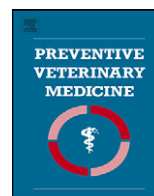




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Factors affecting red deer skin test responsiveness to bovine and avian tuberculin and to phytohaemagglutinin

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ABSTRACT

We studied the effect of management on the responsiveness of red deer (*Cervus elaphus*) to skin testing with mycobacterial and non-mycobacterial antigens. We hypothesized that individuals from populations of the same species under different management conditions would have a different immune responsiveness. Deer sampled in this study included 1041 adult animals from 6 Spanish farms and 111 adult wild deer. We injected four sites of the neck with 0.1 ml bovine purified protein derivative (PPD), 0.1 ml avian PPD, 0.1 ml negative control PBS and 0.1 ml of Phytohaemagglutinin (PHA, containing 250 µg) as positive control, and measured the skin fold increase at time 72 h. Bovine PPD reactors were identified in 5 of 6 farms and among wild deer. Apparent prevalence among wild deer (18.9%) was not significantly higher than among farmed deer (14.5%). Avian PPD reactors were found among all 7 study populations, but apparent prevalence was lower among wild deer (<1%) than among farmed deer (12.6%; $p < 0.001$). Deer management (farmed versus wild) was identified as a key factor affecting deer skin fold thickness increase in response both to mycobacterial (bPPD and aPPD) and non-mycobacterial antigens (PHA). The differences occurred in the same sense, regardless of some interactions; farmed deer showing higher values. The PHA skin fold increase was not affected by the PPD skin test results. We propose that using PHA as a positive control may help in the interpretation of between-population differences in tuberculin responses.

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1. Introduction

Deer farming is a growing activity in Spain. Most farms are devoted to producing red deer (*Cervus elaphus*) for restocking rather than producing venison or velvet. Thus, increasing numbers of deer are released yearly from the farms to the field (usually to fenced estates) for hunting purposes. In addition, over 10,000 wild deer are captured and translocated yearly between hunting estates in Spain (Soriquer et al., 1998). In this context, testing of deer prior to translocation in order to avoid sanitary risks becomes of

paramount importance (European Food Safety Authority, 2008).

Mycobacterial diseases, mainly bovine tuberculosis (bTB) caused by *Mycobacterium bovis* and closely related mycobacteria of the *Mycobacterium tuberculosis* complex, and paratuberculosis (PTB, Johne's disease) caused by *Mycobacterium avium* subspecies *paratuberculosis*, are among the most important health issues in deer farming (Riemann et al., 1979; Chiodini and Vankruiningen, 1983; Clifton-Hadley and Wilesmith, 1991; Griffin and Buchan, 1994; Mackintosh et al., 2004). In Spain, bTB is highly prevalent among wild deer, with 15% of red deer (*C. 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

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red deer (Gortázar et al., 2008). The prevalence of paratuberculosis among Spanish deer is less well known, but a preliminary serosurvey revealed 30% prevalence of antibodies against PPA3 in red deer (Reyes-García et al., 2008), and clinical cases have occasionally been reported in wild fallow deer (*Dama dama*) (Marco et al., 2002; Álvarez et al., 2005) and in farmed red deer (the authors, unpublished data).

Skin-testing with purified mycobacterial protein derived antigens (PPDs) is still the standard test for tuberculosis in man and domestic animals (OIE, 2002). In farmed red deer, studies carried out in New Zealand established 82–86% sensitivity and 46–76% specificity of the comparative skin test (Griffin et al., 1991; Corrin et al., 1993; Norden et al., 1996). No data are available for Spain, since TB testing of farmed or translocated deer is not yet compulsory.

The phytohaemagglutinin (PHA) (Sigma-Aldrich, Missouri, USA) skin test is an inexpensive and easy measurement of in-vivo cellular immune responses (Smits et al., 1999), that can be used in combination with TB skin testing, for example in order to identify anergic individuals, since animals that are immunosuppressed and therefore non-responsive (or less responsive) to PHA (non-specific response) may be also unable to elicit a specific response to mycobacterial antigens (Fernández-de-Mera et al., 2008). PHA, a lectin from *Phaseolus vulgaris*, is a mitogen. 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 (PBS), 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 previous studies, we reported the optimal time and dose for studying the skin fold thickness increase in response to PHA in red deer, and

showed that there was a significant effect of sex but not of age on the increase in skin fold thickness; males tended to have greater increases than females after correcting for other confounding variables. We also evidenced a positive relationship between PHA-responsiveness and body condition (Fernández-de-Mera et al., 2006, 2008).

The aim of this study was highlighting the effect of management on the responsiveness of red deer to skin testing with mycobacterial and non-mycobacterial antigens. We hypothesized that individuals of populations of the same species under different management conditions (captive versus wild; different farms) would have different immune reactivity. We suggest that using PHA as a positive control may help in the interpretation of between-population differences in tuberculin responses.

2. Material and methods

2.1. Sample size, study sites and characteristics of the deer

Deer sampled in this study included 1041 adult animals from 6 Spanish farms and 111 adult wild deer captured by the National Parks Agency in south central Spain. Farmed deer were sampled throughout the period comprised between 2002 and 2007 and wild deer between 2003 and 2005 (Table 1).

Spanish deer farms have a semi-intensive management scheme, with pasture-rotation and year-round food supplementation. These farmed deer are used to human presence and are handled at least twice a year, including physical immobilisation for measurement, sampling, and administration of antiparasitic drugs. In contrast, wild deer captured alive by the National Parks Agency are usually handled only twice in their lives: the first time to take them from the capture corral to the quarantine facility (in or close to the natural area), and a second time when they are sold and translocated. No supplementary feeding takes

Table 1

Deer sampled in this study ($n = 1041$ animals from 6 Spanish farms, and $n = 111$ adult wild deer captured by the National Parks Agency in south central Spain).

Site	Type	Sampling period	n	Deer origin and management	Information on mycobacterial diseases
1	Farm	November 2005	48	Diverse geographic origin, including central Europe.	2 clinical PTB cases confirmed by culture and PCR
2	Farm	February 2004	110	Iberian red deer. Limited introductions from the wild.	One case of subclinical <i>M. avium</i> confirmed by culture and PCR
3	Farm	August 2005	510	Iberian red deer. Limited introductions from the wild.	<i>M. bovis</i> is tested yearly and reactors slaughtered. No diagnosis
4	Farm	September 2002 August 2005	255	Iberian red deer. Limited introductions from the wild.	2 clinical <i>M. bovis</i> cases and several clinical PTB cases, both diagnosed by culture and PCR
5	Farm	September 2007	67	Diverse geographic origin, including central Europe.	TB-compatible lesions and several <i>M. bovis</i> cases diagnosed by culture and PCR
6	Farm	February 2005	51	Deer from Scotland introduced for venison production.	2 clinical <i>M. avium avium</i> cases diagnosed by culture and PCR (González-Fernández et al., 2009)
7	Wild	June to September 2003 and 2005	111	Iberian red deer	TB-compatible lesions highly prevalent and several <i>M. bovis</i> cases confirmed by culture and PCR (Vicente et al., 2006)

place in the areas managed by the National Parks Agency, and deer densities are estimated between 10 and 30 deer per square kilometre (Vicente et al., 2007b).

2.2. Skin testing and sampling procedure

All deer were handled twice during the skin testing experiment, at time 0 h and 72 h. Deer were captured and then immobilised by physical restraint in a crash (hydraulic in the farm, mechanic in the field). Each animal was blinded with a piece of cloth adapted to the forehead with two elastic bands in order to reduce stress and handling risks. Time for handling in the crash was less than 10 min. At time 0, each animal was weighed, eartagged, aged by tooth eruption, and biometric data taken (body length and thorax perimeter). We also collected ectoparasites (mainly ticks), faecal samples and blood. Four areas of 3 cm × 3 cm were shaved at the side of the neck, and 3 times repeated measurements of skin fold thickness were taken at the 4 injection sites. Finally, the intradermal injection was carried out with 0.1 ml avian PPD (Cooper-Zeltia, Spain), 0.1 ml bovine PPD (Cooper-Zeltia, Spain), 0.1 ml negative control PBS and 0.1 ml positive control PHA (containing 250 µg PHA, diluted in PBS). The deer were kept in paddocks (farm) or quarantine facilities (natural areas), with daily observation and food and water provided *ad libitum*. At 72 h, each animal was immobilised again by physical restraint, identified by the eartag number, and the skin fold thickness at each injection site was measured again (3 repeats).

All use of animals in research was approved by Castilla-La Mancha University Animal Ethics Committee. 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 ≥ 2 mm 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 less than 0.5 mm to all 3 antigens were considered anergic animals. In this study, it was not possible to slaughter the positive TB reactors in order to assess the presence of lesions; therefore we must speak of “apparent reactors” or “apparent prevalence”.

2.3. Statistical analysis

We used General Linear Models (GLM) to test the factors affecting PHA, bPPD and aPPD skin fold increase as response variables, respectively. The GLM underlies most of the statistical analyses (e.g. ANOVA, ANCOVA, regression analysis; McCulloch et al., 2008) and allows us to summarize a wide variety of research outcomes. Using GLMs we can test hypotheses concerning the effects of a set of variables (factors and covariates) on a unique dependent variable.

We specified three different GLMs for bPPD, aPPD and PHA increase as dependent continuous variables; respectively. In the first GLM for bPPD skin fold increase (as dependent continuous variable), the model included sex and management type (farm or wild), whereas PBS, aPPD and PHA skin fold increase were included as covariates, respectively. *M. avium* skin testing (positive or negative as aforementioned) was considered as factor. A similar GLM was built for aPPD as dependent continuous variable, but interchanging aPPD and bPPD skin fold thickness increase as response variable and covariate, respectively. In this model, *M. bovis* skin testing (positive or negative as aforementioned) was considered as factor. Finally in the last GLM, PHA increase was considered the dependent variable (continuous), and we included sex, management type (categorical, farm or wild), *M. bovis* skin testing (categorical, positive or negative), and *M. avium* skin testing (categorical, positive or negative) as factors. PBS, aPPD and bPPD skinfold increases were included as covariates (continuous independent variables), respectively.

Spearman rank order correlations were used to check for correlations between reactions to different antigens. We used the Chi square test to compare prevalences between groups. We used the SPSS 14.0 software (SPSS Inc., 1999), *p*-value was set at < 0.05 .

3. Results

Table 2 shows the mean values of skin tests according to site and sex, whereas Table 3 shows the mean values according to sex and management type. In 8 deer, responses to all three agents were less than 0.5 mm. Six out of these were wild deer (5.4%), and only 2 were farmed ones ($\ll 1\%$; $\chi^2 = 32.7$, 1 d.f., $p < 0.001$).

A total of 93 stags (44.1%) and 57 hinds (6.1%) were considered positive reactors to bovine PPD. The difference in apparent prevalence between sexes was significant ($\chi^2 = 220$, 1 d.f., $p < 0.001$). These positive bovine PPD reactors were identified in 5 of 6 farms and among wild deer. Apparent prevalence among wild deer (18.9%) was not significantly higher than among farmed deer (14.5%; $\chi^2 = 1.5$, 1 d.f., $p > 0.05$). One farm (site number 5), where only males were tested, yielded 77.6% apparent prevalence of positive bovine reactors.

Twenty stags (9.5%) and 96 hinds (10.2%) were considered positive reactors to avian PPD ($\chi^2 = 6.1$, 1 d.f., $p < 0.05$). Avian PPD reactors were found among all 7 study populations, but apparent prevalence was significantly lower among wild deer ($< 1\%$) than among farmed deer (12.6%; $\chi^2 = 13.5$, 1 d.f., $p < 0.001$). Farm 1, a site with known clinical PTB cases, and farm 6, a site with known clinical avian TB cases, were the two sites with the highest percentage of aPPD skin test reactors; these clinical cases were diagnosed out of this study, observing the presence of compatible lesions at post-mortem examination and by histology, with culture and PCR confirmation (Table 1).

The outcomes of the GLMs for bPPD, aPPD and PHA skinfold increase, respectively, are shown in Table 4. Fig. 1 shows mean least square values for bPPD, aPPD and PHA skinfold increase after the GLMs. Concerning the model

Table 2

Mean values of skin fold increase according to sampling site and sex (m: males, f: females).

Site	Sex	n	bovis PPD				avium PPD				PHA		
			Mean (0.1 mm)	SD	Range	+ve reactors (n, %)	Mean (0.1 mm)	SD	Range	+ve reactors (n, %)	Mean (0.1 mm)	SD	Range
1	m	0											
	f	48	8.8	7	0–26	0	36.2	18	10–79	17, 35.4	30.3	9	11–56
2	m	51	23.7	34	0–208	18, 35.3	26.7	32	0–167	6, 11.8	64.5	32	8–153
	f	59	7.1	9	0–37	1, 1.7	15.5	16	0–71	4, 6.8	42.2	21	5–93
3	m	32	14.8	17	0–74	6, 18.7	23	19	0–63	3, 9.4	39	20	4–82
	f	478	11.2	11	0–62	24, 5.0	22.8	15	0–78	25, 5.2	61.6	27	3–304
4	m	0											
	f	255	11.1	15	0–102	26, 10.2	17.9	17	0–91	23, 9.0	26.1	18	0–132
5	m	67	69.3	38	0–170	52, 77.6	37.5	35	0–148	7, 10.5	12.9	22	0–98
	f	0											
6	m	3	8	12	0–22	0	66.3	12	53–75	3, 100	47	11	39–59
	f	48	5.5	11	0–56	1, 2.1	23.3	20	0–73	12, 25	43.3	20	0–85
7*	m	58	16.2	26	0–89	16, 27.6	5.8	8	0–31	1, 1.7	25.1	18	0–77
	f	53	6	11	0–51	5, 9.4	4.4	6	0–34	0	19.6	12	1–48
All farms	m	153	41.7	41	0–208	77, 50.3	32	31	0–167	19, 12.4	37	33	0–153
	f	888	10.7	12	0–102	52, 5.86	21.7	16	0–91	93, 10.5	47.1	28	0–304
Tot	m	211	34.9	39	0–208	93, 44.1	25	29	0–167	20, 9.5	33.8	30	0–153
	f	941	10.4	12	0–102	57, 6.1	20.8	16	0–91	96, 10.2	45.6	28	0–304
		1152	14.7	22	0–208	150, 13	21.4	20	0–167	116, 10.1	43.2	29	0–304

* National Parks Agency.

243 fitted for the skin fold increase to bPPD, the outcome
 244 showed that the effects of management and sex were
 245 mediated by their interaction, so that between-sex differ-
 246 ences were evidenced only in farmed deer, whereas wild
 247 animals presented similar responses across sexes (Fig. 1a).
 248 Skinfold increase to bPPD positively related to aPPD and PHA
 249 responses, respectively. The relationship with PHA was
 250 more marked in females ($r_s = 0.12$, $n = 941$, $p < 0.001$) than
 251 in males (significant sex by PHA skin fold increase
 252 interaction, $r_s = -0.19$, $n = 211$, $p < 0.01$). Also, there existed
 253 a statistically significant positive relationship between
 254 bPPD and PHA responses in wild deer, but not in farms
 255 (Fig. 2, significant management by PHA skin fold increase
 256 interaction).

257 Results of the GLM for aPPD showed that management
 258 statistically affected the aPPD response since farmed deer
 259 displayed higher values (Fig. 1b). As previously found, skin
 260 fold increase to aPPD positively related to bPPD. The
 261 relationship with PHA was more marked in females
 262 ($r_s = 0.14$, $n = 941$, $p < 0.001$) than in males (significant
 263 sex by PHA skin fold increase interaction, $r_s = 0.13$, $n = 211$,
 264 $p = 0.05$).

265 Finally, regarding the outcomes of the model on PHA
 266 response, sex (higher values in males) and management
 267 (higher values in farms) statistically related to the skin fold
 268 increase at the PHA injection site, although both effects
 269 were mediated by a significant sex by management
 270 interaction term. This interaction evidenced that
 271 between-sex differences existed only among farmed deer,
 272 whereas wild animals presented similar responses across
 273 sexes (Fig. 1c). Also, there was an impact of management
 274 on bPPD responsiveness, and as aforementioned, there was

275 a statistically significant positive relationship between
 276 bPPD and PHA responses in wild deer, but not in farms
 277 (Fig. 1a and c).

278 The skin fold increase to the injection of the negative
 279 control positively related to PHA response. The farm with
 280 the highest proportion of bovine reactors (number 5)
 281 showed the lowest response to PHA among all the study
 282 sites.

4. Discussion

283
 284 Different species of wild animals including ungulates
 285 such as the red deer have been identified as reservoirs for
 286 bovine tuberculosis and are thought to be responsible for
 287 the failure of eradication programs in cattle throughout
 288 Europe (Caffrey, 1994; Hunter, 1996). Thus, especially in
 289 areas such as central Spain where wild ungulates are
 290 exploited as game species in highly managed environ-
 291 ments, tuberculosis skin-testing is becoming an impor-
 292 tant tool for bTB control (Lloydwebb et al., 1995; Griffin
 293 et al., 2004; Cousins and Florisson, 2005; Palmer et al.,
 294 2006).

295 However, deer skin testing is not yet compulsory
 296 throughout Spain, and standardised procedures are
 297 urgently needed. This study provides useful information
 298 to help designing proper specificity and sensitivity studies,
 299 since the data showed that different factors or conditions
 300 could impact on skin-testing responsiveness in a definite
 301 population or group of animals. Data presented in this
 302 paper belong to different farms and wildlife management
 303 situations and were collected opportunistically in different
 304 seasons.

Table 3
Mean values (\pm standard error) of skin fold increase according to management type (farmed versus wild) and sex (m: males, f: females).

	bPPD (0.1 mm)			aPPD (0.1 mm)			PHA (0.1 mm)			PBS (0.1 mm)		
	m	f	Tot	m	f	Tot	m	f	Tot	m	f	Tot
Farm (n = 1041)	41.69 \pm 1.54	10.66 \pm 0.64	15.22 \pm 0.68	32.03 \pm 1.49	21.73 \pm 0.62	23.24 \pm 0.58	37.04 \pm 2.27	47.12 \pm 0.95	45.60 \pm 0.89	6.37 \pm 0.71	2.57 \pm 0.39	3.44 \pm 0.35
Wild (n = 111)	17.20 \pm 2.50	6.27 \pm 2.62	11.98 \pm 2.07	6.48 \pm 2.42	4.54 \pm 2.58	5.57 \pm 1.79	25.36 \pm 3.68	19.63 \pm 3.89	22.65 \pm 2.69	0.70 \pm 1.03	1.50 \pm 1.09	1.08 \pm 0.76
Total (n = 1152)	34.95 \pm 1.35	10.41 \pm 0.64	14.91 \pm 0.64	25.01 \pm 1.33	20.80 \pm 0.63	21.57 \pm 0.57	33.83 \pm 1.98	45.56 \pm 0.95	43.37 \pm 0.86	4.53 \pm 0.60	2.45 \pm 0.37	3.03 \pm 0.32

Table 4

GLM test statistics of for skin fold increase of PHA, bovine PPD and avian PPD, respectively.

	SS	F [*]	Estimate	p
bPPD				
Management	11504.5	38.7	10.17	<0.01
Sex	36666.6	123.2	12.89	<0.01
aPPD	54966.0	184.7	0.38	<0.01
PHA	1292.4	4.3	0.12	0.04
Management \times Sex	11236.5	37.8	5.83	<0.01
Management \times PHA	6906.2	23.2	-0.27	<0.01
Sex \times PHA	10610.6	35.7	-0.14	<0.01
aPPD				
Management	3373.1	11.7	5.57	<0.01
bPPD	53439.9	184.7	0.37	<0.01
Sex	1498.0	5.2	-2.74	0.02
Sex \times PHA	2690.1	9.3	0.07	<0.01
PHA				
Management**	1738.6	3.9	7.31	0.04
Sex	2254.2	5.1	5.31	0.02
PBS	3418.3	7.7	0.72	0.01
Management \times Sex	1945.3	4.3	2.96	0.04
Management \times bPPD	1732.9	3.9	-0.24	0.04

* F statistic value.

** Management type (farm or wild).

While the effect of age (less relevant) and sex (more relevant) on (PHA) skin testing of deer is known (Fernández-de-Mera et al., 2008), no information is available on the effect of the season on skin testing. Hence, results need to be interpreted with care as regards the skin test reactor prevalences presented in this study. Nonetheless, the percentage of positive bPPD skin test reactors is within the range of prevalence figures given for TB-compatible lesions at necropsy in Spain (e.g. Vicente et al., 2006).

Only limited information exists on paratuberculosis in Spanish deer (Marco et al., 2002; Álvarez et al., 2005; Reyes-García et al., 2008), and avian TB has been reported on very few occasions from wild Spanish deer (González-Fernández et al., 2009). However, avian reactors were detected in all farms tested in this survey, suggesting a widespread and locally important exposure of farmed deer to mycobacteria of the *M. avium* complex, which includes *M. avium paratuberculosis*. This should be taken into account, considering that most farmed deer are sold for restocking of natural areas.

The most consistent result in the present study is that deer management (farmed versus wild) was identified in all three models as a key factor affecting deer skin fold thickness increase in response both to mycobacterial (bPPD and aPPD) and non-mycobacterial antigens (PHA). The differences occurred in the same sense, regardless of some interactions; farmed deer showing higher values (Fig. 1 and Table 3). These findings may relate to three groups of factors. Firstly, differences in condition could be reflected in immunological responsiveness. It is of general knowledge that factors such as nutritional condition and stress may affect immune capacity (Moller et al., 1998; Coop and Kyriazakis, 1999, 2001; Lochmiller and Deereberg, 2000), and both may have been involved in the variability found in this study. Red deer body condition is increased as a consequence of intensive management (which involved artificial provision of high quality food) in

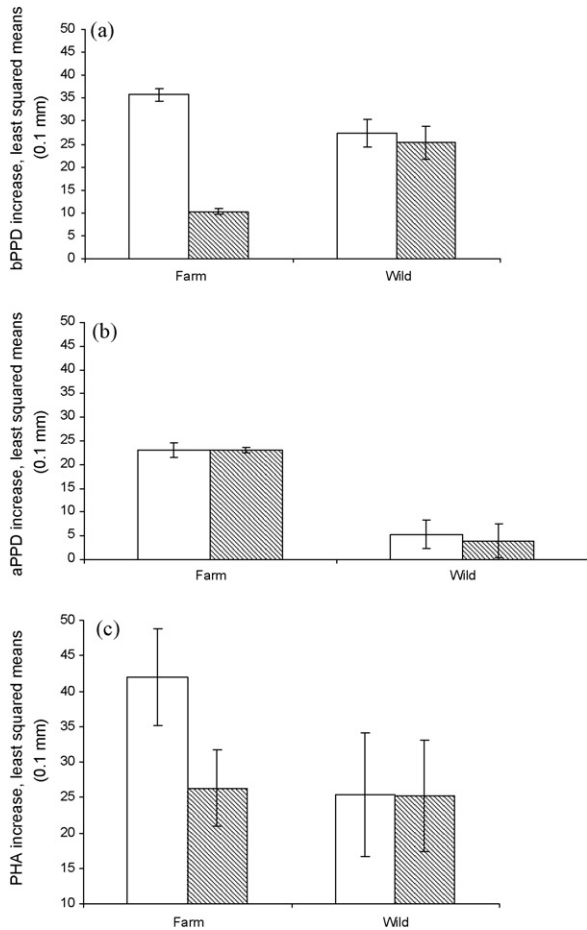


Fig. 1. Differences between wild and farmed red deer in the increase of skinfold thickness after inoculation of bovine PPD (a), avian PPD (b), and PHA (c) in relation to sex (males in white columns and females in the shaded ones). Values are shown as least square means, and therefore are corrected for other variables of the statistical models.

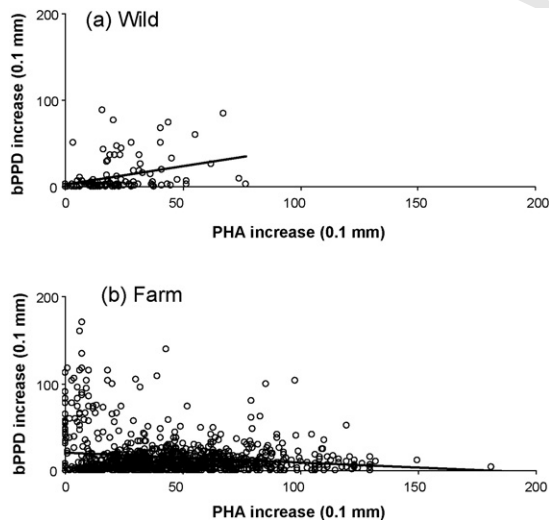


Fig. 2. Relationship between skin-fold thickness after inoculation of PHA and bovine PPD in relation to management (a: wild; b: farm).

our study area (Vicente et al., 2007a). Body condition is closely related to the T-cell mediated immune response as measured by the PHA skin test in birds (e.g. Soler et al., 2003), which is consistent with our own previous findings evidencing a positive relationship between PHA-responsiveness and body condition (Fernández-de-Mera et al., 2006). Stress is also a strong immune suppressor and may alter cellular immune reaction, especially in wild animals when submitted to the stress of handling, thus rendering the skin-testing less reliable (e.g. Tella et al., 2001). In this context, a standard positive control such as PHA would help to correct results of PPD-skin-testing for the immune responsiveness of a particular population.

The second group of factors may relate to differences in the prevalence of mycobacterial infections (or previous contact to environmental or pathogenic mycobacteria), and the subsequent effects on the skin responsiveness against PPDs. We did not evidence any effect of skin testing positivity (neither to aPPD nor to bPPD) on PHA response, which suggests that immune status (measured as PHA response) would determine the responsiveness against PPDs, rather than vice versa. Therefore, the statistical associations between responses to different antigens (especially bPPD and non-mycobacterial PHA) suggest that the skin fold increase to mycobacterial antigens may be affected by the general immunological status of the host. Interestingly, we found that there was an impact of management on bPPD skin fold increase in that there was a statistically positive relationship between bPPD and PHA responses in wild deer, but not in farms (Fig. 2). This is expected to occur if immune system function of the wild animals is limited by resource availability (Hines et al., 2007). This means that under natural conditions in the wild, immune function is at least partly determined by body condition and that, as stated by other authors in birds (Alonso-Álvarez and Tella, 2001), this is readily reflected by variations in PHA responsiveness. In contrast, in farmed deer, animals displayed a high PHA skinfold increase, which could indicate a good body condition and hence more investment in the immune system. Therefore, we hypothesise that once red deer are at a high plane of nutrition and immune capacity, bPPD response would be less determined by (or related to) the body condition mediated immune capacity.

Finally, a third group of factors underlying the differences between farmed and wild deer could relate to red deer life-history (Vicente et al., 2007b). The effect of sex and its interactions in determining the responses to different antigens was very relevant, independently of the mycobacteria infection status. We found, as previously described by Fernández-de-Mera et al. (2008), that sex differences in skinfold increase of the mitogen PHA were marked in farmed deer (in favour to males), but not in the wild. A similar pattern was found for bPPD. As reported by Fernández-de-Mera et al. (2008) most of the females were pregnant when tested. This is an energetically costly period (Clutton-Brock et al., 1984), which can be reflected in a lower activation of the immune function as resources are allocated to reproduction. For instance, some infections are known to occur more frequently during pregnancy (Ramaswamy et al., 2007). We speculate that farmed stags

are probably less affected by resource shortage during the rut than wild stags, which may lead to a better immunological status. Nevertheless we must be cautious because sample size in males was low and future studies are required to account for seasonal effects.

PHA skin fold increase was not affected by the PPD skin test results. This suggests that this measure is highly independent of the mycobacterial infection status of the animals, and underlines its possible use as a positive control of general responsiveness to skin testing. In humans, lack of skin induration to intradermal injection of PPD (PPD anergy) is observed in a subset of patients with active tuberculosis (Delgado et al., 2002). In order to detect anergic individuals, immunocompromised patients are screened not only with tuberculin, but also with *Candida* and mumps antigen (Smirnoff et al., 1998). In domestic animals, the existence of anergic individuals (false negatives in skin-testing) is one of the major limits to the success of bTB eradication programs (Barlow et al., 1997), but to the authors' knowledge no alternative antigens are commonly used as controls. We used PHA as a positive control, expecting to identify those animals that, being negative to both PPDs, do also not respond to the non-mycobacterial antigen PHA. This use of PHA in combination with comparative skin tests needs further research, but it is interesting to observe that several animals with no response to PHA or to the PPD were detected, and that the farm with the poorest PHA response was the one with the highest percentage of bPPD reactors.

In this study, parameters for body condition, size and physiology were not assessed. However, it may be expected that the ad-lib fed farmed deer were in better general condition than wild deer captured in summer, during the dry season. The differences between captive and free-living deer in response to PHA and PPD injection showed that the two populations differed significantly in their immune reaction to these antigens. This may have implications for the interpretation of skin test results: "False-positive" reactions in tuberculosis skin-testing are frequently observed in relation to contact with environmental mycobacteria (Lloydwebb et al., 1995). But in addition to this source of false positives, deer populations with a good response to the non specific PHA antigen may have more false positive reactors to the mycobacterial PPDs, while undernourished or otherwise immunocompromised deer may have more false negative skin-test reactors than healthy deer populations.

5. Conclusion

The results of the present study suggest that a positive control reflecting the immune responsiveness of the tested individual could be useful in tuberculin skin-testing, even in domestic animals, as reactivity may vary between populations. It also underlines that skin-testing parameters standardised in domestic animals, including farmed deer, should not be directly used in wild animals, as handling stress or other factors may considerably alter their immune responsiveness. In this study PHA has proven to be a useful positive control antigen for skin-testing in our model species, the red deer, that (a) allows to

establish cut-off points for the immune responsiveness of a given population, (b) helps detecting anergic reactors suffering from chronic tuberculosis and/or impaired immune function, and (c) helps interpreting non-specific reactions to PPDs that may result from exposure of the animals to unspecific nonpathogenic mycobacteria. Finally, the intradermal reaction to PHA could be a useful management tool for the identification of "risk populations" with view to the amelioration of body condition and thus immunocompetence.

Uncited references

Hawley et al. (2007) and Vicente et al. (2003).

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References

- Alonso-Álvarez, C., Tella, J.L., 2001. Effects of experimental food restriction and body-mass changes on the avian T-cell-mediated immune response. *Can. J. Zool.* 79, 101–105.
- Álvarez, J., De Juan, L., Briones, V., Romero, B., Aranaz, A., Fernández-Garayzabal, J.F., Mateos, A., 2005. A *Mycobacterium avium* subspecies paratuberculosis in fallow deer and wild boar in Spain. *Vet. Rec.* 156, 212–213.
- Barlow, N.D., Kean, J.M., Hickling, G., 1997. A simulation model for the spread of bovine tuberculosis within New Zealand cattle herds. *Prev. Vet. Med.* 32, 57–75.
- Caffrey, J.P., 1994. Status of bovine tuberculosis eradication programmes in Europe. *Vet. Microbiol.* 40, 1–4.
- Chiodini, R.J., Vankruiningen, H.J., 1983. Eastern white-tailed deer as a reservoir of ruminant paratuberculosis. *J. Am. Vet. Med. Assoc.* 182, 168–169.
- Clifton-Hadley, R.S., Wilesmith, J.W., 1991. Tuberculosis in Deer—A review. *Vet. Rec.* 129, 5–12.
- Clutton-Brock, T.H., Albon, S.P., Guinness, F.E., 1984. Fitness cost of gestation and lactation in wild mammals. *Nature* 337, 260–262.
- Coop, R.L., Kyriazakis, I., 1999. Nutrition-parasite interaction. *Vet. Parasitol.* 84, 187–204.
- Coop, R.L., Kyriazakis, I., 2001. Influence of host nutrition on the development and consequences of nematode parasitism in ruminants. *Trends Parasitol.* 17, 325–330.
- Corrin, K.C., Carter, C.E., Kissling, R.C., de Lisle, G.W., 1993. An evaluation of the comparative tuberculin skin test for detecting tuberculosis in farmed red deer. *New Zeal. Vet. J.* 41, 12–20.
- Cousins, D.V., Florisson, N., 2005. A review of tests available for use in the diagnosis of tuberculosis in non-bovine species. *Rev. Sci. Tech. OIE* 24, 1039–1059.
- Delgado, J.C., Tsai, E.Y., Thim, S., 2002. Antigen-specific and persistent tuberculin anergy in a cohort of pulmonary tuberculosis patients from rural Cambodia. *Proc. Natl. Acad. Sci. U.S.A.* 99, 7576–7581.
- Ekkel, E.D., Kuypers, A.H., Counotte, G.H.M., Tielen, M.J.M., 1995. The phytohemagglutinin [PHA] skin-test as an indicator of stress-induced changes in immune reactivity in pigs. *Vet. 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.
- Fernández-de-Mera, I.G., Höfle, U., Vicente, J., García, A., Rodríguez, O., Gortázar, C., 2006. Optimal dose and timing in phytohaemagglutinin skin-testing of deer. *New Zeal. Vet. J.* 54, 357–359.
- Fernández-de-Mera, I.G., 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 Zeal. Vet. J.* 56, 71–73.
- González-Fernández, J., Fernández-de-Mera, I.G., Reyes, L.E., Ferreras, M.C., Pérez, V., Gortázar, C., Fuertes, M., García-Marín, J.F., 2009. Comparison of three immunological diagnostic tests for the detection of avian tuberculosis in naturally infected red deer (*Cervus elaphus*). *J. Vet. Diagn. Invest.* 21, 102–107.
- Gortázar, C., Torres, M.J., Vicente, J., Acevedo, P., Reglero, M., de la Fuente, J., Negro, J.J., Aznar-Martín, J., 2008. Bovine tuberculosis in Doñana Biosphere Reserve: the role of wild ungulates as disease reservoirs in the last Iberian lynx strongholds. (<http://dx.doi.org/10.1371/journal.pone.0002776>). *PLoS ONE* 23;3(7):e2776.
- Griffin, J.F.T., Nagai, S., Buchan, G.S., 1991. Tuberculosis in domesticated red deer—Comparison of purified protein derivative and the specific protein MPB70 for in-vitro diagnosis research. *Res. Vet. Sci.* 50, 279–285.
- Griffin, J.F.T., Buchan, G.S., 1994. Etiology, pathogenesis and diagnosis of *Mycobacterium bovis* in deer. *Vet. Microbiol.* 40, 193–205.
- Griffin, J.F.T., Chinn, D.N., Rodgers, C.R., 2004. Diagnostic strategies and outcomes on three New Zealand deer farms with severe outbreaks of bovine tuberculosis. *Tuberculosis* 84, 293–302.
- Hawley, D.M., Jennelle, C.S., Sydenstricker, K.V., Dhondt, A.A., 2007. Pathogen resistance and immunocompetence covary with social status in house finches (*Carpodacus mexicanus*). *Funct. Ecol.* 21, 520–527.
- Hernández, A., Yager, J.A., Wilkie, B.N., Leslie, K.E., Mallard, B.A., 2005. Evaluation of bovine cutaneous delayed-type hypersensitivity (DTH) to various test antigens and a mitogen using several adjuvants. *Vet. Immunol. Immunopathol.* 104, 45–58.
- Hines, A.M., Ezenwa, V.O., Cross, P., 2007. Effects of supplemental feeding on gastrointestinal parasite infection in elk (*Cervus elaphus*): Preliminary observations. *Vet. Parasitol.* 148, 350–355.
- Hunter, D.L., 1996. Tuberculosis in free-ranging, semi-free-ranging and captive cervids. *Rev. Sci. Tech. OIE* 15, 171–181.
- Kelley, K.W., Greenfield, R.E., Evermann, J.F., Parish, S.M., Perryman, L.E., 1982. Delayed-type hypersensitivity, contact sensitivity, and phytohemagglutinin skin-test responses of heat- and cold-stressed calves. *Am. J. Vet. Res.* 43, 775–779.
- Kollias Jr., G.V., Thoen, C.O., Fowler, M.E., 1982. Evaluation of comparative cervical tuberculin skin testing in cervids naturally exposed to mycobacteria. *J. Am. Vet. Med. Assoc.* 181, 1257–1262.
- Lloydwebb, E.C., Campbell, P.H., Witt, D.J., 1995. The specificity of the single cervical intradermal tuberculosis test in a population of tasmanian fallow deer putatively free of bovine tuberculosis preventive. *Vet. Prev. Med.* 21, 347–353.
- Lochmiller, R.L., Deereberg, C., 2000. Trade-offs in evolutionary immunology: just what is the cost of immunity? *Oikos* 88, 87–98.
- McCulloch, C.E., Searle, S.R., Neuhaus, J.M., 2008. Generalized, Linear, and Mixed Models, second ed. Wiley, New York.
- Mackintosh, C.G., de Lisle, G.W., Collins, D.M., Griffin, J.F.T., 2004. Mycobacterial diseases of deer. *New Zeal. Vet. J.* 52, 163–174.
- Marco, I., Ruiz, M., Juste, R., Garrido, J.M., Lavín, S., 2002. Paratuberculosis in free-ranging fallow deer in Spain. *J. Wildl. Dis.* 38, 629–632.
- Moller, A.P., Christe, P., Eritzoe, J., Mavarez, J., 1998. Condition, disease and immune defence. *Oikos* 83, 301–306.
- Norden, D., Essey, M.A., Meyer, R., 1996. Evaluation of tuberculin skin test in Cervidae. In: *Veterinary Services, Centers for Epidemiology and Animal Health (Eds.)*. CADIA Technical report. USDA, APHIS, Fort Collins, CO, pp. 2–96.
- Office Internationale des Epizooties, 2002. Manual of standards Diagnostic Tests and Vaccines. In: *World Organisation for Animal Health. Part 2 Section 2.3 Chapter 2.3.3* (<http://www.oie.int/>).
- Palmer, M.V., Waters, W.R., Thacker, T.C., Stoffregen, W.C., Thomsen, B.V., 2006. Experimentally induced infection of reindeer (*Rangifer tarandus*) with *Mycobacterium bovis*. *J. Vet. Diagn. Invest.* 18, 52–60.
- Ramaswamy, V., Cresence, V.M., Rejitha, J.S., Lekshmi, M.U., Dharsana, K.S., Prasad, S.P., Vijila, H.M., 2007. *Listeria*—review of epidemiology and pathogenesis. *J. Microbiol. Immunol. Infect.* 40, 4–13.
- Reyes-García, R., Pérez-de-la-Lastra, J.M., Vicente, J., Ruiz-Fons, F., Garrido, J.M., Gortázar, C., 2008. Large scale ELISA testing of Spanish red deer for paratuberculosis. *Vet. Immunol. Immunopathol.* 124, 75–81.
- Riemann, H., Zaman, M.R., Ruppner, R., Aalund, O., Jorgensen, J.B., Worsaae, H., Behymer, D., 1979. Paratuberculosis in cattle and free-living exotic deer. *J. Am. Vet. Med. Assoc.* 174, 841–843.
- Smirnoff, M., Patt, C., Seckler, B., Adler, J.J., 1998. Tuberculin and anergy skin testing of patients receiving long-term hemodialysis. *Chest* 113, 25–27.
- Smits, J.E., Bortolotti, G.R., Tella, J.L., 1999. Simplifying the phytohaemagglutinin skin-testing technique in studies of avian immunocompetence. *Funct. Ecol.* 13, 567–572.
- Soler, J.J., De Neve, L., Pérez-Contreras, T., Soler, M., Sorci, G., 2003. Trade-off between immunocompetence and growth in magpies: An experimental study. *Proc. R. Soc. Lond., Ser. B: Biol. Sci.* 270, 241–248.
- Soriguer, R.C., Márquez, F.J., Pérez, J.M., 1998. Las translocaciones (introducciones y reintroducciones) de especies cinegéticas y sus efectos medioambientales. *Galemys* 10, 19–35.
- Tella, J.L., Forero, M.G., Bertellotti, M., Donazar, J.A., Blanco, G., Ceballos, O., 2001. Offspring body condition and immunocompetence are negatively affected by high breeding densities in a colonial seabird: A multiscale approach. *Proc. R. Soc. Lond., Ser. B: Biol. Sci.* 268, 1455–1461.
- Vicente, J., Höfle, U., Gortázar, C., 2003. Análisis in vivo para el control sanitario de ciervos (*Cervus elaphus*) en el Parque Nacional de Cabañeros, Lugar Nuevo y Selladores - Contadero. Unpublished report to the Nacional Parks Agency, Madrid, Spain.
- Vicente, J., Höfle, U., Garrido, J.M., Fernández-de-Mera, I.G., Juste, R., Barral, M., Gortázar, C., 2006. Wild boar and red deer display high prevalences of tuberculosis-like lesions in Spain. *Vet. Res.* 37, 1–11.
- Vicente, J., Höfle, U., Fernández-de-Mera, I.G., Gortázar, C., 2007a. The importance of parasite life history and host density in predicting the impact of infections in red deer. *Oecologia* 152, 655–664.
- Vicente, J., Pérez-Rodríguez, L., Gortázar, C., 2007b. Sex, age, spleen size and kidney fat of red deer relative to infection intensities of the lungworm *Elaphostrongylus cervi*. *Naturwiss* 94, 581–587.