Testosterone is positively related to the output of nematode eggs in male Alpine ibex (*Capra ibex*) faeces

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ABSTRACT

Question: Does testosterone suppress the immune system of males in a strongly sexually dimorphic and long-lived ungulate?

Immunocompetence handicap hypothesis: Testosterone promotes the development of secondary sexual characteristics and simultaneously suppresses immunological defence.

Organisms: Free-ranging and individually identifiable male Alpine ibex (*Capra ibex*).

Methods: In faecal samples, measure testosterone levels ($ng \cdot g^{-1}$) and the number of parasite eggs per gram of faeces (faecal egg counts). Determine social dominance by observing the outcomes of agonistic interactions in the field. Weigh males at a salt-lick scale.

Data analysis: Path analysis to examine the relationships between testosterone levels, dominance, body mass, age, and faecal egg counts.

Conclusions: We found a strong positive effect of testosterone on the amount of parasite eggs in the faeces of males. The level of parasite infection did not depend on any other tested variable. Testosterone therefore has an immunosuppressive effect in male Alpine ibex, as suggested by the immunocompetence handicap hypothesis.

Keywords: body mass, dominance, faecal egg counts, immunocompetence, immunosuppression, path model.

INTRODUCTION

The handicap principle suggests that exaggerated secondary sexual characters can be an index of male good health if they are costly to produce (Zahavi, 1975). Hamilton and Zuk (1982) suggested that elaborate secondary sexual characters evolve in males because they signal to females the genetic quality of the males and their greater resistance to parasites. The immunocompetence handicap hypothesis [ICHH (Folstad and Karter, 1992)] considers the

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cost of producing and carrying exaggerated secondary sexual characters from an endocrinological perspective. Although a high level of testosterone promotes the development of exaggerated secondary sexual characters and potentially increases the individual mating success, testosterone simultaneously impairs the functioning of the immune system (Folstad and Karter, 1992). Therefore, females should benefit by mating with males that have high levels of testosterone, because only genetically high-quality males can afford or tolerate the costs of decreased defence against parasites and pathogens (Zuk *et al.*, 1995; Peters, 2000). Although the ICHH predictions were made specifically for traits involved in mate choice, they should also apply to intrasexually selected traits (Roberts *et al.*, 2004).

Testosterone is a hormone of the androgen class. Androgens stimulate the production of spermatozoids, reinforce the libido, and stimulate the development of secondary sexual characters (Andersson, 1994). Testosterone is thought to have immunosuppressive effects because of differences in immunocompetence between males and females of many species, including humans (Sthoeger *et al.*, 1988; Zuk, 1996; Stoehr and Kokko, 2006). This idea is supported by the fact that the immune response can be altered by gonadectomy and by sex steroid hormone replacement (Grossman, 1984; Litvinova *et al.*, 2005). Moreover, the organs responsible for the immune response, for example the thymus, contain specific receptors for gonadal steroids (Grossman, 1985; Alexander and Stimson, 1988). Grossman (1985) suggested that the physiological mechanism of the hypothalamic–pituitary–gonadal axis could explain the testosterone immunosuppression. According to this mechanism, increasing levels of testosterone can reduce the release of hormones that are produced by the thymus, which modulate T-cell functions by stimulating their maturation (Grossman, 1985). Because T-cells are regulators of the cell-mediated immune response (Kimball, 1990), testosterone has in turn an immunosuppressive effect.

Testosterone was found to be related to decreased immune investment and/or to increased parasite loads in many bird species (Saino *et al.*, 1995; Zuk *et al.*, 1995; Duffy *et al.*, 2000; Poiani *et al.*, 2000; Buchanan *et al.*, 2003; Mougeot *et al.*, 2004; Peters *et al.*, 2004; Seivwright *et al.*, 2005). However, some studies failed to find clear evidence for an immunosuppressive effect of testosterone in birds (Hasselquist *et al.*, 1999; Peters, 2000; Lindström *et al.*, 2001; Greenman *et al.*, 2005). In mammals, experimental studies for the effects of testosterone on immunity are rare except in rodents (Barnard *et al.*, 1996, 1998; Hughes and Randolph, 2001). The remaining evidence for the ICHH comes from ungulates, and is contradictory (Folstad *et al.*, 1989; Ditchkoff *et al.*, 2001a).

Alpine ibex are highly polygynous, long-lived, and sexually dimorphic mammals (Nievergelt, 1974; Weckerly, 1998; Toïgo *et al.*, 2007). Body and weapon size of males, as in other polygynous ungulates, are probably related to social dominance and reproductive success (McElligott *et al.*, 1998, 2001; Coltman *et al.*, 2002; Preston *et al.*, 2003; Saunders *et al.*, 2005; Bro-Jørgensen, 2007). The large, costly horns of males are used during fights, grow throughout life, and their growth is related to survival (Nievergelt, 1974; Giacometti *et al.*, 2002; von Hardenberg *et al.*, 2004). Horn growth is likely to be related to testosterone levels, as for example in mouflon [*Ovis musimon* (Lincoln, 1998)] and bighorn sheep [*Ovis canadensis* (Henderson and Firebaugh, 1997)]. There is also evidence for a relationship between social dominance rank and testosterone in bighorn sheep and in several deer species (Lincoln *et al.*, 1972; Pelletier *et al.*, 2003; Li *et al.*, 2004). Therefore, polygynous ungulates are ideal models for testing predictions of the ICHH.

Immunocompetence can be assessed by investigating the number of parasite eggs in faeces (Smith *et al.*, 1999). For example, abomasal parasite loads are related to eosinophila, antigens, and other immunological parameters in sheep (*Ovis aries*) and cattle [*Bos taurus* (Stear and Murray, 1994)]. Faecal egg counts can therefore be considered an index of the

combined effects of the intensity of parasitism, and parasite resistance influencing parasite fecundity (Stear *et al.*, 1995, 1997; Coltman *et al.*, 1999). In ibex, the most common parasite eggs in faeces come from Trichostrongylidae intestinal nematodes (Zaffaroni *et al.*, 2000; Pérez *et al.*, 2003).

Horn and body growth in male Alpine ibex occur during the spring–summer period (Giacometti *et al.*, 2002; von Hardenberg *et al.*, 2004) and are likely to be related to testosterone levels as in other ungulate species. The summer is also when the highest burdens of abomasal nematodes are found (Lanfranchi *et al.*, 1995; Zaffaroni *et al.*, 2000), and the peak of nematode egg excretion in the faeces of ibex occurs (A. von Hardenberg, unpublished data). We therefore carried out our investigations during the summer, because this is when a relationship between testosterone, the development of secondary sexual characteristics, and immunocompetence should be evident.

Here we use Alpine ibex males as a model to test the main assumptions of the ICHH. According to the ICHH, we expect a positive relationship between dominance rank and testosterone levels, and a negative relationship between testosterone levels and immuno-competence (measured as parasite faecal egg counts). We then use a path analysis model to test the hypothesis of a direct causal link between testosterone and immunocompetence. Even though correlation does not imply a cause–effect relationship, causation implies a series of partial correlations and constraints on the pattern of covariation between variables (Shipley, 1999). Using path analysis, it is therefore possible to test if the covariation matrix among variables fits the predicted covariation matrix of a specific causal model.

METHODS

Study area and population

We carried out the fieldwork in the Levionaz Basin (45°35′N/07°12′E) of the Valsavarenche valley, in Gran Paradiso National Park (north western Italian Alps) from June to August 2004. There were 98 male Alpine ibex in the study area, and 60 males were marked with ear tags. These animals are part of a long-term study (von Hardenberg, 2005). Ear tags consisted of a unique combination of colours. In addition, six males were radio-collared. All males were between 4 and 15 years old. Age of ibex was determined with precision by counting the annual horn growth annuli during capture (Ratti and Habermehl, 1977). More details about the capture and marking of male ibex in the Gran Paradiso National Park can be found in von Hardenberg (2005).

Faecal collection

We collected faecal samples six times during the summer. Each collection session lasted 2–3 days and between each there was a period of 14–15 days during which no samples were collected. We started collecting on 14 June and stopped on 31 August. We noted the date, time of day, and identity of ibex for every sample. Each faecal sample was separated into two parts to allow both the parasite faecal egg counts and the measurement of testosterone to be carried out later. In total, we collected 266 faecal samples from 51 marked males.

Faecal egg counts

We collected faeces in plastic bags and placed them in the refrigerator at 4°C until the assay was carried out. We analysed all samples within 2 weeks of their collection. A modified McMaster technique was used to estimate the number of parasite eggs per gram of faeces (Thienpont *et al.*, 1979). We mixed 5 g of faeces and 30 ml of a saturated salt solution (density of 1200 kg·m^{-3} at 21°C) in a mortar. The mix was then filtered with a strainer, and the two chambers (10×10 mm) of the McMaster slide were filled with the suspension. We observed the slide with an object-glass ($10\times$) and we counted only the eggs completely entangled in the slide. All the counted eggs were summed and the sum was multiplied by 20 to determine the number of eggs per gram of faeces. We repeated this procedure two times for each sample and if the differences between the two faecal egg counts were higher than 25%, we repeated the procedure a third time. All counts were then averaged to get the faecal egg counts of the sample.

Testosterone levels

We placed approximately 2 g of faecal pellets (three pellets) into tubes (containing 10 ml of absolute ethanol) immediately after defecation and we froze the tubes at −20°C within 3 days of faecal collection (Kraus *et al.*, 1999; Khan *et al.*, 2002; Pelletier *et al.*, 2003). Tubes were placed in a laboratory shaker at 200 rev·min^{-1} overnight and at 26°C for the testosterone extraction. Samples were centrifuged for 1 h at 4000 rev·min^{-1} and at 7°C to remove fine particles. The supernatant (1 ml) was decanted into cryotubes and kept in the freezer (−20°C) until the assay was carried out (Pelletier *et al.*, 2003). We then dried the faecal pellets in an oven at 70°C. Samples were weighed every hour and were considered to be dried when the weight remained stable for at least 1 h.

The testosterone content of the faecal samples was assessed using the kit Testosterone RIA DSL-4100 (Diagnostic Systems Laboratories, Texas, USA; www.dslabs.com). All samples were assayed in duplicate. We repeated the assay for any sample in which the duplicates differed by 15% or more in the counts per minute of the assay (Pelletier *et al.*, 2003). Prior to the analysis, we diluted 100 μl of the extracted supernatant with 100 μl of deionized water. This dilution (50%) was necessary because without it the serum added in the following steps would be denatured. We also added 50 μl of 50% ethanol and 50 μl of the respective standards or control to each of the standard and control tubes. The sensitivity of the RIA DSL-4100 test is 0.05 ng·ml^{-1}. [For assay validation of the DSL-4100 kit, see Pelletier *et al.* (2003).] The manufacturer provided the cross-reactivity of the testosterone antiserum against various compounds as follows: 5α-dihydrotestosterone, 6.6%; 5-androstane-3β,17β-diol, 2.2%; 11-oxotestosterone, 1.8%; androstenedione, 0.9%; 5β-dihydrotestosterone, 0.6%; 5β-androstane-3β,17β-diol, 0.5%; oestradiol-17β, 0.4%; 5α-androstane-3α-ol-17-one, 0.2%. Testosterone antiserum had no detectable cross-reactivity to oestrogens, progestins or corticoids, but it is possible that androgen metabolites other than testosterone were present in faeces and contributed to the assayed results. The radioactivity was determined in a Gamma counter over 3 min and the computer RIA data analysis program calculated a linear-log curve by using the testosterone standard concentrations. All the results were calculated using this curve fit. Concentrations were determined as picograms per tube and then converted to ng·ml^{-1} and divided by the mass of faeces extracted (dry weight). The testosterone level was indicated as ng·g^{-1} dry weight.

Dominance rank and body masses

We carried out behavioural observations from 12 June to 16 August 2004 to determine the dominance ranks of the males. A salt-lick was provided for the ibex and only one animal could access this salt-lick at any time. We collected data on agonistic interactions from animals at or around the salt-lick, and these were subsequently used to calculate the dominance hierarchy. Most observations were carried out late in the evening (19:00–21:00 h) because ibex were more active at the salt-lick during this time. We recorded the identity (or age if not marked) of any male that accessed the salt-lick, and we also identified any male that was around the salt-lick (maximum of approximately 10 m away) during the time a male licked salt. Moreover, we recorded the identity of the two males each time one male displaced another at the salt-lick. A balance, connected to an electronic display suitable for outdoor use, was also placed at the salt-lick. The salt-lick was set up so that each time an ibex wanted access, it also had to stand on the balance. We therefore obtained the body masses of the animals each time they used the salt-lick. More details about the balance set-up can be found in Bassano *et al.* (2003).

Data analysis

Statistical analyses were performed in SPSS 11.5 for Windows and S-PLUS 2000 (MathSoft Inc.). All tests were two-tailed and considered significant if $P < 0.05$. Means are given with standard deviations ($\pm$s.d.).

Analysis of faecal egg counts and testosterone levels

We estimated faecal egg counts for 51 males ($n = 266$ faecal samples). Because it was not possible to collect faeces of all the individuals every time, the sample sizes varied for different males. We considered the 46 ibex for which we collected at least three faecal samples during the whole field season in the analysis. We analysed three sample periods for each animal: the end of June, the end of July, and the end of August. This allowed an overview of the faecal egg counts during the whole summer with three data points for each of the 46 males, and a sample size of 138 repeated measurements. Data were square root transformed to achieve a normal distribution (Kolmogorov-Smirnov = 0.05, d.f. = 138, $P = 0.20$). We measured testosterone levels for 46 males using parts of the same faecal samples investigated for the faecal egg counts ($n = 138$ repeated measurements). Testosterone concentrations were ln + 1 transformed to achieve normality (Kolmogorov-Smirnov = 0.07, d.f. = 138, $P = 0.20$).

Assessment of the dominance ranks

We only used observations from marked males to calculate the *win/loss* matrix and the dominance rank. A male was considered the *winner* if he was able to displace a male that was at the salt-lick, or if he was at the salt-lick and there were some males around him, but none displaced him. In contrast, a male was considered the *loser* if he was at the salt-lick and another male displaced him, or if he was around the salt-lick where there already was a male, but he did not try to displace that male (Côté, 2000). We calculated the proportion of wins by individual *i* in his interactions with another individual *j*. To assess the dominance

rank, we used a modification of David's score (David, 1987), which allows the consideration of differences in the frequency of interactions between dyads (de Vries, 1998; H. de Vries, personal communication). The modified David's score is calculated as follows:

$$Dij = (sij + 0.5)/(nij + 1)$$

where *sij* is the number of times that individual *i* defeats individual *j*, and *nij* is the total number of dominance interactions between *i* and *j*.

$$DS = w + w_2 - l - l_2$$

where *DS* is the David's score, *w* is the sum of all *Dij* values of individual *i*, w_2 is the sum of all *w* values of those individuals with which *i* interacted, *l* is the sum of the *Dji* of individual *i*, and l_2 is the sum of the *l* values of those individuals with which *i* interacted (David, 1987). We calculated the dominance rank of 29 males using a total of 271 interactions. All of these males had at least five interactions with other group members. Subsequently, we examined the effect of a larger sample size on the dominance hierarchy. We calculated the dominance rank for 46 individuals that were involved in at least one interaction with other group members with a total of 339 interactions. The two dominance ranks were highly correlated (Pearson correlation: $r = 0.98$, $n = 29$, $P < 0.001$). Therefore, for further analyses, we used the dominance rank calculated with the larger sample size. Information on faecal egg counts and testosterone levels was available for 41 of the 46 ranked males.

Body mass estimation

We measured the body mass of 27 males several times during the summer and we adjusted them to 1 August 2004. The body masses were adjusted by fitting linear mixed effect models by restricted maximum likelihood implemented in the NLME package of S-PLUS 2000 (Insightful Corp.; Pinheiro and Bates, 2001), and by extracting predicted body masses and slopes (growth rates) following the method described in von Hardenberg (2005). Information on faecal samples was available for 27 weighed males and information on dominance ranks for 26 weighed males.

Model fitting

We fitted linear mixed effect (LME) models and general linear models (GLMs) to explore the relationships between all the investigated parameters. The LME models take repeated measurements into account and allow the influence of a series of covariates on a dependent variable to be assessed. We therefore carried out an LME model (Pinheiro and Bates, 2001) with individual identity as a random grouping factor to assess the effects of testosterone, age, body mass, and dominance on faecal egg counts. A second LME was carried out with the month effect nested within individual identity as a random factor to explore the effects of body mass, age, faecal egg counts, and dominance on testosterone levels. Month was shown to have an effect on testosterone levels. Repeated individual measures were used because faecal egg counts and testosterone levels were considered during three different periods. Generalized linear models were fitted to investigate the relationships between testosterone levels, faecal egg counts, age, dominance rank, and body mass, respectively. Dominance rank and body mass were considered as dependent variables and therefore no repeated individual measures were used in these analyses because we estimated a single dominance
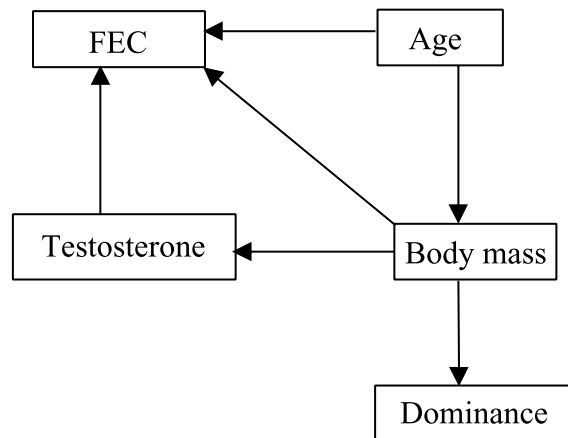
Fig. 1. Path-analysis diagram for the causal relationships between age, body mass, dominance, testosterone, and faecal egg counts (FEC). Goodness of fit of the model, Fisher *C*-test: $C = 3.68$; d.f. = 8; $P = 0.88$.

rank and body mass measure for the whole study period. We fitted in the models the quadratic term of age because age had a quadratic relationship with dominance and body mass. The normality of the residuals for each model was checked.

We then tested for direct causal relationships between testosterone levels, dominance rank, age, body mass, and faecal egg counts using path analysis. The hypothesized causal structure is shown in Fig. 1. The causal claims of this model are captured in its topology (i.e. in the way the variables connect together). The topology can be tested by comparing the predicted patterns of conditional independence between the variables that is implied by it with the actual patterns of conditional dependence and independence in the data. This is done using a d-sep test whose basis set of d-separation claims generated all predicted patterns of conditional independence (Shipley, 2000). Given the hierarchical nature of our data, we tested each hypothesized conditional independency of type X_||_Y | {A,B, ...} by fitting a mixed model of A,B, ... +X on Y and calculated the probability (p_i) that the partial regression coefficient associated with X is zero (i.e. the effect of X on Y conditional on A,B, ...). The overall test of the basis set is given by the Fisher *C*-statistic, where $C = -2*SUM(\ln(p_i))$, which is distributed as a χ^2 variate with $2k$ degrees of freedom (where k is the number of independence tests in the basis set) if all predicted conditional independencies hold in the data. The path model is considered to fit the data when the *C*-value is not significant (*P*-value > 0.05) (Shipley, 2000, 2004).

RESULTS

Faecal egg counts

The mean faecal egg counts of the 46 males over the whole summer was 409 ± 266 eggs per gram of faeces (EPG), and the mean faecal egg counts for the three periods were as follows: 458 ± 283 EPG at the end of June, 435 ± 274 EPG at the end of July, and 327 ± 227 EPG at the end of August. Faecal egg counts between the 3 months did not differ significantly

(ANOVA: $F_{2,135} = 2.85$; $P = 0.06$). Moreover, the variability in faecal egg counts between individuals was greater than the variability within individuals (ANOVA: $F_{45,92} = 4.12$; $P < 0.001$).

Testosterone levels

Testosterone levels ranged from 0.72 to 21.76 ng·g^{-1}. The mean testosterone level of the 46 males over the summer was 4.41 ± 2.92 ng·g^{-1}. The mean levels for the three periods were 6.60 ± 3.55 ng·g^{-1} at the end of June, 3.45 ± 2.14 ng·g^{-1} at the end of July, and 3.18 ± 1.18 ng·g^{-1} at the end of August. The testosterone levels at the end of June were significantly higher than those at the end of July (ANOVA: $F_{2,135} = 24.61$; Bonferroni *post hoc* test, $P < 0.001$) and at the end of August (Bonferroni *post hoc* test, $P < 0.001$). The testosterone levels at the end of July and at the end of August were not significantly different (Bonferroni *post hoc* test, $P = 1.00$). Taking into account the variability among months, the variability in testosterone levels between individuals was greater than within individuals (ANOVA: $F_{45,90} = 1.54$; $P = 0.04$).

Age, social dominance rank, and body mass

The highest ranked male in the dominance hierarchy was 13 years old and the lowest ranked male was 6 years old. Body mass adjusted to 1 August ranged from 55.6 kg (for a 7-year-old male) to 96.4 kg (for a 13-year-old male). The mean body mass was 75.7 ± 10.3 kg ($n = 27$ males).

Model fitting

Age, testosterone, and body mass had a positive effect on faecal egg counts, whereas dominance had no effect (Table 1). Mean testosterone levels showed a positive correlation with mean faecal egg counts (Pearson correlation: $r = 0.33$, $n = 46$, $P = 0.03$; Fig. 2). Moreover, the effect of testosterone on faecal egg counts was at an individual level and was independent of age and body mass (Table 1). Body mass was positively related to testosterone levels, independently of age (Table 2). However, age showed a non-significant tendency to influence the levels of testosterone. No other factor had an effect on

Table 1. Linear mixed effects model with faecal egg counts as the dependent variable and testosterone, dominance, age, body mass, and their interactions as covariates. Individual is the random grouping factor ($n = 78$ repeated measures)

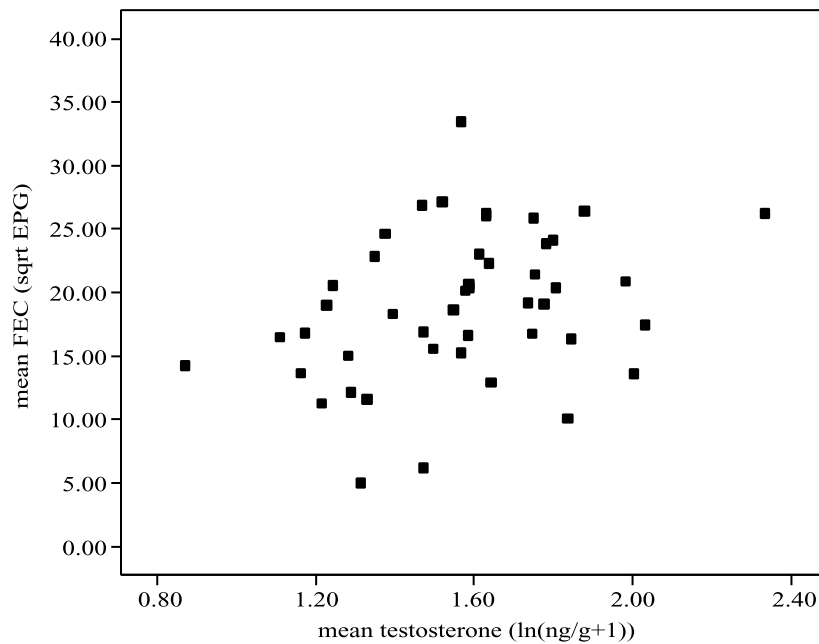| Variables	β	Standard error	d.f.	F	P
Testosterone	4.27	1.43	1	12.33	0.0009
Age	0.42	0.4	1	7.32	0.01
Body mass	0.26	0.13	1	4.03	0.05
Dominance	—	—	1	0.50	0.49
Dominance × body mass	—	—	1	0.35	0.56
Age × testosterone	—	—	1	0.08	0.78
Testosterone × body mass	—	—	1	2.06	0.16

Fig. 2. Mean faecal egg counts (sqrt EPG) in relation to mean testosterone levels (ln (ng/g + 1)). Pearson correlation: $r = 0.33$, $n = 46$, $P = 0.03$.

Table 2. Linear mixed effects model with testosterone levels as the dependent variable and faecal egg counts (FEC), dominance, age, body mass, and their interactions as covariates. Individuals nested within month is the random grouping factor ($n = 78$ repeated measures)

Variables	β	Standard error	d.f.	F	P
Body mass	0.02	0.006	1	11.02	0.001
Age	—	—	1	2.95	0.09
FEC	—	—	1	2.56	0.11
Dominance	—	—	1	1.43	0.24
Age × body mass	—	—	1	2.81	0.11

testosterone (Table 2). Dominance was positively influenced by body mass. No other parameter had an effect on dominance (Table 3). Age and testosterone had an effect on body mass when body mass was fitted as a dependent variable. However, faecal egg counts and dominance were unrelated to body mass (Table 4).

Path analysis

Using path analysis we tested the explicit hypothesis of a causal relationship between testosterone and faecal egg counts assuming other causal relationships linking age to faecal egg counts and body mass, and linking body mass to dominance, testosterone, and faecal

Table 3. Generalized linear model with dominance rank as the dependent variable and faecal egg counts (FEC), age, age^2, body mass, and testosterone as covariates ($n = 26$)

Variables	β	Standard error	d.f.	MS	F	P
Body mass	4.09	2.16	1	32474.56	6.22	0.02
Age	—	—	1	843.66	0.16	0.69
Age^2	—	—	1	9445.28	1.81	0.19
FEC	—	—	1	1828.30	0.35	0.56
Testosterone	—	—	1	8521.62	1.63	0.22

Table 4. Generalized linear model with body mass as the dependent variable and testosterone, age, age^2, faecal egg counts (FEC), and dominance as covariates ($n = 26$)

Variables	β	Standard error	d.f.	MS	F	P
Testosterone	10.65	4.14	1	343.46	7.05	0.01
Age	3.54	4.64	1	538.45	11.05	0.003
Age^2	−0.15	0.23	1	232.79	4.78	0.04
FEC	—	—	1	141.03	2.97	0.1
Dominance	—	—	1	167.90	3.45	0.08

egg counts according to the correlations found with the linear models (Fig. 1). This causal model properly fitted the data (Fisher C-test: $C = 3.68$, d.f. = 8, $P = 0.88$). Partial regressions were computed on the basis of four d-separation statements: FEC∥D|T, BM, A; D∥A|BM; T∥D|BM; T∥A|BM, where FEC = faecal egg counts, D = dominance, T = testosterone, BM = body mass, and A = age. The model states that dominance was d-separated from faecal egg counts given testosterone, body mass, and age (FEC∥D|T, BM, A; dominance: β = N.S., $t_{1,26} = -0.12$, $P = 0.91$; testosterone: $\beta = 4.27$, $t_{1,26} = 2.98$, $P = 0.004$; body mass: $\beta = 0.26$, $t_{1,26} = 2.00$, $P = 0.05$; age: $\beta = 0.42$, $t_{1,26} = 1.04$, $P = 0.31$), and that age was d-separated from dominance given body mass (D∥A|BM; age: β = N.S., $t_{1,26} = 0.39$, $P = 0.69$; body mass: $\beta = 3.42$, $t_{1,26} = 1.95$, $P = 0.06$). Moreover, dominance was d-separated from testosterone given body mass (T∥D|BM; dominance: β = N.S., $t_{1,26} = -1.07$, $P = 0.29$; body mass: $\beta = 0.02$, $t_{1,26} = 3.44$, $P = 0.002$), and age was d-separated from testosterone given body mass (T∥A|BM; age: β = N.S., $t_{1,26} = -0.19$, $P = 0.85$; body mass: $\beta = 0.02$, $t_{1,26} = 2.51$, $P = 0.02$). Therefore, testosterone had a strong effect on faecal egg counts and body mass had an effect on testosterone even when all the other independent variables were held constant. Moreover, dominance was causally linked to body mass but not to testosterone.

DISCUSSION

In this study, we examined the main assumptions of the immunocompetence handicap hypothesis (ICHH) by investigating the relationships between testosterone levels, dominance rank, body mass, age, and faecal egg counts, in a free-ranging population of male Alpine ibex. The major finding was a strong positive effect of testosterone on faecal egg counts at the individual level, which was independent of all the other variables tested. There

was no relationship between testosterone and dominance. Body mass had a positive effect on dominance, testosterone levels, and also on faecal egg counts. Many studies have attempted to test the ICHH in a wide range of vertebrates since the hypothesis was first suggested by Folstad and Karter (1992), but often with equivocal results (Roberts *et al.*, 2004). Our results suggest that testosterone has an immunosuppressive effect in male Alpine ibex, and that body mass mediates the relationship between social dominance rank and testosterone levels.

The link between testosterone levels and faecal egg counts that we detected with the path analysis model indicates that testosterone has an immunosuppressive effect. This is in accordance with results from reindeer (*Rangifer tarandus*), in which gonadally intact males had higher parasite loads than females and castrated males (Folstad *et al.*, 1989), although in this study testosterone levels were not directly investigated. Other studies of ungulates have not provided support for the immunosuppressive effect of testosterone (Ditchkoff *et al.*, 2001a; Pelletier *et al.*, 2005). A meta-analytical study by Roberts *et al.* (2004) clearly supported the ICHH in reptiles and birds, but not in mammals. This may be related to the small number of studies on mammals that were included (Roberts *et al.*, 2004). However, one of the major problems when comparing studies is that the parameters taken into account for the estimation of immunocompetence usually differ between investigations (Adamo, 2004). Moreover, experimental and observational studies are difficult to compare (Roberts *et al.*, 2004).

To date, some of the best evidence for the ICHH in mammals comes from experiments with mice (*Mus musculus*). In male mice, serum steroid hormone concentrations are related to immunocompetence. Subordinate males down-regulate their production of testosterone when faced with an immune challenge to improve long-term survival, whereas dominant males with access to current reproduction opportunities do not (Barnard *et al.*, 1996, 1997, 1998). These results show similarities with ours, even though male Alpine ibex are long-lived with very different life-history strategies to mice (Toïgo *et al.*, 2007). Compared with ibex males, dominant male mice should be more likely to trade-off future survival for short-term reproductive gain because of the high mortality rates that they experience.

The mean faecal egg count in our study was 409 ± 266 EPG (range 10–1640 EPG). In sheep, egg counts between 600 and 2000 EPG are considered to be indicative of moderate worm burdens (McKenna, 1987). The following nematode species are usually found in the faeces of Alpine ibex: *Marshallagia occidentali*, *Marshallagia marshalli*, *Teladorsagia circumcinct*, and *Trichostrongylus axei* (Thienpont *et al.*, 1979; Zaffaroni *et al.*, 2000; Pérez *et al.*, 2003). Some of these are known to be associated with production losses and clinical disease in sheep (Brunsdon and Adam, 1975; Pomroy, 1997). In reindeer, body condition is negatively associated with nematode burdens (Albon *et al.*, 2002). Similarly, in red deer (*Cervus elaphus*), parasite resistance of individuals may vary as body condition varies and faecal egg counts are known to be higher in immunosuppressed individuals experiencing stressful conditions (Vicente *et al.*, 2007).

We found that testosterone was not directly related to social dominance rank, but instead was indirectly related through body mass. This is not consistent with the findings from other ungulates, in which positive relationships between testosterone levels and social dominance were observed (Pelletier *et al.*, 2003; Li *et al.*, 2004). However, our study was carried out during the summer, a time when the levels of testosterone in ungulates are usually low compared with the mating season (Rolf and Fischer, 1990; Ditchkoff *et al.*, 2001b; Pelletier *et al.*, 2003). All other studies that found a positive relationship between testosterone and dominance in ungulates were carried out during the mating season. In the literature, we could not find data for testosterone levels in ibex. In bighorn sheep, Pelletier *et al.* (2003) found a minimal concentration of $2.3 \text{ ng} \cdot \text{g}^{-1}$

dry weight of faeces during the non-mating season and a maximum of 131.8 ng·g^{-1} dry weight of faeces during the pre-rut. In the present study, the mean testosterone level in faeces of male ibex was 4.4 ± 2.9 ng·g^{-1} dry weight of faeces.

The way in which the traits involved in the ICHH interact is likely to be highly complex, and any failure to find a clear relationship between testosterone and parasites in some studies may also reside in the analytical approach used (Shipley, 2000). Traditional statistical methods, including multiple regression and linear mixed models, are of limited use because they cannot distinguish between a correlation due to a third unmeasured variable and one due to a causal relationship. The path-analysis approach we used in this work is appropriate to solve this problem, because it permits testing of hypotheses involving explicit causal relationships (Shipley, 1999, 2000, 2004).

The method we used to assess dominance ranks could have been responsible for the lack of relationship between testosterone levels and dominance in our study, because our dominance calculation was based on the outcomes of aggressive interactions at salt-licks. Côté (2000) found that aggressive interactions at salt-licks in mountain goats (*Oreamnos americanus*) had different outcomes from interactions seen under more natural conditions. If individuals that are licking salt are approached from behind, they often display sub-missive avoidance behaviour. Therefore, a dominant male may show submission to a subordinant male if the subordinant approaches from behind. This would lead to inconsistencies in dyadic dominance relationships (Côté, 2000). However, this kind of submissive behaviour has never been observed in Alpine ibex at salt-licks, and a recent study on the same population shows that dominance ranks estimated at the salt-lick appear to be consistent with dominance ranks estimated from aggressive interactions observed in the field away from the salt-lick (Patrick Bergeron, unpublished data). We also found a positive relationship between body mass and social dominance rank. However, this is not surprising, because body mass is often important for the establishment of dominance hierarchies among males (McElligott *et al.*, 2001; Preston *et al.*, 2003).

Our study is the first to report a strong effect of testosterone on faecal egg counts in individual long-lived mammals, in which males are subject to strong sexual selection. Our finding is important in the context of the immunocompetence handicap hypothesis because it implies that male Alpine ibex are faced with an evolutionary trade-off between sex hormones and immune defence (Hillgarth and Wingfield, 1997). The results also show that ibex males are faced with this trade-off during the very important summer period of growth and body condition development, when testosterone levels are at their lowest. Moreover, it is important to note that the elevated testosterone levels that males experience during the rut coincide with the time when parasite numbers are lowest in the environment (Lanfranchi *et al.*, 1995; Zaffaroni *et al.*, 2000), and males also feed less as a result of rut-related activities (McElligott *et al.*, 2003; Pelletier *et al.*, 2005).

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