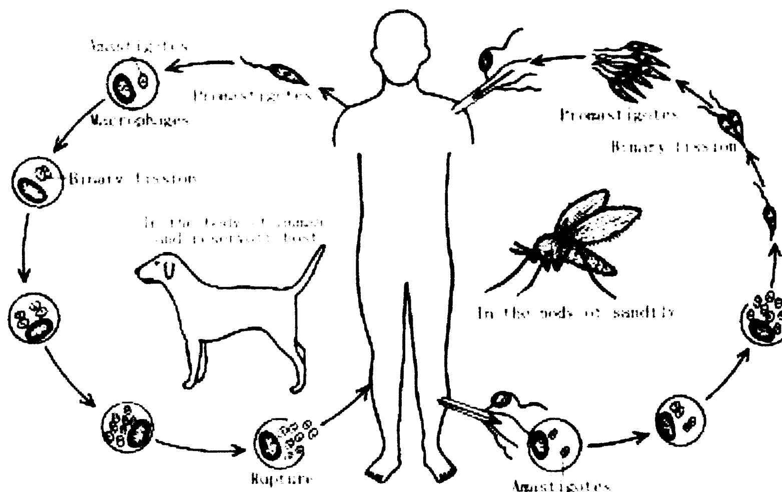

Figure 1. Life cycle of *Leishmania infantum*. In http://www.google.pt/imgres?imgurl=http://jpkc.sysu.edu.cn [12.08.2010].

It has been estimated that at least 2.5 million dogs are infected in southwestern Europe alone (Baneth *et al.*, 2008). It is necessary to understand the potential spill-over of this pathogenic agent toward wild animals, especially the Iberian lynx. Once seroprevalences and parasite DNA have been demonstrated in wildlife, one can thereof conclude autochthony of transmission. The most important animal host species, in which *L. infantum* has been identified, are presented in Table I.

Table I. Species with reported presence of *Leishmania infantum* (adapted from CFSPH, 2009).

| **Species** |
|---|
| Wolves (*Canis lupus*) |
| Hoary zorros (Pseudalopex vetulus) |
| Bush dogs (*Speothos venaticus*) |
| Wild agouti (*Dasyprocta agouti*) |
| White-eared opossums (*Didelphis albiventris*) |
| Egyptian mongooses (*Herpestes ichneumon*) |
| Genets (*Geneta geneta*) |
| Iberian lynx (*Lynx pardinus*) |
| Rodents |
| Bat (*Carollia perspicillata*) |
| Cats (*Felis domesticus*) |

Cats (*Felis domesticus*) are still regarded as unusual hosts for *Leishmania* spp., although the first record of feline leishmaniasis (FL) dates back 1912, when the first infected cat was identified in Algeria (Maroli *et al.*, 2007, Solano-Gallego *et al.*, 2007). Since then, FL cases have been described by different authors in different geographical areas as presented in Table II.

Table II. Leishmaniasis in cats.

| **Prevalence of *Leishmania infantum* in cats (*Felis domesticus*)** | | | |
|---|---|---|---|
| % Positive | Test | Location | Reference |
| 3% | PCR | Spain | Tabar *et al.*, 2008 |
| 10% | PCR | Iran | Hatam *et al.*, 2009 |
| 30.4% | PCR | Portugal | Maia *et al.*, 2008 |
| 0.9% | IFAT | Tuscany, Italy | Poli *et al.*, 2002 |
| 1.3% | IFAT | Madrid | Ayllon *et al.*, 2008 |
| 5.3% | ELISA-IgG | Northern Spain and Balearic Islands | Solano-Gallego *et al.*, 2007 |
| 6.3% | ELISA-prot A | Northern Spain and Balearic Islands | Solano-Gallego *et al.*, 2007 |
| 12.5% | IFAT | Alpines Maritimes, Italy | Mancianti, 2004 |
| 16.3% | IFAT | Abruzzo Region, Italy | Vita *et al.*, 2005 |
| 60% | IFAT | Southern Spain | Martín-Sanchez *et al.*, 2007 |

Although FL in domestic felid infection is unusual, it has been identified as the cause of dermal or systemic disease and often associated with viral infections proven to spread from domestic to wild felids, in particular, the Iberian lynx. FL occurs in endemic areas of canine leishmaniasis and this evidence should be borne in mind when considering the vulnerability of the endangered *Lynx pardinus*.

There are studies carried out throughout the Mediterranean countries, which have documented significant seroprevalences of *L. infantum* in *Vulpes vulpes*. Molecular prevalences ranged from 14.1% (assessed by PCR) in Spain (Sobrino *et al.*, 2008), to 74% (assessed by PCR) in the province of Guadalajara (Criado-Fornelio *et al.*, 2000), and to 40% by PCR in southern Italy (Dipineto *et al.*, 2007). Seroprevalences ranged from 18% (assessed by IFAT-immunofluorescence antibody test and ELISA-enzyme-linked immunosorbent assay) in Italy (Mancianti *et al.*, 1994) to 5.63% (assessed by IFAT) in Arrábida (Abranches *et al.*, 1984) and to 5% (assessed by ELISA) in Israel (Baneth *et al.*, 1998). No significant sex or age differences in prevalence were observed (Sobrino *et al.*, 2008). The majority of foxes, subjected to necropsy, were in a healthy body condition, and can be considered, at least theoretically, as subclinical carriers (Dipineto *et al.*, 2007). Mancianti *et al.* (1994) revealed that experimentally infected foxes that developed the highest IFAT titers also developed overt disease signs such as weight loss, furfuraceous dermatitis, depilation, onychogryphosis and skin ulcers. Table III summarizes all red-fox seroprevalences referred to in the text.

Table III. Prevalence (serological and molecular) of *Leishmania infantum* in red-foxes.

| **Prevalence of *Leishmania infantum* in red-foxes (*Vulpes vulpes*)** | | | |
|---|---|---|---|
| % Positive | Test | Location | Reference |
| 5% | ELISA | Israel | Baneth *et al.*, 1998 |
| 5.63% | IFAT | Arrábida, Portugal | Abranches *et al.*, 1984 |
| 14.1% | PCR | Spain | Sobrino *et al.*, 2008 |
| 18% | ELISA and IFAT | Italy | Mancianti *et al.*, 1994 |
| 40% | PCR | Italy | Dipineto *et al.*, 2007 |
| 74% | PCR | Guadalajara, Spain | Criado-Fornelio *et al.*, 2000 |

Vulpine infection with *L. infantum* in endemic canine leishmaniasis areas is a reflection of vectors infection and spreading (autochtony of transmission). Once susceptibility of other wild species is proven, as for the Iberian lynx in which the infection was detected, knowledge of vulpine *L. infantum* prevalence can be used in predicting the risk to *Lynx pardinus* in a particular situation.

## *Toxoplasma gondii*

*Toxoplasma gondii* is an obligatory intracellular protozoan parasite of worldwide distribution. Virtually all warm-blooded hosts can be intermediate hosts of *T. gondii* (Sobrino *et al.*, 2007), although their susceptibility to infection and especially to disease may considerably differ. Domestic and free-ranging (wild) felids are the only definitive hosts for *T. gondii*. The parasite life cycle is presented in Figure 2.

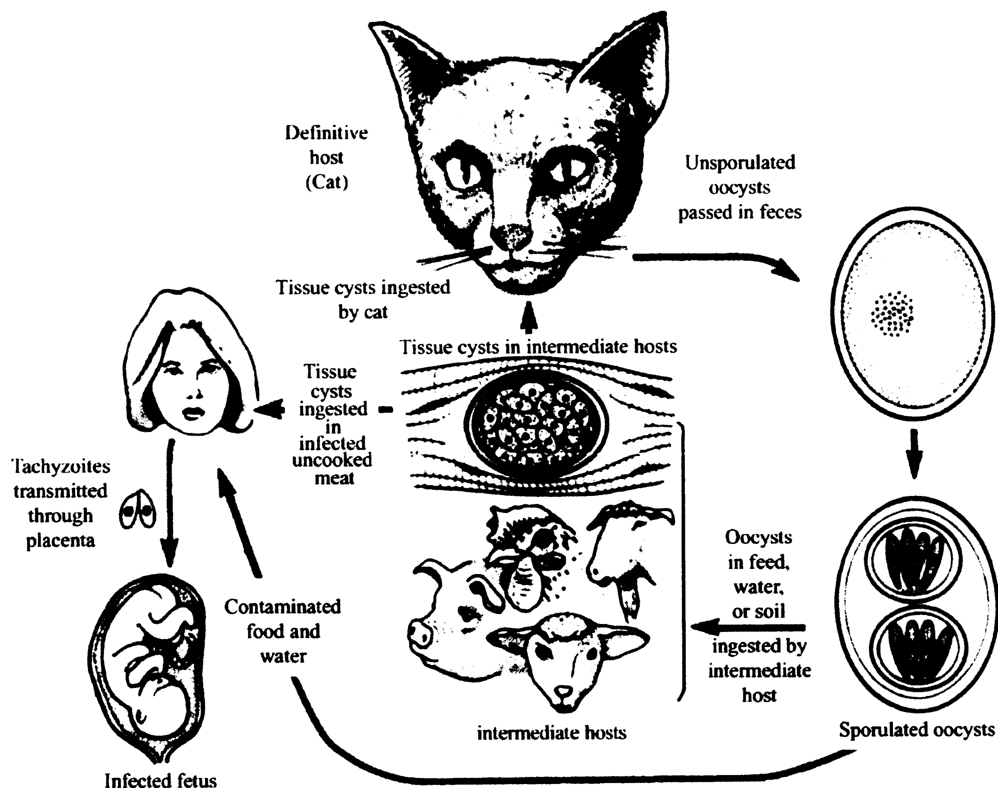


Figure 2. Life cycle of *Toxoplasma gondii* (Hill & Dubey, 2002).

In infected cats, oocysts are produced and shed in feces (Sobrino *et al.*, 2007). Cats may excrete millions of oocysts during a relatively short period (1–2 weeks); however, the enormous numbers shed yeld a widespread

contamination of the environment. Oocysts in soil do not always stay there, as invertebrates such as flies, cockroaches, dung beetles and earthworms can mechanically spread these oocysts and even carry them onto food. Sporulated oocysts survive for long periods under most ordinary environmental conditions. Infection rates in cats reflect the rate of infection in local avian and rodent populations because cats are thought to become infected by eating these creatures. The more oocysts there are in the environment, the more likely it is that prey animals will become infected, and this results in increased infection rates in cats and in carnivorous wild animals that feed on this prey (Hill & Dubey, 2002). Miró *et al.* (2004) studied the seroprevalence in stray, farm and household cats from Spain and detected a level of 36.9%, 33.3% and 25.5%, respectively using IFAT. In Florence, by using MAT, Mancianti *et al.* (2010) identified a 44% seroprevalence of infection in an urban population of colony cats. For the first time, the prevalence of *T. gondii* in feral cats (*Felis silvestris catus*) was identified as 84.7% by the use of MAT (Millan *et al.*, 2009).

Not only the definitive, but also the intermediate host may be infected by ingestion of water or food contaminated with oocysts and by ingestion of tissue cysts or by vertical transmission (Sobrino *et al.*, 2007). Soil is also an environmental source of *T. gondii* infection (Lass *et al.*, 2009) as it offers favorable conditions for the survival of oocysts, such as consistent shade and moisture underground (Afonso *et al.*, 2008). Oocysts may remain infective in soil for more than 1 year and in water for up to 54 months (Lass *et al.,* 2009). *T. gondii* can cause serious disease in humans and seems to be common in wild animals, especially canids (Jakubek *et al.*, 2007). Spatial distribution of areas contaminated by oocysts may be highly heterogeneous in an urban environment, but intermediate levels of contamination can be expected to be frequent in rural environments where cat density is intermediate and territoriality is more pronounced (Afonso *et al.*, 2008). It is undoubtedly necessary to promote surveys of *T. gondii* infection in free-ranging animals which can provide estimates of environmental contamination and circulation of *T. gondii* in domestic and wild ecosystems (Sobrino *et al.*, 2007). Table IV presents prevalence results in different animal species all over the world.

Table IV. Serological and molecular prevalences of *Toxoplasma gondii*.

| Prevalence of *Toxoplasma gondii* | | | | | |
|---|---|---|---|---|---|
| Species | | % Positive | Test | Location | Reference |
| Iberian Lynx (*Lynx pardinus*) | | 62.8% | MAT | Spain | García-Bocanegra *et al.*, 2010 |
| Rodents | *Arvicola terrestris* | 5% | ELISA | Geneva, Switzerland | Reperant *et al.*, 2008 |
| | *Microtus arvalis* | 3.1% | ELISA | Geneva, Switzerland | Reperant *et al.*, 2008 |
| | *Apodemus flavicollis* | 2.5% | ELISA | Geneva, Switzerland | Reperant *et al.*, 2008 |
| | *Mus domesticus* | 59% | PCR | Manchester, UK | Murphy *et al.*, 2008 |
| Wild rabbits (*Oryctolagus cuniculus)* | | 8% | CFT | Czech Republic | Hejlícek *et al.*, 1997 |
| | | 14.2% | MAT | Spain | Almeria *et al.*, 2004 |
| | | 17.4% | IFAT | Victoria, Australia | Cox *et al.*, 1981 |
| | | 21% | Sabine-Feldman dye-test | Norway and Sweden | Kapperud, 1978 |
| Ovine | | 57% | cELISA | Galicia, Spain | Panadero *et al.*, 2010 |
| | | 59% | ELISA | Czech Republic | Bártová *et al.*, 2009 |
| | | 78% | Toxotest-MT® | Central Italian Alps | Gaffuri *et al.*, 2006 |
| | | 84.5% | MAT | Serbia | Klun *et al.*, 2006 |
| | | 97.4% | IFAT | Switzerland | Hassig *et al.*, 2003 |
| | fetus | 15% | PCR | Switzerland | Hassig *et al.*, 2003 |
| | fetus | 18.1% | PCR | Sardinia, Italy | Masala *et al.*, 2007 |
| | placenta | 13.1% | PCR | Sardinia, Italy | Masala *et al.*, 2007 |
| Bovine | | 76.3% | MAT | Serbia | Klun *et al.*, 2006 |
| Wild boars (*Sus scrofa*) | | 26.2% | IFAT | Czech Republic | Bártová *et al.*, 2006 |
| | | 15% | | Czech Republic | Hejlícek *et al.*, 1997 (a) |
| | | 19.3% | | Austria | Edelhofer *et al.*, 1996 (a) |
| | | 33% | | Germany | Rommel *et al.*, 1967 (a) |
| Wild boars (*Sus scrofa*) | | 36.3% | cELISA | Spain | Ruiz-Fons *et al.*, 2006 |

**Prevalence of *Toxoplasma gondii* (continued)**

| Species | | % Positive | Test | Location | Reference |
|---|---|---|---|---|---|
| | | 38.5% | | Slovakia | Catár, 1972 (a) |
| Domestic pigs (*Sus scrofa domestica*) | | 4.7% | IB | Netherlands | Kijlstra *et al.*, 2004 |
| | | 15.6% | MAT | Portugal | Sousa *et al.*, 2010 |
| | | 19% | IFAT | Germany | Damriyasa *et al.*, 2004 |
| | | 28.9% | MAT | Serbia | Klun *et al.*, 2006 |
| | | 95% | MAT | New England, USA | Lehmann *et al.*, 2003 |
| Avian | *Columba livia* | 4.6% | DAT | Lisbon, Portugal | Waap *et al.*, 2008 |
| | Free-ranging chickens (*Gallus domesticus)* | 27.1% | MAT | Portugal | Dubey *et al.*, 2006 |
| | Eurasian Buzzards (*Buteo buteo)* | 79% | MAT | France | Aubert *et al.* 2008 |
| | Tawny Owls (*Strix aluco*) | 50% | MAT | France | Aubert *et al.* 2008 |
| | Barn Owls (*Tyto alba)* | 11% | MAT | France | Aubert *et al.* 2008 |
| Horses (*Equus caballus*) | | 1% | DAT | Sweden | Jakubeck *et al.*, 2006 |
| | Wild horses | 0.4% | MAT | Central Wyoming, USA | Dubey *et al.*, 2003 |

(a) – cited by Bártová *et al.* (2006).

Sobrino *et al.* (2007) detected a seroprevalence of 67.4% infection in red-foxes. Blood samples were collected from the heart or chest cavity of dead animals and from the cephalic vein of live animals; the prevalence of antibodies to *T. gondii* was determined by the MAT with a higher seroprevalence in adults. Reports of *T. gondii* in red foxes in rural Ireland showed a 56% seroprevalence by IFAT (Murphy *et al.*, 2007) and in the USA a 85.9% seroprevalence by MAT (Dubey *et al.*, 1999). Jakubek *et al.* (2007) determined 68% of 337 red foxes to be infected by direct DAT in Hungary and Aubert *et al.* (2010) detected 73.7% of 19 red foxes to be infected by MAT in France. The high seroprevalence for *T. gondii* in foxes might be explained by the widespread occurrence of *T. gondii* in their diets (Jakubek *et al.*, 2007). Red-fox seroprevalences referred to in the text are summarized in Table V.

Table V. Seroprevalence of *Toxoplasma gondii* in red-foxes.

| **Seroprevalence of *Toxoplasma gondii* in red-fox (*Vulpes vulpes*)** | | | |
|---|---|---|---|
| % Positive | Test | Location | Reference |
| 56% | IFAT | Ireland | Murphy *et al.*, 2007 |
| 67.4% | MAT | Spain | Sobrino *et al.* 2007 |
| 68% | DAT | Hungary | Jakubek *et al.* 2007 |
| 73.7% | MAT | France | Aubert *et al.* 2010 |
| 85.9% | MAT | USA | Dubey *et al.*, 1999 |

## *Neospora caninum*

*Neospora caninum* is an apicomplexan intracellular protozoan parasite, with genus and species first described by Dubey and coworkers in 1988 (Dubey *et al.*, 2003). Neosporosis has emerged as a serious infectious disease of cattle and dogs worldwide. Dogs are both the intermediate and definitive host for *N. caninum and* clinical neurologic symptoms of neosporosis (Dubey *et al.*, 2003, Wapenaar *et al.*, 2006) as well as cutaneous signs were reported in dogs (Dubey *et al.*, 2003). The parasite life cycle is presented in Figure 3.

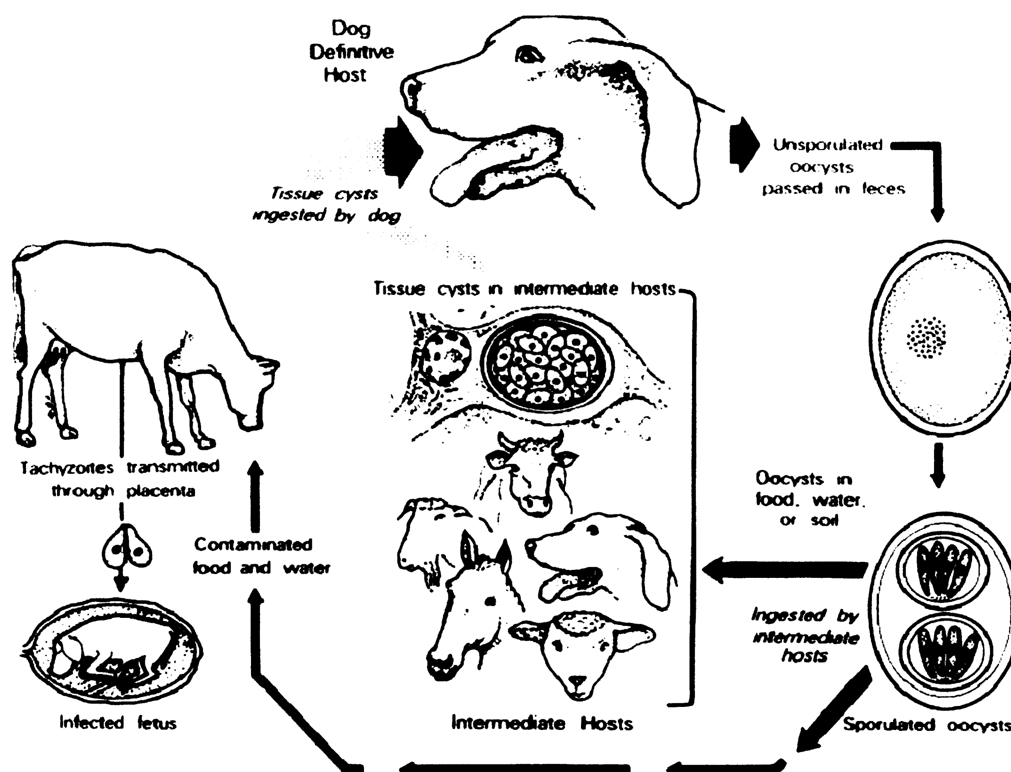


Figure 3. Lyfe cycle of *Neospora caninum* (Dubey, 2003).

There is still a lack of information regarding the frequency of the shedding of oocysts and the survival of the oocysts in the environment (Dubey *et al.*, 2003), but the number of cases reported so far is extremely low. Canine serologic prevalences, reflecting primarily an intermediate host function of the dog, are displayed in Table VI.

Table VI. Seroprevalence of *Neospora caninum* in dogs.

| **Seroprevalence of *Neospora caninum* in dogs** | | | | |
|---|---|---|---|---|
| | % Positive | Test | Location | Reference |
| | 0.5% | ELISA | Sweden | Jakubek *et al.*, 2006 |
| Household dogs | 2.9% | IFAT | Spain | Collantes-Fernández *et al.*, 2008 |
| Household dogs | 6.4% | IFAT | Italy | Cringoli *et al.*, 2002 |
| | 6.7% | IFAT | Brazil | Mineo *et al.*, 2001 |
| Kennel and pet dogs | 10.9% | ELISA | north-eastern Italy | Capelli *et al.*, 2004 |
| | 20.9% | ELISA | southern Italy | Paradies *et al.*, 2007 |
| Stray dogs | 25% | ELISA | Brazil | Gennari *et al.*, 2002 |
| | 37.8% | IFAT | Argentina | Basso *et al.*, 2001 |
| Farm dogs | 51% | IFAT | Spain | Collantes-Fernández *et al.*, 2008 |

*Neospora caninum* causes abortion in both dairy and beef cattle (Hemphill & Gottstein, 2000). Cows of any age may abort from 3-month-gestation to term. Most neosporosis-induced abortions occur at 5-6-month-gestation. Fetuses may die in utero, be resorbed, mummified, autolyzed, stillborn, born alive with clinical signs, or born clinically normal but chronically infected. Neosporosis-induced abortions occur year-round. The age of the dam, lactation number, and history

of abortion generally do not affect the rate of congenital infection but there are reports indicating that in persistently infected cattle vertical transmission is more efficient in younger than older cows (Dubey *et al.*, 2003). Bovine prevalences are studied all over the world and these studies are summarized in Table VII.

Table VII. Serological and molecular prevalence of *Neospora caninum* in cattle.

| **Prevalence of *Neospora caninum* in cattle** | | | | |
|---|---|---|---|---|
| Cattle | % Positive | Test | Location | References |
| dairy | 0.7% | ELISA | Norway | Klevar *et al.*, 2010 |
| beef | 2.8% | ELISA | Netherlands | Loobuyc *et al.*, 2009 |
| dairy | 3.19% | IFAT | Czech Republic | Vaclavek *et al.*, 2003 |
| dairy | 7.9% | ELISA | Germany | Schares *et al.*, 2003 |
| aborted fetus | 10.7 | ELISA and IFAT | Spain | Pereira-Bueno *et al.*, 2003 |
| beef and dairy | 11% | ELISA | Italy | Otranto *et al.*, 2003 |
| breeder bulls | 11.2-13.3% | IFAT | Spain | Caetano-da-Silva *et al.*, 2004 |
| dairy | 15.2% | ELISA | Greece | Sotiraki *et al.*, 2008 |
| aborted fetus | 15.3% | PCR | Spain | Pereira-Bueno *et al.*, 2003 |
| dairy | 15.6% | ELISA | Southest Slovakia | Reiterova *et al.*, 2009 |
| dairy | 15.7% | NAT, ELISA and IFAT | Northwest Spain | Gonzalez-Warleta *et al.*, 2008 |
| dairy | 17.8% | IFAT | South Brazil | Corbellini *et al.*, 2006 |
| aborted fetus | 21% | PCR | Switzerland | Sager *et al.*, 2001 |
| dairy | 26.2% | ELISA | Northern Slovakia | Reiterova *et al.*, 2009 |
| dairy | 28% | DAT | Portugal | Canada *et al.*, 2004 |
| dairy | 30.8% | ELISA | Italian Alps | Rinaldi *et al.*, 2005 |
| dairy | 32% | ELISA | Italian Alps | Rinaldi *et al.*, 2007 |
| beef and dairy | 44% | ELISA | Switzerland | Sager *et al.*, 2001 |
| dairy | 46% | DAT | Portugal | Canada *et al.*, 2004 |
| beef | 55.1% | ELISA | Spain | Quintanilla-Gozalo *et al.*, 1999 |
| dairy | 58% | ELISA | Netherlands | Dijkstra *et al.*, 2008 |
| dairy | 63% (1994) | ELISA | Sweden | Stenlund *et al.*, 2003 |
| dairy | 87%(1999) | ELISA | Sweden | Stenlund *et al.*, 2003 |
| dairy | 83.2% | ELISA | Spain | Quintanilla-Gozalo *et al.*, 1999 |

Clinical neosporosis has additionally been reported in sheep, goats, deer, a rhinoceros, and horses; and antibodies to *N. caninum* have been found in the sera of water buffaloes, red and gray foxes, coyotes, and camels, and felids (Dubey *et al.*, 2003) as well as in HIV immunocompromised humans (Lobato *et*

*al.*, 2006, Robert-Gangneux & Klein, 2009, Tranas *et al.*, 1999). In Table VIII, results of DNA-prevalences and seroprevalences in domestic and wild animals are presented.

Table VIII. Serological and molecular prevalence of *Neospora caninum*.

| **Prevalence of *Neospora caninum*** | | | | | |
|---|---|---|---|---|---|
| Species | | % Positive | Test | Location | Reference |
| iberian lynx (*Lynx pardinus*) | | 21% | ELISA and IFAT | Spain | Millán *et al.*, 2008 |
| wild rabbits (*Oryctolagus cuniculus)* | | 10.5% | PCR | Yorkshire, UK | Hughes *et al.*, 2008 |
| ovine | aborted placenta | 2% | PCR | Sardinia, Italy | Masala *et al.*, 2007 |
| | | 10.1% | cELISA | Galicia, Spain | Panadero *et al.*, 2010 |
| | | 12% | ELISA | Czech Republic | Bártová *et al.*, 2009 |
| caprine | abortion samples | 8.6% | PCR | Sardinia, Italy | Masala *et al.*, 2007 |
| pigs | | 1.1% | ELISA | Germany | Damriyasa *et al.* (2004) |
| wild boars | | 18.1% | cELISA | Czech Republic | Bártová *et al.*, 2006 |
| rodents | *Apodemus sylvaticus* | 3.6% | PCR | Italy | Ferroglio *et al.*, 2007 |
| | *Mus musculus* | 14% | PCR | Italy | Ferroglio *et al.*, 2007 |
| | *Rattus norvegicus* | 14% | PCR | Italy | Ferroglio *et al.*, 2007 |
| Feral cats (*Felis silvestris catus*) | | 6.8% | cELISA | Majorca, Spain | Millan *et al.*, 2009 |
| cats | | 0.5% | IFAT | Hungary | Hornok *et al.*, 2008 |
| horses | | 1% | ELISA | Sweden | Jakubek *et al.*, 2006 |
| | | 23% | | France | Pitel *et al.*, 2001 |

**Prevalence of *Neospora caninum* (continued)**

| Species | | % Positive | Test | Location | Reference |
|---|---|---|---|---|---|
| | | 28% | IFAT | Italy | Ciaramella *et al.*, 2004 |
| | wild horses | 31% | NAT | Central Wyoming, USA | Dubey *et al.*, 2003 |

Jakubek *et al.* (2007) studied the prevalence of the infection in red-foxes in Hungary. Blood samples originating from 337 red foxes were collected during the oral immunization program of foxes against rabies. Antibodies to *N. caninum* were demonstrated by ELISA and a prevalence of 1.5% was detected. Vertical transmission of *N. caninum* similar to that in dogs has not yet been demonstrated in red foxes (Jakubek *et al.,* 2007). Little is known about the epidemiology of *Neospora caninum* infection in humans, but human exposure to *N. caninum* is suggested by surveys using techniques such as IB, IFAT and ELISA (Lobato *et al.*, 2006, Tranas *et al.*, 1999), although clinical implication of exposure to *N. caninum* infection has never been documented in humans.

In the work of Almería *et al.* (2002), in Spain, the first investigating tissue samples from red-foxes, a molecular prevalence of 10.7% was determined by PCR for *N. caninum*. Jakubek *et al.* (2007) refered to other studies such as the one in Belgium with a seroprevalence of 17% by IFAT (Buxton *et al.*, 1997), in Ireland 1.5% (Wolfe *et al.*, 2001) and in the United Kingdom 0.9 (Hamilton *et al.*, 2001). In Sweden, no positive serology was determined for *N. caninum* (Jakubek *et al.*, 2001) in foxes, nor in Austria. The presence of antibodies to *N. caninum* in red-foxes indicates that this parasite species has a sylvatic epidemiological parameter in its cycle. This suggests that red-foxes, as well as other wild canids, may putatively play a role in maintaining this cycle in many European countries, although oocyst shedding by foxes has not been described so far. The infection of red-foxes is likely to originate from *N. caninum* infected prey since antibodies to this obligate intracellular parasite species have been detected in hares. Ingesting sporulated oocysts of *N. caninum* may also infect the foxes (Jakubek *et al.*, 2007). Wapenaar *et al.* (2006) described oocysts morphologically and morphometrically similar to oocysts of *N. caninum* observed in feces of free-ranging foxes. But such oocysts can morphologically

not be discriminated from oocysts originating from e.g. *Toxoplasma gondii* (coprophagy) or *Hammondia* spp. Further studies are needed to investigate the role of wild canids in the epidemiology of neosporosis and to determine whether or not *N. caninum* has an intestinal phase in red foxes resulting in oocyst shedding (Jakubek *et al.,* 2007). Red-fox serological and molecular prevalences of *Neospora caninum* are summarized in Table IX.

Table IX. Serological and molecular prevalence of *Neospora caninum* in red-foxes.

| **Prevalence of *Neospora caninum* in red-foxes (*Vulpes vulpes*)** | | | |
|---|---|---|---|
| % Positive | Test | Location | Reference |
| 0.9 | IFAT | UK | Hamilton *et al.*, 2001 |
| 1.5% | ELISA | Sweden | Jakubek *et al.*, 2007 |
| 1.5% | IFAT | Ireland | Wolfe *et al.*, 2001 |
| 10.7% | PCR | Spain | Almería *et al.*, 2002 |
| 17% | IFAT | Belgium | Buxton *et al.*, 1997 |

Vertical transmission is considered the most important route of *N. caninum* infection (Corbellini *et al.* 2006). Carnivores can acquire infection by ingestion of infected tissues (Dubey *et al.*, 2003). Wild carnivores can be infected by opportunistic feeding on aborted fetuses and placenta, as well as on infected carcasses left in the pasture.

## Material & Methods

Red-foxes were collected from hunting areas in the Alentejo – traditional farms where foxes are hunted from November to February as part of regional predator control policies. Figure 4 schematizes the study area and figure 5 shows it in detail. Table X presents the hunting locations and the fox's numbers captured in each location. Table XI presents the host group characteristics.

Figure 4. Study area.

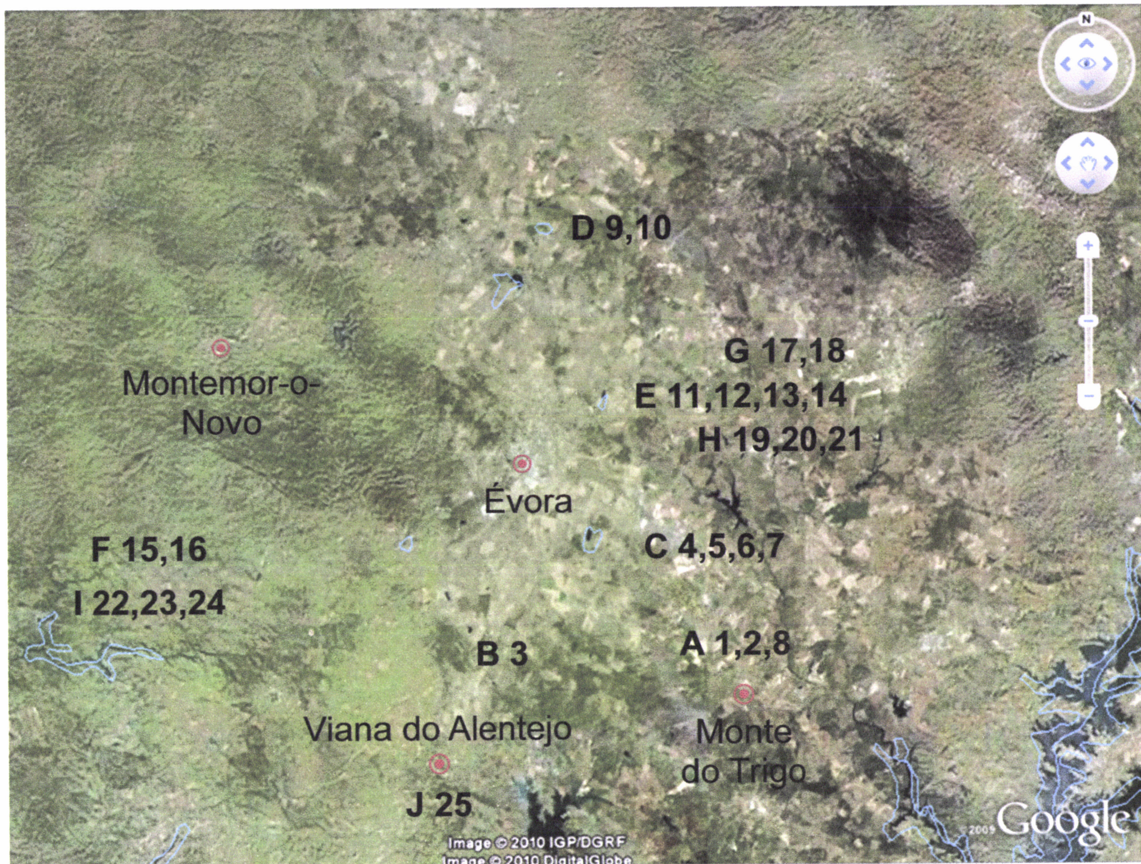


Figure 5. Study area in detail. A – Monte Negro (foxes 1,2,8); B – Monte das Silveiras (fox 3); C – Vale da Moura (foxes 4,5,6,7); D – Comenda Grande (foxes 9,10); E – Herdade do Alpendre (foxes 11,12,13,14); F – Herdade dos Nabos (foxes 15,16); G – Herdade Vale de Melhorado de Baixo (foxes 17,18); H – Herdade da Furada (foxes 19,20,21); I – Foros da Tojeira (foxes 22,23,24); J – Herdade das Prudências (fox 25).

Table X. Red-fox hunting location.

| Hunting location | Foxes number (n = 25) |
|---|---|
| A - Monte Negro - Nossa Sra. de Machede - Évora | 1,2,8 |
| B - Monte das Silveiras - Viana do Alentejo | 3 |
| C - Vale de Moura - Torre de Coelheiros | 4,5,6,7 |
| D - Comenda Grande - Igrejinha - Évora | 9,10 |
| E - Herdade do Alpendre - Monte de Trigo - Évora | 11,12,13,14 |
| F - Herdade dos Nabos - Montemor-o-Novo | 15,16 |
| G - Herdade Vale de Melhorado de Baixo - Évora | 17,18 |
| H - Herdade da Furada - S.Vicente de Pigeiro - Évora | 19,20,21 |
| I - Foros da Tojeira - Montemor-o-Novo | 22,23,24 |
| J - Herdade das Prudências - Viana do Alentejo | 25 |

Table XI. Red-fox group characteristics.

| Vulpes vulpes | Gender | |
|---|---|---|
| | Male | Female |
| n=25 | 14 | 11 |

All animals were adults and in a good body condition. All animals were skinned by hunters except for the last 4, which were infested with fleas. In the months of January and February, females were either pregnant or lactating: 85.7% pregnant and 14.3% lactating.

Necropsies were performed on the hunting day, after collection in the field and immediately upon arrival at the laboratory. Samples of bone marrow, liver, spleen, lymph nodes, muscle, tongue, diaphragm, heart, intra-cardiac blood and brain were frozen (for further DNA extraction by DNeasy Blood & Tissue kit® (Qiagen), modified with 3 cycles of freezing/defreezing and posterior PCR amplification), and smears of liver, spleen, lymph nodes and bone marrow were performed by apposition (after GIEMSA staining for optical observation). It was not possible to collect samples of intra-cardiac blood from 7 specimens, mainly because they had been shot into the heart. Blood smears were performed by extension and GIEMSA stained for optical observation. From those animals from which it was not possible to obtain blood, muscle was collected and frozen for later extraction of muscular juice by centrifugation (5000 rpm for 20 min at 20ºC), for serological detection of antibodies, after use in other serological studies. All organs (except blood) were preserved in formaline 10% for posterior histological preparation with Hematoxyline-Eosine coloration and microscopic observation. Brain samples were digested with HCl and pepsin (30 min at 26ºC) and centrifuged at 770 g for 15 min at 4ºC; the pellet was resuspended in DMEM 10% FCSI and a volume of 700 µl was inoculated onto Vero cell cultures. At 48 h after inoculation, the medium was changed to DMEM 2% FCSI with subsequent medium changes every 3 days. Inoculated cultures were inspected daily using an inverted microscope for the presence of free tachyzoites. Intestinal content preservation was performed for other parasitic studies. Bones of the anterior and posterior limbs were also tagged and frozen for posterior use in the "Ostheo" Library and to further study

their singular-species composition and structure. Antigen preparation were performed for respective test systems, and four serologic tests were carried out (namely ELISA, DAT, IFAT and MAT) as described as follows:

## *Leishmania infantum*

### ELISA

A total of 18 sera and 24 muscular juices were used in this serological test. The *Leishmania*-ELISA included the following steps: the *Leishmania* antigen was a soluble (somatic) extract of in vitro cultivated *Leishmania infantum* promastigotes; coating Dynatech polystyrene plates was done at a concentration of 0.4 mg protein/ml carbonate buffer (pH 9.6) at 4 °C for 24 h. The washing, blocking and serum dilution solution was PBS pH 7.2 containing 0.3% Tween 20 (PBS–Tween). The test and positive/negative control sera were diluted 1:100 in PBS–Tween containing additionally 0.5 mg bovine hemoglobin per ml. Serum incubations were for 45 min at 37 °C. The second antibody was an alkaline phosphatase-conjugated rabbit anti-canine antibody IgG antibody (Sigma Immmunochemicals, cat. no. A 6042) diluted at 1:6000 in PBS–Tween. The conjugate was incubated for 30min at 37°C. The substrate used was 4-nitrophenylphosphate at 1mg/ml. The enzyme reaction was stopped after 30 min with 3N NaOH, and absorbance values were determined at A405 nm using a Bio-Rad CODA Automated EIA Analyzer reader coupled to a computer with the corresponding Dynatech Bio-tek reader. Reading were taken at 5 min, 10 min and 30 min. Positive controls used for the *Leishmania*-ELISA were sera from positive red-foxes and serum from a dog with etiologically proven *L. infantum* infection. Negative controls (n=7) were from local negative dogs presenting no clinical signs of leishmaniasis. Reproducibility of ELISA results was monitored by including a low reactive canine control serum, this in addition to the standard negative and positive control sera. The "blank" value was subtracted from all other absorbance values. The average of negative control (n=7) was performed and 3 standard deviations were defined. This latter value was used as cut-off point to discriminate between "negative" and "positive" serology.

#### DAT

A total of 18 sera were used in this serological test. Serum was diluted in 0.2 M 2-mercaptoetanol (1.56 ml of 2-mercaptoetanol [Sigma M 6250] in 100 ml saline solution). The dilution preparation was placed in the Greiner (96K, V-form 651101) polystyrene plates wells and incubated 1 h at 37ºC. Serial four dilutions were performed and 50 µl of antigen (*Leishmania infantum* promastigotes - KIT Royal Tropical Institute freeze dried *Leishmania*, lot 0905) added to all plates wells. The plate was incubated for 18h at room temperature, after which manual reading was conducted. A 1:320 cut-off is the titer value for dog diagnosis and the same assumed for red-foxes.

### *Toxoplasma gondii*

#### IFAT

A total of 18 sera and 24 muscular juices were used in this serological test. Microspcopic slides coated with the Ag (*T.gondii* RH strain tachyzoizes, in vitro cultivated on Vero cells) were defrozen. A positive and a negative control serum were included. Serial one fold dilution for sera and two fold dilutions for muscular juice in PBS were added and subsequently incubated for 30 min at 37ºC; slides were washed with PBS (three times, 10 min) and droplets were covered with FITC-(Sigma 7884)-conjugated rabbit anti-dog IgG in PBS (1:300), incubated and washed as above. Immunoreactions were detected under 400x magnification using an UV light microscope Olympus BX41 with a blue filter, at a 490 mn wave length.

#### MAT

A total of 18 sera were used in this serological test. The serum samples were analyzed for antibodies to *T. gondii* with the MAT using a direct microagglutination commercial kit (Toxo-Screen DA®, bioMérieux, Lyon, France). Sera were tested at the dilution of 1:20, with whole tachyzoites as antigen and the addition of 2-β-mercaptoethanol. Positive and negative control samples were included in each plate. A cut-off titre of 20 was chosen to maximize both sensitivity and specificity of the test. Positive sera developed

agglutination (at least one half of the well's diameter) that were still visible after 18 h incubation at room temperature.

## *Neospora caninum*

### IFAT

A total of 18 sera and 24 muscular juices were used in this serological test. Microspcopic slides coated with the Ag (*N. caninum* NC1 strain tachyzoizes, in vitro cultivated on Vero cells) were defrozen. A positive and a negative control serum were included. Serial three fold dilutions for sera and three fold dilution for muscular juice in PBS were added and subsequently incubated for 30 min at 37°C; slides were washed with PBS (three times, 10 min) and droplets were covered with FITC-(Sigma 7884)-conjugated rabbit anti-dog IgG in PBS (1:300), incubated for 30 min at 37°C and washed as above. Immunoreactions were detected as described for *T. gondii*.

## Results

### Microscopic reading of Giemsa-stained organ and blood smears

In all of the smears microscopically investigated from different tissues, no parasite could be detected.

### Microscopic reading of Hematoxilin-Eosin-stained histological preparation

In all of the histological preparation microscopically investigated, no parasite could be detected.

### Diagnostic in vitro cultivation of brain specimen

The inoculations of brain samples onto Vero cells and subsequent in vitro cultivation revealed that most specimens were bacterially and fungal contaminated, thus leading to discharge of culture before the stage of expected parasite isolation.

# *Leishmania infantum*

## ELISA

ELISA results are presented in Table XII.

Table XII. ELISA results for *Leishmania infantum*.

| Sample | Number | Elisa (*Leishmania infantum* with manual pipeting) | Blank subtraction | 3 sd subtraction |
|---|---|---|---|---|
| | blank | 0.082 | -0.018 | -0.25 |
| | neg | 0.341 | 0.241 | 0.00 |
| | neg | 0.343 | 0.243 | 0.00 |
| | neg | 0.351 | 0.251 | 0.01 |
| | posit | 1 | 0.969 | 0.74 |
| Fox 5 s | 1 | 0.167 | 0.067 | -0.16 |
| Fox 7 s | 2 | 0.18 | 0.08 | -0.15 |
| Fox 8 s | 3 | 0.123 | 0.023 | -0.21 |
| Fox 9 s | 4 | 0.156 | 0.056 | -0.18 |
| Fox 11 s | 5 | 0.228 | 0.128 | -0.10 |
| Fox 12 s | 6 | 0.18 | 0.08 | -0.15 |
| Fox 13 s | 7 | 0.235 | 0.135 | -0.10 |
| Fox 14 s | 8 | 0.285 | 0.185 | -0.05 |
| Fox 15 s | 9 | 0.176 | 0.076 | -0.16 |
| Fox 17 s | 10 | 0.148 | 0.048 | -0.18 |
| Fox 18 s | 11 | 0.218 | 0.118 | -0.11 |
| Fox 19 s | 12 | 0.151 | 0.051 | -0.18 |
| Fox 20 s | 13 | 0.564 | 0.464 | 0.23 |
| Fox 21 s | 14 | 0.241 | 0.141 | -0.09 |
| Fox 22 s | 15 | 0.167 | 0.067 | -0.16 |
| Fox 23 s | 16 | 0.131 | 0.031 | -0.20 |
| Fox 24 s | 17 | 0.203 | 0.103 | -0.13 |
| Fox 25 s | 18 | 0.156 | 0.056 | -0.18 |
| Fox 25 j | 19 | 0.129 | 0.029 | -0.20 |
| Fox 24 j | 20 | 0.115 | 0.015 | -0.22 |
| Fox 23 j | 21 | 0.122 | 0.022 | -0.21 |
| Fox 22 j | 22 | 0.126 | 0.026 | -0.21 |
| Fox 21 j | 23 | 0 | 0.006 | -0.23 |
| Fox 20 j | 24 | 0.11 | 0.01 | -0.22 |
| Fox 19 j | 25 | 0.096 | -0.004 | -0.24 |
| Fox 18 j | 26 | 0.113 | 0.013 | -0.22 |
| Fox 17 j | 27 | 0.106 | 0.006 | -0.23 |
| Fox 16 j | 28 | 0.098 | -0.002 | -0.23 |
| Fox 15 j | 29 | 0.119 | 0.019 | -0.21 |
| Fox 14 j | 30 | 0.122 | 0.022 | -0.21 |
| Fox 13 j | 31 | 0.103 | 0.003 | -0.23 |
| Fox 12 j | 32 | 0.147 | 0.047 | -0.18 |
| Fox 11 j | 33 | 0.129 | 0.029 | -0.20 |
| Fox 10 j | 34 | 0.098 | -0.002 | -0.23 |
| Fox 9 j | 35 | 0.113 | 0.013 | -0.22 |
| Fox 8 j | 36 | 0.101 | 0.001 | -0.23 |
| Fox 7 j | 37 | 0.133 | 0.033 | -0.20 |
| Fox 6 j | 38 | 0.117 | 0.017 | -0.21 |
| Fox 4 j | 39 | 0.116 | 0.016 | -0.22 |
| Fox 3 j | 40 | 0.116 | 0.016 | -0.22 |

| Sample | Number | Elisa (*Leishmania infantum* with manual pipeting) | Blank subtraction | 3 sd subtraction |
|---|---|---|---|---|
| Fox 2 j | 41 | 0.109 | 0.009 | -0.22 |
| Fox 1 j | 42 | 0.105 | 0.005 | -0.23 |
| Fox 10 a | 43 | 0.695 | 0.595 | 0.36 |
| Fox 11 a | 44 | 0.6 | 0.5 | 0.27 |
| Fox 1 a | 45 | 0.203 | 0.103 | -0.13 |
| Fox 2 a | 46 | 0.352 | 0.252 | 0.02 |
| Fox 3 a | 47 | 1 | 1.382 | 1.15 |
| Fox 5 a | 48 | 0 | 0.245 | 0.01 |
| Fox 5 a | 49 | 0.668 | 0.568 | 0.34 |
| dog k | 50 | 0.282 | 0.182 | -0.05 |
| dog l | 51 | 0.29 | 0.19 | -0.04 |
| dog m | 52 | 0.194 | 0.094 | -0.14 |
| dog n | 53 | 0.243 | 0.143 | -0.09 |
| dog o | 54 | 0.219 | 0.119 | -0.11 |
| dog p | 55 | 0.256 | 0.156 | -0.08 |
| dog q | 56 | 0.254 | 0.154 | -0.08 |
| dog r | 57 | 0.224 | 0.124 | -0.11 |
| dog t | 58 | 0.251 | 0.151 | -0.08 |
| dog u | 59 | 0.226 | 0.126 | -0.11 |
| | blank | 0.105 | 0.005 | -0.23 |
| | blank | 0.127 | 0.027 | -0.20 |
| | blank | 0.113 | 0.013 | -0.22 |
| | blank | 0.101 | 0.001 | -0.23 |
| | blank | 0.101 | 0.001 | -0.23 |
| | blank | 0.096 | -0.004 | -0.24 |
| | blank | 0.091 | -0.009 | -0.24 |
| | blank | 0.104 | 0.004 | -0.23 |
| | neg | 0.336 | 0.236 | 0.00 |
| | neg | 0.363 | 0.263 | 0.03 |
| | neg | 0.346 | 0.246 | 0.01 |
| | neg | 0.358 | 0.258 | 0.03 |
| | posit | 1 | 1.007 | 0.78 |

a) sera from fox with know serological status (DAT positive) s) serum; j) muscle juice; dog k to u) dog serum with a negative immunological status for *Leishmania*; posit – positive control; neg – negative controls; 3 sd – three standard deviation.

There were a total of 5.56% red-foxes with serum seropositivity against *Leishmania infantum,* from the Alentejo area. By comparision, the present level of antibodies presence shows similar results to other studies performed in Portugal (Abranches *et al.*, 1984) by IFAT (vide Table III).

### DAT

DAT results are presented in Table XIII.

Table XIII. DAT results for *Leishmania infantum*.

| Foxes number | Serum 1:50 | Serum 1:100 | Serum 1:200 | Result |
|---|---|---|---|---|
| 1 | na | na | na | na |
| 2 | na | na | na | na |
| 3 | na | na | na | na |
| 4 | na | na | na | na |
| 5 | neg | neg | neg | neg |
| 6 | na | na | na | na |
| 7 | agglutination | neg | neg | neg |
| 8 | agglutination | neg | neg | neg |
| 9 | agglutination | agglutination | neg | Reactivity 1:100 |
| 10 | na | na | na | na |
| 11 | agglutination | agglutination | neg | Reactivity 1:100 |
| 12 | agglutination | agglutination | agglutination | Reactivity 1:200 |
| 13 | agglutination | neg | neg | neg |
| 14 | agglutination | agglutination | agglutination | Reactivity 1:200 |
| 15 | agglutination | agglutination | neg | Reactivity 1:100 |
| 16 | na | na | na | na |
| 17 | agglutination | agglutination | neg | Reactivity 1:100 |
| 18 | neg | neg | neg | neg |
| 19 | neg | neg | neg | neg |
| 20 | agglutination | neg | neg | neg |
| 21 | agglutination | neg | neg | neg |
| 22 | neg | neg | neg | neg |
| 23 | agglutination | agglutination | neg | Reactivity 1:100 |
| 24 | 50 | neg | neg | neg |
| 25 | 50 | neg | neg | neg |

na – not available; neg - negative

At cut-off 1:320 there was no reactivity of any serum sample (muscle juices could not be tested in this assay). When applying the cut-off that is used to assess infection in cats (1:100), a seropositivity rate of 38.9% was obtained. At an intermediate cut-off (1:200), a seropositivity rate was 11.1%.

With this methodical approach, *L. infantum* infection could not be revealed in red-foxes serum from Alentejo. There was also no agreement between ELISA and DAT results for *L. infantum*.

## *Toxoplasma gondii*

### IFAT

IFAT results are presented in Table XIV.

Table XIV. IFAT results for *Toxoplasma gondii.*

| Foxes number | Serum 1:80 | Muscular juice 1:80 | Muscular juice 1:160 |
|---|---|---|---|
| 1 | na | neg | neg |
| 2 | na | neg | neg |
| 3 | na | (+) | (+) |
| 4 | na | neg | neg |
| 5 | (+) | na | na |
| 6 | na | neg | neg |
| 7 | neg | neg | neg |
| 8 | (+) | neg | neg |
| 9 | neg | neg | neg |
| 10 | na | neg | neg |
| 11 | (+) | (+) | (+) |
| 12 | (+) | (+) | (+) |
| 13 | (+) | neg | neg |
| 14 | (+) | (+) | (+) |
| 15 | neg | neg | neg |
| 16 | na | neg | neg |
| 17 | neg | neg | neg |
| 18 | (+) | neg | neg |
| 19 | (+) | neg | neg |
| 20 | neg | neg | neg |
| 21 | (+) | neg | neg |
| 22 | neg | neg | neg |
| 23 | neg | neg | neg |
| 24 | neg | neg | neg |
| 25 | neg | neg | neg |

(+) – positive; na - not available; neg – negative

IFAT result for *T. gondii* in serum samples showed a high rate infection presence with half (50%) the animals being positive.

IFAT result for *T. gondii* using muscular juice samples revealed a seropositivity rate of 22.2%. There was not a good agreement between the tests performed with serum and muscular juice samples.

### MAT

MAT results are presented in Table XV.

Table XV. MAT results for *Toxoplasma gondii.*

| Foxes number | Serum 1:20 |
|---|---|
| 1 | na |
| 2 | na |
| 3 | na |
| 4 | na |
| 5 | doub |
| 6 | na |
| 7 | neg |
| 8 | (+) |
| 9 | neg |
| 10 | na |
| 11 | (+) |
| 12 | (+) |
| 13 | (+) |
| 14 | (+) |
| 15 | (+) |
| 16 | na |
| 17 | neg |
| 18 | (+) |
| 19 | (+) |
| 20 | neg |
| 21 | (+) |
| 22 | neg |
| 23 | neg |
| 24 | neg |
| 25 | neg |

(+) - positive; doub – doubtful; na – not available; neg – negative

With MAT, 50% positivity was detected within serum samples. This result confirms a high level of red-fox infection presence, which reflects widespread environmental contamination.

The statistical assessment of inter-rater agreement Cohen's kappa coefficient was used to evaluate the concordance between serum results of the two serological tests (IFAT and MAT) for *T. gondii.* Table XVI presents the comparative results of both serological tests. There was a good concondance between both test results, with K = 0,889 (an almost perfect agreement). This

result of presence may be considered in concordance with others of seroprevalence developed all over Europe, which demonstrates that the red-fox population is a sentinel species of this parasite presence in the wild.

Table XVI. IFAT and MAT results for *Toxoplasma gondii* (comparative table).

| Foxes number | IFAT for *T.gondii* – Serum 1:80 | DAT for *T.gondii* – Serum 1:20 |
|---|---|---|
| 1 | na | na |
| 2 | na | na |
| 3 | na | na |
| 4 | na | na |
| 5 | (+) | doub |
| 6 | na | na |
| 7 | neg | neg |
| 8 | (+) | (+) |
| 9 | neg | neg |
| 10 | na | na |
| 11 | (+) | (+) |
| 12 | (+) | (+) |
| 13 | (+) | (+) |
| 14 | (+) | (+) |
| 15 | neg | (+) |
| 16 | na | na |
| 17 | neg | neg |
| 18 | (+) | (+) |
| 19 | (+) | (+) |
| 20 | neg | neg |
| 21 | (+) | (+) |
| 22 | neg | neg |
| 23 | neg | neg |
| 24 | neg | neg |
| 25 | neg | neg |

(+) - positive; doub - doubtful ; na – not available; neg – negative

## *Neospora caninum*

### IFAT

IFAT results are presented in Table XVII.

Table XVII. IFAT results for *Neospora caninum*.

| Fox number | Serum 1:80 | Serum 1:160 | Serum 1:320 | Muscular juice 1:80 | Muscular juice 1:160 | Muscular juice 1:320 |
|---|---|---|---|---|---|---|
| 1 | na | na | na | neg | neg | neg |
| 2 | na | na | na | neg | neg | neg |
| 3 | na | na | na | neg | neg | neg |
| 4 | na | na | na | neg | neg | neg |
| 5 | neg | neg | neg | na | na | na |
| 6 | na | na | na | neg | neg | neg |
| 7 | neg | neg | neg | neg | neg | neg |
| 8 | (+) | (+) | (+) | (+) | (+) | doub |
| 9 | neg | neg | neg | neg | neg | neg |
| 10 | na | na | na | neg | neg | neg |
| 11 | neg | neg | neg | neg | neg | neg |
| 12 | neg | neg | neg | doub | doub | neg |
| 13 | (+) | (+) | (+) | doub | neg | neg |
| 14 | neg | neg | neg | neg | neg | neg |
| 15 | neg | neg | neg | neg | neg | neg |
| 16 | na | na | na | neg | neg | neg |
| 17 | neg | neg | neg | neg | neg | neg |
| 18 | neg | neg | neg | neg | neg | neg |
| 19 | neg | neg | neg | neg | neg | neg |
| 20 | neg | neg | neg | neg | neg | neg |
| 21 | (+) | (+) | (+) | doub | doub | neg |
| 22 | neg | neg | neg | neg | neg | neg |
| 23 | neg | neg | neg | neg | neg | neg |
| 24 | neg | neg | neg | neg | neg | neg |
| 25 | neg | neg | neg | neg | neg | neg |

(+) – positive; doub – doubtful; na – not available; neg – negative

IFAT result for *N. caninum* in serum samples: the seroprevalence was 16.7%.

IFAT result for *N. caninum* in muscular juice samples: the seroprevalence was 5.6%.

The comparasition of results did not show a good agreement between serum and muscular juice samples.

Our serum results are nevertheless in a relative concordance with those of other studies carried out in Europe with foxes and using serology and DNA detection. Therefore we conclude that red-foxes from the Alentejo area may indeed be involved in an environmental contamination situation with this parasite.

## Discussion and Conclusions

Red-fox populations have been increasing in size and number throughout Europe (Deplazes *et al.*, 2004). In Portugal there are no concrete data in this respect, but the situation in Central European development could hold true for our own country as well. The red-fox contact with an anthropomorphic environment and domestic animals is documented (Deplazes *et al.*, 2004); this approach and even direct contact with populated areas may enhance parasite dispersal, not only with the problem of wild species-domestic species spill-over but also the inherent spill-back. This exchange of pathogens may be a risk to both or to any other third sympatric species, which shares the wild ecosystem with these wild carnivores, in particular endangered species and/or reestablish ones. Surveillance studies have become essential to establish the level and degree of pathogen presence in wild carnivores and extrapolate possible consequences to any particular species, such as the Iberian lynx, or others in the wild. As an example we know that lynx populations have decreased dramatically in size and distribution in the last four decades, thus becoming increasingly vulnerable to catastrophic events, such as epizooties (Millan *et al.*, 2009). Because of the reduced size of lynx populations, the documented low level of genetic variation coupled with the recently documented state of immune depletion in a majority of necropsied lynx (Roelke *et al.*, 2008), the introduction of pathogenic infection into their small population by other domestic or wild carnivores, as the red-fox, in the same habitats may have severe consequences. It could result in epidemics that have the potential to eradicate lynx populations (Meli *et al.*, 2009). Pathogenic agents originating from wild carnivores, feral cats and stray dogs have already been detected in the Iberian lynx (Millán *et al.*, 2008) and it´s necessary to have current data of pathogens presence among such animal populations. This was the premise to initiate and develop the study of parasitic presence in the red-fox. The purpose established was to determine whether or not protozoan parasites, namely *L. infantum*, *T. gondii* and *N. caninum* were present in the red-fox population in the Alentejo.

For canine leishmaniasis, serology is considered to be a sensitive and useful technique and is well correlated with clinical signs (Solano-Gallego *et al.*, 2001), although asymptomatically *L. infantum*-infected dogs may be

seropositive as well. For vulpine leishmaniasis, we do not yet have similar knowledge. All animals in the study were presented in a good body condition and did not have clinical signs, as in other studies carried out throughout Europe (Mancianti *et al.*, 1994). Results of ELISA in serum revealed a 5.6% seroprevalence for *L.infantum*. The agreement between the results with serum and muscular juice, however, was not provided. A higher dilution of the antibody level and the presence of excessive protein are possible reasons for this disagreement and it may still be necessary to rule them out. For DAT the cut-off for the infection diagnosis in dog's serum is of 1:320; in the present study, a 1:320 titer in serum was not demonstrated for red-fox, but reactivity in lower titers was identified, arising from a previous contact with the parasite. Because reactivity is present, an increasing of the titer during the fox's life could be developed. If a higher titer develops, then clinical signs, similar to those showed by dogs, might develop as happened in experimental studies (Mancianti *et al.*, 1994). Whether this is possible or not would be established only by sampling and testing during the life of the fox. Muscular fluids were tested as well with DAT but the inconsistent positive results created the suspicion of an unspecific reactivity and were excluded from results; this nonspecificity may be due to reactivity with other serum proteins besides *L. infantum* antibodies. Although the seroprevalence level revealed by ELISA in these studied animals is low, our results are nevertheless in agreement with other European and Asian reports (Abranches *et al.*, 1984, Baneth *et al.*, 1998). Undoubtedly, the revelation of infection in red-foxes reflects a shared canid parasitic infection cycle in the Alentejo area, most likely through vector dispersal. The combination of all data, these of the red-fox-presence of the parasite and those reported by other authors about feline infection, even in Portugal (Hatam *et al.*, 2009), should alert us for the possible risk of leishmaniasis to the vulnerable and endangered small population of the Iberian lynx.

Toxoplasmosis is of a special importance due to the high incidence of infections in humans and in animals. Very well studied in humans, but less known in animals, it has to gain significance as an environmental contaminant. Infection in humans it frequently a result of the consumption of raw or undercooked meat containing tissue cysts of the protozoan, but food and water

contaminated with *Toxoplasma* oocysts can generate infection in humans as well. For carnivore animals, either domestic or wild, possible infection routes are the consumption of meat containing tissue cysts in intermediate hosts but also oocysts derived from definitive hosts that produce environmental contamination (water and soil). Foxes have diversified diets that include small mammals, birds and fruits; they do not usually prey on ruminants as other wild canids do, but prefer rabbits and other small rodents as well as birds; these animals have been proven to be carriers of the infection and of tissue-cysts, and transmission occurs. The results of this work confirm the serologic presence of *T.gondii* infection in the red-fox population in Alentejo. A 50% seroprevalence level was obtained by both IFAT and MAT, with a good concordance (with Cohen's kappa coefficient being K=0,889, an almost perfect agreement) between the two tests. This good accordance for serum samples, suggests that both are appropriate analytic techniques for the detection of *T. gondii* infection in red-foxes. The high seroprevalence (50% of the animals tested) is a reflection of prey and habitat contamination and reveals a real risk to the lynx and other wild carnivores that share the same ecosystem and feed on the same prey.

The feeding habits of wild canids seem to be crucial for their exposure to *N. caninum*. The 16.7% seroprevalence result of *N. caninum* in foxes from the Alentejo suggest that these wild carnivores may be involved in sylvatic cycle of the parasite in the area, as Jakubek *et al.* (2007) inferred in their study.

Epidemics can be a serious conservation threat for free-living populations of endangered species and can cause mortality, reduce host fitness and/or alter the dispersal and movement patterns of infected animals; it is important to have the most substancial knowledge of the past and present of parasitic pathogens to infer on new probable pathogens or new hosts emergence within the dynamics of ecossystem biology. Within a dynamic environment, changes in population density or host behavior can affect disease prevalence and disease agents can suddenly boost their virulence and widen their host range. Our present serological findings concerning different protozoans in red-foxes from the Alentejo area must be carefully interpreted in the context of their importance for the Iberian lynx in the Alentejo area. Accordingly, the lynx is undoubtly confronted with an environmental contamination issue, and further studies will

have to elucidate if this situation also represents an effective health threat or problem to the conservation of this animal population.

We have to point at the fact that our serological investigations did only (indirectly) reflect contact and infection with the parasites tested for. Thus, serology does not discriminate between an active and a passed infection. The real presence of the parasite in an active infection requires the etiological demonstration of the presence of the parasite. Such information may be obtained with DNA detection (e.g. by PCR) of the parasites genome, we anticipate to include such an approach for different body tissues samples in future studies.

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