

## The effects of sex and age on phytohaemagglutinin skin-testing of deer

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### Abstract

**AIM:** To determine if there are sex- or age-related differences in the increase in skinfold thickness in response to the mitogen phytohaemagglutinin (PHA) in red deer.

**METHODS:** One dose of 250 µg of PHA was injected intradermally in the right side of the neck, and phosphate buffered saline (PBS) was injected at a second site as a control, in 110 (51 males and 59 females) captive Iberian red deer (*Cervus elaphus hispanicus*), ranging in age from 21 months to ≥5 years. Skinfold thicknesses were measured immediately before and 72 h following injection.

**RESULTS:** There was a significant effect of gender on the average increase in skinfold thickness; males had greater increases (8.8 (SEM 0.57) mm) than females (4.23 (SEM 0.39) mm) after correcting for other confounding variables. No age-related differences were evident, but differences between sexes were more marked with increasing age.

**CONCLUSIONS AND CLINICAL RELEVANCE:** Effects of gender, probably due to differences in energetic and reproductive constraints in red deer, should be taken into account when interpreting skinfold-test data, both in ecology and in the control of tuberculosis (Tb). Males tend to have a thicker skin than females, so skinfold increase relative to the thickness of the skin, rather than skinfold increase *per se*, should be used as a more appropriate measure of skinfold increase. This may also have clinical relevance in the interpretation of tuberculin skin testing.

**KEY WORDS:** Cellular immunity, *Cervus elaphus*, mammalian immune response, wildlife bioindicators, phytohaemagglutinin skin test

### Introduction

Measurement of immune reactivity is an important tool in defining how animals cope with environmental demands (Hessing et al 1995), and is also valuable as a complement to diagnostic tests based on the immune response. The PHA skin test is an inexpensive and easy measurement of cellular immune responses *in vivo* (Smits et al 1999). Phytohaemagglutinin, a lectin from *Phaseolus vulgaris*, causes agglutination of erythrocytes, and growth, division, and non-specific activation of T-cells. 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 PBS, or the increase in swell-

ing measured after injection of PHA (Kelley et al 1982; Ekkel et al 1995; Smits et al 1999; Hernández et al 2005).

In a previous study (Fernández-de-Mera et al 2006), we demonstrated *in vivo* a dose-dependent response in red deer hinds to PHA injected intradermally, and a variation in response over time after injection, that required the establishment of a fixed time for measurement of response. Reasonable reliability was obtained in deer using both a fixed protocol and the same observer for the measurement of skin thickness. The results showed that a dose of 250 µg gave distinct and long-lasting responses. A time interval of 72 h was proposed for reading PHA skin-testing of deer, since tuberculin skin tests are also read at 72 h post-injection (Waters et al 2004), and both techniques would most likely be used concurrently, particularly under field conditions, with the purpose of detecting possible false-negatives in the tuberculin skin test, since PHA facilitates the detection of anergic animals.

It is also necessary to determine whether differences in reliability might depend on the sex and age of the individuals tested. Hence, in this study we used a large sample of farmed Iberian red deer of different age groups and both sexes, to test the effects of these factors on the skin-test response to PHA.

### Materials and methods

#### Study animals

A group of 110 healthy Iberian red deer (51 males and 59 females), comprising 45 21-month-old individuals (yearlings; 24 males and 21 females), 13 3-year-old adults (11 males and two females), 19 4-year-old adults (11 males and eight females), and 33 ≥5-year-old adults (five males and 28 females), was kept on the experimental farm of the University of Castilla-La Mancha, Albacete, Spain. The deer were accustomed to handling, and showed no outward signs of behavioural stress. They were individually identified with an ear tag and transponder. All deer were kept in open-air enclosures during the course of the study, which was conducted in February 2003. At this time of the year, late winter in Spain, the majority of stags drop their antlers, and most hinds are pregnant. The farm is free of Tb, as no animals have tested positive to the comparative cervical skin test (CCT) and there have been no clinical cases or post-mortem evidence of the disease.

The deer were immobilised in an hydraulic crush for no more than 5 min each, for injection and measuring skin thicknesses. Two areas of skin on the right side of the neck, each measuring 3 x 3 cm, were shaved with an electric shaver (Moser Avalon 1290; Moser, Valencia, Spain) prior to intradermal injection. On Day

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CCT	Comparative cervical skin test
PBS	Phosphate buffered saline
PHA	Phytohaemagglutinin
Tb	Tuberculosis

1, all animals were weighed to an accuracy of 50 g using a digital balance (KC 300 S; Mettler-Toledo SAE, Silla, Spain). All use of animals in this research was approved by the Research Ethics Commission of Castilla-La Mancha University Animal Ethics Committee, Albacete, Spain.

### Skin-testing of cell-mediated immunity with PHA

The animals were injected intradermally at one of two sites with 0.1 ml of 250 µg of PHA (Sigma-Aldrich, Missouri, USA), and with 0.1 ml PBS at the other site, as a control. One-ml syringes fitted with a 25-G ½-inch needle were used. Immediately prior to and 72 h after injection, skinfold thicknesses were measured twice, to the nearest 0.1 mm, using a digital calliper (Mitutoyo, Cardiff, UK), by the same person. The reliability of double measures was previously assessed by Fernández-de-Mera et al (2006).

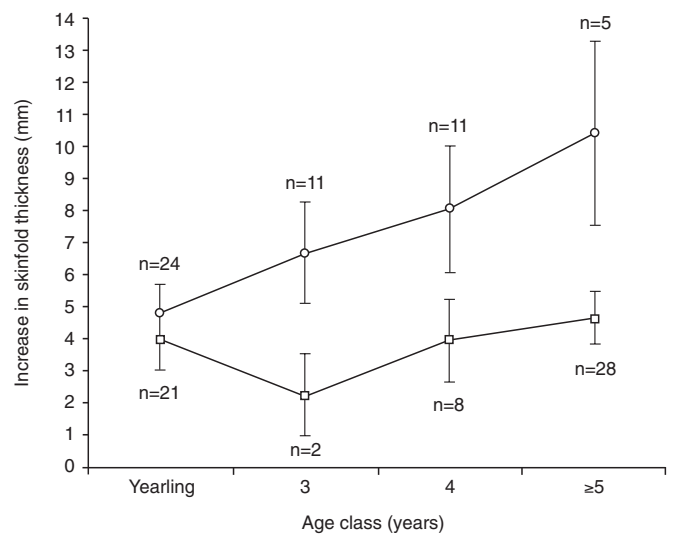
### Statistical analysis

The effect of sex and age on increases in skinfold thickness (response variable), while controlling for bodyweight, was tested using a GLM (Glimmix procedure, SAS v9; SAS Institute Inc, Cary NC, USA). Sex and age (four levels: 21-month-old yearlings; and 3-, 4-, and ≥5 year-old adults), and the two-way interaction, were explanatory categorical factors; live bodyweight was included as an explanatory continuous variable. Skin thickness prior to injection and total body length (to the nearest 0.1 cm) were also included in the model. Finally, the increase in skin thickness measured at the negative control site was also included in the model, to control for any possible increase as a consequence of mechanical irritation.

The residuals of the regression of increases in skinfold thickness on bodyweight across sex-by-age classes are presented, in order to visualise the increase in skinfold thickness once it was corrected for the initial thickness of the skinfold. The residuals are the difference between the observed value of the variable and the value suggested by the regression model, and provide a measure of the relative deviation (positive or negative, in relative, rather than absolute terms) of the dependent variable (increase in skinfold thickness) with respect to explanatory factors (initial skinfold thickness). The standardised residuals, i.e. the residual divided by the SE, are presented. Statistical uncertainty was expressed through 95% CI and SEM.

## Results

Detectable increases in skinfold thickness were evident in all deer injected with PHA and no aberrant reactions were seen. Figure 1 shows the average increase in skinfold thickness in response to PHA as a function of sex and age. There were differences between sexes ( $F=4.75$ ,  $p=0.03$ ,  $R^2$  for the model = 0.41); males had greater mean skinfold increases (8.8 (SEM 0.57, range 0.85–15.30) mm) than females (4.23 (SEM 0.39, range 0.50–9.35) mm) after correcting for other explanatory variables. Figure 2 shows the average standardised residuals of the regression of increase in skinfold thickness on bodyweight across sex-by-age classes. No age-related differences were evident ( $F=0.95$ ,  $p=0.4$ ), but differences between sexes were more marked with increasing age (sex-by-age interaction:  $F=2.62$ ,  $p=0.05$ ; Figures 1 and 2). Increases in skinfold thickness after injection of PHA was not affected by initial skin thickness ( $F=1.33$ ,  $p=0.3$ ), increase in skinfold thickness due to PBS ( $F=0.37$ ,  $p=0.5$ ), bodyweight ( $F=1.06$ ,  $p=0.3$ ), or body length ( $F=2.24$ ,  $p=0.13$ ).



**Figure 1.** Average increase in skinfold thickness ( $\pm$  95% CI) in the neck of Iberian red deer 72 h after injection of 250 µg of phytohaemagglutinin, in relation to sex (○=males; □=females) and age class.

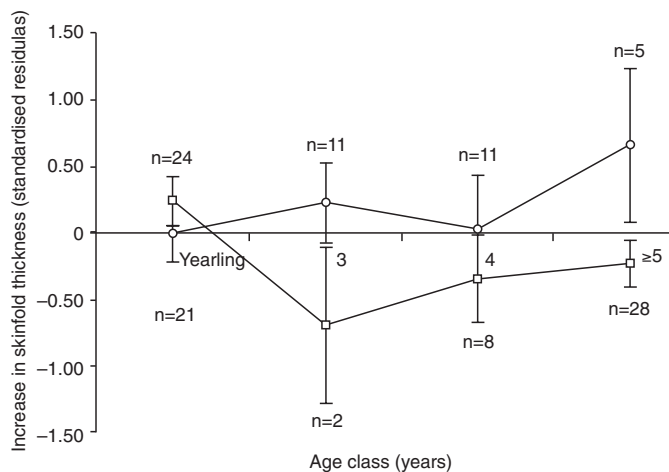
## Discussion

In this study, sex-by-age variations in the increase of the skinfold thickness after intradermal injection of the mitogen PHA was described for the first time in red deer. Reference values for a dosage and time interval for measurement are provided that previously were tested and that are in accordance with the logistics of performing CCT on red deer. The reported increases in skinfold thickness of individuals in good health, as the deer in this study were, are useful baseline data for development of a complementary diagnostic tool for specific tests against diseases such as Tb.

The use of PHA in conjunction with CCT may facilitate the detection of anergic individuals and therefore could improve the reliability of diagnostic tests for Tb in red deer *in vivo*. Animals that are immunosuppressed and therefore non-responsive (or less responsive) to PHA (non-specific response) may be unable to also elicit a specific response to Tb antigens. Future research should examine this relationship, as the number of red deer translocated in Spain is high, and undiagnosed Tb could result in uncontrollable spread of the disease to new areas (Vicente et al 2006).

Tuberculosis is an important disease in red deer in Spain (Vicente et al 2007a) as well as many other countries where red deer are farmed (de Lisle et al 2001). In Spain, testing wild Iberian red deer from areas with endemic Tb demonstrated a correlation between the skin-test responses to PHA and to bovine tuberculin (data not shown). Our results suggested that the response to tuberculin could vary with response to PHA, according to the immunological status of the deer; but more research is needed in this area. Additionally, the PHA test could be useful in the future for monitoring the general health of populations of red deer, and for ecological studies quantifying immune response. To the best of our knowledge, no adverse effects of PHA on mammals have been reported, and in our experience there is no interaction between PHA and tuberculin antigens during skin-testing of deer.

Males had greater increases in skinfold thickness than females, after controlling for the effects of weight, body size and skin thickness. Thus, the effect of gender should be taken into account when comparing the PHA test with reference values. Cellular immune responses may differ between males and females in red deer as a



**Figure 2.** Average standardised residuals of the regression of the increase in skinfold thickness ( $\pm$  95% CI) on bodyweight in Iberian red deer, 72 h after injection of 250  $\mu$ g of phytohaemagglutinin, in relation to sex (○=males; □=females) and age class.

result of differences in reproductive effort and energy expenditure (Clutton-Brock et al 1982; Vicente et al 2007b).

Our study was conducted in February, at which time the males were recovering from the rutting period (September–October), an energetically demanding time (Johns et al 1984) even for animals in captivity. Therefore, immune function capacity was probably increasing. In the case of the females, although pregnancy rates were unknown, most were expected to be pregnant, which is also an energetically costly period (Clutton-Brock et al 1984). This may be reflected in lower activation of immune function as resources are allocated to reproduction. The fact that gestation is especially costly in primiparous hinds (Landete-Castillejos et al 2004) could explain the decrease evidenced in the 3-year-old females (Figure 2), and the significant sex-by-age interaction, supporting the hypothesis that early reproductive investment in young hinds which are still growing may negatively affect cellular immune responses. These results are in agreement with recent data on age-by-sex variation on the size of the spleen, an indirect measure of immune capacity (Corbin et al 2008), in wild Iberian red deer (Vicente et al 2007b). The apparent absence of gender differences in non-reproductive individuals (yearlings) is also consistent with this hypothesis. Nevertheless, caution must be exercised in interpreting our data, due to the small number of 3-year old animals. Future research is needed in order to determine seasonal differences in PHA testing of red deer, which may vary between sexes.

In conclusion, gender and age should be taken into account when measuring PHA skin test responsiveness in deer. Our findings also suggest that, as skinfold thickness varies among individuals (e.g. male skinfold tend to be thicker than female), increase in skinfold thickness relative to thickness of skin, rather than increased thickness of skinfold *per se*, should be considered a more appropriate measure of 'true skinfold increase'. These factors could also apply to the interpretation of skin reactions after injection of tuberculin. Future research should focus on this issue, from immunological, biometrical and histopathological aspects.

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