U.PORTO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

Relatório Final de Estágio

Mestrado Integrado Medicina Veterinária

RELATIONSHIPS BETWEEN PARENTAL AND OFFSPRING CELLULAR IMMUNE RESPONSE IN RED DEER (*Cervus elaphus*): IMPLICATIONS FOR THE CONTROL OF MYCOBACTERIAL DISEASES

João Luís Teixeira de Queirós

Orientador:
Professor Paulo Manuel Rodrigues Martins da Costa

Co-orientadores:
Professor Paulo Célio Pereira Martins Alves
Doutor Christian Gortázar Schmidt

Porto, Abril 2010

U.PORTO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

Relatório Final de Estágio

Mestrado Integrado Medicina Veterinária

RELATIONSHIPS BETWEEN PARENTAL AND OFFSPRING CELLULAR IMMUNE RESPONSE IN RED DEER (*Cervus elaphus*): IMPLICATIONS FOR THE CONTROL OF MYCOBACTERIAL DISEASES

João Luís Teixeira de Queirós

Orientador:

Professor Paulo Manuel Rodrigues Martins da Costa

Co-orientadores:

Professor Paulo Célio Pereira Martins Alves

Doutor Christian Gortázar Schmidt

Porto, Abril 2010

RESUMO

O presente trabalho tinha como objectivo estudar a relação entre a resposta imune celular parental e das suas crias, assim como o sua potencial influência nos resultados ao teste intra-dérmico. A hipótese proposta era que indivíduos de diferentes pais teriam diferente resposta imune. Para tal, utilizaram-se 366 crias, 17 machos (pais) e 143 fêmeas (mães) de veado de uma exploração espanhola. Em três locais do pescoço de cada animal, respectivamente, injectaram-se 0,1 ml de *M. bovis* PPD, 0,1 ml de *M. avium* PPD e 0,1 ml de PHA como controlo positivo, e mediu-se o incremento da espessura da pele após 72 horas. Com estes resultados, realizamos 3 modelos estatísticos: I) relacionamos o incremento de pele aos diferentes antigénios entre si, nas crias (GLM); II) relacionamos o incremento de pele das crias aos antigénios *M. avium* PPD, *M. bovis* PPD e PHA, em função do pai; III) relacionamos a resposta imune celular das crias com a dos seus pais. Também, realizamos a necrópsia de todos os animais suspeitos (n=22) e histopatologia, cultura micobacterial e PCR dos 11 animais mais positivos. A prevalência aparente ao teste intra-dérmico e ELISA aos antigénios micobacterianos não foi confirmada à necrópsia (lesões compatíveis com bTB e PTB), histopatologia, cultura micobacterial e PCR. Evidenciaram-se efeitos parentais significativos (ou relações empíricas), incluindo efeitos da idade maternal na resposta ao teste intra-dérmico nas crias e efeitos da resposta imune paternal entre as crias fêmea. Estes efeitos parentais deveriam ser considerados em conjunto com os outros factores de variação da resposta ao teste intra-dérmico conhecidos, aquando da interpretação dos resultados do teste em veados. Os nossos resultados apoiam a hipótese da base genética da resposta imune celular em veados, o que deveria ser confirmado por estudos de “heritability”.

Palavras-chave: Doença micobacteriana; Efeito parental “heritability”; Phytohaemagglutinin; Resposta imune celular; Veado (*Cervus elaphus*)

ABSTRACT

The aim of this work was to study the relationships between the cellular immune response in parents and their offspring in red deer, as well as its potential influence on the results of the tuberculin skin-test. We hypothesized that individuals from different fathers would have a different immune responsiveness. Deer sampled in this study included 366 calves, 17 stags and 143 hinds from a Spanish farm. We injected three sites of the neck with 0.1 ml *M. bovis* PPD, 0.1 ml *M. avium* PPD and 0.1 ml of PHA as positive control, and measured the skin fold increase at time 72 h. We performed 3 statistical models to test: I) the relationship between the skin fold increases to different antigens (GLM); II) the differences between the skin fold increases to *M. avium* PPD, *M. bovis* PPD and PHA in calves as a function of the stag (GLMMs); III) the relationship between the cellular immune response of the calves and those of the assigned parents (GLMMs). Also, we performed the necropsy of all suspect positive calves (n=22) and histopathology, mycobacterial culture and PCR for the 11 highest positive animals. Apparent prevalence in skin-test to mycobacterial antigens was not confirmed in necropsy (bTB and PTB compatible lesions), histopathology, mycobacterial culture and PCR. Significant parental effects (or empirical relationships) were evidenced, including effects of maternal hind age on calf skin-test responsiveness, and effects of the stag immune response only among female calves. These effects should be considered along with other known factors causing variation in skin-test responsiveness when interpreting skin-test results in red deer. Our findings provided support to a genetic basis of immune cellular response in farmed red deer, which should be confirmed by heritability studies.

Key Words: Cellular immune response; Heritability; Mycobacterial disease; Phytohaemagglutinin; Red deer (*Cervus elaphus*).

AGRADECIMENTOS

Apesar de querer partilhar muitas coisas do que aprendi ao longo de todos estes anos de estudo, concretizados com o culminar deste trabalho, não posso deixar de escrever a toda a gente que me acompanhou ao longo da minha vida pessoal, escolar, profissional... e que a todos lhes devo uma retribuição:

Meus pais, *José e Maria*

Graças a eles nasci, cresci, estudei…me formei tanto como pessoa como profissional. Tudo isto Lhes devo a Eles. Não há palavras que possam expressar tudo o que sinto por Eles e por isso espero continuar a retribuir-Lhes com os meus actos.

Minha irmã*, Cátia*

Sempre e para sempre ao meu lado…desejo retribuir-lhe tudo o que fez por mim.

Minha namorada*, Filipa*

Sempre me acompanhou durante quase metade da minha vida e por conseguinte durante este percurso académico. Sempre me compreendeu nas alturas de estudar, me apoiou e me deu força para fazer sempre melhor, o melhor de mim! Muito do que sou também lhe devo a ela.

Restante família

Toda me apoiou durante toda a minha vida académica….Mas não posso deixar de mencionar: Minha avó – Amélia, Meus tios – Maria e Neca; Mário e Laurinda; José e Emilia, Meus primos João, Ivo e Sara.

Meios-irmãos, *Sérgio e Igor*

Vivemos sempre juntos durante este percurso e criamos um laço muito forte aprendendo a viver juntos! Espero que este nunca se desfaça. Já tenho saudades de viver Convosco!!!

Meus amigos

A todos lhes quero deixar uma palavra de Amigo…nada melhor do que fazerem parte do meu trabalho como fazem da minha vida,

Bruno, Joana, Paulo, Sara, Patrícia, Cláudia, Suzete, João, tive o gosto de conviver quase durante toda a minha vida de estudante.

Sérgio, Dinis, Joaquim, Manel, Duarte, Zenaide, João, Bruno, Alex, Sofia, Tita, Raquel, Bianca, Juliana, Diana, Magui, Mónica, Bea, etc...que tive o prazer de conhecer e conviver durante esta etapa da minha vida.

Ao IREC

Um local com um misto de cultura e conhecimento incrível, do qual aprendi e continuo a aprender muito a cada dia. A todos as pessoas do IREC quero dizer, não se vão ver livre de mim…vão ter que aguentar com este Português por mais um tempo.

Dr. Christian,

Co-orientador deste relatório e ao qual devo esta oportunidade de fazer este trabalho. Pessoa exemplar pela sua dedicação ao trabalho e aos seus estudantes. Graças a si tive a oportunidade de fazer este estágio e também o meu primeiro emprego. Espero continuar a retribuir-lhe toda esta confiança.

Joaquín, Tânia e Raquel,

Meus instrutores durante as diferentes fases de execução do meu trabalho e com os quais aprendi muito e em diversas áreas.

Jose António Ortiz,

Veterinário da exploração de veados e grande companheiro de trabalho. Iste trabalho só foi possível pela sua grande colaboração, disponibilidade e empenho.

Mauricio,

Companheiro de viagem…. meu instrutor de Espanhol…

Mariana e Sandra,

Vivi convosco os meus primeiros tempos em Espanha e tenho-vos enorme gratificação.

A todos os alunos do Máster,

Convivemos durante todo este estágio…quer nas aulas como nas cervejas….Muito importantes na minha integração na cultura espanhola.

Elisa,

Portuguesa de gema e quem me ajudou a vir para este centro.

Meus professores,

Tive muitos durante todo o percurso académico e a todos eles devo uma retribuição pelo o que cada um em seu momento me ensinou. Não menciono nomes porque todos eles foram importantes, quer como Professores, Amigos ou Exemplos que tive e tenho com grande admiração.

Peço desculpa se me esqueci de alguém.

A todas a estas pessoas, quero dizer um MUITO OBRIGADO por tudo e todas ELAS têm a minha consideração.

CONTENTS

FIGURE INDEX

TABLE INDEX

ABREVIATION LIST

bTB - Bovine tuberculosis
CCT - Comparative cervical test
df - Degrees freedom
DNA - Deoxyribonucleic acid
EFSA - European Food Safety Authority
ELISA - Enzyme-linked immunosorbent assay
e. g. - E*xempli gratia*
F - F-stastistic
FFPE - Formalin-fixed paraffin-embbeded
GLM - General Linear Model
GLMM - Generalized Mixed Linear Models
h - Hours
h^2 - Heritability
LNs - Lymph nods
MAP - *Mycobacterium avium paratuberculosis*
MAPIA - Multiantigen print immunoassay
***M. avium* PPD -** Avium purified protein derivative
***M. bovis* PPD -** Bovine purified protein derivative
min - Minutes
ml - Millilitre
MPB70 - *M. bovis*-derived protein antigen MPB70
***M. bovis* -** *Mycobacterium bovis*
n - Number
OD - Optical density
OIE - *Office International Epizooties*
***p* -** Probability

PCR - Polymerase Chain Reaction

PHA - Phytohemagglutinin

PPA3 - Paratuberculosis protoplasmatic antigen 3

PPD - Purified protein derivative

PTB - Paratuberculosis

SE - Standard error

SST - Single intradermal tuberculin test

TB – Tuberculosis

µl - Microlitre

µm - Micrometre

µM - Micromole

V_A - Additive genetic variance

V_E - Environmental variance

V_P - Phenotypic variance

***β* -** *Beta*

% - Odds

ºC - Celsius degrees

= - Equal

< - Lower

> - Upper

1. INTRODUCTION

1.1 RED DEER (*Cervus elaphus*)

Taxonomy

Kingdom: Animalia

Phylum: Chordata

Class: Mammalia

Order: Artiodactyla

Suborder: Ruminantia

Family: Cervidae

Sub-family: Cervinae

Species: *Cervus elaphus*

Number chromosomes: (2n)=68.

Figure I: Red deer (*Cervus elaphus* Linnaeus, 1758)
(Source: Christian Gortázar – IREC)

Red deer, *Cervus elaphus* (Figure I), is originated from Eurasian lines and it is one of forty species of cervids that exist worldwide. In Europe, there are thirteen subspecies of red deer, being *Cervus elaphus hispánicus* (Erxleben 1777) the subspecies that inhabits in south-western Spain (Soriguer *et al.* 1994). In the Iberian Peninsula, red deer is an ecologically and economically important game species, with an increasing geographical distribution, principally in last decades (Gortázar *et al.* 2000; Heitor 2004; Salazar 2009). These widespread expansions have been facilitated by numerous factors, including regulation of exploitation and control of poaching (Gortázar *et al.* 2000), rural exodus and abandonment of agricultural land (Acevedo *et al.* 2006), introductions and reintroductions (Gortázar *et al.* 2000), the loss of top predators (Tellería & Royuela 1985; Jedrzejewska *et al.* 1997) and creation of protected areas (Alados 1997). However, anthropogenic expansion, primarily carried out for hunting purposes, is the key factor responsible for the expansion of large herbivore populations (Gortázar *et al.* 2000; Whittaker *et al.* 2001; Acevedo & Cassinello 2008). This requirements of animals for hunting purposes prompted increase of industrial deer farms in Spain, particularly after the eighties (Carranza 1999; Martínez 2000).

1.2 FARMED RED DEER

Deer have been farmed for centuries in several countries and under a variety of farming systems, including farms, extensive ranching conditions, hunting parks, zoological parks and private estates (Mackintosh *et al.* 2004). Farmed deer are most common in New Zealand (1.6 million), China (1 million), Russia (400,000) and United states (250,000) (Griffin & Mackintosh 2000). In Europe, Fletcher (2004a) estimated 410,000 farmed deer, mainly red and fallow deer. There are substantial regional differences in farmed deer industry, relating to species farmed, management, methods of slaughter and venison marketing (Fletcher 2004a). In Spain, deer

farming is a growing activity and most of these farms are devoted to producing red deer (*Cervus elaphus*) for restocking purposes, usually for fenced estates for hunting purposes (Fernández-de-Mera *et al.* 2009). Both farms and fenced semi-free ranging populations of the deer are managed through artificial watering and feeding. The difference being that in farms most of the food is provided artificially, whereas in hunting estates artificially feeding is usually only offered in limiting seasons, such as summer. This artificial management causes overabundance and animal aggregation (Figure II) with numerous associated sanitary problems (Gortázar *et al.* 2006), particularly bovine tuberculosis (Vicente *et al.* 2006, 2007).

Figure II: Farmed red deer - group of the hinds with the calves (Source: Joaquín Vicente Baños - IREC).

The fact that many wildlife diseases are shared with man or with domestic livestock, with deer having close contact to domestic cattle, sheep and goats and being a source of meat, makes the surveillance of diseases such as tuberculosis and paratuberculosis a priority in wildlife management and sanitary control (Fredriksen *et al.* 2004; Gortázar *et al.* 2007). Furthermore, over 10,000 wild deer are captured and translocated yearly between hunting estates in Spain (Soriguer *et al.* 1998), which increase the risk of dispersion and maintenance of these diseases. In this context, testing of deer either in farms as in fence areas in order to avoid sanitary risks becomes of principal importance (European Food Safety Authority 2008).

1.3 IMPORTANCE MYCOBACTERIOSIS IN WILDLIFE/LIVESTOCK/HUMANS

1.3.1 TUBERCULOSIS

Tuberculosis (TB) is one the most widespread diseases of the humanity and animals and has recently reemerged as a major health concern. Each year, approximately 2 million persons worldwide die of tuberculosis and 9 million become infected (Centers for Disease Control and Prevention 2007). The prevalence of TB is continuing to increase because of the increased number of patients infected with human immunodeficiency virus, bacterial resistance to

medications, increased international travel and immigration from countries with high prevalence and the growing numbers of the homeless and drug abusers (Goldrick 2004). Even though most of the cases of human TB are caused by *Mycobacterium tuberculosis*, a small proportion is caused by *Mycobacterium bovis* (*M. bovis*) (O'Reilly *et al.* 1995). In Europe, 120 cases of TB due *to M. bovis* were reported in humans, in 2006. The highest proportions of reported and confirmed cases occurred in Germany and the United Kingdom (67.5%) (European Food Safety Authority 2009). *M. bovis*, the etiological agent of bovine tuberculosis (bTB), can infect a wide range of domestic and wild animals (Grange *et al.* 1987; Morris *et al.* 1994; O'Reilly *et al.* 1995; De Lisle *et al.* 2002). The infection of a diversity free-ranging wildlife species with *M. bovis* can cause problems for biodiversity and species conservation, and in several cases, like Eurasian badgers (*Meles meles*) in Great Britain and Ireland (Clifton-Hadley *et al.* 1993; Griffin *et al.* 2005), white-tailed deer (*Odocoileus virginianus*) in the United States (Schimtt et all. 1997; O'Brien *et al.* 2002, 2006a), brushtail possums (*Trichosurus vulpecula*) in New Zealand (Coleman *et al.* 2006; Porphyre *et al.* 2007), wild boar (*Sus scrofa*) in Spain and Portugal **(**Gortázar *et al.* 2003; Naranjo *et al.* 2008; Duarte et al 2008; Santos *et al.* 2009), African buffalo (*Syncerus caffer*) in south Africa (Michel *et al.* 2006) or the bison (*Bison bison*) in Canada (Choquete *et al.* 1961), infection is self-sustaining, providing a reservoir of infection that can spill over to livestock with economic and zoonotic consequences (Aranaz *et al.* 2004; Chambers 2009. For Ryan *et al.* (2006), farmed deer are also considered a maintenance host for bTB, in the absence of infection in other hosts, in contrast to the wild deer situation. However, others authors may consider deer a reservoir per se. In Spain, bTB is highly prevalence among wild deer, with 15% of red deer (*Cervus elaphus*) from southern Spain showing bTB compatible lesions at necropsy (Vicente *et al.* 2006). Local *M. bovis* infection prevalence up to 27% has been recorded in wild red deer (Gortázar *et al.* 2008). In Portugal (Idanha-a-Nova county), the prevalence of red deer with bTB compatible lesions at necropsy was 11.5%, arriving this value to 27.8% in the south part of the county (Alberto 2009).

1.3.2 PARATUBERCULOSIS

Paratuberculosis (PTB) or Johne's disease represents a mycobacterial disease of major importance to the livestock farming industry after elimination of bTB (O'Brien *et al.* 2006b). Its distribution is worldwide and the disease is causing severe financial losses among cattle producers (Hasanova & Pavlik 2006), sheep and goat producers (Dhand *et al.* 2007), deer farmers and game rancher (de Lisle *et al.* 2003). Cervids, like red deer (*Cervus elaphus*), fallow deer (*Dama dama*) and roe deer (*Capreolus capreolus*) (Machackova *et al.* 2004; Alvarez *et al.* 2005; Robino *et al.* 2008), and other wild ruminants have frequently been identified as PTB hosts, and high prevalence along with clinical disease is reported in some cases (Balseiro *et al.* 2008). Moreover, *Mycobacterium avium* subspecies *paratuberculosis* (MAP) was isolated from

wild boar (*Sus scrofa*), foxes (*Vulpes vulpes*), stoats (*Mustela erminea*), weasels (*Mustela nivalis*), badgers *(Meles meles*), wood mice (*Apodemus sylvaticus*), rats (*Rattus norvegicus*), brown hares (*Lepus europaeus*), jackdaws (*Corvus monedula*), rooks (*Corvus frugilegus*), crows (*Corvus corone*), wild rabbits (*Oryctolagus cuniculus*) and among other wildlife (Greig *et al.* 1997, 1999; Beard *et al.* 1999, 2001a,b; Álvarez *et al.* 2005). Strain similarities with isolates from domestic ruminants strongly suggest interspecies transmission (Florou *et al.* 2008; Robino *et al.* 2008). Furthermore, wildlife PTB reservoirs may further limits the success of PTB control in domestic ruminants (Daniels *et al.* 2003). In addition, this bacterium has received a progressively more wide interest because of a rapid growing body of scientific evidence which suggests that human infection with MAP may be causing cases of Crohn's disease (Naser *et al.* 2004; Uzoigwe *et al.* 2007). However, the infection of humans with this bacteria and possible association with Crohn's disease remains a controversial issue and requires more study (Feller *et al.* 2007; Nacy *et al.* 2008). In Spain, the prevalence of PTB among red deer is less well known, but preliminary study revealed 30% prevalence of antibodies against PPA3 in red deer (Reys-García *et al.* 2008) and clinical cases have occasionally reported in farmed red deer (Fernández-de-Mera *et al.* 2009). In Portugal (Contenda perimeter forest), Maio (2009) reported two cases of PTB in red deer by PCR analysis to mesenteric lymph nodes (of 10 animals analyzed).

1.4 MYCOBACTERIOSIS IN RED DEER

1.4.1 AETHOLOGY

Mycobacterium is the only genus listed in the Family Mycobacteriaceae in Bergey's Manual of Systematic Bacteriology (Wayne & Kubica 1986), but the genus is considered to be closely related to the other mycolic acid-containing genera of cell wall chemotype IV: *Caseobacter, Corynebacterium*, *Nocardia* and *Rhodococcus*, within Actinomycetales (Goodfellow & Cross 1984**).** The number of *Mycobacterium* species has increased from about 40 in 1980 (Skerman *et al.* 1980) to about 110 in 2004 (Hartmans *et al.* 2006). The description of novel species is paralleled by the development of molecular methods and by the increased recognition that slow growing mycobacteria are clinically important and fast-growing mycobacteria are ecologically important (Hartmans *et al.* 2006). *Mycobacterium* species can be divided into two groups based upon their growth rate in culture (Shinnick & Good 1994). The slow-growing species require more than 7 days to form visible colonies on solid media, whereas the rapid-growing species require less than 7 days. Slowly growing species are often pathogenic for humans or animals (e.g. *Mycobacterium tuberculosis* complex, *Mycobacterium avium* complex) while rapidly growing species are usually considered nonpathogenic for humans, although important exceptions exist (Shinnick & Good, 1994). Mycobacteria are aerobic, acid-fast actinomycetes

that usually form slightly curved or straight non-motile rods (0.2–0.6; 1.0–10 μm). Branching and mycelium-like growth may take place with fragmentation into rods and coccoid elements. Many species form whitish or cream-colored colonies, but especially among the rapid growers, there are also many bright yellow or orange species containing carotenoid pigments (David 1984). In some cases, the pigments are only formed in response to light (photochromogenic species), but most pigmented species also form these pigments in the dark (scotochromogenic species) (Hartmans *et al.* 2006). Phylogenetic trees are available which depict genetic relatedness based on homology of the 16S ribosomal gene sequence. Mycobacteria that have highly homologous rRNA sequences are closely related and are on neighboring branches of the tree (Figure VIII) (Shinnick & Good 1994)**.** In addition, within the genus *Mycobacterium* a number of species are grouped into complexes (e.g., *M. avium* and *M. tuberculosis* complexes) that include bacterial species that have a high degree of genetic similarity as well as cause similar disease syndromes (Shinnick & Good, 1994).

1.4.2 PATHOGENY

The disease pathogenesis begins with the infection that occurs mainly in the terminal bronchioles, pharynx, or guts depending of route of transmission, either by respiratory route or by oral ingestion. However, it can also reach the organism through continuity solutions or bite wounds (Clifton-Hadley *et al.* 2001; Santos, 2006). The cellular immune response is main response of the host to infection with mycobacteria and initiates immediately after infection. Thus, the mycobacteria is phagocyted by macrophages but, instead of being eliminated, they survive due to mechanisms that inhibit the fusion of the lisossomes to the phagossome or by blocking the acidification of the phagolisossome and multiply themselves originating the macrophage death and liberation of more mycobacteria. The repetition of this process with the arrival of more macrophages to the region creates a granuloma known as a tubercle (Whipple & Palmer 2000; Pollock *et al.* 2006; Skoric *et al.* 2007). The location of this first lesion reflects the entrance path and it's called primary infection. This primary infection can evolve towards agent elimination, sequestration in a granuloma or generalization throughout the entire organism depending on the virulence of the agent and the host capacity to activate an effective immune response that is mainly related to T-cells. In this context, T-cells release cytokines that upregulate macrophage microbicidal activity. Once activated, these macrophage can develop to epithelioid cell and then to giant Langhans cell. The periphery of the infected area usually contains a cell layer of lymphocytes and monocytes which is subsequently encapsulated by fibrosis, leading to a tubercle like lesion. (Whipple & Palmer 2000; Clifton -Hadley *et al.* 2001; Neill et al, 2001; Pollock *et al.* 2001; Cooper 2002; Cassidy 2006; Pollock *et al.* 2006; Skorick *et al.* 2007). After this first phase of the immune response, come the “anergic” phase for the reason that of a loss of detectable reactivity to the tuberculin test. In this stage, the immune

response is dominated by formation of circulating antibodies and correlates with severe progression of the disease which is characterized by an increasingly large number of mycobacteria (Figure III) (EFSA, 2008).

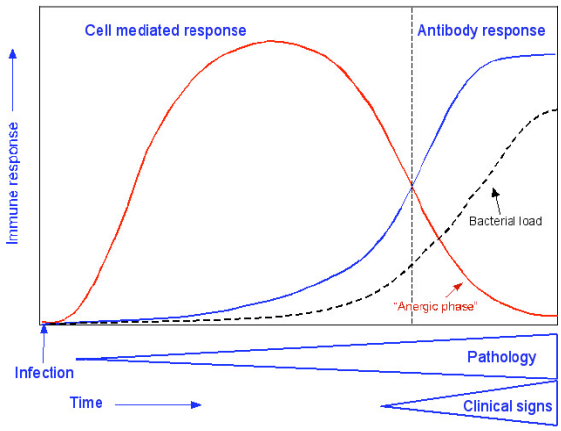


Figure III: Different phases of the immune response and the development of pathological changes in deer following experimental infection with *Mycobacterium bovis*. (Source: EFSA 2008).

1.4.3 CLINICAL-PATHOLOGY

1.4.3.1TUBERCULOSIS

The course of TB in deer is variable, since this associated clinical signs, distribution and severity of lesions (Clifton Hadley & Wilesmith 1991). Some animals are showing clinical signs within 6 months of infection, whereas others may survive for several years without apparent evidence of infection (Fedoseev *et al.* 1982; Williams *et al.* 1987). If lesions are confined to internal lymph nodes or restricted areas of lung, the animals can have no clinical signs throughout their life. Generalized disease involving the lungs may result in emaciation (Basak *et al.* 1975; Jorgensen *et al.* 1988). When an infected animal shows clinical evidence of disease, death will occur within 1-2 weeks (Griffin & Buchan 1994). Nonspecific signs have been described, including retardation of antler growth, sexual indifference in stags in the rutting season and failure of hinds to come into estrus (Friend *et al.* 1963; Fedoseev *et al.* 1982). Coughing and respiratory rales, although sometimes present, are not typical features of the disease in deer (Krucky *et al.* 1982). A more common clinical finding is enlargement of one or more superficial lymph nodes in the throat, axilla and groin areas as abscesses develop (Beatson 1985; Jorgensen *et al.* 1988). These may form sinuses and discharge pus through the skin or mucosal surfaces (Beatson *et al.* 1984; Fleetwood *et al.* 1988). TB presents as a spectrum of pathological conditions at post mortem, ranging from no obvious gross lesions (Kaneene *et al.* 2002; de Lisle *et al.* 2001) to liquefactive abscessetion of lymph nodes (more typically associated with acute pyogenic bacterial infection) or classical caseo-granulomatous lesions, as are found in cattle (Griffin & Mackintosh 2000). Abscesses in deer tend to have a thinner wall containing pus with multiple bacilli and minimal calcification or fibrosis. Tuberculosis

lesions are usually found in the lymph nodes draining the nasopharynx, lung or mesenteric tissue (Figure IV a), b) and d), respectively), most likely reflecting the different routes of transmission, either by respiratory route or by oral ingestion (Griffin 1988). In deer farms, the lymphoreticular tissues of the head and neck, particularly the tonsil and retropharyngeal lymph nodes are most commonly involved (Lugton *et al.* 1998; Palmer *et al.* 2002; O'Brien *et al.* 2004), while in wild deer, head or neck lesions are as common as mesenteric lymph nodes lesions

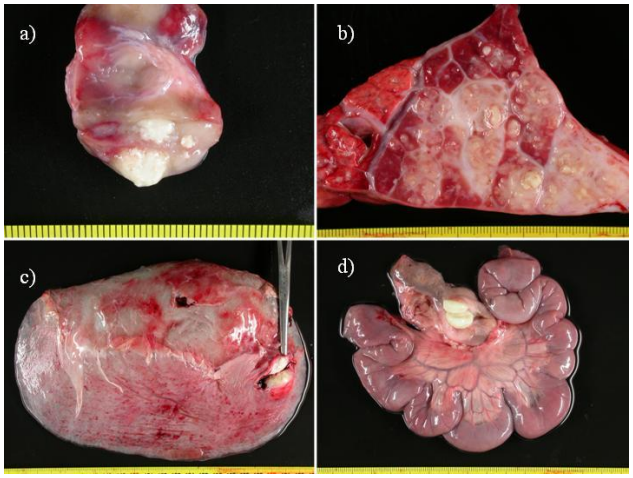


Figure IV: Bovine tuberculosis compatible lesions on lymph node retropharyngeal (a), lung (b), spleen (c) and mesenteric tissue (d) in a red deer.

(Martín-Hernando *et al.* 2010). Lesions range from a single granuloma in one lymph node to a general anatomic distribution involving many nodes and internal organs (Griffin & Buchan 1994). Given the spectrum of presentations, it is not possible to distinguish tuberculosis by the other diseases, such as those caused by other mycobacteria, particularly MAP, other bacteria and even parasites (Griffin & Mackintosh 2000). Indeed, some authors suggest that all abscesses in deer should be considered as tuberculous until proven otherwise (Beatson 1985).

1.4.3.2 PARATUBERCULOSIS

In deer, as in cattle and sheep, PTB presents as a chronic inflammatory disease of the lower intestinal tract, which can lead to loss of condition due to impaired gut digestive and absorptive functions. In severe cases, mortality can occur in clinically affected adult animals. However, in contrast to the case for cattle and sheep, Johne's disease may often be diagnosed in yearlings and can cause death in deer less than 1 year old (Machackova-Kopecna *et al.* 2005).

At post mortem, PTB macroscopic lesions cannot be distinguished of the others causes, as commented above for TB. However, histopathological lesions have been described in sheep (Stamp & Watt 1954; Carrigan & Seaman 1990; Pérez *et al.* 1996), goats (Paliwal *et al.* 1985; Corpa *et al.* 2000), cattle (Buergelt *et al.* 1978; González *et al.* 2005) and fallow deer (Balseiro *et al.* 2008). In ovine paratuberculosis, Pérez *et al.* (1996) described small ''tuberculoid'' granulomas in the ileocaecal lymphoid tissue (Peyer's patches). This type of lesion, also

referred as a ‘‘focal lesion’’, was later reported in goats (Corpa *et al.* 2000), cattle (González *et al.* 2005) and fallow deer (Balseiro *et al.* 2008). A ‘‘multibacillary’’ form, in which macrophages were filled with numerous mycobacteria, was also described (Carrigan & Seaman, 1990; Pérez *et al.* 1996; Clarke 1997; Corpa *et al.* 2000; González *et al.* 2005; Balseiro *et al.* 2008). The immune response plays an important role in determining the histopathological type of PTB. Whereas ‘‘tuberculoid’’ types are associated with a strong peripheral cellular immune response, ‘‘multibacillary’’ types are associated with a marked humoral immune response (Clarke *et al.*,1996; Pérez *et al.* 1997, 1999; Corpa *et al.* 2000; González *et al.* 2005).

1.4.4 DIAGNOSTIC TESTS

The diagnosis of mycobacterial diseases in wildlife animals is complicated both for particularities of these microorganisms and for the diagnosis tests. Several new tests are recently available to be used in wildlife species (e.g. Griffin *et al.* 2004). The diagnosis of mycobacteriosis infection in an animal may be based: on direct identification of the agent by polymerase chain reaction test (PCR) or culture test; indirect identification of the agent by tests based on detection of cell mediated immune response, as intradermal tuberculin test, gamma interferon test or lymphocyte stimulation, or by tests based in detection of antibodies as enzyme-linked immunosorbent assay (ELISA), rapid test (RAPID), multiantigen print immunoassay (MAPIA), complement fixation (CF) test, and agarose gel immunodiffusion (AGID) test; and also by necropsy and histopathology (EFSA 2008; Sockett *et al.* 1992). These tests can be used separately or in most of cases are used in combination. Thus, the sensibility and specificity of the diagnostic varies with the test or tests that are applied. Isolation by culture and subsequent identification of tubercle bacilli by PCR is considered the gold standard for diagnosis mycobacterial infection in deer as reported by several authors (de Lisle *et al.* 1985; Rhyan *et al.* 1992; Rohoncsy *et al.* 1996; Palmer 2004; Griffin *et al.* 2004;). However, the long time for the mycobacterial growth can cause undue delays in the implementation of control measures.

1.4.4.1 DIAGNOSIS IN FARMED RED DEER

Mycobacterial disease surveillance systems for farmed deer include a range of potential system components. Screening test(s) are conducted initially, followed by confirmatory test(s). In live animals, single intradermal tuberculin test (SST), comparative cervical test (CCT) and/or ELISA are generally used as screening test, and histology and culture, generally preceded by necropsy, as confirmatory tests. At post-mortem, necropsy is used as screening test, and histology and culture as confirmatory tests (More *et al.* 2009).

1.4.4.1.1 PURIFIED PROTEIN DERIVATIVE TUBERCULIN SKIN-TEST

Tuberculin is the name given to extracts of *Mycobacterium tuberculosis*, *Mycobacterium bovis*, or *Mycobacterium avium* used to skin test animals in order to identify those with TB. Several types of tuberculin have been employed for this purpose. The most important is purified protein derivative (PPD) tuberculin (Tizard 2000). The PPD tuberculin skin-test has been used worldwide as the standard method designated by World Organization for Animal Health (OIE, Paris, France) for diagnosis of bTB. It is used for controlling or eradicating bTB as a strategy for eradicating human TB (Liu *et al.* 2006). When tuberculin is injected into the skin of a normal animal, there is no significant response. If it is injected into an animal sensitized by infection with mycobacteria, a delayed hypersensitivity response will occur. This tuberculin reaction is an immunologically specific inflammatory reaction mediated by T cells (Tizard 2000).

The SST, based on the bTB test in cattle, was developed to diagnostic TB in farmed deer (Corrin *et al.* 1987), following first cases of bTB detected in farmed deer in New Zealand in 1978 (Tweedle *et al.* 1994). Nevertheless, deer routinely develop sensitization to others mycobacteria, like *M. avium*/*intracellulare* complex, *M. avium paratuberculosis* and saprophytic mycobacteria, due to the large antigenic cross reactivity among mycobacterial species, which may cause false positive reactions (de Lisle *et al.* 1985). In order to reduce false positive skin-test reactions, a modified comparative cervical test was developed to improve the specificity (Corrin *et al.* 1993). In this case the test is performed as a CCT using both bovine and avium PPD tuberculin. Although the CCT has improved specificity, compared with the SST, it has a reduced sensitivity for TB diagnosis (Griffin *et al.* 2003). To overcome the limitations of moderate test sensitivity or specificity, tests can be used in combination, either in series and/or parallel. In present study, we used the comparative cervical test in parallel with ELISA. Moreover, other combinations were performed, such as skin-test/ELISA with HIST/CULT/PCR in series.

1.4.4.2 ENZYME-LINKED IMMUNOSORBENT ASSAY

In case of TB, to date no assay detecting circulating antibodies to *M. bovis* has shown adequate sensitivity or specificity for standalone routine diagnostic use in deer (Harrington *et al.* 2008). Nevertheless, these tests can be used as complement to other tests. The situation is different in the case of PTB, as the serologic tests developed allow the detection of positive animals with acceptable levels of sensitivity and specificity. Nonetheless, there are few references on the application of these diagnostic tests in wildlife, making it difficult to establish the cut-off points and make a correct interpretation of results (Reyes-García *et al.* 2008). Among serological tests that can be used for the diagnosis of mycobacterial disease, ELISA test is a most commonly employed in laboratory techniques, due to their flexibility, low cost, and ease of automation (Reyes-García *et al.* 2008). ELISA vary between laboratories (e.g. in the use of

antigen and of microtiter plates) (EFSA, 2008). The antigens that can be used in this test vary from the more commonly used antigen, PPD tuberculin (PPD bovis and PPD avium) until the most recently developed antigens, such as ESAT-6, MPB83, MPB70, CFP10, among others for the case tuberculosis (Harrington *et al.* 2008), and PPA3 for the case of paratuberculosis (Reyes-García *et al.* 2008). The use of these recent antigens increases the specificity of ELISA assay, however decreasing the sensitivity. Several studies have been done in the last years with goal to determine the sensitivity and specificity of ELISA test in infected deer with *M. bovis*. Sutton *et al.* 1985 based only on PPD bovis ELISA test had a sensitivity of approximately 70%, but low specificity, in natural and infected deer. Griffin *et al.* (1994) based on PPD bovis, PPD avium and MPB70 ELISA test had a high specificity but a low sensitivity when used the ELISA test alone, in deer farms with low and high prevalence of bTB. However, when the ELISA tests were performed 10 days after intradermal tuberculin skin-test the sensitivity increased (Griffin *et al.* 1994). Griffin *et al.* (2006) and Waters *et al.* (2006) recommend used the ELISA test 2-4 weeks after intradermal tuberculin skin-test, because the intradermal tuberculin skin-test leads to a sharp rise in antibody levels in animals in first stage of infection, which it allow to detect the anaemnestic animals. Moreover, the intradermal skin-test and ELISA results may be combined to improve the overall test performance. The sensitivity of single intradermal tuberculin skin-test (82%) and ELISA (85%, post-intradermal tuberculin skin-test) could be increased to 95%, when these tests were interpreted in parallel (Griffin *et al.* 1994).

1.5 PHYTOHEMAGGLUTININ SKIN-TEST

Although several techniques have been used to measure immune function in natural contexts, the phytohemagglutinin (PHA) skin-swelling test has become one of the most popular. The PHA induced skin swelling test is classically used in vivo in immunoecological studies and provokes infiltration and proliferation of several types of immune cells (Martin *et al.* 2006). When purified mammalian lymphocytes are exposed to PHA, mitosis of T lymphocytes is preferentially stimulated. This has led to PHA being commonly used as a T cell mitogen in human and mouse immunology (Kennedy & Nager 2006). PHA is a lectin derived from red kidney beans, *Phaseolus vulgaris*. It causes agglutination of erythrocytes, growth, division and a non-specific activation of T-cells (Smits *et al.* 1999). It is an inexpensive and easy method that can be used in combination with comparative cervical skin-test, as a positive control of general responsiveness to the skin-test (Fernández-de-Mera *et al.* 2008). Wild animals are particularly susceptible to stress of handling, and the stress is a strong immune depressor and may alter the cellular immune reaction, thus rendering the skin-testing less reliable (e.g. Tella *et al.* 2001). So, a standard positive control such as PHA would help to correct results of PPD-skin-testing for the immune responsiveness of a particular population (Fernández-de-Mera *et al.* 2008). The skin-test comprises injecting PHA intradermally and measuring the change in skin thickness.

The immune response is considered to be proportional to the difference in swelling between the site injected with PHA and a control site injected with phosphate buffered saline, or the increase in swelling before and after injection of PHA (Kelley *et al.* 1982; Ekkel *et al.* 1995; Smits *et al.* 1999; Hernández *et al.* 2005).

In red deer, several studies were performed by Fernández-de-Mera *et al.* (2006, 2008, 2009). These authors assessed the PHA reaction only or in combination with PPD tuberculin skin-test, both in wild red deer and farmed red deer. Initially, they demonstrated the optimal time of reading the PHA skin reaction (72 hours after application the mitogen intradermal) and the best dose of the PHA (0,1ml PHA, 250 µg) (Fernández-de-Mera *et al.* 2006) using only the PHA in an experimental study. Afterwards, in a similar study, the authors showed that there was a significant effect of sex (more relevant) and age (less relevant) on the increase in skin fold thickness (males tended to have greater increases than females after correcting for other confounding variables) (Fernández-de-Mera *et al.* 2008). And finally, these authors proved that PHA skin fold increase was not affected by the PPD tuberculin skin-test in wild and farmed red deer, but showed that farmed deer had higher values of skin fold thickness both to mycobacterial and non-mycobacterial (PHA) antigens compared with wild red deer. Recently, this team also established that the season year has a great importance of the PHA skin fold increase, this being these more relevant in males than in females (Jaroso *et al.* in prep.). The PHA skin-test allows identifying non-responsive animals suffering from chronic tuberculosis and those with a impaired immune function, and helps to interpret non-specific reactions to *M. avium* PPD that may result from exposure of wild animals to nonpathogenic mycobacteria (Fernández-de-Mera *et al.* 2009). All these studies performed in red deer also evidenced a positive relationship between PHA-responsiveness and body condition. All these studies performed in red deer evidenced a positive relationship between PHA-responsiveness and body condition.

1.6 HERITABILITY OF IMMUNE RESPONSE

Heritability is an important component of the ability of a trait to respond to natural or artificial selection. The heritability (h^2) of a trait is defined as a proportion of the total phenotypic variance that can be attributed to additive genetic variance (V_A) (Falconer 1989), which is equivalent, the ratio of additive genetic variance to total phenotypic variance (V_P) ($h^2= V_A/V_P$). Genetic contributions to the total phenotypic variance of parasite resistance and immune response have mostly been demonstrated in domestic or laboratory species (Lillehoy *et al.* 2007). The genetic variation underlying those traits is however harder to detect in natural populations (Kilpimma *et al.* 2005), in part because of environmental variance (V_E) (Sorci *et al.* 1997), but also because fitness traits have a lower heritability than non-fitness traits (Mousseau & Roff 1987). Furthermore, differing levels of non-additive genetic variance (dominance and epistatic

variance) will also affect heritability estimates (Barton & Turelli 1989), and this variance may vary consistently among traits (Lynch & Walsh 1998; Merilä & Sheldon 1999).

Several studies have found that exposure of traits to selection can vary with environmental conditions due to differences in trait heritability, suggesting that the rate of evolutionary change can vary among populations (Mousseau & Roff 1987; Houle 1992; Roff *et al.* 2002; Charmantier & Garant 2005). Interestingly, the influence of additive genetic variance on the heritability of the immune response varies depending on the strength of selection (Nath *et al.* 2001; Zekarias *et al.* 2002; Davison 2003), which suggest that a single assessment may not accurately reflect heritability across a species (Ardia & Rice, 2006). Although strong directional selection should deplete additive genetic variance, there may be several other processes maintaining genetic variation in life history traits (Charlesworth & Hughes, 2000). In a study about heritability of fitness in a wild red deer (*Cervus elaphus*) population, Kruuk *et al.* (1999) reported a negative correlation between the heritability of a trait and its association with fitness; there was no evidence of significant heritability of total fitness; coefficients of additive genetic variance differed distinctly between traits, but highly skewed measures, such as male breeding success, generally had greater coefficients of variance than morphometric traits; and there were significant maternal effects in a range of traits, particularly for females.

Immune defense has become a central research topic in evolutionary ecology (Sheldon & Verhulst 1996; Westneat & Birkhead 1998; Mallon *et al.* 2003; Schmid-Hempel 2003; Schmid-Hempel & Ebert 2003) whereas immune function reflects an important aspect of an individual's ability to cope with parasites and diseases (Kilpimaa *et al.* 2005). Resistance to disease can be divided into two categories: innate resistance or immunity and acquired resistance or immunity, both of which are under genetic control (Roitt *et al.* 1996; Wakelin & Apanius 1997). For hosts that are exposed to parasites and successfully colonized by them, the expression of acquired resistance is the most important way in which the infection load may be controlled (López 1997). Hosts that had the genetic ability to build up an effective immune response will be protected by the new level of acquired resistance; thus, they will face lower costs in subsequent encounters with these parasites than hosts without such a genetic ability. Because of these differential costs that individuals will incur during different stages of resistance development, it can be predicted that costly secondary sexual characters of individual hosts will be adjusted following the handicap principle, which suggest that male secondary sexual characters may represent genetic quality signaling traits (Zahavi 1975, 1977; Grafen 1990). As Hamilton and Zuk (1982) suggested, resistance to parasites may be one important genetic qualify revealed through exaggerated secondary sexual characters. The immunohandicap hypothese offers a mechanistic extension to the Hamilton and Zuk handicap version (Folstad & Karter 1992; Wedekind & Folstad 1994). It is based on the fact that circulating testosterone enhances the

expression of many male secondary sexual characters and behaviors, but at the same time suppresses the immune system (Malo *et al.* 2009). Therefore, males producing well-developed secondary sexual characteristics may be indicating their superior parasite resistance. Ornaments represent handicaps that potentially reveal a male's ability to allocate sufficient resources to both immunocompetence and the maintenance of sexual characters (López 1997). In this scenario it means that once males have been exposed to parasites, each individual host will adjust the showiness of sexual characters according to his ability to build and maintain an effective resistance. However, there exist no studies relating the expression of secondary sexual characters to an individual's level of acquired immunity (López 1997).

In birds, the genetic control of immune function has been largely studied in poultry industry through the use of PHA. Laboratory studies demonstrated that PHA response is heritable in poultry (Cheng & Lamont, 1988; Sundaresan *et al.* 2005) while in wild populations just could estimates of heritability of PHA response because cannot separated additive genetic effects from dominance variance and environmental effects (Lynch & Walsh, 1998).

In red deer the genetic resistance *Mycobacterium bovis* was firstly studied by Mackintosh *et al.* (1999). These authors reported a strongly heritable basis for the resistance and susceptibility of deer to experimental infection with *M. bovis*. The offspring showed patterns of response to *M. bovis* challenge similar to those of their sire, providing evidence for a strong genetic basis of resistance to TB, with an estimated heritability of 0.48 (standard error, 0.096). Cellular reactivity and antibody responsiveness results showed two distinct patterns of immunity that differed markedly between the resistant calves. Resistant calves developed cellular reactions that were cross-reactive for *M. bovis* and *M. avium* tuberculin (*M. bovis* PPD and *M. avium* PPD, respectively), while all infected animals developed specific cellular reactivity to *M. bovis* tuberculin, between 4 and 8 weeks post-challenge. Patterns of humoral immunity (ELISA) for the resistant and susceptible animals also differed. Resistant calves did not produce antibody to tuberculin at any time post-challenge. By contrast, all susceptible calves produced *M. bovis*-specific ELISA reactivity 4 to 8 weeks after challenge. The level of antibody correlated directly with the severity of disease in the infected calves. Therefore, the immune response in resistant animals involves both acquired immune response and innate immune response (Mackintosh *et al.* 1999).

2. OBJECTIVES

The aim of this work was to study, as a proxy to heritability, the relationships between parental and offspring cellular immune response in red deer (Cervus elaphus), as well as its potential influence on the results of the tuberculin skin-test. For this, we proposed to perform the skin-test using the mycobacterial antigens and non-mycobacterial antigens and the posterior analyze of these results through the use of the statistical models. Furthermore, we proposed to perform the ELISA test for all calves as well as the necropsy, histopathology, mycobacterial culture and PCR of the 11 highest suspect animals. Thus, we hypothesized that individuals (calves) from different fathers would have a different immune responsiveness and hence we could potentially select animals with a great immune response regarding to control of mycobacterial diseases.

3. MATERIAL AND METHODS

3.1 STUDY SITE, CHARACTERISTICS OF DEER AN SAMPLE SIZE

The present study was carried out in a red deer farm in Cádiz, southern Spain. It is a red deer farm with a semi-intensive management scheme, with pasture-rotation and year-round food supplementation. These farmed deer are handled at least twice a year, including physical immobilization to perform the skin-test, measurement, sampling and administration of antiparasitic drugs. Furthermore, deer are separated by age in calves, yearling hinds (females), adult hinds, yearling stags (males) and adult stags. Calves born in May and June 2009 were separated from their mothers in August 2009 and kept in two paddocks (groups 1 and 2) from August to December 2009. Deer sampled in this study included 366 calves, 143 adult hinds and 17 adult stags. Farmed deer were sampled throughout the period comprised between December 2009 and January 2010.

3.2 FIELD PROCEDURES

3.2.1 SKIN-TEST AND SAMPLING PROCEDURES

The skin test and sampling procedure was performed during December 2009 (fawns) and January 2010 (hinds and stags). Animals were handled twice during the procedure, at time 0 h and 72h. Deer were moved from the paddocks to the farm enclosures and then immobilized by physical restraint, in a hydraulic crush. At time 0, each animal was identified by the ear-tag number, weighed and blood samples were collected. Three areas of 3 cm x 3 cm were shaved at the right side of the neck (Figure V a)), with an electric shaver (Moser Avalon 1290; Moser, Valencia, Spain), and 3 times repeated measurements of skin fold thickness were taken at the 3 injection sites, to the nearest 0.1 mm, using a manual caliper (Mitutoyo, Cardiff, UK), by the same observer. The intradermal injection was carried out with 0.1 ml purified protein derivative (PPD) of *Mycobacterium avium* (*M. avium* PPD; Cooper- Zeltia, Spain), 0.1 ml *M. bovis* PPD (*M. bovis* PPD; Cooper-Zeltia, Spain) and 0.1 ml of the plant derived mitogen phytohaemagglutinin (PHA; Sigma-Aldrich, Missouri, USA) as positive control, containing 250 mg PHA, diluted in phosphate buffered saline (PBS) (Figure V b)). One-ml syringes fitted with a 25-G ½-inch needle were used. At 72 h, each animal was immobilized again by physical restraint, identified, and the skin fold thickness at each injection site was measured again (3 repeats). Handling procedures and sampling frequency were designed to reduce stress and health risks for subjects, according to European (86/609) and Spanish laws (RD 223/1988; RD 1021/2005), and current guidelines for ethical use of animals in research (ASAB, 2006). Any deer with a skin fold increase > 2mm to *M. bovis* PPD and > 1 mm larger than the skin fold increase to *M. avium* PPD were considered positive bTB reactors (Griffin *et al.* 1991; Corrin *et al.* 1993; Norden *et al.* 1996). Any deer with a skin fold increase to *M. avium* PPD > 3 mm and larger than the one to

M. bovis PPD were considered positive *M. avium* reactors (Kollias *et al.* 1982). Deer with skin fold thickness increases of < 0.5 mm to all 3 antigens were considered anergic animals.

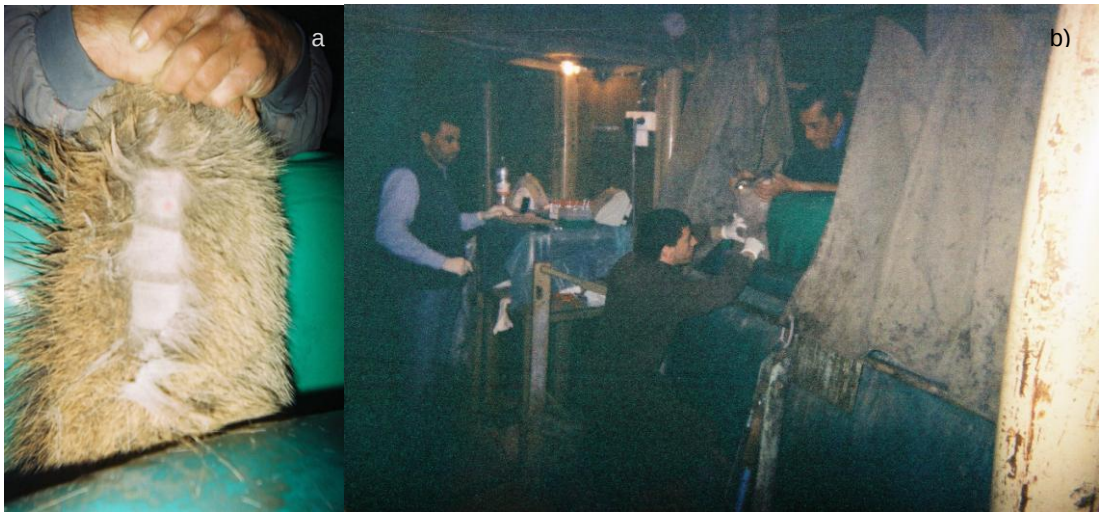


Figure V: Skin-testing in a red deer farm. a) Three areas of 3 cm x 3 cm shaved at the right side of the deer neck, b) Intradermal injection of different antigens in shaved areas, after 3 times repeated measurements of skin fold thickness.

3.2.2 NECROPSY AND SAMPLING PROCEDURES

The necropsy of 22 animals slaughtered was performed in the field, after assessing the results of the skin-test and the ELISA assay. Gross lesions observed in deer carcasses were recorded, attention being focused on the gut and associated lymph nodes. Of the total of animals culled, we chose 11 animals in which had higher skin fold increase to *M. bovis* PPD (n= 5) and *M. avium* PPD (n= 6). These animals, samples for histopathology were taken from the proximal and distal jejunum, proximal and distal ileum, ileocaecal valve, and associated lymph nodes, which were fixed in 10 % neutral buffered formalin. We also collected the medial retropharyngeal LNs and the tonsil; the mediastinal and the left tracheobronchial LNs; and the right kidney, spleen and mesenteric LNs, were taken to the laboratory for detailed examination. These samples were kept 4 ºC.

3.3 LABORATORY PROCEDURES

3.3.1 PATHOLOGY/HISTOPATHOLOGY/ MYCOBACTERIAL CULTURE

Tissue samples were dissected and examined carefully for macroscopic lesions. One pool of these tissues (of the 11 animals): medial retropharyngeal, mediastinal, left tracheobronchial and mesenteric LNs, and tonsil, were refrigerated and delivered to the VISAVET laboratory (Universidad Complutense, Madrid), for mycobacterial culture. Paired tissue samples fixed in 10% neutral buffered formalin were dehydrated through graded alcohols and xylol before being embedded in paraffin wax. Several sections (4 µm) were cut from each sample and stained with haematoxylin and eosin and by Ziehl - Neelsen's method for acid-fast bacteria .

3.3.2 ENZYME-LINKED IMMUNOSORBENT ASSAY

The adsorbed ELISA test was performed to detect antibodies against *M. bovis* PPD, *M. avium* PPD , paratuberculosis protoplasmatic antigen 3 (PPA3; Allied Monitor, Fayette, MO, USA) and *M. bovis* specific protein MPB70 (Lionex GmbH, Germany) following protocols reported for PTB in red deer (Reyes-García *et al.*, 2008).

Briefly, high absorption capacity polystyrene microtiter plates were coated with 50 ml/well of 0.02 mg/ml solution of PPA-3 in carbonate–bicarbonate buffer (Sigma, Barcelona, Spain). The serum samples were left at 4 ºC overnight. After an overnight incubation at 4 ºC, the coated plate was blocked with 200 µl/well of blocking solution (5% nonfat dried milk in PBS containing 0.05% Tween 20). The adsorbed sera were diluted (1:10, v/v) in blocking solution and 100 ml/well was added into duplicate wells of the antigen-coated plate. After a 1-h incubation period at room temperature, the plates were washed three times with a washing solution (PBS containing 0.05% Tween 20). Protein G horseradish peroxidase conjugate (Sigma) was added (0.002 mg/ml in blocking solution) and incubated at room temperature for 1 h. After three washes, 200 ml/well of substrate solution (Fast OPD, Sigma) was added. The reaction was stopped with 50 ml/well of H_2SO_4 3N and optical density (OD) was measured in a spectrophotometer at 450 nm.

Deer negative control sera were included in every plate in duplicate. OD values of animals between different plates were normalized according to the values of the negative controls included in each plate. The negative sera were obtained from an experimental facility belonging to the University of Castilla - La Mancha with no history of PTB and repeated negative culture of deer. Sera from culture – confirmed PTB positive deer and culture – confirmed *M. bovis* infected deer were includes as positive controls.

3.3.3 POLYMERASE CHAIN REACTION

DNA for PCR confirmation of *Mycobacterium avium paratuberculosis* and of *Mycobacterium bovis* was extracted from formalin-fixed paraffin-embedded (FFPE) intestinal tissue of 11 animals slaughtered in field. Three FFPE sections (10 µm each) were cut, introduced in a microfuge tube, and submitted to DNA extraction using the WaxfreeTM DNA extraction kit (Trimgen, MD). A negative extraction control was included in all steps to exclude contaminations during the extraction procedure. The final extract (50 µl) was diluted (1:10) in DNase-RNase-free water and used in a triplex real-time PCR targeting the IS900 (Herthnek *et al.*, 2006) and ISMAP02 sequences of MAP, and an internal amplification control (IAC) to rule out inhibition of the reaction. The 50 µl PCR mixture contained 2.5 µl of the diluted DNA extract, 1x TaqMan Universal MasterMix (Applied Biosystems, CA), 0.4 µM (each) of primers co-amplifying ISMAP02 and the IAC, 0.3 µM (each) of IS900 primers, 0.2 µM (each) of ISMAP02, IAC and IS900 probes and, 2 µl of IAC template DNA. Amplification was carried out in an

Applied Biosystems 7500 Real-Time PCR System under the following standard conditions: one cycle at 95ºC for 10 min and 45 cycles with two steps of 95ºC for 15 sec and 60ºC for 1 min.

3.4 STATISTICAL ANALYSES

The values of the skin fold increase to *M. bovis* PPD, *M. avium* PPD and PHA were $\log_{10}$-transformed prior to statistical analysis in order to normalize them, which was assessed by means of the Kolmogorov-Smirnov test.

We tested the effect of calf sex (categorical independent variable) on the *M. avium* PPD, *M. bovis* PPD and PHA skin fold increases (as dependent variables, respectively) using General Mixed Linear Models (GLMM), considering the calf group and the father as random factors in order to avoid pseudo-replication. Following, more inferential statistics were performed in order to test: (i) The relationship between the skin fold increases to different antigens. For this purpose, the response against one antigen (*M. avium* PPD, *M. bovis* PPD and PHA) was set as dependent variable, and the response against the two other antigens as independent variables. We also included live weight (n=366). We modeled using a General Linear Model (GLM); (ii) Since the paternity of a variable number of calves could be assigned to specific stags, we tested the differences between the skin fold increases to *M. avium* PPD, *M. bovis* PPD and PHA in calves (n=327) as a function of the stag. We included one of the skin fold increases as dependent variable (*M. avium* PPD, *M. bovis* PPD and PHA, respectively). As independent variable we included the stag (as categorical, only including those for which paternity was assessed for at least 7 calves, and therefore 14 stags where involved in the analysis). We modeled using Generalized Mixed Linear Models (GLMMs), considering the calf group as a random factor. We used an identity link and a normal error; (iii) Finally, we tested the relationship between the cellular immune response of the calves (skin fold increase to PHA, n=143) and these of the assigned parents (n=10 and 143 for stags and hinds, respectively). For this purpose, the calf skin fold increase to PHA was set as dependent variable and these of the parents (and their interaction) were included as independent variables. We included the age class of the mother (1- 2 and 3 years old; 2- 4 to 9 years old; 3-10 to 14 years old) as categorical predictor and its interaction with the PHA skin fold increase of the mother. We modeled using a GLMM, considering the calf group and the assigned father as random factors. We used a identify link and a normal error. We only included those female hinds in which the PHA skin fold increase was measured. The age of the fathers were not include in this model, once that they ranged from 5 to 8 years old and we assume that not exist differences of the cellular immune response among these closely related age classes.

All those inferential analyses (i, ii and iii) were separately carried out for each calf sex. We used SPSS 17.0 software, the *P*-value was set at < 0.05 and all *P*-values refer to two tailed tests.

4. RESULTS

4.1 FIELD PROCEDURES

The skin fold increase values for the different antigens in the stags are shown in Table I (for 10 stags the skin-tests were performed) and the mean values for calves according to the parental stag and sex are displayed in Table II. In this table, comparing for example stag 8 and

Table I. Values of skin fold increase in response to *M. avium* PPD, *M. bovis* PPD and PHA in stags (mm).

Stags	*M. avium* PPD	*M. bovis* PPD	PHA
2	35.0	19.7	32.3
3	52.0	30.3	58.0
4	50.0	50.3	42.7
5	28.0	9.0	37.0
7	18.3	3.7	51.7
8	41.3	20.3	38.3
9	32.7	8.7	18.3
10	30.7	4.7	35.3
12	0.0	4.0	28.3
16	11.3	8.0	55.0

stag 2, the differences in the skin fold increase of their calves become clear. In the case of the *M. avium* PPD antigen, the difference between skin fold increase is almost 10 mm (stag 8/2: males, n= 31/6, mean=14.5/6.6 mm, range 0-73/2-15 mm; females, n=32/9, mean =16.2/6.8 mm, range 0-85/0-15 mm, respectively). For the same animals, the skin fold increase to *M. bovis* PPD differs but less (stag 8/2: males, n= 31/6, mean=11.5/8.1 mm, range 0-48/2-19 mm; females, n=32/9, mean =10.7/6.3 mm, range 0-44/0-16 mm, respectively). In the case of PHA skin fold increase, the differences observed between calves of these two stags is also evident 14.5 mm (stag 8/2: males, n= 31/6, mean=35.7/50.2 mm, range 6-85/25-67 mm; females, n=32/9, mean =37.9/39.2 mm, range 12-57/21-66 mm, respectively). A total of 13 calves (3.6%) were considered positive reactors to *M. avium* PPD. No statistical differences were evidenced for *M. avium* PPD prevalence between sexes (Chi^2 = 0.692, 1df.,p = 0.40). All of these animals were in the second group (6.2%) and significant differences occurred between groups (Chi^2 = 10.52, 1df., p<0.01). Nine calves (2.5%) were considered positive reactors to *M. bovis* PPD, and the prevalence did not statistically differ between sexes (Chi^2 = 0.11, 1df., p = 0.73). Five of these animals belonged to the first group (3.2%) and 4 animals to the second group (1.9%). No significant differences occurred between groups (Chi^2 = 0.11, 1df., p = 0.74).

4.2 LABORATORY PROCEDURES

The cut-off values for each antigen in the ELISA test were arbitrarily calculated after analyzing all odds values of the population, separately for each antigen.

Table II. Mean values (0.1 mm) of the skin fold increases to *M. avium* PPD, *M. bovis* PPD and PHA in calves as a function of the sex (m: males, f: females) and the parental stag.

Stag	Sex	n	*M. avium* PPD				*M. bovis* PPD				PHA		
			Mean	SE	Range	%	Mean	SE	Range	%	Mean	SE	Range
1	m	10	7.7	1.69	0-16	0	9.6	1.38	2-17	0	39.3	4.37	22-61
	f	13	9.7	3.91	0-54	7.7	9	1.77	0-22	0	36.6	3.65	20- 56
2	m	6	6.6	1.90	2-15	0	8.1	2.56	2-19	0	50.2	6.37	25-67
	f	9	6.8	1.77	0-15	0	6.3	1.81	0-16	0	39.3	4.45	21-66
3	m	11	10.9	4.09	0-45	9.1	7.2	1.52	0-16	0	50.1	10.07	9-127
	f	16	8.0	1.63	0-18	0	7.9	1.37	0-21	0	45.1	5.77	18-108
4	m	9	12.0	3.76	0-39	11.1	8.7	1.89	3-21	0	55.8	7.16	34-96
	f	6	8.1	2.25	1-15	0	7.2	2.01	2-13	0	43.6	5.46	30-61
5	m	15	11.3	1.82	0-26	0	11.6	3.10	0-50	6.7	30.6	3.69	12-73
	f	10	5.9	1.61	0-14	0	4.3	1.52	0-14	0	23.0	2.20	15-37
6	m	9	10.5	4.78	0-7	10	8.5	2.32	1-19	0	39.3	5.22	14-60
	f	9	9.4	2.08	2-21	0	8.0	1.77	2-20	0	39.3	4.52	16-57
7	m	30	7.6	1.10	0-27	0	8.0	0.91	0-22	3.3	40.4	2.36	10-78
	f	24	9.9	2.31	0-53	4.2	8.4	1.14	0-25	0	45.6	3.67	12-89
8	m	31	14.5	3.23	0-73	6.5	11.5	1.96	0-48	3.2	35.7	3.14	6-85
	f	32	16.2	3.72	0-86	15.6	10.7	1.63	0-44	0	37.9	2.04	12-57
9	m	9	7.1	1.99	1-19	0	4.5	1.27	0-9	0	38.3	3.61	22-54
	f	9	7.3	1.49	0-12	0	8.7	1.99	2-21	11.1	45.0	4.92	22-72
10	m	10	6.5	1.50	0-13	0	7.1	1.22	2-13	0	38.6	4.42	22-66
	f	5	4.5	1.05	2-8	0	2.5	1.23	0-7	0	28.7	4.28	19-44
11	m	5	8.3	1.43	5-12	0	8.7	1.44	4-12	0	47.5	7.13	32-72
	f	8	16.6	5.95	0-56	12.5	11.3	2.50	2-26	0	60.6	8.12	30-108
12	m	6	7.8	1.96	0-14	0	5.4	1.55	0-12	0	38.2	8.80	4-72
	f	8	6.8	2.07	0-16	0	4.7	0.71	1-7	0	50.1	6.69	24-72
13	m	8	10.6	2.06	3-17	0	10.0	2.40	4-22	12.5	42.8	4.52	27-58
	f	14	9.4	2.06	0-24	0	13.1	2.56	1-39	14.3	45.3	3.46	18-63
14	m	16	6.5	1.03	0-14	0	6.8	1.22	0-18	0	46.1	2.69	34-63
	f	10	7.3	0.89	3-12	0	10.1	3.56	1-39	10	38.8	3.31	20-59
15	m	4	8.3	3.58	0-17	0	6.6	2.21	3-13	0	27.3	2.64	24-35
	f	3	5.3	2.22	2-9	0	4.6	3.89	0-12	0	44.3	10.63	25-62
16	m	3	16.6	3.13	12-23	0	15.4	2.12	13-20	0	39.1	7.78	25-51
	f	4	9.5	1.77	7-15	0	11.4	2.95	4-18	0	45.9	9.36	27-68
17	m	2	4.5	1.17	3-6	0	2.8	1.17	2-4	0	27.3	20.33	7-48
	f	2	5.7	2.33	3-8	0	18.0	9.33	9-27	50	34.0	3.67	30-38
Total	m	184	9.7	0.76	0-73	2.7	8.7	0.54	0-50	2.2	40.42	1.27	4-127
	f	182	9.9	0.89	0-86	4.4	8.8	0.55	0-44	2.7	41.45	1.22	12-108
		366	9.8	0.58	0-86	3.6	8.8	0.38	0-50	2.5	40.93	0.88	4-127

The cut-off values are represented in Figure VI. These values are relatively smaller compared the cut-offs values that normally whether consider the positive animals (cut-off ≤

100).In this work, we considered possible positive the animals whose cut-off for each antigen were above of the calculated value. So, the apparent prevalence for each antigen were to MPB70 = 4.6%, *M. bovis* PPD = 4.4%, PPA3 = 5.7% and *M. avium* PPD= 3.3%.

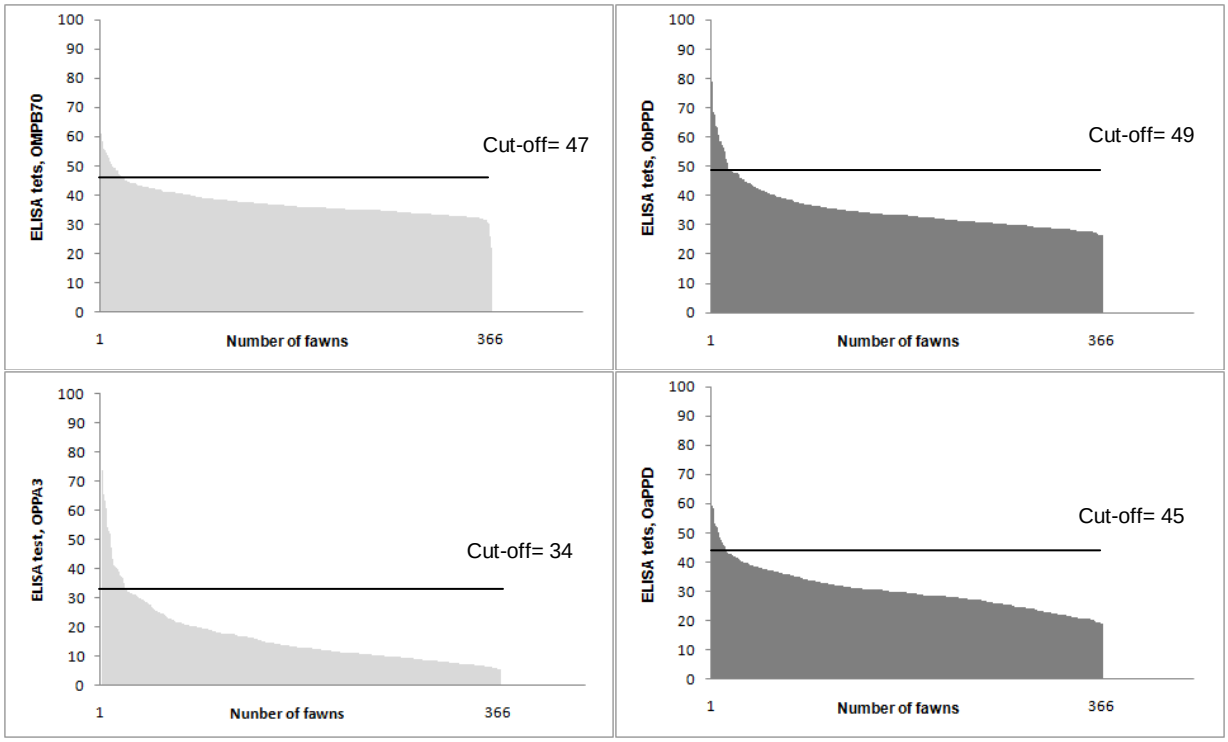


Figure VI: Distribution of odds value of the ELISA test for different antigens. The calculated cut-off value for each antigen is represented with horizontal lines.

No macroscopic bTB or PTB compatible lesions were recorded during the 22 necropsies. Moreover, the tissue samples collected during 11 of these necropsies also revealed negative histopathology, mycobacterial culture and negative PCR results.

4.3 STATISTICAL ANALYSES

No statistical differences were found in the skin fold increase to any antigen in relation to sex after controlling by the group and the father (F=2.95, 1/159 df., p=0.09; F=0.01, 1/197df., p=0.94; F=0.58, 1/227 df., p=0.45; for *M. avium* PPD, *M. bovis* PPD and PHA, respectively). Concerning the GLM on the relationships between the skin fold increases to different antigens (Table III), we evidenced that, after controlling by weight, skin fold increase to *M. bovis* PPD (as dependent variable) positively related to *M. avium* PPD and PHA in male calves and only with *M. avium* PPD skin fold increase in female calves. The model on skin fold increase to *M. avium* PPD confirmed the statistical association with increase to *M. bovis* PPD in both sexes and similarly the model on skin fold increase to PHA confirmed a statistical association with the increase to *M. bovis* PPD in male calves. Nonetheless, no statistical associations between *M. avium* PPD and PHA skin fold increases were found in both calf sexes. Also, no statistical

association between *M. bovis* PPD and PHA skin fold increase were evidenced in any model for male calf.

Table III. GLM test statistics for skin fold increase in response to *M. bovis* PPD, *M. avium* PPD and PHA.

Dependent variable	Sex	Covariates	Estimate	df	*F*	*p*
***M. bovis* PPD**	**m**	weight	0.004	1	3.559	0.061
		M. avium PPD	0.397	1	48.605	**<0.001**
		PHA	0.268	1	6.637	**<0.05**
	f	weight	0.000	1	0.001	0.972
		M. avium PPD	0.432	1	67.72	**<0.001**
		PHA	0.207	1	2.496	0.116
***M. avium* PPD**	**m**	weight	0.004	1	2.964	0.087
		M. bovis PPD	0.543	1	48.605	**<0.001**
		PHA	-0.022	1	0.03	0.862
	f	weight	0.005	1	3.754	0.054
		M. bovis PPD	0.646	1	67.72	**<0.001**
		PHA	0.214	1	1.782	0.184
PHA	**m**	weight	-0.003	1	3.441	0.065
		M. bovis PPD	0.135	1	6.637	**<0.05**
		M. avium PPD	-0.008	1	0.03	0.862
	f	weight	-0.002	1	2.925	0.089
		M. bovis PPD	0.068	1	2.496	0.116
		M. avium PPD	0.047	1	1.782	0.184

Regarding the GLMMs on the calf skin fold increases as a function of the stag (*M. avium* PPD, *M. bovis* PPD and PHA as dependent variables, respectively), statistical differences were found for PHA and *M. bovis* PPD skin fold increase in females (males: F=0.876, 13/128df., p=0.58; F=1.66, 13/95df., p=0.08; F=1.64, 13/132df., p=0.08; for *M. avium* PPD, *M. bovis* PPD and PHA, respectively; females: F=0.63, 13/133df., p=0.82; F=1.99, 13/135df., p<0.05; F=3.30, 13/142df., p<0.001; for *M. avium* PPD, *M. bovis* PPD and PHA, respectively). The differences among the stags in their offspring increases to the different antigens are shown in Figure VII as marginal means of the models. Finally, results concerning the relationship between the cellular immune response of the calves and those of the assigned parents are shown in Table IV. In the case of male calves, statistical differences were found for age of their mother and the interaction between age and PHA skin fold increase of their mothers. For female calves, statistical differences were separately found for PHA skin fold increase of their parents, and their interaction. Also statistical differences were found regarding the age of the mothers, as well as the interaction between age and PHA skin fold increase of the mother. In the case of the

interaction between PHA skin fold increase in parents, when regressing the predicted values of the GLMM (as dependent) and hinds PHA response (as independent) separately for three

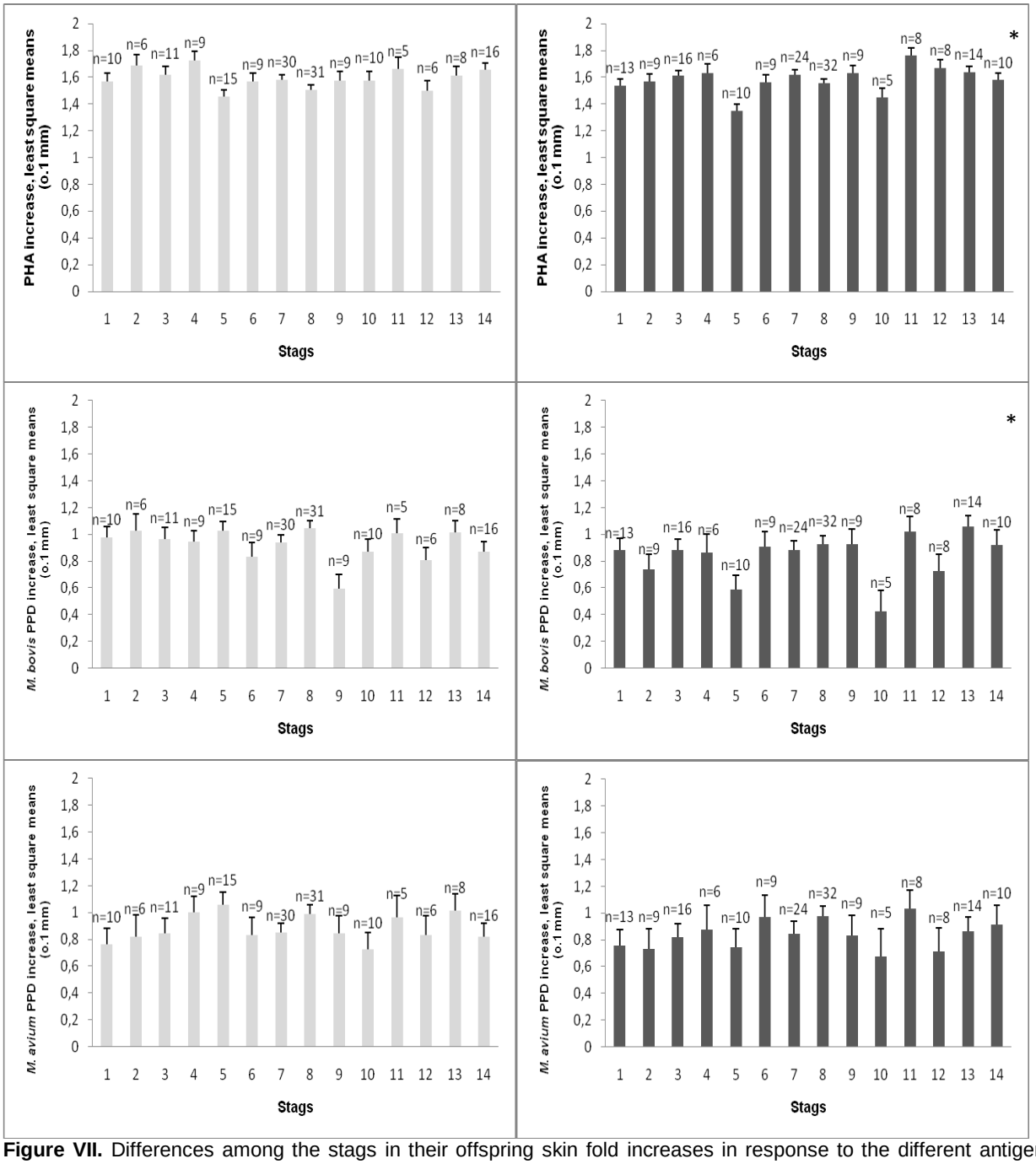


Figure VII. Differences among the stags in their offspring skin fold increases in response to the different antigens (males in graphs on the left and females on the right). Values are shown as least square means of the GLMMs.
* means statistical significant differences among stags.

different categories of PHS skin fold increase for stags (established according to the 33 and 66 percentiles, n= 3, 4 and 3, respectively), statistically significant results were only found for the father middle category (positive slope: β=0.272, SE= 0.104, p<0.05), whereas no relationship was found for the other two extreme categories (β=-0.363, SE= 0.181, p=0.06; β=-0.369, SE=

0.211, *p*= 0.10; for the lower and upper categories, respectively) (Figure IX), even though there was a tendency.

Table IV: GLMMs test statistics concerning the relationship between the cellular immune response of the calves and those of the assigned parents.

Dependent variable	Sex	Covariates		Estimate	SE	df *	*F*	*p*
PHA	m	PHA-Stag		1.698	1.315	1, 18.1	1.665	0.213
		PHA-Hind		1.089	1.496	1, 16.7	1.333	0.265
		PHA Stag*PHA Hind		-0.949	0.874	1, 17.4	1.178	0.293
		Age classes Hind	1	-1.749	0.412	2, 27.1	9.02	**<0.01**
			2	-0.521	0.423			
			3	0a	0			
		Age classes Hind*PHA Hind	1	1.203	0.282	2, 28.0	9.103	**<0.01**
			2	0.445	0.276			
			3	0a	0			
	f	PHA-Stag		2.488	1.029	1, 12.45	5,846	**<0.05**
		PHA-Hind		3.578	1.013	1, 11.57	7,956	**<0.05**
		PHA Stag*PHA Hind		-1.785	0.688	1, 12.87	6,719	**<0.05**
		Age classes Hind	1	0.059	0.374	2, 13.1	30.163	**<0.001**
			2	2.153	0.279			
			3	0a	0			
		Age classes Hind*PHA Hind	1	-0.130	0.259	2, 12.3	33.682	**<0.001**
			2	-1.544	0.188			
			3	0a	0			

*df: numerator, denominator

a. This parameter is set to zero because it is redundant.

In the case of interaction between age class and PHA skin fold increase in hinds, in both sexes, when regressing the predicted values of the GLMM (as dependent) and hinds (as independent) separately for three different age categories of hind (young hinds, adult hinds, older hinds, n= 26, 33 and 15 for males, n=26, 29 and 14 for females, respectively), statistical differences were found for all age classes (1, 2 and 3) and for both sexes (males: 1- β=0.716 SE= 0.041, p<0.001; 2- β=0.184, SE= 0.075, p<0.05; 3- β= -0.531 SE= 0.048, p<0.001; females: 1- β=0.6 SE= 0.06, p<0.001; 2- β=-0.738, SE= 0.08, p<0.001; 3- β= 0.734 SE= 0.062 p<0.001) (Figure X).

5. DISCUSSION

The control of mycobacterial disease (TB and PTB) in farmed deer has become an important issue regarding to global control programs for TB, due to the fact that red deer can be a reservoir for bTB and that TB and PTB are among the most important health issues in deer farming (Riemann *et al.* 1979; Chiodini & Vankruiningen 1983; Clifton-Hadley & Wilesmith 1991; Griffin & Buchan 1994; Mackintosh *et al.* 2004). These facts are thought to contribute to the failure of eradication programs in cattle throughout Europe (Caffrey 1994; Hunter 1996). The eradication programs for TB using PPD tuberculin skin-test have been used worldwide as the standard method for diagnosis of bTB (OIE, Paris, France) and in farmed red deer like in cattle herds this test is an important tool for bTB control. However, this test does not identify anergic animals suffering from chronic TB and those with an impaired immune function (Fernández-de-Mera *et al.* 2009). This increases the false negative animals (decreasing sensibility) and hampers control programs for tuberculosis in farmed red deer. Therefore, using the positive control (PHA skin-test) that reflects the immune reactivity of the tested individual or group should be imperative in tuberculin skin-testing in each species, because it allows to establishing cut-off points for the immune reactivity in a given population under given circumstances as needed (Fernández-de-Mera *et al.* 2009). In last years, important knowledge about PHA skin-test in red deer has been published, but nobody assessed the relationship between the cellular immune response of the offspring (skin fold increase to PHA) and those of the assigned parents. In this sense, we can estimate, as a proxy to heritability of the cellular immune response in a specific red deer farm and over specific environment effects, the relationship between the immune response between parents and calves. Knowing that the cell mediated immune response is main response of the host to infection with mycobacteria and that PHA skin fold increase is a response mainly mediated by T cell, we can determine which animals have a better cellular immune response and thus contribute for a positive selection of animals with stronger cellular immune responses. Indirectly, we can also to be select animals more resistant to infection with mycobacteria.

The variability of the skin-test depends on several factors: the time of reading of the skin fold thickness after intradermal application (Fernández-de-Mera *et al.* 2006); the dose of the antigen applied and the place where are applied (Fernández-de-Mera *et al.* 2006); the sex and age of individuals (Fernández-de-Mera *et al.* 2008); the season of year that the antigen is applied (in press); body condition of the animals (Fernández-de-Mera *et al.* 2006, 2008, 2009); origin of the animals (farmed versus wild) (Fernández-de-Mera *et al.* 2009); effects of the person that performed the skin test; and individual ability of the cellular immune response, partly determined by genetic factors and partly determined by environment effects. In this sense, the same

procedures of management of the animals were used in this study, which means that of the factors that influence the skin-test only the age, body condition, and the ability of immune response of the animals can vary among the stags. Nonetheless, the body weight, despite having a positive relationship with skin fold increase to PHA (immune capacity) (Moller *et al.* 1998; Coop & Kyriazakis 1999, 2001; Lochmiller & Deeremberg 2000; Fernández-de-Mera *et al.* 2006), in the case of farmed red deer has less relevance than in wild red deer (Fernández-de-Mera *et al.* 2009), because in farms all animals are artificially fed, which increases the nutritional condition and the capacity to mobilize energy for immune response. Environmental conditions were equal for all stags and therefore this factor was minimized. Although the age of stag is a factor that can influence the PHA skin fold increase (Fernández-de-Mera *et al.* 2008), in our study all stags were adults and their age was similar. The age range of the age of hinds was wider (between 2 and 14 years old), so we needed to establish age classes: young hinds, middle hinds and older hinds. Although no statistical differences were found in the skin fold increase to any antigen in relation to the sex after controlling by the group and the parental father in the calves, we performed the inferential analyses separately for each calf sex, because as it well knows in polygynous species. Males and females may differ in their allocation priorities, as females may obtain greater lifetime reproductive success by investing more in self-maintenance (such as growth or immunity) and rearing offspring (Landete-Castillejos *et al.* 2004), whereas males may tend to follow a “live hard, die young” strategy (Promislow & Harvey 1990; Carranza *et al.* 2004), allocating more resources to displaying and mate attraction than to immunity (Zuk & Stoehr 2002; Malo *et al.* 2009).

We found a high variability in the skin-fold increase response to the different antigens in the stags. This high variability of skin-fold increase to PHA in stags can be related with genetic differences in the cellular immune response, since these animals were in same conditions of environment and management. In relation to the skin-fold increase to *M. avium* PPD and *M. bovis* PPD antigens the high variability can be related with the frequency of contact or/and stage of infection with mycobacteria and/or the ability to mount an immune response in each individual as commented above for PHA.

Among calves, the prevalence of reactors to *M. avium* PPD was 3.5 % and to *M. bovis* PPD was 2.5%. Similar prevalence was found for ELISA test (MPB70= 4.6%, *M. bovis* PPD= 4.4%, PPA3= 5.7% and *M. avium* PPD= 3.3%), following the cut-off arbitrarily chose. This apparent prevalence to *M. avium* PPD in skin-test was lower as compared with a previous study in the same farm performed 3 years ago, where the seroprevalence in this farm in deer (all ages) was the 26% (Reís-Garcia *et al.*, 2008). This fact can be explained by the measures taken to control mycobacterial infections in this farm in last years and by the lower probability (less lifespan) of

the calves to contact with mycobacteria than adult animals. The same occurred with TB. In this case the control measures were even more intensive with several animals culled throughout these years. However, the results of these tests were no proofed during the necropsy (22 animals), histopathology, mycobacterial culture and PCR of the tissues samples collected from 11 highest positive animals. These results suggest that some red deer could have a general high cellular immune response and hence being positive reactors to mycobacterial antigens. Alternatively, these results could be due to cross reactive response with other nonpathogenic mycobacteria

Regarding the association between the skin fold increases to different antigens we evidenced, after controlling by weight, a bidirectional positive association between skin fold increase to *M. bovis* PPD and *M. avium* PPD in both calves sexes. Moreover, we proofed a positive association between skin fold increase to *M. bovis* PPD and PHA in male calves, but not in females. This association was yet proofed by other authors in wild animals in both sexes but not in farmed red deer, where the animals are artificially fed and therefore have no limited resources (Fernández-de-Mera *et al.*, 2009). No statistical association between *M. avium* PPD and PHA skin fold increases in both calves were evidenced in any model as well as between skin fold increase to *M. bovis* PPD and PHA in female calves. These associations between antigens enhance the idea that the skin fold increase to mycobacterial antigens may be affected by common causes, such as unspecific responses or a general high responsiveness.

Concerning the differences among the stags in their offspring skin fold increase in response to the different antigens, statistical differences were found for PHA and *M. bovis* PPD skin fold increase in females. This should be interpreted with care due to the fact that this model did not include other variables (e.g. PHA responsiveness of the hind). However, we managed to show the great variability of the response to different antigens in calves, in function of their fathers. This could be indicative of the differences among stags in the transmission of the genetic characteristics. Statistical differences were found for PHA and *M. bovis* PPD skin fold increase in females, which can be indicative of the importance of the male in transmission of characteristic to the female offspring.

Finally, the last model described the relationship between the cellular immune response of the calves and those of the assigned parents. In the case of male calves, statistical differences were found for age of their mother, and the interaction between age and PHA skin fold increase of their mothers. In this sense, when regressing the predicted values of the GLMM (as dependent) and hinds (as independent) separately for three different age categories of the hinds, statistical differences were found for all age classes (Figure X), being the PHA skin fold increase in young hinds the most associated with PHA skin fold increase of the male calves.

This result could mean that the genetic characteristics related with cellular immune response in male calves are enhanced in young hinds as compared to adult and old hinds, which can be due to capacity of the hind for reared the calf, which increases with maternal age (Clutton-Brock *et al.* 1982). Calves of younger females that are reared in worse conditions needed to express the genotype characteristics more than those of adult females, where the phenotype characteristics are enhanced. Furthermore, the reproductive effort of the female deer change with age groups, where middle reproductive age groups might be expected to be in better condition compared to younger growing animals that are partitioning resources into growth (Albon *et al.* 1983). All this can explain the need of male offspring of the younger females to express genotype characteristics as compared with calves of adult and old hinds. No age by PHA response interaction could be tested for stags, and therefore we cannot discard that this effect (which was evident for female and calve models, Table IV model) could also exist regarding fathers.

Regarding, the female calves, statistical differences were found for the PHA skin fold increase of their parents (and their interaction) and with age of the mothers as well as the interaction between age and PHA skin fold increase of their mothers. In the case of interaction between PHA skin fold increase in their parents, when regressing the predicted values of the GLMM (as dependent) and hinds (as independent) separately for three different categories of PHA skin fold increase for stags (established according to the 33 and 66 percentiles, n= 3, 4 and 3, respectively), the only statistical significant result was found for the middle category, whereas no relationship was found for the other two extreme categories, even though there was a tendency (Figure IX). This result means that the PHA skin fold increase in hinds was more relevant for the PHA skin fold increase of the female calves, when the response to PHA antigen in the father was medium. This suggests that the PHA skin fold increase in the calf is a result of both parents genetics, and that extreme PHA values (resembling immune response) in one parent may be greatly influential. In the case of interaction between age classes and PHA skin fold increase in hinds, when regressing the predicted values of the GLMM (as dependent) and hinds (as independent) separately for three different categories of hind age (young hinds, adult hinds, older hinds), statistical differences were found for all age classes, being the older hinds more associated with the PHA skin fold increase in female calves, followed by young hinds (Figure X). Briefly, in this model we can tell that the genotype characteristics of cellular immune response are enhanced in calves, which are offspring's of the young and old hinds. Hinds in the peak of their reproductive age have less influence on the cellular immune response of their offsprings. This may be because they are in better conditions than other age classes of females and have less dependence on the expression of genetics characteristics (as aforementioned).

We only observed association between cellular immune response in stags and offspring in the case of the female calves. The genetic characteristics of the fathers may have more relevance in case of the female compared to male calves because the phenotipic effect of hind (the mothers) in life traits of the calves may be more evident in male claves (the most cooslty offspring), and therefore genetic effects of stags can be hidden in male compared to female calves. Therefore, although this research provided novel insights into the relationship between the cellular immune response in red deer calves and their progenitors, more research in this area is needed to clarify the true heritability of the immune response in red deer in farm conditions. Given the special natural history in red deer, greatly influenced by sexual selection, it would be interesting to study the relationships between secondary sexual characters and level of acquired immunity in stags, testing the Handicap principle in a controlled environment.

6. CONCLUSIONS

As a proxy to heritability, we investigated the relationships between the cellular immune response in parents and their offspring in farmed red deer, as well as its potential influence on the results of the tuberculin skin-test. Significant parental effects (or empirical relationships) were evidenced, including effects of maternal hind age on calf skin-test responsiveness, and effects of the stag immune response only among female calves. Our results are greatly conditioned by the natural history of red deer, which is mainly determined by sexual selection. This is evident in different live strategies in immune response investment among sexes and resource allocation to calves as a function of their sex. These effects should be considered along with other known factors causing variation in skin-test responsiveness when interpreting skin-test results in red deer. Our findings provided support to a genetic basis of immune cellular response in farmed red deer, which should be confirmed by heritability studies. Although complex interactions relating to parents traits and those of offspring depending on sex were detected when studying the immune response in calves, we recommend that the selection of reproductive stags as a function of their immune response can be incorporated as a parameter to consider in management plans in farmed deer since: most of the offspring usually come from a few fathers, father cellular immunity seems to be transmitted to females calves, and those hinds (which would inherit immunity from high standing males (according to immunity) are influential to both male and female offspring.

7. REFERENCES

Alberto J (2009) "Study of Bovine Tuberculosis (*Mycobacterium bovis*) in large game hunted in Idanha-a-Nova county" **Tese de Mestrado Medicina Veterinária,** Universidade de Trás-os-Montes e Alto Douro Vila Real

Albon SD, Mitchell B, Staines BW (1983) "Fertility and body weight in female red deer: a density-dependent relationship" **Journal Animal Ecology** 52, 969-980

Álvarez J, de Juan L, Briones V, Romero B, Aranaz A, Fernández-Garayzábal JF, Mateos A (2005) "*Mycobacterium avium* subspecies *paratuberculosis* in fallow deer and wild boar in Spain" **Veterinary Record** 156, 212-213

Aranaz A, de Juan L, Montero N, Sanchez C, Galka M, Delso C, Alvarez J, Romero B, Bezos J, Vela AI, Briones V, Mateos A, Dominguez L (2004) "Bovine Tuberculosis (*Mycobacterium bovis*) in Wildlife in Spain" **Journal Clinical Microbiology** 42, 2602-2608

Ardia DR, Rice EB (2006) "Variation in heritability of immune function in the tree swallow" **Evolutionary Ecology** 20, 491-500

Balseiro A, García-Marín JF, Solano P, Garrido JM, Prieto JM (2008) "Histopathological classification of lesions observed in natural cases of paratuberculosis in free-ranging fallow deer (*Dama dama*)" **Journal Comparative Pathology** 138, 180-188

Barton NH, Turelli M (1989) Annu Rev Gen 23, 337-370

Basak DK, Chatterjee MK, Neogi MK, Samanta DP (1975) "Tuberculosis in captive deer" **Indian Journal of Animal health** 14, 135-137

Beard PM, Daniels MJ, Henderson D, Pirie A, Rudge K, Buxton D, Rhind S, Greig A, Hutchings MR, Mckendrick I, Stevenson K, Sharp JM (2001a) "Paratuberculosis infection of non-ruminant wildlife in Scotland" **Journal Clinical Microbiology** 39, 1517-1521

Beard PM, Henderson D, Daniels MJ, Pirie A, Buxton D, Greig A, Hutchings MR, Mckendrick I, Rhind S, Stevenson K, Sharp JM (1999) "Evidence of paratuberculosis in fox (*Vulpes vulpes*) and stoat (*Mustela erminea*)" **Veterinary Record** 145, 612-613

Beard PM, Rhind SM, Buxton D, Daniels MJ, Henderson D, Pirie A, Rudge A, Greig A, Hutchings MR, Stevenson K, Sharp JM (2001b) "Natural paratuberculosis infection in rabbits in Scotland" **Journal Comparative Pathology** 124, 290-299

Beatson NS (1985) "Tuberculosis in red deer in New Zealand: Biology of deer production" **Royal Society of New Zealand** 22, 147-150

Beatson NS, Hutton JB, de Lisle GW (1984) "Tuberculosis test and slaughter - Proceedings of a deer course for Veterinaries" **Palmerston North: Deer Branch of the New Zealand Veterinary Association** volume 1, 18-27

Buergelt CD, Hall C, McEntee K, Duncan JR (1978) "Pathological evaluation of paratuberculosis in naturally infected cattle" **Veterinary Pathology** 15, 196-207

Carrigan MJ, Seaman JT (1990) "The pathology of Johne's disease in sheep" **Australian Veterinary Journal** 67, 47-50

Centers for Disease Control and Prevention World TB (2007) **MMWR Morb Mortal Wkly Rep** 56, 245

Charlesworth B, Hughes KA (2000) "In Evolutionary Genetics" **Cambridge University Press Cambridge U K**, 369-392

Charmantier A, Garant D (2005) Environmental quality and evolutionary potential: lessons from wild populations" **Proc Roy Soc B-Biology Science** 272, 1415-1425

Cheng S, Lamont SJ (1988) "Genetic analysis of immunocompetence measures in a white leghorn chicken line" **Poultry Science** 67, 989-995

Chiodini RJ, Vankruiningen HJ (1983) "Eastern white-tailed deer as a reservoir of ruminant paratuberculosis" **Journal Animal Veterinary Medicine Association** 182, 168-169

Choquete LP, Gallivan JF, Byrene JL, Pilipavicius J (1961) "Parasites and disease of bison in Canada: tuberculosis and some other pathological conditions in bison at wood buffalo and elk island national parks in the fall and winter of 1959-60" **Canadian Veterinary Journal** 2, 168-174

Clarke C J, Patterson IA, Armstrong KE, Low JC (1996) "Comparison of the absorbed ELISA and agar gel immunodiffusion test with clinicopathological findings in ovine clinical paratuberculosis" **Veterinary Record** 139, 618-621

Clarke CJ (1997) "The pathology and pathogenesis of paratuberculosis in ruminants and other species" **Journal of Comparative Pathology** 116, 217-261

Clifton-Hadley R, Sauter-Louis C, Lugton I, Jackson R, Durr P, Wilesmith J (2001) "Mycobacterium infections" *in* Williams E, Barker I (eds) "Infectious diseases of wild mammals" **The veterinary Press London** 3rd edition, 340-361

Clifton-Hadley RS, Wilesmith JW (1991) "Tuberculosis in deer: a review" **Veterinary Record** 129(1), 5-12

Clifton-Hadley RS, Wilesmith JW, Stuart FA (1993) "*Mycobacterium bovis* in the European badger (Meles meles): epidemiological findings in tuberculous badgers from a naturally infected population" **Epidemiology Infection** 111, 9-19

Clutton-Brock TH, Guinness FE, Albon SP (1982) "Reed deer: behavior and ecology of two sexes" **University of Chicago**

Coleman JD, Coleman MC, Warburton B (2006) "Trends in the incidence of tuberculosis in possums and livestock associated with differing control intensities applied to possum populations" **New Zealand Veterinary Journal** 54, 52-60

Corpa JM, Garrido J, García-Marín JF, Pérez V (2000) "Classification of lesions observed in natural cases of paratuberculosis in goats" **Journal of Comparative Pathology** 122, 255-265

Corrin KC, Carter CE, Kissling RC, de Lisle GW (1987) "Short interval skin testing in farmed red deer (*Cervus elaphus*) inoculated with *Mycobacterium bovis"* **New Zealand Veterinary Journal** 35, 204-207

David HL (1984) "Carotenoid pigments of the mycobacteria" in Kubica GP, Wayne LG (Eds) "The Mycobacteria" **A Source Book Part A Marcel Dekker New York,** 537-545

Davison TF (2003) "The immunologists' debt to the chicken" **British Poultry Science** 44, 6-21

de Lisle GW, Bengis RG, Schmitt SM, O'Brien DJ (2002) "Tuberculosis in free-ranging wildlife: detection diagnosis and management" **Rev Science Tech** 21, 317-334

de Lisle GW, Mackintosh CG, Bengis RG (2001) "*Mycobacterium bovis* in free-living and captive wildlife including farmed deer" **Rev Science Tech** 20, 86-111

de Lisle GW , Yates GF, Montgomery RH (2003) "The emergence of *Mycobacterium paratuberculosis* in farmed deer in New Zealand - a review of 619 cases" New Zealand Veterinary Journal 51, 58-62

Dhand NK, Eppleston J, Whittington RJ, Toribio JA (2007) "Risk factors for ovine Johne's disease in infected sheep flocks in Australia" **Preventive Veterinary Medicine** 82, 51-71

Ekkel ED, Kuypers AH, Counotte GHM, Tielen MJM (1995) "The phytohemagglutinin [PHA] skin-test as an indicator of stress-induced changes in immune reactivity in pigs" **Veterinary Q** 17, 143-146

European Food Safety Authority (2008) "Tuberculosis testing in deer - Scientific Opinion of the Panel on Animal Health and Welfare" **EFSA-Q-2006**-179

European Food Safety Authority (2009) "The Community Summary Report on Trends and Sources of Zoonoses and Zoonotic Agents in the European Union in 2007" **The EFSA Journal**, 223

Falconer DS (1989) “Introduction to Quantitative Genetics” **Longman New York**

Fedoseev VS, Rubtsova LN, Omarbekov EO, Kirilenko NG (1982) “Anti-tuberculosis measures in maral rearing” **Veterinarya (Moscow)** 4, 35-36

Feller M, Huwiler K, Stephan R, Altpeter E, Shang A, Furrer H, Pfyffer GE, Jemmi T, Baumgartner A, Egger M (2007) “*Mycobacterium avium* subspecies *paratuberculosis* and Crohn's disease: a systematic review and meta-analysis” **Lancet Infections Disease** 7, 607-613

Fernández-de-Mera IG, Höfle U, Vicente J, García A, Rodríguez O, Gortázar C (2006) “Optimal dose and timing in phytohaemagglutinin skin-testing of deer” **New Zealand Veterinary Journal** 54, 357-359

Fernández-de-Mera IG, Vicente J, Höfle U, Rodríguez O, Gaspar-López E, Gortázar C (2008) “The effects of sex and age on phytohaemagglutinin skin-testing of deer” **New Zealand Veterinary Journal** 56, 71-73

Fernández-de-Mera IG, Vicente J, Höfle U, Fons FR, Ortiz JA, Gortázar C (2009) “Factors affecting red deer skin test responsiveness to bovine and avian tuberculin and to phytohaemagglutinin” **Preventive Veterinary Medicine** 90, 119-126

Fleetwood AJ, Stuart FA, Bode R, Sutton JP (1988) “Tuberculosis in deer” **Veterinary record** 123, 279-280

Fletcher J (2004) “Deer veterinary issues in the United Kingdom and Europe” **Proceedings of the 1st World Deer Veterinary Congress and the Deer Branch of the New Zealand Veterinary Association** 21, 5-8

Folstad I, Karter AJ (1992) “Parasites bright males and the immunocompetence handicap" **Animal Nature** 139, 603-622

Florou M, Leontides L, Kostoulas P, Billinis C, Sofia M, Kyriazakis I, Lykotrafitis F (2008) “Isolation of *Mycobacterium avium* subspecies *paratuberculosis* from non-ruminant wildlife living in the sheds and on the pastures of Greek sheep and goats” **Epidemiology Infection** 136, 644-652

Fredriksen B, Djønne B, Sigurdardóttir O, Tharaldsen J. Nyberg O, Jara J (2004) “Factors affecting the herd level of antibodies against *Mycobacterium avium* subspecies *paratuberculosis* in dairy cattle” **Veterinary Record** 154, 522-526

Friend M, Kroll ET, Gruft H (1963) “Tuberculosis in a wild white-tailed deer” **New York Fish and Game journal** 10, 118-123

Gaborick CM, Salman MD, Ellis RP, Triantis J (1996) “Evaluation of a five-antigen ELISA for diagnosis of tuberculosis in cattle and Cervidae” **Journal Animal Veterinary Medicine Association** 209, 962-966

Goldrick BA (2004) “Once dismissed still rampant: tuberculosis the second deadliest infectious disease worldwide” **Animal Journal Nurs** 104, 68-70

González J, Geijo MV, García-Pariente A, Verna A, Corpa JM, Reyes LE, Ferreras MC, Juste RA, García-Marín JF, Pérez V (2005) “Histopathological classification of lesions associated with natural paratuberculosis infection in cattle” **Journal of Comparative Pathology** 133, 184-196

Goodfellow M, Cross T (1984) “Classification” in Goodfellow M, Mordarski M, Williams ST (Eds) “The Biology of the Actinomycetes” **Academic Press London UK** 8-164

Gortázar C, Ferroglio E, Höfle U, Frölich K, Vicente J (2007) “Diseases shared between wildlife and livestock: a European perspective” **Eur J Wildl Res** 53, 241-256

Gortázar C, Herrero J, Villafuerte R, Marco J (2000) “Historical examination of the distribution of large mammals in Aragón Northeastern Spain” **Mammalia** 61, 411-422

Gortázar C, Vicente J, Gavier-Widen D (2003) “Pathology of bovine tuberculosis in the European wild boar” **Veterinary Record** 152, 779-780

Grange JM, Collins CH (1987) “Bovine tubercle bacilli and disease in animals and man” **Epidemiology Infection** 99, 221-234

Greig A, Stevenson K, Henderson D, Perez V, Hughes V, Pavlick I, Hines ME, Mckendrick I, Sharp M (1999) “Epidemiological study of paratuberculosis in wild rabbits in Scotland” **Journal Clinical Microbiology** 37, 1746-1751

Greig A, Stevenson K, Perez V, Pirie AA, Grant JM, Sharp JM (1997) “Paratuberculosis in wild rabbits (*Oryctolagus cuniculus*)” **Veterinary Record** 140, 141-143

Griffin JF (1988) “The aetiology of tuberculosis and mycobacterial diseases in farmed deer” **Irish Veterinary Journal** 42, 23-26

Griffin JF, Chinn DN, Rodgers CR (2004) “Diagnostic strategies and outcomes on three New Zealand deer farms with severe outbreaks of bovine tuberculosis” **Tuberculosis 84**, 293-302

Griffin JF, Buchan GS (1994) Etiology pathogenesis and diagnosis of *Mycobacterium bovis* in deer” **Veterinary Microbiology** 40, 193-205

Griffin JF, Mackintosh CG (2000) “Tuberculosis in deer: perceptions problems and progress” **Veterinary Journal** 160, 202-219

Griffin JF, Rodgers CR, Liggett S, Mackintosh CG (2006) “Tuberculosis in ruminants: Characteristics of intra-tonsilar *Mycobacterium bovis* infection models in cattle and deer” ***Tuberculosis* 86**, 404-418

Griffin JM, Williams DH, Kelly GE, Clegg TA, O’Boyle I, Collins JD, More SJ (2005) The impact of badger removal on the control of tuberculosis in cattle herds” **Ireland Preventive Veterinary Medicine** 67, 237-266

Haagsma J (1993) “Working Paper on Recent Advances in the Field of Tuberculosis Control and Research” **World Health Organization Meeting on Zoonotic Tuberculosis with Particular Reference to *Mycobacterium bovis*,** 15 November 1993 Geneva, Switzerland

Hasanova L, Pavlik I (2006) “Economic impact of paratuberculosis in dairy cattle herds: a review” **Veterinary Medicine Czech** 51, 193-211

Harrington NP, Surujballi OP, Prescott JF, Duncan JR, Waters WR, Lyashchenko K, Greenwald R (2008) “Antibody Responses of Cervids (*Cervus elaphus*) following Experimental *Mycobacterium bovis* - Infection and the Implications for Immunodiagnosis” **Clinical Vaccine Immunology** Nov, 1650-1658

Hartmans S, Bont JA, Stackebrandt E (2006) “The Genus *Mycobacterium*-Nonmedical” **Prokaryotes** 3, 889-918

Hernández A, Yager JA, Wilkie BN, Leslie KE, Mallard BA (2005) “Evaluation of bovine cutaneous delayed-type hypersensitivity (DTH) to various test antigens and a mitogen using several adjuvants” **Veterinary Immunology and Immunopathology** 104, 45-58

Houle D (1992) “Comparing evolvability and variability of quantitative traits” **Genetics** 130, 195-204

Jorgensen JB, Husum P, Sorense I (1988) “Bovin tuberculose I en hjortefarm” **Dansk Veterinaertideekrift** 71, 806-808

Kaneene JB, Vanderklok M, Bruning-Fann CS, Palmer MV, Whipple DL, Schmitt SM, Miller R (2002) “Prevalence of *Mycobacterium bovis* infection in cervids on privately owned ranches” **JAVMA** 220, 656-659

Kelley KW, Greenfield RE, Evermann JF, Parish SM, Perryman LE (1982) “Delayed-type hypersensitivity contact sensitivity and phytohemagglutinin skin-test responses of heat- and cold-stressed calves” **Animal Journal Veterinary Resource** 43, 775-779

Kennedy MW, Nager RG (2006) “The perils and prospects of using phytohaemagglutinin in evolutionary ecology” **Trends in Ecology & Evolution** 21, 653-655

Kilpimaa J, Van-de-Casteele T, Jokinen I, Mappes J, Alatalo RV (2005) “Genetic and environmental variation in antibody and T-cell mediated responses in the great tit” **Evolution** 59, 2483-2489

Kruuk lEB, Clutton-Brock TH, Salte J, Pemberton JM, Brotherstone S (1999) Heritability of fitness in a wild mammal population” **PNAS** 97, 698-703

Krucky JD, Zajicek D, Pavlista J (1982) “Vyskyt tuberkulosy u jeleni zvere” **Veterinarstvi** 32, 501-503

Lillehoj HS, Kim CH, Keeler CL, Zhangt S (2007) “Immunogenomic approaches to study host immunity to enteric pathogens” **Poultry Science** 86, 1491-1500

Liu S, Guo S, Wang C, Shao M, Zhang X, Guo Y, Gong Q (2007) "A novel fusion protein-based indirect enzyme-linked immunosorbent assay for the detection of bovine tuberculosis" **Tuberculosis 87**, 212-217

López-Olvera JR, Höfle U, Vicente J, Fernández-de-Mera IG, Gortázar C (2006) Effects of parasitic helminths and ivermectin treatment on clinical parameters in the European wild boar (*Sus scrofa*)" **Parasitology Research** 98, 582–587

López S (1997) "Acquired resitance affects male sexual display and female choice in guppies" **Real Society London** B 265, 717-723

Lugton I W, Wilson PR, Morris RS, Nugent G (1998) "Epidemiology and pathogenesis of *Mycobacterium bovis* infection of red deer (*Cervus elaphus*) in New Zealand" **New Zealand Veterinary Journal** 46,147-156

Lynch M, Walsh B (1998) "Genetics and Analysis of Quantitative Traits" **Sinauer Sunderland MA**

Machackova M, Svastova P, Lamka J, Parmova I, Liska V, Smolik J, Fischer OA, Pavlik I (2004) "Paratuberculosis in farmed and free-living wild ruminants in the Czech Republic (1999–2001)" **Veterinary Microbiology** 101, 225-234

Machackova-Kopecna M, Bartos M, Straka M, Ludvik V, Svastova P, Alvarez J, Lamka J, Trcka I, Treml F, Parmova I, Pavlik I (2005) "Paratuberculosis and avian tuberculosis infections in one red deer farm studied by IS*900* and IS*901* RFLP analysis" **Veterinary Microbiology** 105, 261-268

Mackintosh CG, de Lisle GW, Collins DM, Griffin JF (2004) "Mycobacterial diseases of deer" **New Zealand Veterinary Journal** 52, 163-174

Mallon EB, Loosli R, Schmid-Hempel P (2003) "Specific versus nonspecific immune defense in the bumblebee (*Bombus terrestris)"* **Evolution** 57,1444-1447

Malo AF, Roldan ERS, Garde JJ, Soler AJ, Vicente J, Gortazar C, Gomendio M (2009) "What does testosterone do for red deer males?" **Proceedings Royal Society B** 276, 971-980

Martín-Hernando MP, Torres MJ, Aznar J, Negro JJ, Gandía A, Gortázar C (2010) "Distribution of Lesions in Red and Fallow Deer Naturally Infected with *Mycobacterium bovis"* **Journal of Comparative Pathology** 142, 43-50

Martin LB, Han P, Lewittes J, Kuhlman JR, Klasings KC, Wikelski M (2006) "Phytohemagglutinin-induced skin swelling in birds: histological support for a classic immunoecological technique" **Funct Ecology** 20, 290-299

Merilä J, Sheldon BC (1999) "Heredity" **Heredity 83,** 103-109

Michel AL, Bengis RG, Keet DF, Hofmeyr M, Klerk LM, Cross PC, Jolles AE, Cooper D, Whyte IJ, Buss P, Godfroid J (2006) "Wildlife tuberculosis in South African conservation areas: implications and challenges" **Veterinary Microbiology** 112, 91-100

Mitchell RJ (1992) "Testing evolutionary and ecological hypothesis path analysis and structural equation modeling" **Funct Ecology 6**, 123-129

More SJ, Cameron AR, Greiner M, Clifton-Hadley RS, Rodeia SC, Bakker D, Salman MD, Sharp JM, De Massis F, Aranaz A, Boniotti MB, Gaffuri A, Have P, Verloo D, Woodford M, Wierup M (2009) "Defining output-based standards to achieve and maintain tuberculosis freedom in farmed deer with reference to member states of the European Union" **Preventive Veterinary Medicine** 90, 254-267

Morris RS, Pfeiffer DU, Jackson R (1994) "The epidemiology of *Mycobacterium bovis* infections" **Veterinary Microbiology** 40,153-157

Mousseau TA, Roff DA (1987) "Natural-selection and the heritability of fitness components" **Heredity** 59, 181-197

Nacy C, Buckley M (2008) "*Mycobacterium avium paratuberculosis*: Infrequent human pathogen or public health threat? In Report from the American Academy for Microbiology" **American Academy for Microbiology Washington DC**

Naranjo V, Höfle U, Vicente J, Martin MP, Ruiz-Fons F, Gortazar C, Kocan KM, de la Fuente J (2006) "Genes differentially expressed in oropharyngeal tonsils and mandibular lymph nodes of tuberculous and nontuberculous European wild boar naturally exposed to *Mycobacterium bovis"* **FEMS Immunology Medicine Microbiology** 46, 298-312

Naser SA, Ghobrial G, Romero C, Valentine JF (2004) "Culture of *Mycobacterium avium* subspecies *paratuberculosis* from the blood of patients with Crohn's disease" **Lancet** 364, 1039-1044

Nath M, Singh B, Saxena V, Singh R, Roy A (2001) "Genetic analysis of concanavlin-A response in broilers" **Journal Appl Animal Resource** 20, 171-180

Neill S, Bryon D, Pollock J (2001) "Pathogenesis of tuberculosis in cattle" **Tuberculosis** 81, 79-86

O'Brien DJ, Schimitt SM, Berry DE, Fitzgerald SD, Vanneste JR, Lyon TJ, Masig D, Fierke JS, Cooley TM, Zwick LS, Thomsen BV (2004) "Estimating the true prevalence of *Mycobacterium bovis* in hunter-harvested white-tailed deer in Michigan" **Journal Wildlife Disease** 40, 42-52

O'Brien DJ, Schmitt SM, Fierke JS, Hogle SA, Winterstein SR, Cooley TM, Moritz WE, Diegel KL, Fitzgerald SD, Berry DE, Kaneene JB (2002) "Epidemiology of *Mycobacterium bovis* in free-ranging white-tailed deer Michigan USA 1995–2000" **Preventive Veterinary Medicine** 54, 47-63

O'Brien DJ, Schmitt SM, Fitzgerald SD, Berry DE, Hickling GJ (2006a) "Managing the wildlife reservoir of *Mycobacterium bovis*: the Michigan USA experience" **Veterinary Microbiology** 112, 313-323

O'Brien R, Mackintosh CG, Bakker D, Kopecna M, Pavlik I, Griffin JF (2006b) "Immunological and molecular characterization of susceptibility in relationship to bacterial strain differences in *Mycobacterium avium* subsp *paratuberculosis* infection in the red deer (*Cervus elaphus*)" **Infection Immunology**, 74, 3530-3537

O'Reilly LM, Daborn CJ (1995) "The epidemiology of *Mycobacterium bovis* infections in animals and man: a review" **Tuberculosis Lung Disease** 76, 1-46

Palmer MV (2007) "Tuberculosis: a reemerging disease at the interface of domestic animals and wildlife" **Curr Top Microbiol Immunol** 315, 195-215

Palmer MV, Waters WR, Whipple DL (2002) "Aerosol delivery of virulent *Mycobacterium bovis* to cattle" **Tuberculosis** 82, 275-282

Pérez V, García-Marín JF, Badiola JJ (1996) "Description and classification of different types of lesion associated with natural paratuberculosis infection in sheep" **Journal of Comparative Pathology** 114, 107-122

Pérez V, Tellechea J, Badiola JJ, Gutiérrez M, García-Marín JF (1997) "Relation between serologic response and pathologic findings in sheep with naturally acquired paratuberculosis" **American Journal of Veterinary Research** 58, 799-803

Pérez V, Tellechea J, Corpa JM, Gutiérrez M, García Marín JF (1999) "Relation between pathologic findings and cellular immune responses in sheep with naturally acquired paratuberculosis" **American Journal of Veterinary Research** 60, 123-127

Pittius NC, Sampson SL, Hyeyoung Lee H, Kim Y, Helden PD, Warren RM (2006) "Evolution and expansion of the *Mycobacterium tuberculosis* PE and PPE multigene families and their association with the duplication of the ESAT-6 (*esx*) gene cluster regions" **BMC Evolutionary Biology** 6, 95

Pollock J, McNair J, Welsh M, Girvin R, Kennedy H, Machie D, Neill S (2001) "Imune responses in bovine tuberculosis" **Tuberculosis** 81, 103-107

Pollock J, Rodgers J, Welsh M, McNair J (2006) "Pathogenisis of bovine tuberculosis: the role of experimental models of infection" **Veterinary Microbiology** 112, 141-150

Porphyre T, McKenzie J, Stevenson M (2007) "A descriptive spatial analysis of bovine tuberculosis in intensively controlled cattle farms in New Zealand" **Veterinary Resource** 38, 465-479

Reyes-García R, Pérez-de-la-Lastra JM, Vicente J, Ruiz-Fons F, Garrido JM, Gortázar C (2008) "Large-scale ELISA testing of Spanish red deer for paratuberculosis" **Veterinary Immunology and Immunopathology** 124, 75-81

Riemann H, Zaman MR, Ruppanner R, Aalund O, Jorgensen JB, Worsaae H, Behymer D (1979) "Paratuberculosis in cattle and free-living exotic deer" **Journal Animal Veterinary Medicine Association** 174, 841-843

Robino P, Nebbia P, Tramuta C, Martinet M, Ferroglio E, De Meneghi D (2008) "Identification of *Mycobacterium avium* subsp *paratuberculosis* in wild cervids (Cervus elaphus hippelaphus and Capreolus capreolus) from North-Western Italy" **Eur J Wildl Res** 54, 357-360

Roff D, Mostowy S, Fairbairn D (2002) "The evolution of trade-offs: testing prediction on response to selection and environmental variation" **Evolution** 56, 84-95

Roitt IM, Brostoj J, Male DK (1996) "Immunology" **Immunology** 4th edn London, Mosby

Ryan TJ, Livingstone PG, Ramsey DSL, de Lisle GW, Nugent G, Collins DM, Buddle BM (2006) "Advances in understanding disease epidemiology and implications for control and eradication of tuberculosis in livestock: the experience of New Zealand" **Veterinary Microbiology** 112, 211-216

Sams MG, Lochmiller RL, Qualls CW, Leslie DM, Payton ME (1996) "Physiological correlates of neonatal mortality in an overpopulated herd of white-tailed deer" **Journal of Mammalogy** 77, 179-90

Santos N (2006) "A tuberculose no javali em Portugal" **Tese de Mestrado em Saúde Pública Veterinária,** Faculdade de Medicina Veterinária Universidade Técnica de Lisboa, 109

Santos N, Correia-Neves M, Ghebremichael S, Kallenius G, Svenson S B, Almeida V (2009) "Epidemiology of *Mycobacterium bovis* infection in wild boar (*Sus Scrofa*) from Portugal" **Journal Wildlife Disease** 45, 1048-1061

Salazar DC (2009) "Distribuição e estatuto do Veado e Corço em Portugal" **Tese de Mestrado em Biologia,** Universidade de Aveiro

Schmid-Hempel P (2003) "Variation in immune defence as a question of evolutionary ecology" **Proc R Soc Lond B** 270, 357-366

Schmid-Hempel P, Ebert D (2003) "On the evolutionary ecology of specific immune defence" **Trends Ecology Evolution** 18, 27-32

Schmitt SM, Fitzerald SD, Cooley TM, Bruning-Fann CS, Sullivan L, Berry D, Carlson T, Minnis RB, Payeur JB, Sikarskie J (1997) "Bovine tuberculosis in free-ranging whitetailed deer from Michigan" **Journal Wildlife Disease** 33, 749-758

Sheldon BC, Verhulst S (1996) "Ecological immunology: costly parasite defence and trade-offs in evolutionary ecology" **Trends Ecology Evolution** 11, 317-321

Shinnick TM, Good RC (1994) "Mycobacterial taxonomy" **Eur J Clin Microbiol Infect Dis** 13, 884-901

Smits JE, Bortolotti GR, Tella JL (1999) "Simplifying the phytohaemagglutinin skin-testing technique in studies of avian immunocompetence" **Funct Ecology** 13, 567-572

Sockett DC, Conrad TA, Thomas CD, Collins MT (1992) "Evaluation of four serological tests for bovine paratuberculosis" **Journal Clinical Microbiology** 30, 1134-1139

Sorci G, Moller AP, Boulinier T (1997) "Genetics of host-parasite interactions" **Trends Ecology Evolution** 12, 196- 200

Soriguer RC, Márquez FJ, Pérez JM (1998) "Las translocaciones (introducciones y reintroducciones) de especies cinegéticas y sus efectos medioambientales" **Galemys** 10, 19-35

Skoric M, Shitaye E, Halouzka R, Fictum P, Trcka I, Heroldova M, Tkadlec E, Pavlik I (2007) "Tuberculous and tuberculoid lesions in free living small terrestrial mammals and the risk of infection to Humans and animals: a review" **Veterinary medicine** 54,144-161

Stamp JT, Watt JA (1954) "Johne's disease in sheep" **Journal of Comparative Pathology** 64, 26-40

Tella JL, Forero MG, Bertellotti M, Donázar JA, Blanco G, Ceballos O (2001) "Offspring body condition and immunocompetence are negatively affected by high breeding densities in a colonial seabird: A multiscale approach" **Biology Science** 268, 1455-1461

Tizard IA (2000) "Veterinary Immunology" **An Introduction Veterinary Immunology** 6 edi, W B Saunders Company

Tweedle NE, Livingstone P (1994) "Bovine tuberculosis control and eradication programs in Australia and New Zealand" **Veterinary Microbiology** 40, 23-9

Uzoigwe JC, Khaitsa ML, Gibbs PS (2007) "Epidemiological evidence for Mycobacterium avium subspecies paratuberculosis as a cause of Crohn's disease" **Epidemiology Infection** 135, 1057-1068

Vicente J, Höfle U, Garrido JM, Fernández-De-Mera IG, Acevedo P, Juste R, Barral M, Gortazar C (2007) "Risk factors associated with prevalence of tuberculosis-like lesions in wild boar and red deer in South Central Spain" **Veterinary Resource** 38, 451-464

Vicente J, Höfle U, Garrido JM, Fernández-De-Mera IG, Juste R, Barral M, Gortazar C (2006) "Wild boar and red deer display high prevalences of tuberculosis-like lesions in Spain" **Veterinary Resource** 37, 107-119

Wakelin D, Apanius V (1997) "Immune defence: genetic control" in "Host-parasite evolution: general principles and avian models" **Oxford University Press**, 30-58

Waters WR, Palmer MV, Bannantine JP, Greenwald R, Esfandiari J, Andersen P, McNair J, Pollock JM, Lyashchenko KP (2005) "Antibody responses in reindeer (*Rangifer tarandus*) infected with *Mycobacterium bovis"* **Clin Diag Lab Immun** 12, 727-735

Wayne LG, Kubica GP (1986) "Genus Mycobacterium Lehmann and Neumann" *in* Sneath PHA, Mair NS, Sharpe ME, Holt JG "Bergey's Manual of Systematic Bacteriology" **Williams & Wilkins Baltimore MD** 2, 1436-1457

Wedekind C, Folstad I (1994) "Adaptive or non-adaptive immunosuppression by sex hormones?" **Animal Nature** 143, 936-938

Westneat DF, Birkhead TR (1998) "Alternative hypothesis linking the immune system and mate choice for good genes" **Proc R Soc Lond B** 265, 1065-1073

Williams ES (1987) "Bovine tuberculosis in deer: what to look for and what to do" **Deer** 7, 143

Whipple D, Palmer M (2000) "Re-emergence of tuberculosis in animals in the United States" i*n* Brown C, Bolin C "Emerging diseases of animals" **ASM Press Washington**, 281- 299

Zekarias B, Huurne A, Landman W, Rebel J, Pol J, Gruys E (2002) "Immunological basis of differences in disease resistance in the chicken" **Veterinary Resource** 33, 109-125

ANNEX

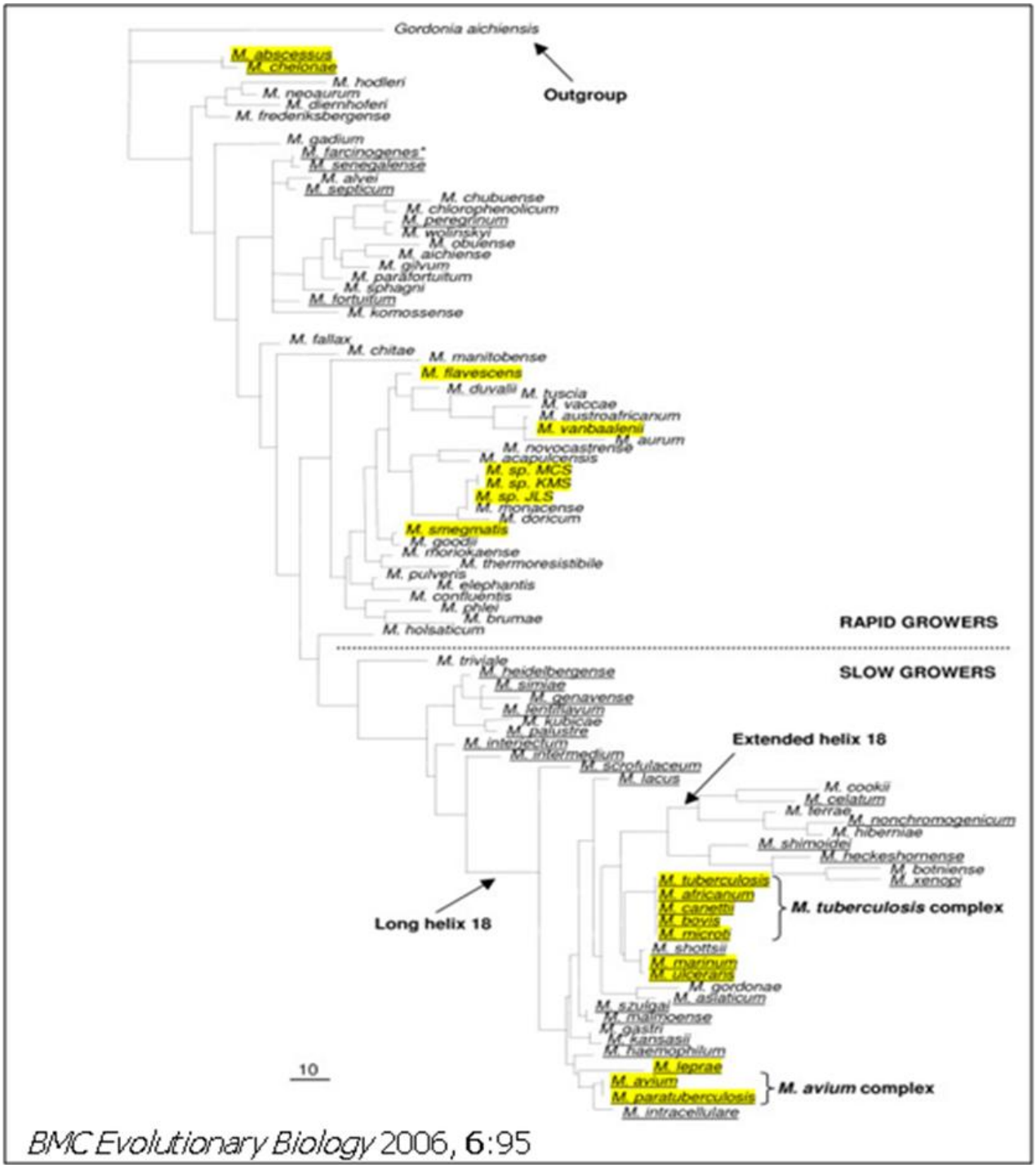
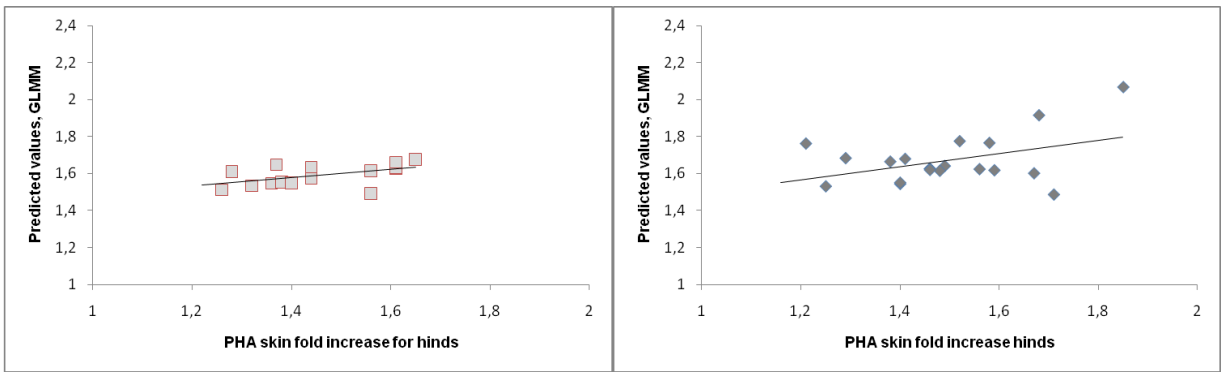
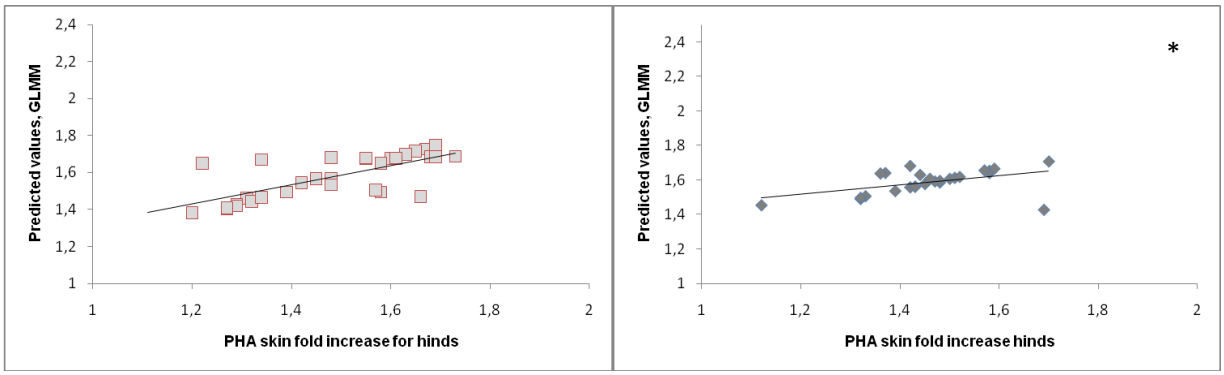


Figure VIII: Phylogenetic tree of genus *Mycobacterium*. The division between fast and slow-growing species is indicated by a dotted line. The members of the *M. tuberculosis* complex and the *M. avium* complex are indicated. Underlined species are considered pathogens (Source: BMC Evolutionary Biology. 6:95.).

CATEGORY 1



CATEGORY 2



CATEGORY 3

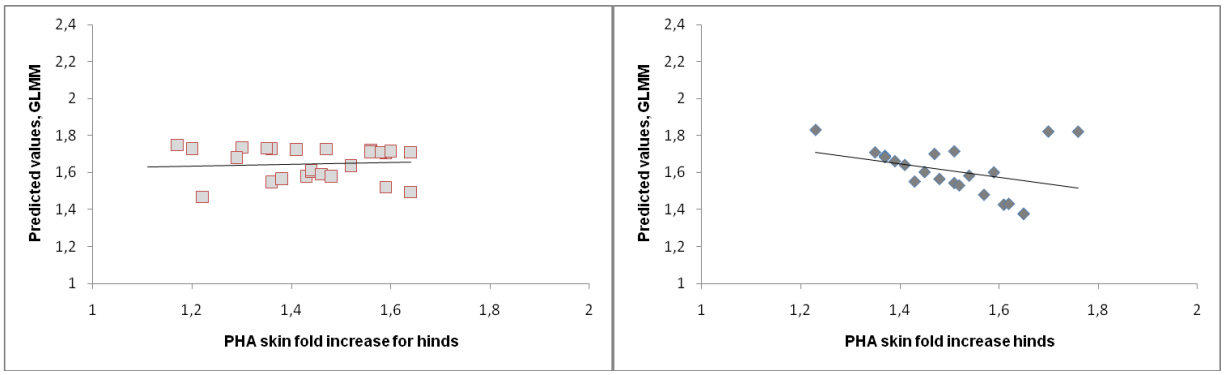
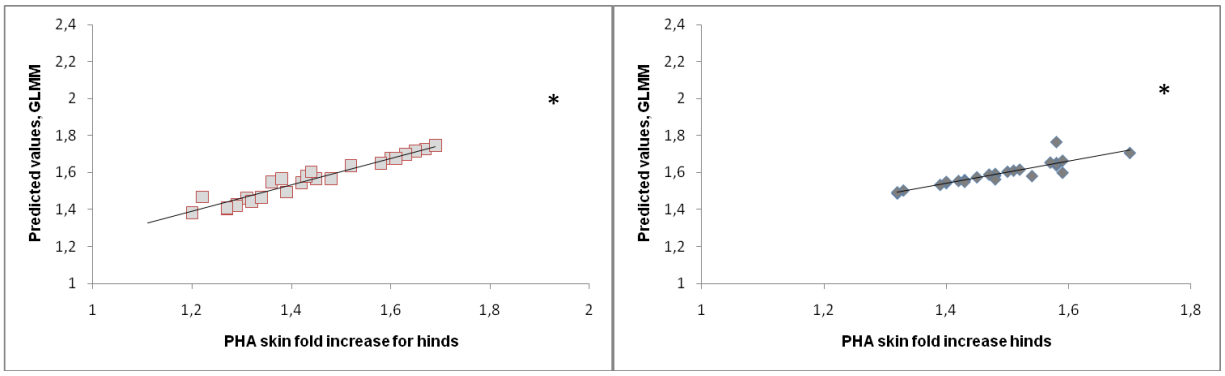
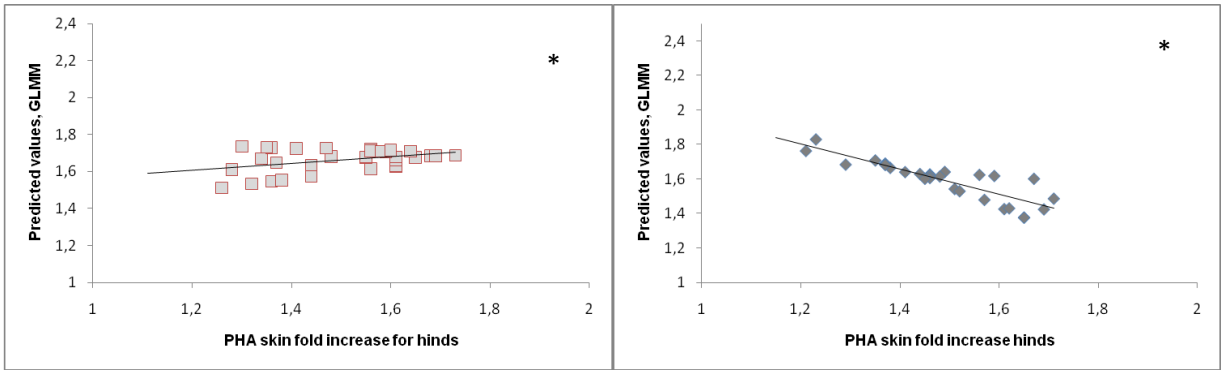


Figure IX: Regression between the predicted values of the GLMM and PHA skin fold increase of the hinds, in function to the three categories of PHA skin fold increase of the stags (1, 2 and 3, respectively; male calves in graphs on the left and female calves on the right). * means statistically significant differences.

CATEGORY 1



CATEGORY 2



CATEGORY 3

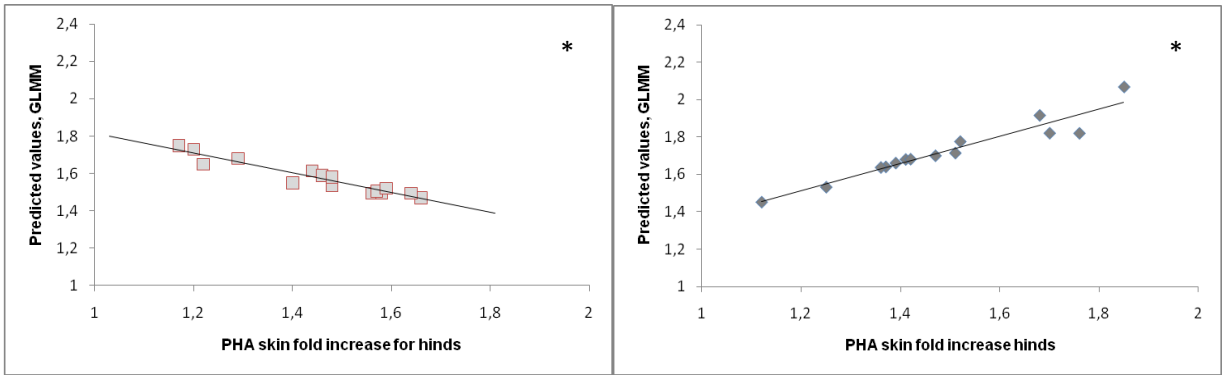


Figure X: Regression between the predicted values of the GLMM and PHA skin fold increase of the hinds, in function to the three age categories of the hinds (1, 2 and 3, respectively; males in graphs on the left and females on the right). * means statistically significant differences.