



UNIVERSIDADE DE ÉVORA

ESCOLA DE CIÊNCIAS E TECNOLOGIA

DEPARTAMENTO DE BIOLOGIA

Pseudorabies and Tuberculosis:

serologic evidence of infection in wild boar populations in southeast Portugal and associated risks for Iberian lynx conservation

Vanessa Alexandra vieira Soeiro

Orientador: Professor Paulo Célio Pereira Martins Alves

Co-orientador: Doutor Christian Gortázar Schmidt

Mestrado em Biologia da Conservação

Dissertação

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Pseudorraiva e Tuberculose: evidência serológica de infecção em Javali no Sudeste de Portugal e riscos associados para a conservação do Lince-ibérico

Resumo

Em Portugal foi recentemente iniciado um programa que visa o restabelecimento da população de Lince-ibérico. O rastreio do estado sanitário das populações selvagens é essencial para qualquer programa de reintrodução. O presente estudo teve como principal objetivo estimar em javalis caçados a taxa de contacto com os agentes etiológicos da pseudorraiva (também conhecida por doença de Aujeszky) e da tuberculose bovina, na área com maior potencial para a reintrodução de Lince-ibérico no sudeste de Portugal. Para o efeito foram realizados dois testes diferentes de ELISA para deteção de anticorpos contra o vírus da pseudorraiva (PrV) e o complexo *Mycobacterium tuberculosis*. Os resultados evidenciaram uma taxa de seroprevalência para PrV de 45,9% que está entre as mais altas documentadas até à data. Os anticorpos contra *Mycobacterium bovis* foram detetados em 9,9% das amostras, o que se encontra dentro do intervalo de valores para taxas de infeção por *M. bovis* previamente referidos. A prevalência destas doenças numa espécie reservatório, abundante, simpátrica e, ocasionalmente, presa do Lince-ibérico, pode constituir um importante problema para o programa de reintrodução devendo, por esse facto, ser implementado um sistema de vigilância sanitária e exploradas opções de prevenção.

Palavras-chave: Doença de Aujeszky; Complexo *Mycobacterium tuberculosis*; ELISA; Javali; Lince-ibérico Pseudorraiva; Reintrodução; Tuberculose.

Pseudorabies and Tuberculosis: serologic evidence of infection in wild boar populations in Southeast Portugal and associated risks for Iberian lynx conservation.

Abstract

An important effort has been recently endeavoured to promote the reestablishment of Portuguese Iberian lynx population. The awareness of the sanitary status of wild populations is essential in any reintroduction program, and the present study aimed to estimate the rates of contact with causative agents of pseudorabies (also known as Aujeszky's disease) and bovine tuberculosis in hunted wild boar from the foremost Iberian lynx potential releasing site in Southeast Portugal. To achieve this purpose two different ELISA tests were performed in order to detect serum antibodies against pseudorabies virus (PrV) and *Mycobacterium tuberculosis* complex. Results evidenced a seroprevalence rate for PrV of 45,9%, which is amongst the highest so far documented. *Mycobacterium bovis* antibodies were detected in 9,9% of the samples, which is within the range of previously reported *M. bovis* infection rates. The prevalence of contact with both disease agents in a sympatric, abundant, reservoir and occasionally prey species can represent a serious problem for the Iberian lynx reintroduction program, and thus a monitoring scheme should be implemented and prevention options explored.

Key words: Aujeszky's disease; ELISA; Iberian lynx; *Mycobacterium tuberculosis* complex; Pseudorabies; Reintroduction; Tuberculosis; Wild boar.

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Abbreviations

AD	Aujeszky's disease
ADV	Aujeszky's disease virus
bPPD	Bovine tuberculin purified protein derivative
bTB	Bovine tuberculosis
CI	Confidence interval
df	Degrees of freedom
E%	ELISA percentage
<i>e. g.</i>	<i>Exempli gratia</i>
ELISA	Enzyme-Linked Immunosorbent Assay
FelV	Feline leukaemia virus
G ²	Chi-square value calculated by the log-linear method
IUCN	International Union for Conservation of Nature
neg	Negative
OD	Optical density
<i>P</i>	Probability
PBS	Phosphate buffered saline
pos	Positive
PrV	Pseudorabies virus
SSC	Species Survival Commission
TMB	Tetramethylbenzidine
Unrec.	Unrecorded
%IN	Percentage of inhibition
μl	Microliter
°C	Celsius degrees

1. Introduction

The role of captive breeding as a tool for the recovery of endangered species is a controversial subject. Rather than a long-term or prophylactic solution, Snyder *et al.* states that such approach should be viewed as the last resort in a species recovery strategy. Nevertheless in several conservation programs captive breeding for reintroduction¹ is considered an option (despite its known limitations) and therefore justifiable when other alternatives are not available (Snyder *et al.*, 1996).

Difficulties in keeping self-sustainable captive populations, strict management conditions settings not to condition behaviour (loss of ability to survive in the wild – wild fitness), disease monitoring and other more political and financial features should be evaluated before deciding whether a recovery plan should or should not include any animal movement. Moreover the reduced survival probability amongst captive-born individuals, when in contact with endemic diseases in the releasing site is a significant risk (due to decreased immunological capacity to survive diseases that prevail in the wild). Jule and co-authors found evidence that the survival rates were higher for wild caught individuals than for captive-born ones, particularly in carnivore reintroduction programs. Captive-born carnivores are more susceptible to starvation with a rate of over 50% of fatal incidents being directly associated with human activities (Jule *et al.*, 2008). This data is in agreement with authors who describe high mortality rates in captive-born Canada lynx (*Lynx canadensis*) caused by starvation early after release into the wild, apparently due to the lack of hunting competencies (Shenk *et al.*, 2009).

¹ According to the IUCN/SSC (1998) reintroduction is “an attempt to establish a species in an area which was once a part of its previous historical range”

It is, therefore, of great concern when the conservation efforts towards an endangered species rely almost exclusively on captive breeding for reintroduction, because along with it, efforts should be made in order to save the wild population and restore or preserve its habitat (Millán *et al.*, 2009). Consequently both *in situ* and *ex situ* actions must be engaged, to promote adequate measures to improve species viability within the historical distribution area. Furthermore, reintroduction is considered a high risk activity for wildlife, thus, surveillance and monitoring programs should be designed in order to gather reliable information. Disease surveys and monitoring must be comprised in reintroduction programs.

Until recently diseases were considered a secondary factor in the dynamics and decline of wild animal populations. Small, fragmented and isolated wildlife populations are defiantly threatened by any stochastic event such as a disease outbreak, therefore more vulnerable to extinction (Delahay *et al.*, 2009). To better understand the effects and dynamics of infectious agents in wild populations, it is fundamental to integrate the study of demographic, reproductive and mortality aspects associated with disease surveillance and the host-pathogen interaction.

Disease surveillance can be more objectively applied when focused on more abundant species or prey ones, being easier to establish management actions for disease control and risk reduction (Artois *et al.*, 2009).

The Iberian lynx (*Lynx pardinus*) is an interesting case study since a captive-born reintroduction program is being considered for this endemic species in the Iberian Peninsula. It is also the world's most endangered felid species. This wild felid was distributed throughout the Iberian Peninsula (Palomares, 2009), but by 1950 its distribution area was reduced and it almost disappeared from the northern middle of and eastern regions of Spain (Rodríguez, 2004). By this time, in Portugal it was already extinct from the northern and central regions of the country and a drastic reduction in the remaining population also occurred in the

same decade. Figure 1 reports to the historical distribution of the Iberian lynx after the mentioned period.



Figure 1: Historical distribution of the Iberian lynx, adapted from Sarmiento *et al.* (2009a): 1 - Algarve–Odemira–Sado valley; 2 - Gata–Malcata–San Pedro–S. Mamede; 3 - Western Sierra Morena–Guadiana; 4 - Alberche; 5 – Gredos; 6 - Subbéticas; 7 – Doñana; 8 - Central Sierra Morena; 9 - Central population.

During this century's first decade a new Iberian census was conducted and no evidence of the species presence was found in Portugal, placing the Iberian lynx population in this country in the verge of extinction (Sarmiento *et al.*, 2009b). Thus, currently this species only occurs in Spain.

More recently this species population in the Iberian Peninsula was estimated in less than two hundred individuals, mostly grouped in two subpopulations in the region of Andalusia (Palomares *et al.*, 2010; Gil-Sánchez & McCain, 2011), both in Doñana and Eastern Sierra Morena (Palomares, 2009) (Figure 2).



Figure 2: Current Iberian lynx distribution in Spain adapted from Sarmiento *et al.* (2009a): 1 - Doñana; 2 - Cardeña-Andujár.

The Iberian lynx's basic prey is the wild rabbit (*Oryctolagus cuniculus*) and both species evolved together and inhabit the same landscape (Real *et al.*, 2009; Rodríguez *et al.*, 2011). Habitat changes (due to altered agricultural practices and subsequent habitat loss) and viral diseases, namely myxomatosis in the 1950s and rabbit hemorrhagic disease (RHD) at the end of the 1980's, caused consecutive abrupt declines in wild rabbit numbers (Cabezas-Díaz *et al.*, 2009; Calvete, 2009). Wild rabbit populations have declined 55% in three decades in Spain (1973-2002) (Cabezas-Díaz *et al.*, 2009) and in Portugal this reduction is estimated in the last decade in 30% (Ferreira & Alves, 2009). According to Ferreras and co-authors, the reduction of Iberian lynx's staple prey, has had a direct impact in this predator abundance and led to high rates of non-natural mortality (Ferreras *et al.*, 1992).

The confluence of events such as rabbit number decline, lynx population fragmentation, habitat loss and non-natural mortality, confined the Iberian lynx species to the present situation (Meli *et al.*, 2009a; Palomares, 2009). Moreover the observed change in forestry management affected negatively both predator and prey species, which in association with other economic activities (artificial feeding for hunting purposes) enhanced ungulate species such as the wild boar (*Sus scrofa*) and the red deer (*Cervus elaphus*) occupying/ destroying the rabbit's territorial patches (Cabezas-Díaz *et al.*, 2009).

A widespread decrease in environmental favorability for the rabbit within lynx's areas, as described by Real *et al.* (2009), led to the scarcity of its basic prey and probably forced the Iberian lynx to look for alternative resources (Gortázar *et al.*, 2007). Since ungulates populations are currently increasing, they can easily become alternative or complementary prey (Briones *et al.*, 2000).

The Iberian lynx is also known for its habitat specificity. Apart from the shrubland cover structure, dominant in Mediterranean scrubland landscape where the Iberian lynx still occurs and breeds (Fernández *et al.*, 2007), rocky areas with some scrubland are also described as suitable for this species to establish its territory (Fernández *et al.*, 2006). Furthermore, important elements (other than food availability) must be present, namely breeding dens (such as old hollow trees or rock cavities) (Fernández & Palomares, 2000), and permanent water sources during the dry season (Palomares *et al.*, 2001).

Habitat loss and fragmentation has also been determinant in this top predator constraining range. Changes on soil uses, related to agricultural and forestry altered practices as well as the construction of large infrastructures disrupted the landscape structure and connectivity, consequently narrowing potential lynx territories (Ferrerías, 2001; Ferrerías *et al.*, 2004). Moreover the inadequate management practices of livestock and hunting estates are overpressuring this species distribution (Calzada *et al.*, 2007). Regarding the dispersal of young lynxes outside the National Park in Doñana, the 85% mortality rate was mostly due to predator control practices (Ferrerías *et al.*, 1992), and the

stable population within this National Park may be attributable to the protection status and cessation of predator persecution (Rodríguez, 2004).

Another study comprised radio-tracking of more than 50 individuals and revealed that 21% of the deaths were due to direct shooting, another 21%, to illegal trapping with leg-hold traps and snares and hunting with dogs, 17% were road accident casualties and 4% drowned in water wells (Palomares, 2009). This reflects the relevance of non-natural mortality over the Iberian lynx population, even after the legal protection of the species (Rodríguez & Delibes, 2004). Furthermore, the recent evidence that local extinctions have occurred before disease outbreaks within the wild rabbit populations, suggests that the primary cause of Iberian lynx decline was directly or indirectly related to human persecution (Gil-Sánchez & McCain, 2011).

Despite this catastrophic scenario, recent data (2001 – 2006) are more optimistic, indicating some improvements, particularly regarding the increase of population in Sierra Morena (Simón *et al.*, 2009; Simón *et al.*, 2012).

Concerning to the Portuguese claimed extinction of the Iberian Lynx (an unfortunate but expected outcome), the importance of a National Conservation Action Plan was highlighted, and it was legally approved in 2008 (Despacho nº 12697/2008, de 6 de Maio). The contemplated guidelines in this document aim to implement *in situ* and *ex situ* actions in order to preserve both habitat and prey populations, to develop pre-release activities (IUCN /SSC, 1998) and strengthen reintroduction viability in Portugal. For this purpose these actions should be carefully planned, and the multidisciplinary team, committed to establish the necessary connection between *in situ* and *ex situ* actions (Vargas *et al.*, 2009).

As in any long-term conservation plan, the evaluation of disease prevalence is an important aspect to consider, especially in small and isolated populations, in which the vulnerability to stochastic events such as infectious diseases is increased (Gortázar *et al.*, 2007; Simón *et al.*, 2009) and genetic variability is reduced, potentially compromising individuals immunocompetence (Godoy *et al.*, 2009).

Within the scope of disease epidemiology, it is less likely, due to Iberian lynx's solitary social system (Palomares, 2009) that disease transmission occurs during intra-species, rather than inter-species contacts, particularly when reservoir species are maintaining the disease in the wild (Power & Mitchell, 2004; Millán *et al.*, 2009). In order to better understand the role of sympatric wild and domestic species, potentially acting as disease reservoir, several studies regarding disease prevalence in those species were endeavoured within the Iberian Peninsula (Aranaz *et al.*, 2004; Martin-Atance *et al.*, 2005; Millán *et al.*, 2007; Sobrino *et al.*, 2007; Millán *et al.*, 2009; Santos *et al.*, 2009a). That is the case of wild ungulates which have been extensively studied for their role in tuberculosis epidemiology (Vicente *et al.*, 2007; Gortázar *et al.*, 2008; Naranjo *et al.*, 2008; Santos *et al.*, 2009b). On other hand, for pseudorabies (also named Aujeszky's disease), the major concern is the economic loss as an outcome from disease transmission to livestock, but the risks to carnivore conservation are also in evidence (Vicente *et al.*, 2005).

Therefore, it is mandatory to establish an epidemiological surveillance program for the Iberian lynx and associated species in its current range and in potential reintroduction sites (Roelke *et al.*, 2008; Martínez *et al.*, 2009).

Different pathogens have been identified in Iberian lynxes, namely: parasitic (Luaces *et al.*, 2005; Millán *et al.*, 2007; Sobrino *et al.*, 2007; Roelke *et al.*, 2008; Millán *et al.*, 2009; Acosta *et al.*, 2011), bacterial (Briones *et al.*, 2000; Aranaz *et al.*, 2004; Martin-Atance *et al.*, 2005; Willi *et al.*, 2007; Meli *et al.*, 2009a; Millán *et al.*, 2009) and viral agents (Roelke *et al.*, 2008; López *et al.*, 2009; Meli *et al.*, 2009b; Meli *et al.*, 2010; López *et al.*, 2011; Palomares *et al.*, 2011). Amongst these, few are known to be lethal to infected individuals, but the scrutiny of all possible diseases affecting wild and domestic felids, as well as other sympatric species, comprise an important tool to the Iberian lynx conservation plan.

Within the Iberian Peninsula the infectious diseases described with utmost relevance in lynx's mortality rate are from bacterial and viral origin, respectively:

tuberculosis (Briones *et al.*, 2000; Pérez *et al.*, 2001) and feline leukaemia (López *et al.*, 2009; Meli *et al.*, 2009a). The death of five Iberian lynxes between 1998 and 2007, in Spain, was reported to be due to bovine tuberculosis (Meli *et al.*, 2009a). This disease was also described in free ranging lions, cheetahs and leopards in South Africa (Keet *et al.*, 1996) and other free-ranging and captive carnivore species (Carbin, 1982; Bruning-Fann *et al.*, 1998; Helman *et al.*, 1998; Lantos *et al.*, 2003) and in these cases feeding on infected carcasses or meat was probably the primary infection route. Emaciation and multiple granulomas within the lungs and regional lymph nodes are common findings.

Even though considered a rare disease in nondomestic felids, feline leukaemia virus (FeLV) infection has been reported in free-ranging and captive felids (Jessup *et al.*, 1993; Leutenegger *et al.*, 1999; Sleeman *et al.*, 2001; Ostrowski *et al.*, 2003; Cunningham, 2005) and recently a severe epidemic outbreak struck the Doñana's Iberian lynx population; starting in December 2006, and during a 6 month period, 7 individuals died (López *et al.*, 2009; Meli *et al.*, 2010; López *et al.*, 2011). This was evidence to the susceptibility of small sized populations associated with low genetic variability regarding the introduction of new diseases by other sympatric animals.

Pseudorabies or Aujeszky's disease (AD) is also a viral infection already confirmed and reported in a dead Florida panther (*Felis concolor coryi*) (Glass *et al.*, 1994). Despite the scarce information about this particular disease in wild felids, it was fairly studied in domestic cats (Hagemoser *et al.*, 1980), hunting dogs (Müller *et al.*, 2010; Müller *et al.*, 2011), racoons (Thawley & Wright, 1982), reported in captive carnivores (Raymond *et al.*, 1997; Zanin *et al.*, 1997), and surveyed in other wild free-ranging carnivore species (Garcelon *et al.*, 1992; Roelke *et al.*, 1993; Mainka *et al.*, 1994; Dunbar *et al.*, 1998; Bakker *et al.*, 2006). The members of suidae family are the natural hosts for pseudorabies virus thus acting as main potential reservoirs.

Within the suidae from the Old World, the wild boar is the species with the widest distribution. It is the second most abundant ungulate in Europe (Müller *et al.*, 2011), with great adaptive plasticity and prolific nature (Fonseca *et al.*,

2004). This is particularly true in areas where there is food availability all year round and quality vegetation cover is present (Fonseca *et al.*, 2004; Santos *et al.*, 2006).

In Portugal the wild boar populations experienced a severe bottleneck in the middle of 19th century, mainly due to African swine fever outbreak and over hunting (Ferreira *et al.*, 2009). The increase in wild boar abundance started after the abandonment of agricultural lands (1960), and the banning of wild boar hunting in 1967. A notorious recovery in this species population was observed in 1980's, being currently found throughout all of Portugal (Lopes & Borges, 2004; Fonseca, 2006). In 1986 a new hunting law was published (Lei nº 30/86, de 27 de Agosto) and along with the increase of game areas, the interest of hunters in this species also grew (Lopes & Borges, 2004), acquiring an economically important value as game species (Lopes & Borges, 2004; Ferreira, 2005; Fonseca, 2006). This economic relevance is present in all Iberian Peninsula, but particularly more noticeable in South and Central Spain where an important commercial hunting industry is developing (Vicente *et al.*, 2006). In these areas, artificial management of this ungulate populations either by fencing or by providing feeding and watering in artificial points, as well as translocation of individuals², have been suggested as possible causes of re-emergence of some diseases (Vicente *et al.*, 2007).

In contrast, wild boar breeding farms have little importance in Portugal. Nevertheless, wild ungulate translocation for hunting purposes is increasing and circumstantial evidence was found of the introduction of the bovine tuberculosis infectious agent in two areas by ungulate translocation, out of its previous distribution (Santos *et al.*, 2009b).

Diseases like tuberculosis and pseudorabies are fairly studied and it was evidenced that overabundance ("overpopulation") boosts infectious disease transmission risk, because of its effect in host aggregation and consequent increase in contact rate, either intra-species or inter-species (Gortázar *et al.*,

²According to the IUCN/SSC (1998), translocation is the "deliberate and mediated movement of wild individuals or populations from one part of their range to another".

2006). Therefore, high disease prevalence and incidence can be expected in areas where a given wildlife species is overabundant. Artificial management with fencing, creation of feeding and watering points promotes aggregation, consequently enhancing infection transmission (Vicente *et al.*, 2004; Gortázar *et al.*, 2006). These and other human interventions (such as predator control and preventative treatments), interfere with natural mechanisms of wildlife population control, thus, in predator absence, hunting is an important regulation mechanism for these ungulate populations (Gortázar *et al.*, 2006).

Diseases can affect not only the fitness of a given species, but also public health, livestock health, and the conservation of endangered species (Gortázar *et al.*, 2006; Roelke *et al.*, 2008). The aforementioned, underlines the importance of disease surveillance, wildlife abundance monitoring and ecology. The wild boar is an excellent example of direct correlation between overabundance and disease prevalence, and is also an interesting species for wildlife diseases epidemiological research (Ruiz-Fons *et al.*, 2008a).

The increasing range and density of this species contributed to the spread of diseases like classical swine fever (Ruiz-Fons *et al.*, 2008a), pseudorabies (Ruiz-Fons *et al.*, 2008b), post weaning multisystemic wasting syndrome caused by the porcine circovirus type 2 (Vicente *et al.*, 2004), and bovine tuberculosis (Santos *et al.*, 2012). This wild host's role in these diseases epidemiology still endeavours scientific investigations, suggesting or confirming the species as wildlife reservoir (Acevedo *et al.*, 2007; Ruiz-Fons *et al.*, 2008a). Whenever confirmed, serious complications arise for diseases eradication programs in livestock (Cunha *et al.*, 2011; Vieira-Pinto *et al.*, 2011; Boadella *et al.*, 2012). As some previous studies have suggested, disease prevalence is most probably related to wildlife's farm management schemes promoting overabundance and aggregation (Vicente *et al.*, 2004), also interfering with natural regulation mechanisms of wildlife populations (Gortázar *et al.*, 2000; Gortázar *et al.*, 2006).

Pseudorabies is a viral disease caused by an alphaherpesvirus that infects not only natural hosts as domestic pig, feral pig and wild boar, but also other domestic and wild mammals (Romero *et al.*, 2001; Vengust *et al.*, 2005; Ruiz-

Fons *et al.*, 2007; Müller *et al.*, 2011). This virus is distinguished by its rapid lytic growth in cell culture, its neurotropism, and its latency in neurons (Mettenleiter, 2000). After oronasal infection, the first viral replication occurs in the upper respiratory epithelium, with a later infection of the tonsils and lungs, from where the virus disseminates to the entire organism, either in a free form or associated with leucocytes. The virus easily progresses through the nervous terminations of the trigeminus and olfactive nerves to the Central Nervous System (Mettenleiter, 2000; Ruiz-Fons *et al.*, 2007; Allepuz *et al.*, 2008). Pigs and the wild boar are the only animals capable of surviving a productive infection, becoming infection reservoirs and carriers (Vengust *et al.*, 2005; Allepuz *et al.*, 2008). Major sites of pseudorabies virus latency are the trigeminal ganglion, the olfactory bulb, and the tonsil (Mettenleiter, 2000).

The reactivation of the latent viral replication can occur when animals are subjected to any stimulus, including stressful situations (Allepuz *et al.*, 2008). The infection can result in neurological signs (like encephalitis) and respiratory signs, being more frequent in young and adult animals respectively (Vengust *et al.*, 2005; Vicente *et al.*, 2005; Ruiz-Fons *et al.*, 2007). Even though very young wild swine seem to be more resistant to pseudorabies virus infection than domestic ones (probably due to earlier maturation of the CD8 T cells), high mortality rates have been reported in young wild individuals (Müller *et al.*, 2011).

The clinical presentation of an Aujeszky's disease outbreak in a free-ranging wild boar population was described as typical nervous affliction (inability to stand on hind legs, tremor and incoordination) (Gortázar *et al.*, 2002).

Disease transmission occurs by several possible routes, namely:

- respiratory route (favoured by social gregariousness) among females and offspring;
- the venereal route that may be particularly important for males during the breeding season;
- the digestive infection route can occur by consumption of infected carcasses (Romero *et al.*, 2001; Müller *et al.*, 2011).

The gastrointestinal route is of most concern for wild boar predators like Iberian wolf, Iberian lynx, and other opportunistic scavengers (Vicente *et al.*, 2005).

Pseudorabies is nowadays as distributed as the wild boar is, and once present it raises economic concerns due to pig industry and international pig product trade. For this reason several serosurvey studies were conducted all over Europe in countries with ongoing eradication plans (Müller *et al.*, 2011).

Tuberculosis is a bacterial disease generally chronic, progressive, caused by mycobacteria from *Mycobacterium tuberculosis* complex, affecting a broad range of species including humans. Some of the members of this complex like *Mycobacterium tuberculosis*, *M. africanum* and *M. canetti* are predominantly human pathogens, *M. microti* affects rodents, whereas *M. bovis* and *M. caprae* have a broader host selection (Good & Duignan, 2011). Within this complex the *Mycobacterium bovis* species is the one that presents a wider range of hosts (Biet *et al.*, 2005; Mostowy *et al.*, 2005). In the infected host the disease is characterized by the development of pyogranulomatous lesions in lungs and lymph nodes, but has potential to disseminate to other organs (Bengis, 1999).

For its chronic and progressive character, clinical signs are only evident in advanced stages of infection and include anorexia, weakness, depression and progressive emaciation. In advanced pulmonary disease, cough, reduced exercise tolerance and progressive dyspnea may be present (Bengis, 1999).

Even though the direct contact amongst animals is the most efficient route of infection, this obligate intracellular organism can survive for long periods out of the animal host in the environment, directly or indirectly contaminated by infected animal discharges. This suggests other possible transmission routes (Biet *et al.*, 2005). According to Bengis, the bovine tuberculosis agent can be transmitted by:

- respiratory route by aerosolized infectious particles;
- digestive route by ingestion of contaminated food (e.g. infected carcasses) or water;

- vertical or intra-uterine transmission and pseudo-vertical transmission by infected milk in lactants.

Respiratory route infection requires a lower number of infectious agents when compared with digestive route (Bengis, 1999).

Several authors refer that the tuberculosis re-emergence and the failure in its eradication in livestock in many countries is related with the maintenance of reservoirs of disease in wild populations (Alexander *et al.*, 2002; Pavlik *et al.*, 2002; Naranjo *et al.*, 2008; Cross *et al.*, 2009).

According to Biet *et al.*, (2005), a host species can become a maintenance species through the confluence of aspects like physiopathogenesis (capacity to excrete the infectious agent), ethology (e.g. social behaviour) and ecology (feeding, population density and interactions between species). In reservoir hosts the infectious agent can be horizontally transmitted in the absence of other sources.

The wild boar and red deer (*Cervus elaphus*) are referred by some authors as being nowadays reservoir hosts, while formerly they were only affected by bovine tuberculosis in a sporadic way (Aranaz *et al.*, 2004; Santos, 2006; Trcka *et al.*, 2006; Vicente *et al.*, 2006; Gortázar *et al.*, 2007; Naranjo *et al.*, 2008).

Wild felids are very sensitive to *Mycobacterium bovis* infection (Gortázar *et al.*, 2007), and the severe course of pseudorabies infection in domestic cats is well known (Hagemoser *et al.*, 1980). It is thus logical to speculate that wild felines are also susceptible to pseudorabies virus.

This can be a problem for the Iberian lynx reintroduction program since both of these diseases etiological agents are present in other sympatric species, but furthermore important because they are prevalent in an abundant, reservoir and occasionally prey species – the wild boar. These diseases' transmission to top predators such as the Iberian lynx will probably occur when they feed over infected carcasses.

The main goals of the present study are:

- to conduct a survey on antibody seroprevalence against pseudorabies virus and to monitor bovine tuberculosis agent within a wild boar population in a potential area for Iberian lynx reintroduction of the Portuguese portion of the Guadiana valley.
- the evaluation of the adequacy of the chosen sampling and test methodology (the use of hunting bags for serum collection and ensuing ELISA testing) for large scale surveys on the sanitary status of a given wild boar population.
- to use the wild boar as an indicator species of the risk of infection with both disease agents for Iberian lynx, as well as other wild carnivore species.
- to contribute to other long-term ongoing disease monitoring studies by collecting tissue samples.

To our knowledge this was the first study on pseudorabies virus seroprevalence in this geographical area.

2. Materials and methods

2.1 Study area

This study was conducted in four municipalities within Beja's district and in one of the Évora's district, all included in Alentejo's region in southeast Portugal (Figure 3).

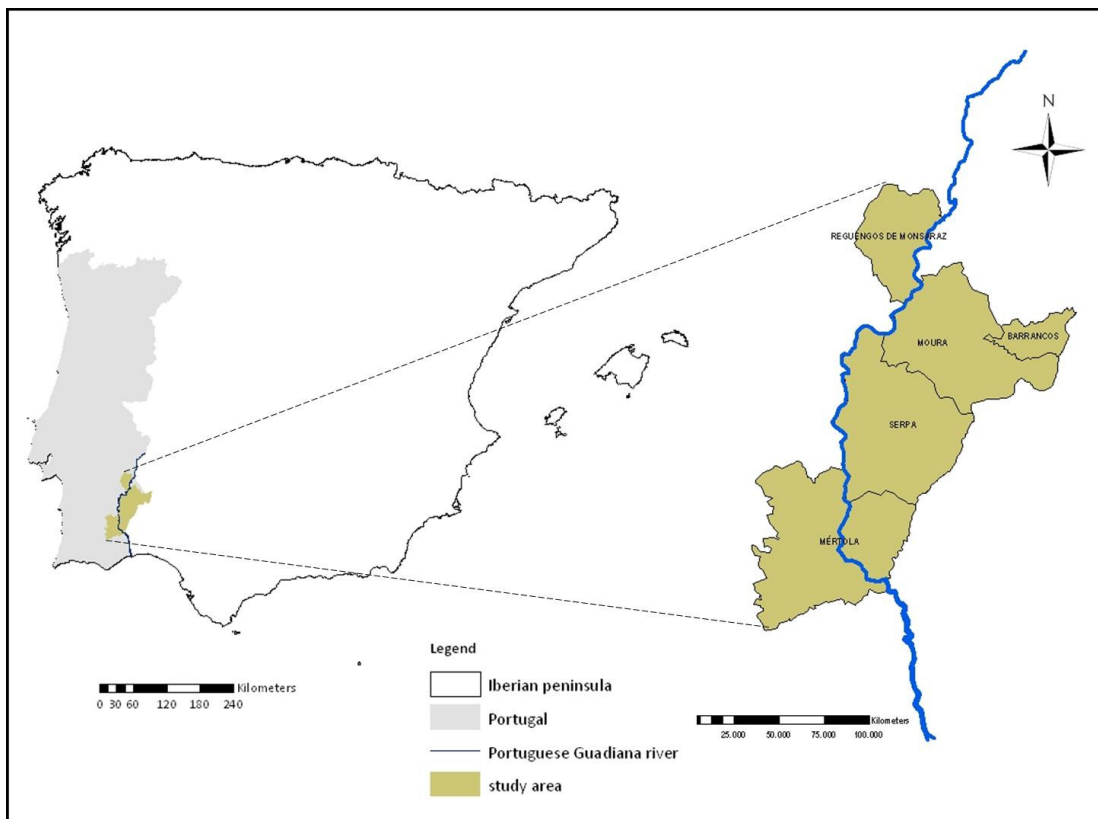


Figure 3: Geographic localization of the study area and sampled municipalities.

This region is characterized by its habitats diversity, with shrublands, scrublands and well preserved forest, with either cork-oaks (*Quercus suber*) “montados” or holm-oaks (*Quercus ilex* subsp. *rotundifolia*) predominance with or without shrubby undercover. The sub-shrubby vegetation cover is mostly composed by rockroses (*Cistus spp.*), as well as brooms (*Genista spp.*), heathers (*Erica spp.*), among others. These areas are intercalated by open areas with pastures and cereal cultures creating a mosaic (Figure 4 and 5).



Figure 4: Characteristic “Montado” landscape within the study area.



Figure 5: Permanent water source in the study area

The study area has Mediterranean characteristics with hot summers and cold, rainy winters. Economic activities in the area are mainly agriculture, livestock, and hunting.

2.2 Sampling

The chosen sampling area was defined based on the targeted areas for future reintroductions.

Serum samples were collected from hunted wild boars in five municipalities in Guadiana river valley between November 2010 and February 2011. The eleven sampling sites were grouped according to their belonging municipalities and all except one were located in the left margin of the Guadiana River. In every hunting estate sampled the wild boar was free-living except in a fenced one where animals were artificially managed.

Sampled wild boars were classified in two different age classes. Animals less than 24 months were classified as juveniles and those over 24 months as adults based on external characteristics (Ruiz-Fons *et al.*, 2007).

Pertaining to field work constraints, for some of the animals sampled it was not possible to determine either sex or age classes or both. Therefore only 50 samples had complete data and the remaining 61 were incomplete.

Blood samples were collected directly from the thoracic cavity into 15 ml sterile tubes, after the animal evisceration (Figure 6). They were kept refrigerated until arrival to laboratory. Serum was obtained by centrifugation and stored at -20°C in 0,5 ml aliquots until use.

Alongside with blood collection other tissues were collected, namely submandibular and mesenteric lymph nodes in order to contribute to other long-term ongoing studies. (Figure 7 and 8)



Figure 6: Hunted wild boars and procedures to collect blood samples



Figure 7: Tissue sample collection.



Figure 8: Submandibular lymph node collection.

2.3. Serology testing

Two different Enzyme-Linked Immunosorbent Assay (ELISA) tests were used to detect specific antibodies against pseudorabies virus and *Mycobacterium tuberculosis* complex infectious agents in the collected serum samples. Positive and negative controls were tested in duplicate in each plate and XREAD PLUS program version 2.10 was used to read the plates. This test is based in the interaction between antibody and antigen.

2.3.1 Detection of serum antibodies against Pseudorabies/ Aujeszky's disease virus

The ELISA test chosen to perform pseudorabies (gpl antigen) antibodies detection is commercially available as a kit (IDEXX HerdCheck Anti-ADV gpl, IDEXX Inc., USA).

The protocol was followed according to the manufacturer's instructions. Briefly, the sample was diluted (1:2) in sample solvent. This diluted sample (100 µl) was deposited in each well (except the control ones) in a PrV-antigen coated plate and incubated for 1 hour at room temperature. After a washing step, an anti-PrV gpl monoclonal antibody conjugate (100 µl) was added and left to incubate for 20 minutes at room temperature. The wells were washed again and a Tetramethylbenzidine (TMB) chromogen solution (100 µl) was added and incubated for 15 minutes at room temperature, generating a blue colour when interacting with the enzyme. This reaction was then interrupted by the deposition of 50 µl of Stop solution in each well. Optical density (OD) was measured in a spectrophotometer at 650 nm. The percentage of inhibition (%IN) was calculated to express the results (%IN) value using the following formula: [%IN = (mean negative control OD - mean sample OD/mean negative control OD) × 100]. The quantity of antibodies against PrV gpl was inversely proportional to the OD and directly proportional to the %IN. According to manufacturer's instructions samples were considered positive when respective %IN values were equal to or greater than 40%. For presented %IN values between 30 and 40%, samples were considered doubtful and samples with %IN values under 30% were negative. According with the manufacturer this test has a sensitivity of 95-98% and a specificity of 97–99% for domestic pigs. It also has been broadly used in wild boars.

2.3.2 Detection of serum antibodies against Mycobacterium tuberculosis complex.

The selected method for bovine tuberculosis antibody detection was based on previously validated Indirect ELISA (Aurtenetxe *et al.*, 2008).

Bovine tuberculin purified protein derivative (bPPD)³ was used as antigen to coat the wells of 96 well plates by incubating for 18 hours at room temperature. The wells were then washed with PBS and later blocked unspecific binding sites

³CZ Veterinaria SL, Porriño, Lugo, Spain.

by incubating at 37°C for 1 hour with 5% skim milk in PBS with 0.05% Tween 20. The plate was washed again and then the serum samples were directly added to the wells (100 µl/ well), at a concentration of 1:200 in PBS. Again, the wells were washed with PBS. It was then added the conjugate solution of immunoglobulin-binding protein G and horseradish peroxidase⁴. After washing 100 µl per well of o-phenylenediamine dihydrochloride (SIGMAFAST™ OPD) was added as revelation solution. The reaction is interrupted by adding 50 µl per well of sulphuric acid (H₂SO₄; 3N). The colour intensity (Figure 9) was then measured at 450 nm. The following formula expresses sample results as an ELISA percentage (E%): [sample E% = (mean sample OD/2 x mean negative control OD) x100]. Samples with E% values greater than 100 were considered positive. This test sensitivity was 79.2% and the specificity 100% (Boadella *et al.*, 2011a).

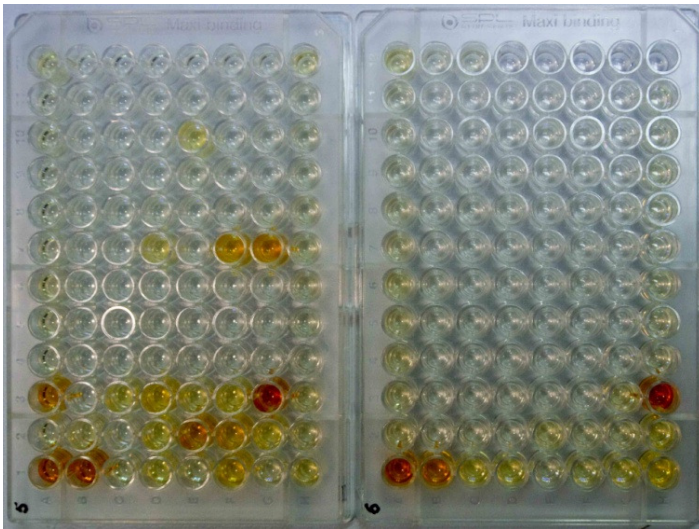


Figure 9: Two 96 well plates ready after colorimetric reaction.

2.4 Statistical analysis

Data was organized in an excel database and statistical analysis performed using VassarStats: Website for Statistical Computation, a statistical computation tool, available freely on line in <http://vassarstats.net/>. The 95% confidence intervals (CI 95%) were calculated using a method without a

⁴Sigma-Aldrich Quimica SA, Madrid, Spain.

correction for continuity. In order to test differences in seroprevalence, between different sexes and age groups and their interactions, Log-Linear Analysis was used. Three-Way Contingency Tables were built using two rows for factor A (Gender: Female/Male), two columns for factor B (Age: Adult/Juvenile) and two layers for factor C (Antibodies: Positive/Negative). All variables investigated were included in the initial model (saturated model) and then removed in a stepwise way. ABC represents the 3-way interaction between factors A, B and C. AB(C), AC(B) and BC(A) represent interactions between pairs of variables when the effects of the third variable are removed. For example, AB(C) represents the interaction between gender and age when the effects between each and the presence/absence of the antibodies are removed.

3. Results

From the 111 sampled wild boars, 32 were females, 23 were males and 56 with undetermined sex. Thirty eight animals were adults; twenty three were juveniles and the remaining 50 of undetermined age. The eleven sampling sites were grouped according to their belonging municipalities and the respective seroprevalence of antibodies against pseudorabies virus and bovine tuberculosis are shown in Table 1.

Table 1: Number of samples, number of seropositive samples to pseudorabies virus (PrV) and bovine purified protein derivative (bPPD) and respective prevalence (%) with 95% confidence intervals (95% CI), in each sampled municipality.

Municipality	Samples (n°.)	Pseudorabies + (PrV)		Bovine tuberculosis + (bPPD)	
		N	%(95% CI)	N	%(95% CI)
Barrancos	35	20	57,1 (40-70)	2	5,7 (0-20)
Mértola	5	0	–	0	–
Moura	45	22	48,9 (40-60)	8	17,8 (10-30)
Serpa	22	8	36,4 (20-60)	1	4,5 (0-20)
Reguengos	4	1	25,0 (0-70)	0	–
Total	111	51	45,9 (40-60)	11	9,9 (10-20)

Regarding to the distribution of seroprevalence for pseudorabies antibodies in a municipal level, Barrancos presented the highest prevalence, followed by Moura, Serpa and Reguengos. In Mértola, no tested sample presented antibodies for this disease agent.

Concerning the bovine tuberculosis agent distribution, the municipality that presented higher antibodies seroprevalence was Moura, followed by Barrancos and Serpa. Mértola and Reguengos showed no positive samples (Figure 10).

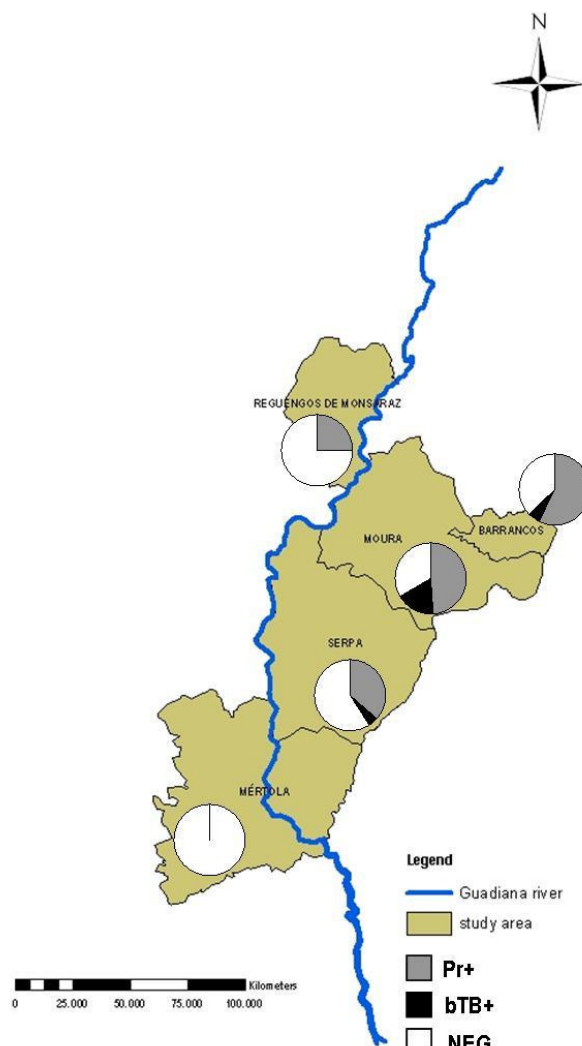


Figure 10: Seroprevalence distribution, in each sampled municipality. Seropositivity for antibodies against Pseudorabies virus (Pr+) and bovine tuberculosis agent (bTB+); Absence of seropositivity (NEG).

Seventeen out of 32 females were seropositive to pseudorabies antibodies, as well as 11 out of 23 males and 23 out of 56 of unrecorded sex. Regarding the 11 seropositive samples for bovine tuberculosis (bTB) antibodies, 4 belonged to females; 2 were from males and 5 from undetermined sex. Seropositivity rates for gender classes were in females for PrV and for bTB agent respectively 53,1% (CI 95%= 40-70) and 12,5% (CI 95%= 5-28), in males 47,8% (CI 95%= 29-67) and 95%= 40-70) and 12,5% (CI 95%= 5-28), in males 47,8% (CI 95%= 29-67) and

8,7% (CI 95%= 2-26) and for unrecorded gender 41,1% (CI 95%= 30-54) and 8,9% (CI 95%= 4-2) (Figures 11 and 12).

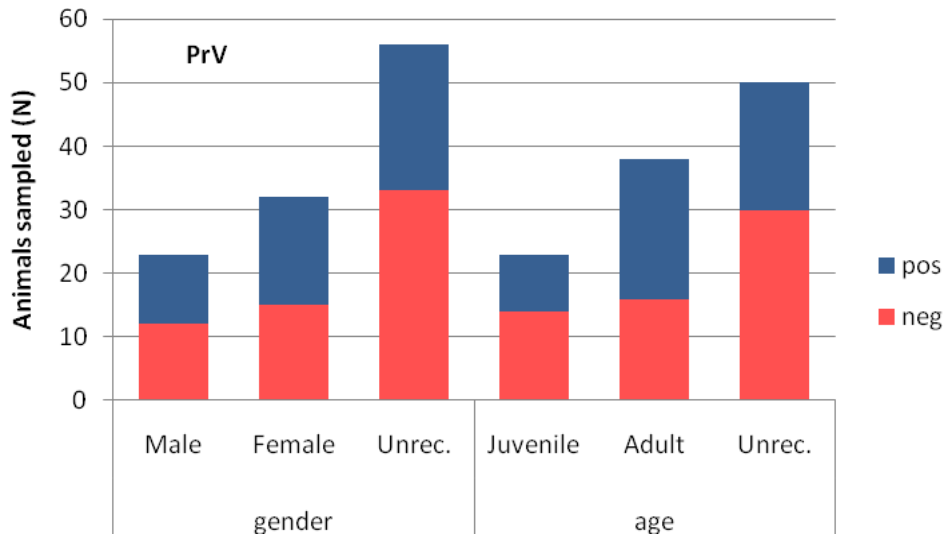


Figure 11: Wild boar serology results for pseudorabies virus antibodies depending on age and gender classes.

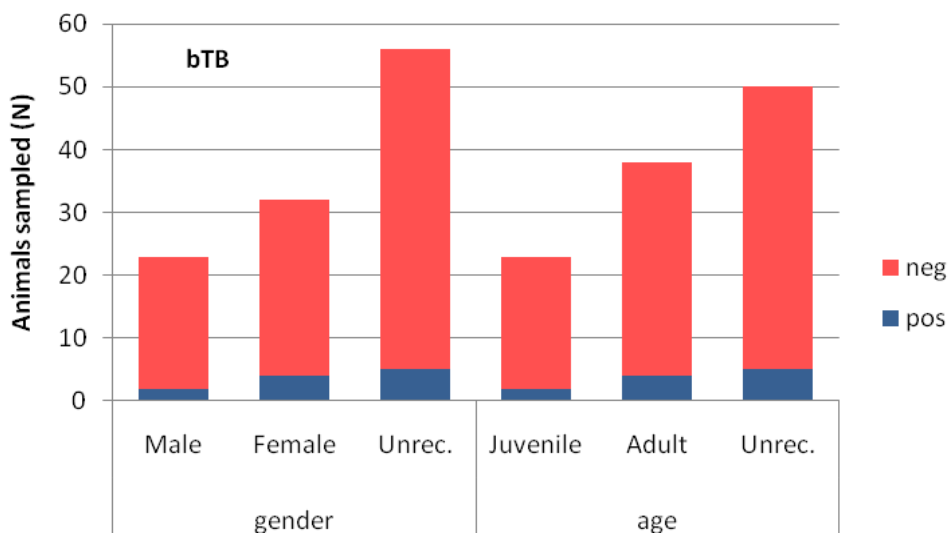


Figure 12: Wild boar serology results in which antibodies against bovine tuberculosis agent (*Mycobacterium bovis*) were detected depending on age and gender classes.

Of 38 adult animals, 22 tested positive to PrV and 4 to bTB antibodies. Of the 23 juveniles, 9 were seropositive to PrV and 2 to bTB antibodies. Within the animals with unrecorded age (n=50), 20 were seropositive to pseudorabies and 5 to bovine tuberculosis antibodies. Seropositivity rates for age classes were for PrV and bTB respectively 57,9% (CI 95%= 42-72) and 10,5% (CI 95%= 4-24) in adults; 39,1% (CI 95%= 22-59) and 8,7% (CI 95%= 2,4-2,6) in juveniles and finally 40% (CI 95%= 27-53) and 10% (CI 95%= 4-21) in undetermined age samples.

In the overall 111 serum samples, 51 tested positive for pseudorabies virus antibodies, 11 for bovine tuberculosis antibodies, with seropositivity prevalences of 45,9% (CI95%= 40-60) and 9,9% (CI95%= 10-20), respectively. Five blood samples tested positive for contact with both diseases agents (prevalence was 4,5% and CI95%= 0-10).

To perform the Log-Linear Analysis only the samples with complete data for all variables were used. Log-Linear Analysis results for evidence of contact with pseudorabies and bovine tuberculosis agents are shown in Tables 2 and 3, respectively.

Table 2: Log-Linear Analysis results for pseudorabies ELISA test in sampled wild boar

Source	G ²	df	P
ABC	4.68	4	0.3217
AB(C)	3.34	2	0.1882
AC(B)	0.76	2	0.6839
BC(A)	0.74	2	0.6907

Table 3: Log-Linear Analysis results for bovine tuberculosis ELISA test in sampled wild boar

Source	G ²	df	P
ABC	3.82	4	0.4309
AB(C)	3.38	2	0.1845
AC(B)	0.46	2	0.7945
BC(A)	0.22	2	0.8958

The results obtained showed that there are no significant interactions among any of the variables (gender, age, and antibodies), since each one is independent from the others, for the established level of significance of $\alpha=0.05$.

4. Discussion

This work is the first reported survey for pseudorabies virus seroprevalence in the wild boar in the Guadiana valley, the principal area for the reintroduction of Iberian lynx in Portugal.

The decision to study this particular geographic area was related to its high support capacity (Sarmiento *et al.*, 2009b), not only for the Iberian lynx, but also for other carnivores like the wild cat (*Felis silvestris*).

Therefore, considering that there is already an Action Plan in place in the Natura 2000 Network in Moura-Barrancos (Special Conservation Zone); the Adiça area, contiguous to Contenda-Barrancos has a high habitat suitability and high lynx's staple prey density; and the Iberian lynx corridor definition and its presence in western Sierra Morena, it is reasonable to recognise that these border areas in the Guadiana valley are probably the ones with more adequate characteristics to attend to the reintroduced Iberian lynx needs. As a result, it is of the utmost importance to be aware of the sanitary status of wildlife within this area. This is an important aspect, considered in the Iberian Lynx Conservation Strategy II (Grupo de trabajo del Lince Ibérico, 2008). In this work, both pseudorabies and bovine tuberculosis are referred as important infections to be surveyed in sympatric animals, namely wild and domestic ungulates as well as wild and domestic carnivores.

Pseudorabies and bovine tuberculosis have been reported in a wild potential reservoir (wild boar) in several European countries (Meng *et al.*, 2009). In areas like the Mediterranean region of Spain, where the hunting industry promoted a more intensive management of game ungulates for economic reasons, and in some patches of the Portuguese territory, special epidemiological circumstances arose and are associated with higher prevalences of these diseases (Aranaz *et al.*, 2004; Cunha *et al.*, 2011; Müller *et al.*, 2011).

A wide range of pseudorabies seroprevalence (0,3% to 66%) is reported in different regions of Europe (Müller *et al.*, 2011). The seroprevalence obtained in the present study area (45,9%) is amongst the highest rates so far documented. According to Müller *et al.* (2011) the countries that reported higher seropositivity rates using ELISA method were Croatia (54,55%), Romania (55,18%), France (up to 54% in some continental areas and 53% in Corsica) and Spain. In this latter country the seroprevalence in South-central region is at the same level (45,95%) as the one observed in Portuguese Guadiana valley. Nevertheless, pseudorabies virus seroprevalence has a higher value (66,06%) when the surveyed animals come from intensively managed populations for hunting purposes (Ruiz-Fons *et al.*, 2007). Probably, due to residual or absent management actions concerning the increase of game populations, Canado *et al.* (2012) reported lower pseudorabies seroprevalence (8%) in a Portuguese northern municipality (Vinhais).

Considering the sample from this work representative of the hunting bags', a higher proportion of contact rate with the causative agent occurs in adults (57,9%) than in juveniles (39,1%). The present results are consistent with other researches (Vicente *et al.*, 2005; Lari *et al.*, 2006; Pannwitz *et al.*, 2012) and are most likely to be justified by the fact that older animals have had a longer time frame for possible exposure.

The difference in pseudorabies seroprevalence between sexes is not noticeable in this work but still the value in females (53,1%) is higher than in males (47,8%). Higher seroprevalence in females have also been reported in Spain (Vicente *et al.*, 2005), even though this difference has not been described for other European countries (Lari *et al.*, 2006; Pannwitz *et al.*, 2012).

Within the considered study area, wild boar and Iberian pig share the same landscape, thus, the high contact rate with pseudorabies virus evidenced in the present work may raise economical concerns.

Bovine tuberculosis monitoring in wildlife has become a major concern since wildlife reservoirs can maintain the disease among livestock despite the eradication plans implemented in several countries (Pavlik *et al.*, 2002; Cross *et al.*, 2009).

In Portugal until recently, bovine tuberculosis in cattle (subject of a national eradication plan) was decreasing, but since 2008 the number of infected animals and herds has grown, increasing concern about the success of the control program (Cunha *et al.*, 2011). This is probably the reason why potential wildlife reservoirs are being subject of attention by sanitary authorities.

The information concerning bovine tuberculosis prevalence in the wild boar in Portugal is scarce, but an overall prevalence of 11,1% and high rate (63%) of *Mycobacterium bovis* isolation in Eastern-central region have been reported (Santos *et al.*, 2009b; Cunha *et al.*, 2011).

In the present study the seroprevalence found for *Mycobacterium bovis* antibodies (9,9%) is within the range of previously reported *M. bovis* infection rates in tuberculosis epidemiological risk area in Eastern South-central Portugal (Santos *et al.*, 2009b; Vieira-Pinto *et al.*, 2011). Also, an higher evidence of exposure to bovine tuberculosis agent was found for females (12,5%) than for males (8,7%), which is in agreement with Santos' results. This is coherent with the more gregarious nature of females, increasing the probability of infection. Age differences in seroprevalence rates (although slight) were also present, and adults had higher antibody prevalence (10,5%) than juveniles (8,7%). This fact can be explained by the chronic character of the disease (Bengis, 1999). This data is in compliance with results from other surveys (Vicente *et al.*, 2006) but contrasting with others (Santos *et al.*, 2009b).

The present results should be interpreted with caution. Despite the small sample size, sample collection from hunted wild boars can be considered as random and representative of the study area, even though for larger sample

sizes differences between variables could be statistically significant. Misinterpretation of the seroprevalence results can be induced by biased sampling, serological assay or investigation period, being desirable a longer study period and a larger sample size.

Tests based on antibody detection may fail to detect some of the recently infected animals (Ruiz-Fons *et al.*, 2007), as well as those with delayed seroconversion (Müller *et al.*, 2011) or in a latency period. So, under-detection of infected individuals is a concern (Ruiz-Fons *et al.*, 2007). In contrast crossed reactions with related members of the *Mycobacterium tuberculosis* complex may occur increasing the proportion of positive detected samples (Boadella *et al.*, 2011b). Regarding the ELISA test used for detection of bovine tuberculosis antibodies in this work, cross reactions between different mycobacteria (*Mycobacterium bovis*, and *Mycobacterium avium*) have already been studied and a high specificity was confirmed (Boadella *et al.*, 2011a).

Serologic tests are easy to perform, sensitive, specific, reproducible and not expensive to perform, thus highly recommendable for large-scale research (Sedlak *et al.*, 2008; Boadella *et al.*, 2011b; Boadella *et al.*, 2012). The implementation of large-scale serosurveys with potential wild reservoir species may bring enlightenment about the diseases occurrence in Portuguese territory, with benefits both economical and conservationist.

In this work the wild boar was chosen as the targeted species for several reasons:

- its abundance in the destination reintroduction site;
- the possibility to sample carcasses from hunting bags;
- the possibility to become an alternative prey for the Iberian lynx;
- for its already recognizable role as a maintenance or reservoir host for Pseudorabies virus and *Mycobacterium bovis*, and possibility to spread these disease agents to other wildlife species.

The evidence of exposure to pseudorabies and bovine tuberculosis agents within a potential reservoir species, suggests the presence of both diseases in

the environment. This can pose considerable risks of infection to some endangered species mainly concerning top predators like the wild cat and lynxes or other carnivores like bears and wolves (Lari *et al.*, 2006; Sedlak *et al.*, 2008; Boadella *et al.*, 2012).

The control of these diseases in wildlife is most certainly related with game management control. The adopted measures should focus on balanced abundance thresholds, the discouragement of practices that promote aggregation and strict supervision of any animal translocation. Notwithstanding, to reduce the risk that the presence of diseases within the lynx reintroduction site represents, it is fundamental to discuss a significant reduction in wild boar population. Nevertheless, to assure these measures effectiveness it is important to gather information about the species diversity, its populations, as well as its sanitary status.

Although vaccination is not an option at present, it will probably acquire greater importance in future years.

5. Conclusions

Despite the relative small number of animals sampled in some municipalities to get any reliable conclusion about these diseases' agents seroprevalence, as a whole it is possible to have a spatial perspective of its distribution.

It is reasonable to assume that pseudorabies is highly prevalent in wild boar population within the study area (the main potential place for the Iberian lynx reintroduction) and the observed seroprevalence data is amongst the highest reported in Europe. This study also reports seroprevalence rates for bovine tuberculosis similar to those previously described for the same geographical area.

Notwithstanding the use of other more sensitive methods in those previous studies, the serologic test performed in this work confirms the appropriateness for continuous regular surveys at a lower cost and may be a valuable tool for disease surveillance and consequently for the establishment of control measures and evaluation of their effectiveness.

Considering the study area as a possible Iberian lynx reintroduction site, there is an increased risk of infection for future reintroduced animals. Given the similarity of ecological characteristics of the bordering Spanish territory, inference about both studied diseases epidemiological cycles can be made to implement a disease control program.

Hunting bags can represent important and convenient resources of information although field constraints were particularly disappointing, whereas in some of the hunting bags it was very difficult to catch up and get all necessary data for each animal. It is therefore advisable for future studies that imply sample collection on hunted animals to have a larger working team.

In this work the study of these two economically important diseases focus on its conservation implications. Nevertheless the role of the wild boar in these diseases' epidemiology should be assessed both in livestock and public health. Even if not in the scope of this work, it is noticeable that the low standards of health and safety procedures among hunters and carcass handlers may place them in major infection risk.

Future prospects

Portugal should develop an effective nationwide disease monitoring program, enabling the integration of information in an international epidemiological surveillance network. To accomplish this task, a multidisciplinary working team should be assigned to establish specific priorities, to design surveillance programs, to establish adequate protocols for sample collection and testing and to keep the highest standards in scientific production.

This study results suggest the need to intensify sample collection by enlarging the sampling area and sample size. The present work also reinforces the usefulness of serological tests in fieldwork. These practices when performed in a regular basis may provide long-term surveillance information about both diseases' dynamics. It is also urgent to determine wild boars role in these diseases' epidemiology. The surveillance programmes developed in the future should also encompass other ungulate and non-ungulate species, when pseudorabies and tuberculosis are considered.

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