What does testosterone do for red deer males?
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Testosterone has been proposed to have a dual effect, enhancing sexual traits while depressing parasite resistance in males. Here, we test this hypothesis in red deer, examining males from captive populations during the whole annual cycle and males from natural populations during the breeding season. We first explored the effects of body size, age and sampling date on testosterone to avoid confounding effects. Our results show that in captive populations seasonal changes in testosterone levels were mirrored by changes in testes size, and that during the rut there was a strong correlation between both. In natural populations, males with higher testosterone levels had larger testes, improved sperm quality, smaller burr diameter, stronger antlers, higher haematocrit levels, and increased nematode parasite load. By contrast, no significant relationship was found between testosterone and spleen size or tick parasite load. We conclude that testosterone (i) improves males’ reproductive investment and physical stamina, (ii) improves antler strength but reduces burr diameter, and (iii) imposes a cost in terms of depressed parasite resistance.

**Keywords:** immunocompetence handicap hypothesis; testosterone; antler; haematocrit; testes; sperm quality

1. INTRODUCTION
The immunocompetence handicap hypothesis (Folstad & Karter 1992) proposes that testosterone (T) has a dual role: on the one hand, it enhances the expression of sexual traits and, on the other hand, it depresses the immune system. The hypothesis suggests that this duality leads to honest signalling because if low quality males produce larger amounts of T than they can afford, they would still enhance their sexual traits, but would experience a cost in terms of immunocompetence.

Experimental studies have shown that males to which exogenous T is given have poorer immunocompetence and more parasites than non-treated males (Saino et al. 1995; Salvador et al. 1996; Hughes & Randolph 2001); that selection for low immune levels results in higher T levels and larger ornaments (Verhulst et al. 1999); and that testosterone limits the production of B and T-lymphocytes (Jacobson & Ansari 2004). However, observational studies carried out in natural populations have not detected negative effects of T upon immunity/parasites (Weatherhead et al. 1993; Ditchkoff et al. 2001a). Thus, although experimental increases in T result in depressed immunocompetence, observational studies have found no evidence of a trade-off (for a review see Roberts et al. 2004).

Among seasonal breeders, the timing of the events throughout the reproductive cycle is driven by hormonal changes which, in turn, are influenced by environmental (e.g. photoperiod, rainfall, food availability) or social cues. Among male mammals, seasonal changes in T are associated with key events in the reproductive cycle. The main effect upon male reproduction is that T levels peak just before the breeding season starts, triggering spermatogenesis in males. Seasonal changes in T levels are associated with changes in testes size (Lincoln 1971; Neal et al. 1977). However, little is known about inter-individual differences in natural populations, i.e. whether males with higher T levels have larger testes and enhanced sperm production. In addition, the possibility that T may not only influence sperm production, but also influence sperm quality, has not been explored.

The timing of key events in the deer antler cycle is also known to be regulated by T levels, since changes in T levels throughout the year are associated with the annual casting of antlers, pedicle growth, antler hardening and velvet shedding (Lincoln 1992; Price & Allen 2004). However, T levels are minimal during antler growth and the widely accepted assumption that T levels directly determine growth rates and antler size has been challenged. Experimental studies show that T may, in fact, inhibit antler growth rates among red deer (Suttie et al. 1994; Li et al. 1999) and studies in natural populations have found conflicting results, reporting an effect of T in subadult white tailed deer that disappeared when including the effect of insulin-like growth factor I (IGF1) (Ditchkoff et al. 2001a,b). An untested possibility...
is that T could influence antler size through its effects on the timing of antler casting which would determine the period of time available for antler growth. Males with higher levels of T may cast their antlers earlier and as a consequence will have more time to grow their new antlers before the breeding season starts.

However, size is not the only antler component that could have a positive effect on reproductive success. During intrasexual combats males clash their antlers repeatedly and may also interlock the antlers and push against each other with great force. Antler strength is hence crucial in withstanding the tension generated by both males without breaking, and in transferring the forces generated by the hind quarters to the opponent. In fact, antler bone has been shown to be up to three times more resistant than ordinary bone (Zioupos et al. 1996), suggesting that this trait might have been under strong selective pressures. Stags with broken antlers have been shown to lose more combats and drop in the hierarchy (Espmark 1964; Lincoln et al. 1970). Testosterone has been shown to have a strengthening effect on the bone tissue (Beld et al. 2000; Prevrhal et al. 2007) and T injections have an effect on bone density (Wang et al. 1996; Leder et al. 2003). However, the possibility that T levels have a positive effect on antler strength enabling them to support the physical pressure exerted upon antlers during agonistic encounters has not been tested before.

Testosterone could also have a major influence on male reproductive investment by enhancing physical strength and endurance, since males engage in energetically demanding activities (fighting and mating) during the breeding season which result in losses in physical condition. This possibility is supported by evidence indicating that androgens increase the haematocrit (Gardener & Besa 1983; Salvador et al. 1996), thus improving the capacity for oxygen transport to the muscles. The effects of testosterone on the number of red blood cells have not been considered before, despite the effect it could have on the ability of males to maintain high levels of physical activity during the breeding season. In this study, haematocrit levels are used as surrogate of physical stamina.

We studied a captive population of Iberian red deer (Cervus elaphus hispanicus) sampled throughout the year to explore the effect of age and season on T levels, the association between T and testes size and the association between T levels and antler casting date. In addition, we studied natural populations of Iberian red deer during the breeding season to test the relationship between T and (i) sperm production and sperm quality, (ii) antler size and strength (iii) spleen size and parasite load, and (iv) haematocrit levels, accounting for the effects of population, age and body condition.

The Iberian red deer is a good model species to test for these associations because (i) it develops particularly exaggerated sexual characters (antlers) that are grown every year, (ii) investment in T and sperm production is limited to the reproductive season facilitating the comparison between males, and (iii) the mating season is energetically costly for males, since they move large distances in search for females, defend harems/territories, engage in fights with other males and mate with females, all while having low feeding rates. Thus, any potential trade-offs are likely to arise during the mating season.

2. MATERIAL AND METHODS

The study sample included captive and free-ranging Iberian red deer stags (C. e. hispanicus). All animal manipulations were performed in accordance with the Spanish Animal Protection Regulation, RD1201/2005, which conforms to European Union Regulation 2003/65/CE.

(a) Captive population data collection

Stags from the captive population (n = 18) were housed under semi-natural conditions and maintained under natural day length conditions at the University of Castilla-La Mancha (Albacete, Spain, latitude 38° 57′ N). Samples were collected from November 1999 to September 2002.

Testes size in live stags was measured by the circumference of the scrotum and blood was collected for T analyses and processed and analysed as described below. To examine the relationship between T and age (treated as a factor including 2 to 6-year-old males), only individuals sampled in September were used. Antler casting date was also recorded in the captive population.

(b) Natural population data collection

The wild group was composed of 128 culled stags that were sampled during the breeding season (October–December) from 1999 to 2001, in seven different natural populations from the south of Spain (latitude range, 38° 10′ N–39° 30′ N; longitude range, 4° 40′ W–3° 5′ W). In this region, the breeding season begins in September–October and lasts for three months as shown by calving dates in Spring (Sanz & Rodriguez 1993; Garcia et al. 2002), suggesting that it lasts for longer than in other populations from northern Europe, probably because differences between seasons are less marked, so females need not give birth at the same time. Populations were sampled in October (A), November (B, C, D and E) and December (F and G). The temporal effect of sampling populations as the reproductive season progresses is coded as a variable from day 1 (first population sampled, A), onwards (B = 15, C = 20, D = 24, E = 27, F = 34 and G = 50). The average number of stags per population was 18 (s.d. = 3; range 13–21).

(c) Age

In order to determine age (range 3–12), lower jaw incisor teeth were collected in the field and tooth sections conducted (Klevezal & Klinenbergen 1967). Only five populations had values recorded for age and T. As many individuals could not be aged, fitting age as a default covariate in the models would drastically reduce the degrees of freedom and the statistical power of the analyses, so age was only fitted in the models to be aged, fitting age as a default covariate in the models would drastically reduce the degrees of freedom and the statistical power of the analyses, so age was only fitted in the models with T when there was a close to or significant effect of age on the dependent variable considered (p ≤ 0.1). Age had a significant effect on testes volume (r = 0.24, p = 0.029, n = 81), on the three antler size measures (r = 0.65, p < 0.001; r = 0.62, p < 0.001; r = 0.39, p < 0.001, n = 78–79), on the nematode load (r = 0.24, p = 0.035, n = 80), tick number (r = 0.44, p = 0.002, n = 46), an effect close to significance on the haematocrit (r = −0.18, p = 0.1, n = 60) and a non-significant effect on the percentage of normal sperm, percentage of sperm with intact acrosomes (p > 0.1 and p = 0.7, respectively, for n = 82), sperm swimming velocity (p = 0.35, n = 48) and spleen mass (p = 0.2, n = 59).

Regarding the effects of age on T levels, an ANOVA model pooling all stags from natural populations together showed that there was no effect because all males were 3 years or older.
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testes and epididymides were removed from the scrotum, the
either (reported and in our data such an association was not found
No effect of body condition upon sperm quality has been
model testing the effects on the antler size (Malo
transported at 20–21°C and (ii) testes were removed (in the scrotum) in the field and
calculated by dividing the weight of kidney
beginning of the tail (sacrococcygeal articulation) following
measured body length of the culled stags on the field and used it as an index of body size. Body length was recorded in centimetres as the distance from the tip of the muzzle to
the field and conditioned by dividing the weight of kidney
weight remaining after trimming by kidney weight. Based on
previous research showing the effects of body condition
on a number of traits, body condition was included as a
covariate in the models testing the effects of T levels on the
testes size (Schulte-Hostedde & Millar 2004), on parasite load
(nematodes and ticks; Zuk & Stoehr 2002; Vicente et al. 2007)
and on immunocompetence (spleen size; Lochmiller et al.
1993; Deerenberg et al. 1997), but it was not included in the
model testing the effects on the antler size (Malo et al. 2005b).
No effect of body condition upon sperm quality has been
reported and in our data such an association was not found either
(p>0.11).

Testes size
We used testes size as an indicator of sperm production. Both
testes were removed (in the scrotum) in the field and
transported at 20–21°C to the laboratory. In the laboratory, the
testes and epididymides were removed from the scrotum, the
three diameters (length, height and width) of both testes were
measured with a calliper to the nearest 0.1 mm, radii were
calculated (\( r_h, r_n \) and \( r_w \)) and its volume (in cm\(^3\)) determined
with the formula of the ellipsoid \( 4/3\pi r_h r_n r_w \). Testes volume determines the size of the scrotum, so it was included as a
covariate when testing the effects of T levels on the number of
ticks per scrotum. In order to test in one single step the effect of
T on sperm production (testes size) and the effects of sperm
depletion which are also known to be an important
determinant of testes size (Preston et al. 2001), we included
sampling date as a covariate instead of population origin.

Sperm quality
Time elapsed between animal death and sperm analyses
ranged from 3 to 6 hours, an adequate time interval for
evaluating sperm parameters, as it has been shown that within
12 hours of the death of a stag sperm quality remains
unaltered (Garde et al. 1998). Spermatozoa were recovered from the cauda epididymides and evaluated following the
methodology described in previous studies (Malo et al. 2005a,b, 2006). The following variables were included: the proportion of morphologically normal spermatozoa (percentage of normal sperm), the proportion of spermatozoa with an
intact acrosome (% NAK), and sperm swimming velocity
(straight-line velocity, VSL). Sperm swimming velocity measures were only available for stags from three populations.

Body size
Body length was measured with a calliper in centimetres as the distance from the tip of the muzzle to
the beginning of the tail (sacrococcygeal articulation) following
the dorsal vertebrae. This variable was included in the models
to control for allometry in testes, antler and spleen size.

Body condition
Kidney fat index was used as a reliable indicator of body
condition (Riney 1955; Van Rooyen 1993). It was recorded in
the field and calculated by dividing the weight of kidney
fat remaining after trimming by kidney weight. Based on
previous research showing the effects of body condition
on a number of traits, body condition was included as a
covariate in the models testing the effects of T levels on the
testes size (Schulte-Hostedde & Millar 2004), on parasite load
(nematodes and ticks; Zuk & Stoehr 2002; Vicente et al. 2007)
and on immunocompetence (spleen size; Lochmiller et al.
1993; Deerenberg et al. 1997), but it was not included in the
model testing the effects on the antler size (Malo et al. 2005b).
No effect of body condition upon sperm quality has been
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(p>0.11).

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Antler size and strength
Three measurements were recorded: (i) antler length (AL),
length of the main beam from the base of the burr to the
farthermost tines on the crown (cm), (ii) antler diameter
(AD), measured at the burr (mm), and (iii) antler points
(AP), total number on both sides. The average of both sides of
the antlers was used for the analyses. However, males for
which only one antler was measured were also included in the
analyses to increase sample size (proportion of the individuals
with only one side of the antler measured: AL=8%,
AD=0%, AP=0.7%). The three measures were strongly
correlated between them before
(t=0.5–0.8, p<0.001, n=75) and after accounting for the effect of age (r=0.35–0.67, 0.003>p>0.001, n=75).

Given that males over 9 years old showed a decrease in
their T levels (figure 1a), and this decrease is a sign of ageing,
males from 3 to 9 years old were selected to test the effects of
T levels on antler size. In order to test whether there was an
effect of T levels on antler strength, we recorded the existence
of broken frontal or middle tines in either side of the antler.
This measure of antler strength was recorded as a binomial
variable (1–0).

Haematocrit
Blood was extracted with a vacutainer needle by cardiac
puncture on the culled stags. Samples were taken to the labora-
tory at 4°C, cells were separated from plasma by centri-
fugation and the haematocrit was assessed as a percentage.

Evaluation of parasite intensity
Parasitic larvae detached directly from the rectum during field
necropsy in order to quantify the faecal larval
excretion load of Elaphostrongylus cervi (Nematoda: Meta-
strongyloidea), these parasitic worms of the central nervous
system and skeletal muscles of red deer (Mason 1995; Lankester 2001), commonly called extra-pulmonary lung-
worms. Details of the methodology and parasitic cycle can be
found in previous studies (Vicente & Gortázar 2001).

Ecotoparasites
The number of ixodid ticks (Genera Hyalomma and
Rhipicephalus) on the scrotum was recorded in the laboratory
before testes dissection.

Evaluation of immunocompetence
We used spleen mass as our measure of immunocompetence as
(i) spleen tissue is involved both in humoral and cellular
immune responses, (ii) it has previously been shown to correlate
negatively with nematode parasite burden in the red deer
(Vicente et al. 2007; Corbin et al. 2008).

Testosterone
Blood was extracted from a vacutainer needle from a vein on
the captive deer or by cardiac puncture on the culled stags.
Samples were taken to the laboratory at 4°C, plasma fraction
was separated from cells by centrifugation, frozen at −80°C
and shipped to the Laboratoire de Dosages Hormonaux,
Institut National de la Recherche Agronomique (Tours,
France) for analyses. After thawing, T plasma concentrations
were assessed in duplicate by radioimmunoassay, with a test
sensitivity for T of 0.1 ng ml\(^{-1}\) (as described by

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were used to test the relationship between T levels and testes, antler size, haematocrit, spleen size and ticks in the natural population. To obtain the reduced models, a stepwise deletion of terms from full models was conducted. A logistic regression model was used to test the effect of T levels on the presence of broken tines in the antlers. Maximum-likelihood chi-square (ML-$\chi^2$) was used to test for differences between populations in the frequency of broken tines. A log-linear model was used to test the effect of T levels on nematode load (count data), including body condition and age as covariates. Nematode larvae count was fitted as a Poisson distribution (Wilson et al. 1996) and corrected for over dispersion adjusting the scale parameter, so that the ratio of the residual deviance and its degrees of freedom was equal to unity ( Crawley 1993).

To allow for maximum statistical power, age and/or population were only fitted in the models when their effect on the dependent trait in question had been previously ascertained. Population (up to seven levels, depending on data availability for each population) was included in order to account for confounding ecological factors. All analyses were performed in STATISTICA v. 6.0 computer package (StatSoft 2001).

### 3. RESULTS

Overall, there was no significant difference in T levels between the captive (mean $\pm$ s.e., $2.41 \pm 0.41$ ng ml$^{-1}$) and natural populations ($2.32 \pm 0.33$ ng ml$^{-1}$) of red deer during the reproductive season (independent samples t-test $= -0.04$, d.f. = 162, $p = 0.96$). Table S1 (see the electronic supplementary material) shows the descriptive statistics of the variables studied from the natural populations’ dataset.

#### (a) Age and seasonal effects on T levels of captive and natural deer populations

Among captive males, T levels vary significantly with sampling date ($F_{4,29} = 13.28$, $p < 0.001$, $r^2 = 0.65$) and age (2–6 years old; $F_{3,31} = 3.60$, $p = 0.016$, $r^2 = 0.12$). Regarding seasonality, T levels remain low until July, then begin to rise and reach a peak in September when the breeding season starts; thereafter, T levels start to decline (figure S1a in electronic supplementary material). During September (when T levels are high), 2-year-old males had significantly lower T levels than older males, but no difference was found between males aged 3–6 years old (figure S1b in electronic supplementary material).

Males from natural populations did not show either linear or nonlinear significant relationships between age (3–12) and T levels (figure 1a).

#### (b) Relationship between T and testes size among captive deer

When we consider all captive males sampled, changes in T levels and in testes size followed the same annual pattern: low levels during the first months of the year, a gradual increase starting around June, a drastic peak during September, and a gradual decline thereafter, with levels reaching low values again by January (figure 2). Within seasons, we tested the existence of association between T and testes size between males. Our results show that there was only a positive significant relationship in September, when T levels are at its maximum (September, $r = 0.60$, $p = 0.019$; January, March, April, July, November, $p > 0.30$). For September, a general linear mixed model

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**Figure 1.** (a) Nonlinear relationships between age (x) and antler length (AL = $21.454 + 12.016x - 0.587x^2$, $r = 0.68$, $p < 0.0001$, n = 79), and between age (x) and T levels (T levels (log) = $0.08 - 0.108x - 0.007x^2$, $r = 0.17$, $p = 0.4$, n = 64) in male red deer from natural populations (black circles and solid line) and captive males (open circles and solid line) (Hochereau-De-Reviers et al. 1990). The average of both measures was used in the analyses.

**Figure 1 (cont.)** (b) Relationship between testosterone and burr diameter for 4-year-old red deer stags from natural populations ($r = 0.55$, $p = 0.014$, $y = 54.878 - 18.266x$). (c) Nematode larvae count was fitted as a Poisson distribution (Wilson et al. 1996) and corrected for over dispersion adjusting the scale parameter, so that the ratio of the residual deviance and its degrees of freedom was equal to unity ( Crawley 1993).

**Figure 1 (cont.)** (k) Statistical analyses

Variables were transformed if required (log, square root or arcsine). A mixed model was used in the captive population data when testing the effects of T on testes size. Linear models...
showed a significant effect of T on testes size ($F_{1,35}=4.18, p=0.038$) after accounting for male effects as a random factor ($F_{18,35}=4.18, p=0.010$; global model $R^2=0.64$, estimated using fixed effects general linear model).

(c) Relationship between T and testes size in deer from natural populations

We fitted a linear model including testes volume as the dependent variable, T levels as the predictor, age, body length, body condition and day of sampling as the covariates. The result was a significant full model ($F_{5,47}=0.04, p<0.0001$, $r^2=0.43$) in which sampling date had a negative effect on testes size ($F_{1,47}=5.53, p=0.02$, $\beta=-0.27 \pm 0.11$), body size and T levels presented a positive effect upon testes size ($F_{1,47}=4.94, p=0.03$, $\beta=0.27 \pm 0.12$ and $F_{1,47}=8.39, p=0.006$, $\beta=0.34 \pm 0.12$, respectively), and age and body condition were not significant ($p=0.32$ and $p=0.54$). The reduced model excluding the non-significant variables did not show major differences ($F_{5,47}=14.18, p<0.0001$, $r^2=0.43$; sampling date: $F_{1,47}=6.94, p=0.01$, $\beta=-0.27 \pm 0.10$; T levels: $F_{1,47}=9.46, p=0.003$, $\beta=0.32 \pm 0.10$; body size: $F_{1,47}=4.94, p=0.03$, $\beta=0.41 \pm 0.10$).

(d) Relationship between T and sperm quality in deer from natural populations

To test for the relationship between T and sperm quality, we constructed three general linear models including percentage of normal sperm and on the percentage of sperm with intact acrosomes (table 1 for linear models and figure 3a,b, respectively, for correlations), but did not have an effect on sperm velocity (table 1).

(e) Relationship with antler casting date in captive deer

Data from two different years were used. Two regression models including antler casting date as the dependent variable and T levels as the predictor show that there is no detectable effect of T on antler casting date for the first year ($r=0.4, p=0.36, n=7, 1999–2000$) nor for the second ($r=-0.12, p=0.77, n=8, 2000–2001$).

(f) Relationship with antler size in natural populations

In order to test the relationship between T and antler size, males from 3 to 9 years old were selected. Three different ANOVAs showed that there was no difference between natural populations in the three antler size traits considered (AL, $F_{4,61}=2.02, p=0.10$; AD, $F_{4,61}=1.99, p=0.11$; AP, $F_{4,61}=1.90, p=0.12$). Three linear regressions were subsequently conducted to test the association between T and antler size, including age and body size as covariates (table 2). The three multiple regression models conducted show that, controlling for the effects of age and body size, T is negatively associated with the three antler size measures, reaching statistical significance only in the case of burr diameter (AD; table 2; figure 1b shows one age class to eliminate any potential effects of age) but not for antler length and number of points.

(g) Relationship with antler strength in natural populations

In order to test the relationship between T levels and antler strength we first confirmed that there was no significant difference in the frequency of broken tines between populations ($ML-\chi^2=4.75, d.f.=4, p=0.31$). A total of 44 stags had no broken tine and 14 had at least one broken tine (two had two broken tines). We conducted one logistic regression model including the presence of broken tines (0–1 as the dependent binomial variable (using the logit link function) and T levels, age and antler length as the independent variables. The resulting model was non-significant ($\chi^2(3)=6.41, p=0.09$), where T levels was significant (Wald’s $\chi^2=4.73, p=0.029$) but age and antler length were not ($p>0.3$). The reduced model after deleting non-significant terms was significant ($\chi^2(1)=6.07, p=0.01$) showing that increased T levels reduced the probability of having broken tines ($p$) in the antlers ($p=1/1+e^{(-0.20-2.41 \times \log \text{testosterone})}$; Wald’s $\chi^2=5.37, p=0.02$). This shows that age and antler length did not influence antler strength and were not confounding the association between T levels and antler strength. As figure 1c shows, stags with broken antlers had lower T levels than stags with unbroken antlers.

(b) Relationship with haematocrit

We tested the relationship between T and the haematocrit by constructing a linear model where haematocrit was included as the dependent variable, and where T levels and age were included as covariates and population as a factor (table 3; figure S2 in electronic supplementary material for a correlation). This analysis rendered a significant full model that accounted for half of the variance in the haematocrit, where T had a positive and significant association with the haematocrit ($F_{1,51}=11.2, p=0.0015$, $\beta=0.36 \pm 0.11$) but age did not ($F_{1,51}=0.45, p=0.50$), after accounting for the significant effect of population upon haematocrit ($F_{4,51}=7.80, p<0.0001$). The reduced model excluding age was equally significant and explanatory and, although slightly increasing the significance level of T levels and population, did not show a change in the slope ($\beta=0.37 \pm 0.10$).
Table 1. General linear models for the proportion of normal spermatozoa (Full model: \( r^2 = 0.21, F_{6,70} = 3.09, p = 0.009 \)), for the proportion of spermatozoa with intact acrosome (Full model: \( r^2 = 0.21, F_{6,70} = 3.16, p = 0.008 \)) and for sperm swimming VSL (Full model: \( r^2 = 0.04, F_{3,10} = 0.39, p = 0.76 \)). (Testosterone and proportion of normal sperm were log and arcsine transformed, respectively.)

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</table>

(i) **Relationship with spleen size and parasite load**

The linear model conducted including spleen size as the dependent variable and T levels, body length, body condition and population (categorical) as the independent variables rendered a full significant model (\( F_{7,33} = 7.04, p < 0.0001 \), \( r^2 = 0.63 \)) entirely due to the significant effect of population (\( F_{5,33} = 10.63, p < 0.0001 \)). There was no significant effect of T levels (\( F_{1,33} = 0.3, p = 0.59 \)) or the other two covariates (\( p > 0.34 \) in both cases).

The log-linear model conducted shows that there was a significant positive effect of T upon nematode load after accounting for the significant effect of population. Age and body condition remained as non-significant (table 4).

We further tested the relationship between T levels (as the independent variable) and number of ticks per scrotum (as the dependent variable) by means of a linear model including testes volume, body condition and age as covariates, and population as a categorical factor. The resulting model was not significant (\( F_{7,22} = 1.46, p = 0.23 \)), only age having an effect on tick number (\( F_{1,22} = 6.55, p = 0.02 \)), while the remaining three variables did not (\( p > 0.36 \)). A reduced model just retaining age and the variable of interest, T levels (\( F_{2,34} = 6.72, p = 0.003 \), \( r^2 = 0.28 \)), showed that older males have more ticks per scrotum (\( F_{1,34} = 11.61, p = 0.001, \beta = 0.49 \pm 0.15 \)) and that T levels have no effect on ectoparasite numbers (\( F_{1,34} = 1.74, p = 0.19, \beta = 0.20 \pm 0.15 \)).

4. DISCUSSION

The present study shows that T is strongly associated with sperm production and some aspects of sperm quality in the Iberian red deer. The effects of T upon antlers are novel since we find a significant negative effect and two negative trends upon different size components, and a positive effect upon antler strength. T is also associated with an increase in nematode load, giving support to the immunocompetence handicap hypothesis. By contrast, no relationship was found between T and ectoparasite load and spleen size. Our findings reveal for the first time a positive significant relationship between T and the haematocrit.

To examine the effects of T, we collected data on a captive population where males could be sampled periodically during the whole annual cycle, and on a wild population to understand how T levels may influence a number of fitness-related traits during the breeding season. The absence of differences in T levels between males from the captive and the natural populations during the breeding season suggests that this captive population can be used as a valid reference to examine seasonal changes in Mediterranean environments.

Within individuals T levels vary greatly with age and season. In our captive population T levels increase significantly from 2 to 3-year-old males, and are similar for males aged between 3 and 6 years. Seasonal variation in T levels is common in red deer and other seasonally breeding ungulates: T levels remain low during spring, increase during the summer reaching a peak just before the

![Figure 3. Relationship between testosterone and two sperm quality traits: (a) the proportion of normal spermatozoa (arcsine transformed; \( r = 0.248, p = 0.029, y = 1.092 + 0.101x \)) and (b) the proportion of spermatozoa with intact acrosome (\( r = 0.239, p = 0.035, y = 83.699 + 7.266x \)).](image_url)

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breeding season starts, and decline gradually during the winter (Lincoln 1971, 1972). In our captive population, the difference in T levels from the rut to the non-breeding season was over 20-fold, showing that T follows a highly seasonal pattern in red deer inhabiting Mediterranean ecosystems. This seasonal fluctuation pattern mirrored changes in size of testes, the main organ responsible for the changes in T serum levels.

In captive populations, comparisons between males revealed that T and testes size were only associated during September, the month when both reach maximum values. On the contrary, in the natural population we were able to detect this relationship during the whole reproductive season. However, there is a negative effect of sampling date on testes size, which suggests that the effect of T on testes size will be stronger earlier in the breeding season. However, there is a negative effect of sampling date on testes size. In addition, higher T levels among males in natural populations were associated with some of the sperm traits examined. There was a significant relationship between T and both the proportion of normal sperm and sperm with intact acrosomes, but not with sperm swimming velocity. This result has far-reaching implications. We have previously shown that proportion of normal sperm is a determinant of fertility in this species (Malo et al. 2005a), so T is likely to affect fertility in the red deer, through its effects on not only sperm numbers (i.e. quantity) but also sperm quality. To the best of our knowledge, this is the first report of an effect of T on sperm quality in a wild mammal.

T controls the timing of key events in the antler cycle (Lincoln 1973; Suttie et al., 1984): an increase in T triggers pedicle growth, maximum T levels lead to antler mineralization, and a drop in T levels results in antlers being cast. The influence of seasonal changes in T levels upon these events seems to have led to the conclusion that T levels also influence antler growth rates, so antler size has been widely assumed to be positively influenced by T levels (James 2007; Leblanc 2007). However, this seems unlikely given that T levels remain low during the phase of antler growth, and thus do not seem to be necessary to stimulate growth rates. In fact, experimental studies both in vivo and in vitro show that T is not necessary for antler growth. Castrated males grow antlers of similar size, although these antlers do not mineralize and shed the velvet (Li et al. 2003; Price & Allen 2004). In addition, stags treated with anti-androgen grow larger antlers with species-specific antler shapes (Suttie et al. 1995), suggesting that T has an inhibitory effect upon antler growth. Experimental evidence shows that antler growth rate is under the control of growth factors such as IGF1 (Suttie & Fennessy 1992) and that T reduces IGF1-stimulated antler growth probably by influencing IGF1 binding (Suttie et al. 1994; Price & Allen 2004). Our results from natural populations support the idea that T has an inhibitory effect upon antler growth, since we have detected a significant negative relationship between T levels and number of points. In addition, we found no relationship between T levels and casting date which could have influenced antler size by giving males more time to grow their antlers before the breeding season starts. Only one study has examined in natural populations the effect of T upon antler size during the breeding season on a different deer species reporting a positive association between an index of antler size and T levels (Ditchkoff et al. 2001a). However, this study included very young males (1–3 years) so the apparent relationship may be due to the effects of age, and it used an antler score that weights antler asymmetry negatively so it may not reflect antler size accurately. A different study by the same authors showed that when IGF is considered, the effects of T on antler size are no longer found (Ditchkoff et al. 2001b).

Our results show that T levels have a strong influence upon the probability of antler breakage and thus strength. This may be the consequence of the influence of T levels on antler mineralization (Fennessy & Suttie 1983; Bubenik et al. 2005). Thus, males with higher levels of T may be able to outcompete other males in agonistic encounters, since their antlers will be more resistant to the high

Table 2. Multiple regression models for three antler variables, antler length, burr diameter and number of points for males 3–9 years old (Model summary for antler length: $r^2=0.53, F_{3,45}=16.8, p<0.001$, AD diameter $r^2=0.49, F_{3,44}=14.20, p<0.001$, AP $r^2=0.25, F_{3,44}=4.91, p<0.005$). (Testosterone and body size were log and square root transformed, respectively.)

| predictor variables | antler length | | | | | | burr diameter | | | | | | number of points | | | |
|---------------------|---------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
|                     | parameter estimate (s.e.) | $p$-value | parameter estimate (s.e.) | $p$-value | parameter estimate (s.e.) | $p$-value |
| testosterone        | -0.14 (0.10) | 0.19 | -0.29 (0.10) | 0.011 | -0.08 (0.13) | 0.53 |
| age                 | 0.64 (0.11) | <0.001 | 0.54 (0.11) | <0.001 | 0.36 (0.14) | 0.02 |
| body length         | 0.18 (0.11) | 0.11 | 0.22 (0.11) | 0.07 | 0.25 (0.14) | 0.09 |

Table 3. General linear model for the haematocrit (Full model: $r^2=0.49, F_{6,51}=8.14, p<0.0001$). (Testosterone and age were log and square root transformed, respectively.)

<table>
<thead>
<tr>
<th>dependent variable</th>
<th>independent variable</th>
<th>d.f.</th>
<th>$F$</th>
<th>parameter estimate (s.e.)</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>haematocrit</td>
<td>age</td>
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<td>0.50</td>
<td>-0.023 (0.034)</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>testosterone</td>
<td>1</td>
<td>11.19</td>
<td>0.095 (0.028)</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>population</td>
<td>4</td>
<td>7.80</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>residual</td>
<td>51</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
pressures exerted by clashes with other males. When testing the effect of T levels on antler strength, we assumed that all males where exposed to the same intensity of antler wear through combats. However, stags with high T levels are more aggressive (Lincoln et al. 1972; Mooradian et al. 1987), and thus are more prone to engage in combats, so they would be expected to have a higher proportion of broken tines. Our results showing that males without broken tines present significantly higher T levels than males with one or two broken points are hence conservative, and if biased would probably be underestimating the effects of T on antler strength. Previous results from the longitudinal study in the island of Rum have shown that stags with heavier antlers have a higher reproductive success (Kruuk et al. 2002). Heavier antlers could reflect larger antler size, increased bone density, or both, so the relative contributions of antler strength and size on lifetime reproductive success still remain to be assessed.

Red deer stags need physical strength and endurance to be able to cope with the huge energetic demands that they face during the mating season. The positive significant effect of T on the haematocrit revealed in this study suggests a clear pathway from the physiological to the behavioural level. Since increased haematocrit improves oxygen supply to the muscles, it seems likely that during the rut, males with high T levels will be able to afford more intense physical exercise in terms of increased endurance during male to male combats and also an increase in the metabolic efficiency needed to search for, copulate with and defend females. The effects of T on the haematocrit have not been predicted or studied before but it may have important consequences for male breeding success through its likely effects upon physical stamina.

Contrary to other studies carried out in natural populations (Ditchkoff et al. 2001a), we have found a positive relationship between T and nematode load. This result shows that increased T levels have trade-offs predicted by the immunocompetence handicap hypothesis (Folstad & Karter 1992). However, no relationship was found between T and spleen size or ectoparasite load (ticks).

In conclusion, our results suggest that accounting for seasonal, ecological and age effects, there are inter-individual differences in T levels which translate into (i) fertility variance between stags, mediated through increased sperm production and some aspects of sperm quality, (ii) stronger antlers with higher probability of not breaking during combat, and (iii) haematocrit differences that are likely to affect physical performance and stamina during the rut. Also, previously reported effects of T on physical activity stimulation (Marler & Moore 1988), and aggressive behaviour (Pelletier et al. 2003), suggests that T is a physiological trait which may have a major influence upon male reproductive success in natural populations, mainly by increasing their ability to win fights with other males and defend harems/territories. Thus, T seems to improve traits that are needed in male/male competition. However, our results show that there is a clear cost associated with high T levels, such as (iv) higher parasite loads and (v) reduced antler size.

Taken together, the findings from this and our earlier studies (Malo et al. 2005a,b; Gomendio et al. 2007a,b) lead us to conclude that T influences mainly traits that improve a male’s ability to win agonistic encounters, such as antler strength and haematocrit, and may also improve male/male competition after copulation by enhancing sperm production and sperm morphological traits. Traits that enhance male fighting ability are likely to be honest signals, since they are constantly tested by other males in the breeding season during combats.

By contrast, T had a negative effect upon antler size and no influence on sperm swimming velocity. We have previously shown that sperm swimming velocity, which is the main determinant of fertility in this species (Malo et al. 2005a), is honestly advertised by antler size (Malo et al. 2005b). However, our findings show that the relationship between antler size and sperm swimming velocity is not mediated by T. We suggest that, in addition to their role as armaments (relevant for intrasexual competition), antlers may have a role as ornaments which may play a role in female choice. Thus, males with large antlers also have faster sperm swimming speed that is the main determinant of male fertilization success both in competitive and non-competitive contexts (Birkhead et al. 1999; Moore et al. 2002; Malo et al. 2005a), so females may benefit from choosing males with large antlers (Malo et al. 2005b).

In conclusion, T plays a major role in improving a male’s ability to win fights, have physical endurance and avoid sperm depletion, but it does not seem to have any role in influencing antler traits that may be the target of visual female choice.

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REFERENCES


Table 4. Log-linear model for nematode load (modelling as a Poisson distribution using a log link function). (ΔDev gives the percentage of increase in deviance for the significant terms when they were not fitted in the model.)

<table>
<thead>
<tr>
<th>dependent variable</th>
<th>independent variables</th>
<th>d.f.</th>
<th>$\chi^2$</th>
<th>p-value</th>
<th>parameter estimate (s.e.)</th>
<th>ΔDev</th>
</tr>
</thead>
<tbody>
<tr>
<td>nematode load</td>
<td>testosterone</td>
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<td>10.75</td>
<td>0.001</td>
<td>1.50 (0.74)</td>
<td>25%</td>
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<td></td>
<td>body condition</td>
<td>1</td>
<td>2.90</td>
<td>0.09</td>
<td>-0.46 (1.09)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>age</td>
<td>1</td>
<td>1.83</td>
<td>0.18</td>
<td>0.36 (0.29)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>population</td>
<td>4</td>
<td>21.52</td>
<td>&lt;0.001</td>
<td></td>
<td>18%</td>
</tr>
</tbody>
</table>
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Neal, J., Murphy, B. D., Moger, W. H. & Oliphant, L. W. 2001 Reproduction in the male ferret: gonadal activity during the annual cycle: recrudescence and matura-


