Molecular Ecology (2007) **16**, 1969–1980 doi: 10.1111/j.1365-294X.2006.03221.x

Age-dependent genetic effects on a secondary sexual trait in male Alpine ibex, *Capra ibex*

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Abstract

Secondary sexual traits, such as horns in ungulates, may be good indicators of genetic quality because they are costly to develop. Genetic effects on such traits may be revealed by examining correlations between multilocus heterozygosity (MLH) and trait value. Correlations between MLH and fitness traits, termed heterozygosity–fitness correlations (HFC), may reflect inbreeding depression or associative overdominance of neutral microsatellite loci with loci directly affecting fitness traits. We investigated HFCs for horn growth, body mass and faecal counts of nematode eggs in wild Alpine ibex (*Capra ibex*). We also tested if individual inbreeding coefficients (f') estimated from microsatellite data were more strongly correlated with fitness traits than MLH. MLH was more strongly associated with trait variation than f'. We found HFC for horn growth but not for body mass or faecal counts of nematode eggs. The effect of MLH on horn growth was age-specific. The slope of the correlation between MLH and yearly horn growth changed from negative to positive as males aged, in accordance with the mutation accumulation theory of the evolution of senescence. Our results suggest that the horns of ibex males are an honest signal of genetic quality.

Keywords: Alpine ibex, heterozygosity–fitness correlations, horns, multilocus heterozygosity, senescence, ungulates

Received 20 August 2006; revision accepted 30 October 2006

Introduction

Inbreeding depression is the decrease in fitness of offspring born from matings among related individuals (Charlesworth & Charlesworth 1987). It has been typically measured by regressing fitness-related phenotypic traits on individual inbreeding coefficients. Inbreeding coefficients are generally estimated from complete pedigree data, which are rarely available for wild animals. Alternatively, researchers have used individual multilocus heterozygosity (MLH), the standardized average observed heterozygosity among all microsatellite marker loci in one individual, as a proxy for individual inbreeding. Associations between MLH and fitness-related traits, hereafter termed heterozygosity–fitness correlations (HFC), have been widely reported in wild vertebrate populations in recent years (review in Coltman & Slate 2003).

Three main hypotheses have been proposed to explain HFC: (a) direct overdominance, whereby marker loci directly affect fitness; (b) associative overdominance, whereby marker loci are in linkage disequilibrium with loci directly influencing fitness traits; (c) identity disequilibria, whereby marker heterozygosity reflects genome-wide heterozygosity (Hansson & Westerberg 2002). Hypothesis (a) is possible for allozyme markers, while it can usually be ruled out for nonfunctional and selectively

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neutral microsatellite markers. Hypothesis (b), which is also termed a local effect, assumes that individuals heterozygous at a marker locus, physically linked to an overdominant or dominant locus affecting fitness, tend to be heterozygous also at the latter locus. The identity disequilibria hypothesis (c) assumes that individuals heterozygous at marker loci tend to be heterozygous also at unlinked fitness-influencing loci. Identity disequilibrium is expected to arise only in populations showing interindividual variance in inbreeding, therefore when there is variance in inbreeding a positive HFC may reflect inbreeding depression (Slate *et al*. 2004). Therefore, hypothesis (c) is also referred to as the inbreeding hypothesis.

Microsatellites are simple DNA sequences repeated in tandem that exhibit variation in repeat unit number. Because most microsatellite loci lie outside of expressed genes, it is assumed that they are selectively neutral (Scribner & Pearce 2000). Mutation rates for microsatellites are extremely high because of slipped-strand mispairing in DNA replication, making them ideal markers for assessing individual genetic variability. Even though microsatellite MLH has been widely used as an indicator of inbreeding, it is not equivalent to the true inbreeding coefficient, which measures the probability that two alleles at a locus are identical by descent. Instead, MLH scores all homozygous loci that are identical by state, regardless of whether they are inherited from a common ancestor. Slate *et al*. (2004) showed that MLH was only weakly correlated with inbreeding coefficient in a large sample of domestic sheep from a population where inbreeding was common. In the same study, the inbreeding coefficient estimated from pedigree data detected evidence of inbreeding depression in morphological traits, while MLH did not (Slate *et al*. 2004).

Recently developed statistical methods allow the estimation of individual inbreeding coefficients (f) from microsatellite data in the absence of complete pedigrees (Ritland 1996a; Lynch & Walsh 1998). We will use the notation f' to indicate inbreeding coefficients estimated from microsatellite data to distinguish them from true inbreeding coefficients (f) estimated from pedigree data. The estimated inbreeding coefficient f' should, in theory, be superior to MLH for detecting inbreeding depression, as it also includes information on allele frequency (Ritland 1996b).

Here we investigate the relationship between individual heterozygosity (MLH and f') and three fitness-related traits: faecal counts of nematode eggs (FEC, an index of the combined effects of intensity of gastrointestinal nematode parasitism and host resistance influencing parasite fecundity; Coltman *et al*. 1999), summer body mass (a determinant of winter survival and reproductive success in mountain ungulates; Festa-Bianchet *et al*. 1997; Coltman *et al*. 2002) and horn growth (a sexually selected trait related to reproductive success in mountain ungulates; Coltman *et al*. 2002) in wild Alpine ibex (*Capra ibex*) males in the Gran Paradiso National Park (GPNP), Italy. We do so using parameters estimated from a large data set obtained from amplification of 32 microsatellite loci.

We predicted that MLH would be negatively correlated with FEC, and positively correlated with body mass and horn size. As the sign of expected relationships between f' and fitness-related traits is opposite to that expected for MLH, we predicted correlations of the opposite sign for f'. According to the handicap principle (Zahavi 1975; Zahavi & Zahavi 1997) exaggerated secondary sexual traits in males that are costly to produce, such as large horns, reliably advertise genetic quality. Secondary sexual traits are usually more phenotypically variable than other traits (Pomiankowski & Møller 1995). A comparison of standardized linear selection gradients on quantitative traits in natural populations suggested that sexual selection is stronger than viability selection (Hoekstra *et al*. 2001). Moreover, it has been shown that additive genetic variation is higher in sexually selected traits than in other traits (Pomiankowski & Møller 1995). Secondary sexual traits should therefore be more strongly affected by genetic effects (inbreeding depression or local effects) than other morphometric and physiological traits. We therefore expected stronger effects of genome-wide genetic diversity (MLH and f') on horn growth than on mass and FECs.

Alpine ibex males grow a distinct horn segment each year. Horn growth stops over winter, producing a very clear ring (annulus) around each horn. At GPNP the probability of over-winter mortality can be predicted from the size of the yearly horn growth increments (von Hardenberg *et al*. 2004). Horn growth decreases by up to 19% compared to the age-specific mean in each of the last two years prior to death; therefore yearly horn growth is an indicator of the onset of senescence (von Hardenberg *et al*. 2004). The two main hypotheses for the evolution of senescence are: (i) the mutation accumulation theory (Medawar 1952), according to which age-specific deleterious mutations accumulate because selection is more efficient at removing those expressed early in life; (ii) the antagonistic pleiotropy hypothesis (Williams 1957), according to which selection favours genes that enhance early performance at the expense of fitness later in life. The mutation accumulation theory predicts that inbreeding effects increase with age, while the antagonistic pleiotropy hypothesis does not predict such an increase (Charlesworth & Hughes 1996). We therefore tested if the effect of individual heterozygosity (MLH and f') on horn growth was age-specific (increasing for MLH and decreasing for f'). Because ibex horns show obvious yearly increments, they are ideal to test this hypothesis in a long-lived, iteroparous organism.

Finally, we explored the correlation between MLH and inbreeding in the GPNP Alpine ibex population and estimated the power to detect this correlation.

Methods

Study area and population

The study was conducted in the Gran Paradiso National Park (northwestern Italian Alps; 45°25′N, 07°34′E). Samples for genetic analyses were collected in three of the five main valleys of the Park (Orco, Cogne and Valsavarenche) from 116 live-captured and tagged males and from 37 untagged males found dead. Preliminary analyses, using both conventional population-based methods (F_{ST}) and Bayesian methods implemented in STRUCTURE (Pritchard *et al*. 2000), showed no evidence of population substructure among the three valleys (A. von Hardenberg, unpublished). Horn growth was measured on 109 males captured or found dead. Data on FEC were collected from 53 tagged males and summer mass gain was available for 50 tagged males in the study area of Levionaz, Valsavarenche (GPNP) (von Hardenberg 2005). Most of the Levionaz study area lies above tree line, between 2300 and *c*. 3300 m above sea level and is characterized by high-altitude alpine meadows (mainly *Festuca varia*), moraines, rock cliffs and glaciers. It is used by ibex from the end of May to December. The winter range (at 1700–2300 m above sea level) is characterized by pastures (near villages) and a mixed forest of *Larix decidua*, *Picea abies* and *Pinus cembra*. During this study, about 200 adult ibex were in the study area.

Field methods

Details about capture and marking methods are provided in von Hardenberg (2005). Individual males in Levionaz were repeatedly weighed from June to September each year with an electronic platform scale baited with salt (Bassano *et al*. 2003). Body mass was adjusted to the 1st of August of each year as described in von Hardenberg (2005).

Faecal egg counts

FEC were estimated twice a month from June to September for most tagged males in Levionaz in 2000–2004. Animals were observed from a distance of 10–50 m and at least 20 g of faeces were collected within 1–5 min of defecation. Faecal pellets were kept in plastic bags at 4 °C prior to analysis. FEC followed a modified McMaster technique (Ministry of Agriculture *et al*. 1971) and were expressed as number of eggs per gram of fresh faeces (EPG). At least two counts per sample were carried out in order to minimize measurement error. EPG was determined to the nearest 20 eggs/gram of faeces. Individuals experimentally treated with antihelmintics (von Hardenberg 2005), were excluded from analyses in the year of treatment. The arithmetic mean of EPG measured for each individual from June to September was used as a yearly index of infection as described in von Hardenberg (2005).

Horn growth measurement

The length of each yearly growth annulus was measured for both horns, along a central line on the front of the horn using a precision calliper to the nearest 1 mm. The average length of the left and right annuli was used for analysis. More details on horn growth measurement can be found in von Hardenberg *et al*. (2004).

Microsatellite analysis

A sample of cutaneous tissue for microsatellite analysis was collected from each live-captured animal and from carcasses of animals found dead during winter. Using a sterile blade, we cut a tiny piece of tissue (< 2 mm^3) from one ear and stored it at room temperature in collection tubes with 1–5 mL of 95% (w/v) ethanol solution. DNA was extracted from about 20 mg of tissue using the DNeasy Kit (QIAGEN GmbH) following the manufacturer's instructions. Polymerase chain reaction (PCR) were used to amplify 39 polymorphic microsatellite loci: MAF209, MAF36, OarHH35, BMS1350, TGLA441, Texan4, IDVGA30, BM81124, OarHH62, McM73, CSSM47, BM4208, RT1, HUJ1177, McM152, OarJMP29, BM121, BM1225, HEL1, SR6Q, SR26, ETH10, BM1818, SR25, ILST029, INRA185, FCB48, AE54, TGLA122, TGLA126, SR8, HAUT27, ILST030, SR24, SR15, BM4505, OMHC1, OarkP6, TGLA387. Details on methods used for PCR and electrophoresis are described in Maudet *et al*. (2001, 2002).

Data analysis

Hardy–Weinberg proportions and gametic disequilibrium for all possible pairs of loci were tested using the program GENEPOP version 3.4 (Raymond & Rousset 1995). Individual standardized multilocus heterozygosity was calculated as the ratio of the heterozygosity of an individual to the mean heterozygosity of those loci at which the individual was typed. The standardization avoids confounding because of possible systematic differences in loci used between individuals (Coltman *et al*. 1999). Individual inbreeding coefficients (f') were estimated from microsatellite data using SPAGEDI version 1.1 (Hardy & Vekemans 2002). This coefficient, calculated as intra-individual kinship coefficient (Ritland 1996b), estimates the kinship between alleles within an individual at a locus averaged over all loci according to the following formula:

$$f' = \Sigma_{i,l}\,[(S_{il} - P_{il})/\mathrm{P}_{il}]^2/n - 1$$

where S_{il} is an indicator variable with $S = 1$ if the individual is homozygous for allele i at locus l and $S = 0$ if it is heterozygous; P_{il} is the frequency of allele i at locus l and n is the number of typed loci for the individual. The sign of expected relationships between f' and fitness-related traits is opposite to that expected for MLH (for example, a negative relationship with horn size is expected for f' while a positive one is expected for MLH).

To avoid pseudoreplication because of measurements of the same individuals taken in different years, we fitted linear mixed-effects models (LME) implemented in the NLME package of S-PLUS 2000 (Pinheiro & Bates 2001) to test the relationship between MLH or f' and FEC and body mass. The LME function in S-Plus also allows models to be fitted with heteroscedastic within-group errors. We fitted LME models with the appropriate variance function whenever the within-group errors appeared to have unequal variances. We followed the model-building approach suggested by Pinhero & Bates (2001) for all fitted LME models. The significance of fixed terms was assessed using conditional *F*-tests. For LME models with different random terms, we chose the model with the lowest Akaike information criterion (AIC). To normalize error terms, we transformed the FEC data to ln(EPG + 1) prior to analyses. Treatment contrasts were used to assess differences within factors. Age was centred at 8 years to reduce multicollinearity whenever a quadratic term was included. To minimize the risk of type I error from multiple tests, all probabilities were corrected with the sequential Bonferroni method (Rice 1989). The effect of MLH and f' was tested as a single factor and as an interaction with age for all fitness traits.

Associations between alleles of specific loci and phenotypic traits were assessed under a dominance and an additive model. For each allele, we scored 1 if the allele was present and 0 if absent for the dominance model. We scored 0 if an allele was absent, 1 if one copy of the allele was present, and 2 if two copies of the allele were present for the additive model. We then fitted the same LME models as for MLH, but with the alleles as genetic terms instead of MLH (once for the dominance and once for the additive model). We fitted k-1 alleles because the k^{th} allele is non-independent. The significance of genetic associations was tested comparing the fit of a model with all genetic terms to one with no genetic terms.

The correlation between MLH and true inbreeding was estimated according to Slate *et al*. (2004) as:

$$r(\mathrm{MLH},f) = -\sigma(f)/\{[1 - \mathrm{E}(f')]\sigma(\mathrm{MLH})\}$$

where E(f') is the mean estimated inbreeding.

The variance in true inbreeding $\sigma^2(f)$ was estimated from the covariance in marker estimates of inbreeding as suggested by Ritland (1996b) using SPAGEDI version 1.1 (Hardy & Vekemans 2002). Power analyses followed Zar (1998).

Results

Hardy–Weinberg and linkage disequilibrium

Six of 39 loci (BM81124, OarJMP29, BM121, SR25, TGLA126 and SR24) that deviated significantly from Hardy–Weinberg proportions after Bonferroni correction were removed from all further analyses. Two loci (OMHC1 and TGLA387) were in linkage disequilibrium (and thus highly correlated) and we excluded TGLA387 from analyses, leaving 32 loci for subsequent analyses.

Faecal egg counts

We found no significant relationship between MLH and FEC or between f' and FEC after accounting for identity, age and year effects (Tables 1 and 2). The interactions MLH × age and f × age did not affect FEC. When we analysed the effect of each single locus on FEC (Fig. 1) we found that heterozygotes had lower mean FEC than homozygotes at 11 of 32 loci. However, the difference was statistically significant at only one locus (ETH10) after sequential Bonferroni correction.

Body mass

Body mass was not related to MLH or f' after accounting for identity, age and year effects (Tables 3 and 4). The interactions MLH × age and f' × age did not affect mass. When we analysed the effect of each locus separately (Fig. 1) we found that at 14 of 32 loci heterozygotes appeared to be heavier than homozygotes. However, no difference was significant after sequential Bonferroni correction.

Table 1 Wald test for the significance of terms included in a linear mixed effects (LME) model with faecal output of parasite eggs [ln(EPG + 1)] of Alpine ibex as the dependent variable, ibex identity as a grouping factor, a general positive-definite within-group error structure, a random slope for age (AIC = 244.87 for the model with a random slope for age vs. AIC = 246.35 for a model with a random intercept only), and age, year and MLH as independent variables. Data were collected at Levionaz (Gran Paradiso National Park, Italy), from 2000 to 2004. Standard errors in parentheses. Model is based on 164 observations from 53 individual ibex

	Coefficient	d.f.	*F*	*P*
Terms included in the model:				
Age	+0.21 (0.08)	1.105	20.23	≤ 0.0001
Year		4.106	20.22	≤ 0.0001
Rejected terms:				
MLH	+1.54 (0.09)	1.51	0.76	0.39
Age × MLH	–0.14 (0.08)	1.105	2.88	0.09

Table 2 Wald test for the significance of terms included in an LME model with faecal output of parasite eggs [ln(EPG + 1)] of Alpine ibex as the dependent variable, ibex identity as a grouping factor, a general positive-definite within-group error structure, a random slope for age (AIC = 242.84 for the model with a random slope for age vs. AIC = 244.97 for a model with a random intercept only), and age, year and inbreeding coefficient *f′* as independent variables. Data were collected at Levionaz (Gran Paradiso National Park, Italy), from 2000 to 2004. Standard errors in parentheses. Model is based on 164 observations from 53 individual ibex

	Coefficient	d.f.	*F*	*P*
Terms included in the model:				
Age	+0.06 (0.01)	1.105	17.78	≤ 0.0001
Year		4.105	20.09	≤ 0.0001
Rejected terms:				
f′	–0.86 (1.19)	1.51	2.18	0.15
Age × *f′*	+0.04 (0.14)	1.105	0.09	0.76

Table 3 Wald test for the significance of terms included in an LME model with body mass (kg) as the dependent variable, identity as a grouping factor, a general positive-definite within-group error structure, a random intercept, and age, age^2, year and MLH as independent variables for male Alpine ibex in Levionaz (Gran Paradiso National Park, Italy), 2000–2004. Standard errors in parenthesis. Model is based on 122 observations from 50 individuals

	Coefficient	d.f.	*F*	*P*
Terms included in the model:				
Age	+16.33 (1.86)	1.65	121.82	≤ 0.0001
Age^2	– 0.70 (0.06)	1.65	135.60	≤ 0.0001
Year		4.65	13.70	≤ 0.0001
Rejected terms:				
MLH	– 3.19 (13.49)	1.48	0.33	0.57
Age × MLH	– 0.05 (1.19)	1.65	0.002	0.96

Horn growth

Neither MLH nor *f′* affected horn annulus length as single factors (Tables 5 and 6), but their interactions with age had significant effects on annulus length (Tables 5 and 6). The slope of the regression between MLH and annulus length increased with age (Fig. 2 and Table 7). The opposite pattern was found for *f′*. Regressing MLH against annulus length at each age revealed a significant positive relationship at 9 years after sequential Bonferroni correction. The relationship between *f′* and annulus length at 9 years of age was not significant after sequential Bonferroni correction. The correlations between MLH and the length of annuli grown at 6–8 and at 10–12 years also showed positive trends (Table 7). The correlations between inbreeding coefficient *f′* and horn annulus size at 7–12 years of age showed negative trends which, however, were not significant after sequential Bonferroni correction (Table 7). When we analysed the effect of each single locus on horn annulus length we found that in 17 of 31 loci

Table 4 Wald test for the significance of terms included in an LME model with body mass (kg) as the dependent variable, identity as a grouping factor, a general positive-definite within-group error structure, a random intercept, and age, age^2, year and *f′* as independent variables in Alpine ibex males in Levionaz (Gran Paradiso National Park, Italy), 2000–2004. Standard errors in parentheses. Sample size was 122 observations from 50 individuals

	Coefficient	d.f.	*F*	*P*
Terms included in the model:				
Age	+16.23 (1.15)	1.65	121.89	≤ 0.0001
Age^2	– 0.69 (0.06)	1.65	136.23	≤ 0.0001
Year		4.65	13.76	≤ 0.0001
Rejected terms:				
f′	+9.22 (18.22)	1.48	0.03	0.86
Age × *f′*	– 1.36 (1.99)	1.65	0.47	0.50

Table 5 Wald test for the significance of terms included in an LME model with horn annulus length (mm) as the dependent variable, identity as a grouping factor, a general positive-definite within-group error structure, a random intercept, and age, year, MLH and the interaction term age × MLH as independent variables in Alpine ibex males from the Gran Paradiso National Park, Italy 2000–2004. Standard errors in parentheses. Sample size was based on 750 observations from 109 individuals

	Coefficient	d.f.	*F*	*P*
Terms included in the model:				
Age	– 5.79 (1.87)	1.623	747.59	≤ 0.0001
Year		16.623	4.59	≤ 0.0001
MLH	+8.51 (5.00)	1.107	0.171	0.67
Age × MLH	+3.54 (0.92)	1.623	14.83	0.0001

Table 6 Wald test for the significance of terms included in an LME model with horn annulus length (mm) as the dependent variable, identity as a grouping factor, a general positive-definite within-group error structure, a random intercept, and age, year, *f′* and the interaction term age × *f′* as independent variables in Alpine ibex males from the Gran Paradiso National Park, Italy 2000–2004. Standard errors in parentheses. Sample size was based on 750 observations from 109 individuals

	Coefficient	d.f.	*F*	*P*
Terms included in the model:				
Age	– 5.54 (0.25)	1.623	743.41	≤ 0.0001
Year		16.623	4.51	≤ 0.0001
f′	+4.22 (6.26)	1.107	1.89	0.17
Age × *f′*	– 3.91 (1.35)	1.623	8.30	0.0041

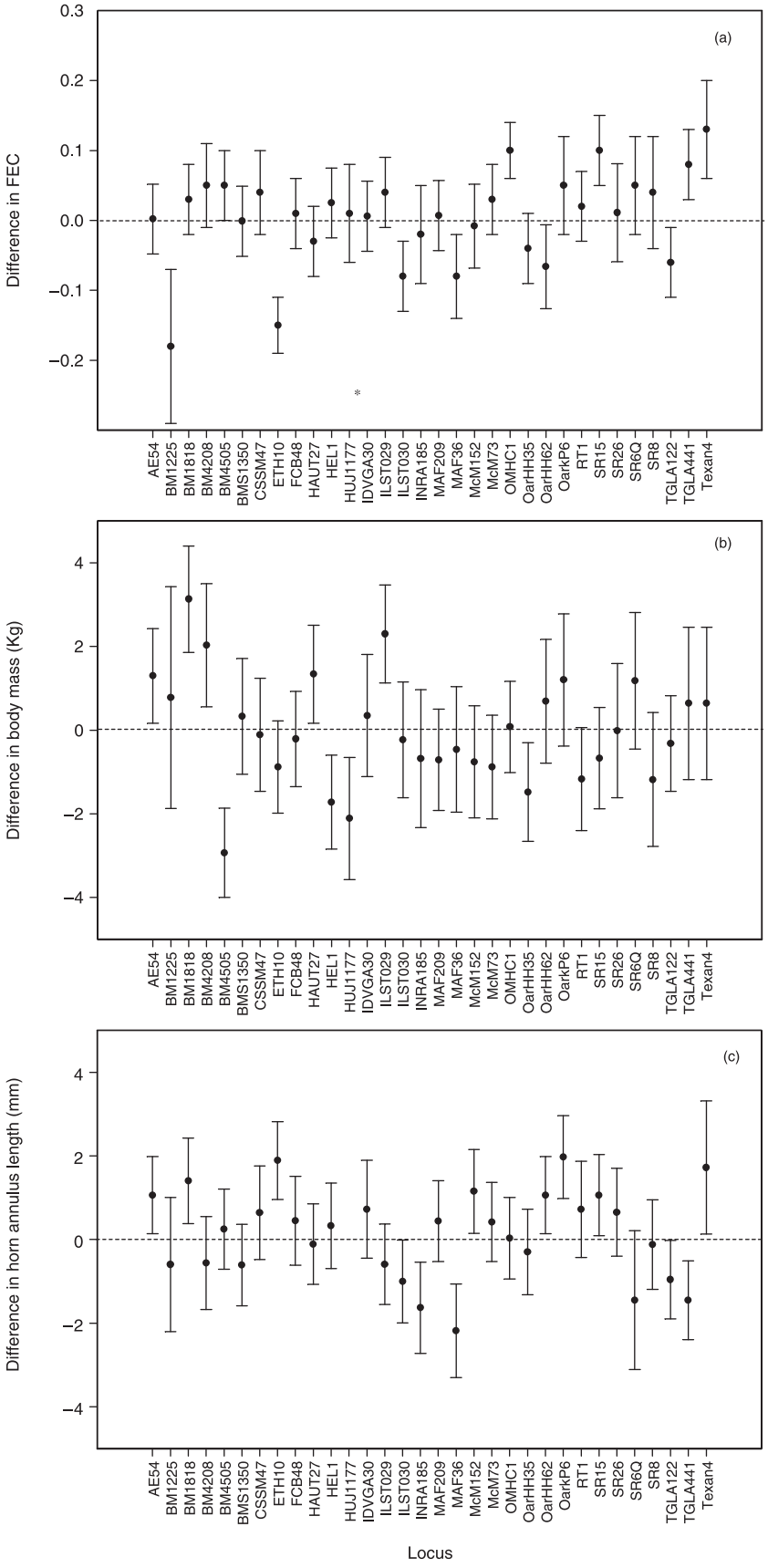

Fig. 1 Difference in FEC, body mass, and horn annulus length between heterozygotes and homozygotes at 32 microsatellite loci for adult male Alpine ibex (*Capra ibex*) in Levionaz (Gran Paradiso National Park, Italy). Values higher than '0' indicate a larger value in heterozygotes compared to homozygotes. Error bars represent Standard Errors. Values and significances have been estimated with Linear mixed effects models with ID as grouping factor, a general positive-definite within-group error structure, and the following fixed terms structures: Body mass ~ Age + Age^2 + Year + Locus; FEC ~ Age + Year + Locus; Annulus length ~ Age + Year + Locus. Significance levels were corrected using the Sequential Bonferroni Method. Significant differences are marked with an asterisk. The model could not be computed for HUJ1177 on horn annulus size because of singularity in backsolving.

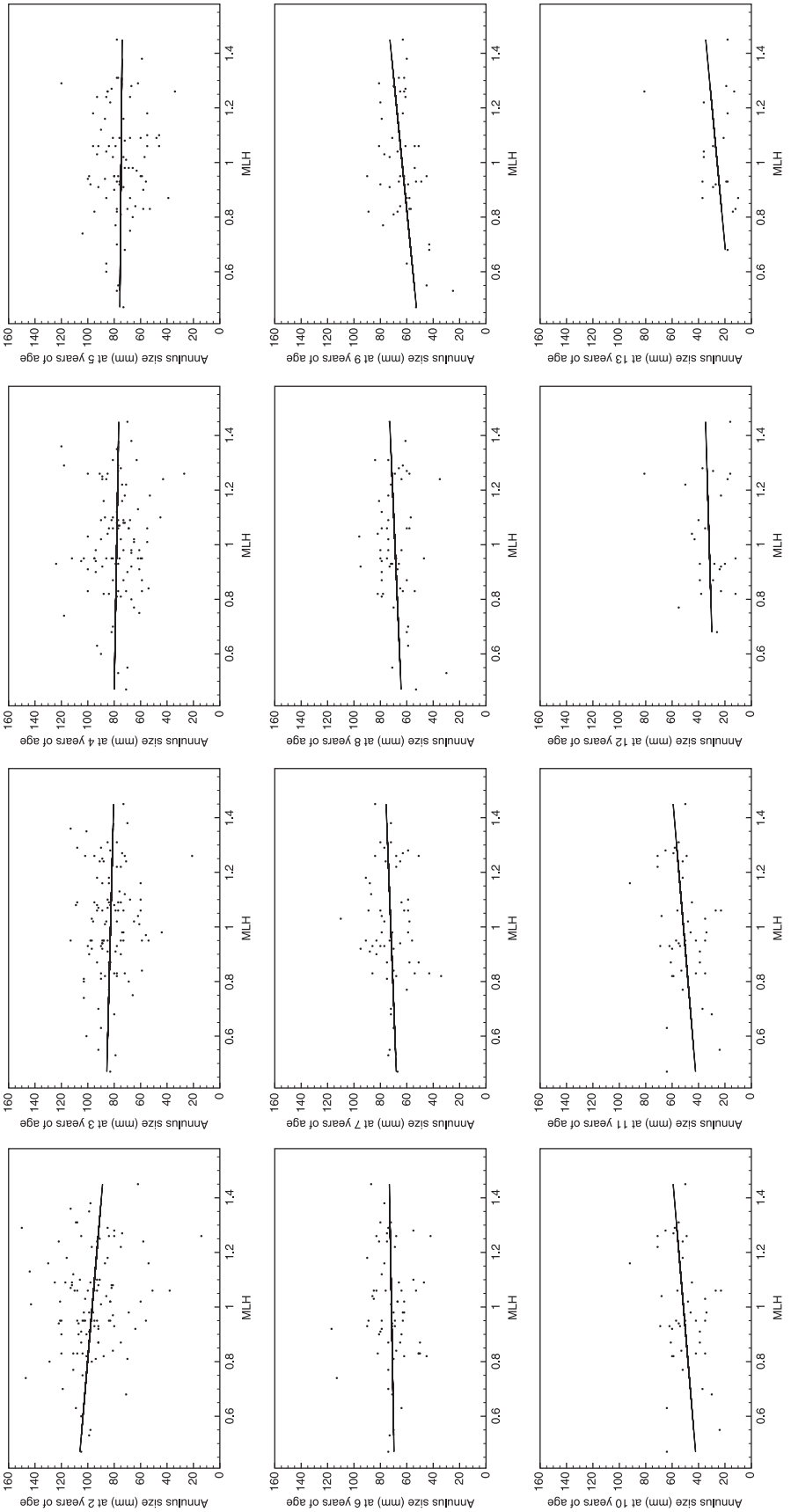

Fig. 2 Relationship between MLH and horn annuli grown at 2–13 years of age in male Alpine ibex in Gran Paradiso National Park (Italy).

Table 7 Relationships between MLH, *f* and annulus size (Ln transformed) grown at different ages in Alpine ibex in the Gran Paradiso National Park. *P* (Bonf.) is the *P* value after Bonferroni sequential correction (Rice 1989). Significant results are underlined

Annulus	Value	Standard error	*F*	d.f.	*P*	*P* (Bonf.)	R^2
MLH							
2	– 0.2765	0.1445	3.662	1107	0.0583	NS	0.03309
3	– 0.1087	0.1079	0.2141	1105	0.3158	NS	0.009584
4	– 0.0825	0.1141	0.2237	1100	0.4716	NS	0.005196
5	– 0.0604	0.1219	0.2453	1.81	0.6217	NS	0.003019
6	+0.0573	0.1164	0.4921	1.64	0.6243	NS	0.00377
7	+0.1194	0.1243	0.9233	1.56	0.3407	NS	0.01622
8	+0.1724	0.1288	1.339	1.52	0.1864	NS	0.03333
9	+0.4113	0.129	10.16	1.47	0.0025	0.05	0. 1778
10	+0.3738	0.2063	1.8114	1.43	0.0771	NS	0.0709
11	+0.5119	0.4097	1.2495	1.30	0.2211	NS	0.04947
12	+0.0167	0.4994	0.0334	1.23	0.9736	NS	4.86E-05
f'							
2	+0.2607	0. 1869	1.947	1107	0.1658	NS	0.01787
3	+0.2753	0.1368	4.047	1105	0.0468	NS	0.03712
4	+0.2136	0.1524	1.964	1100	0.1642	NS	0.01926
5	+0.3124	0.1612	3.755	1.81	0.0561	NS	0.04431
6	+0.0748	0.1588	0.2219	1.64	0.6392	NS	0.003455
7	– 0.1637	0.1853	0.7808	1.56	0.3807	NS	0.01375
8	– 0.2675	0.1901	1.979	1.52	0.1654	NS	0.03666
9	– 0.4975	0.198	6.313	1.47	0.0155	NS	0.1184
10	– 0.3996	0.3093	1.669	1.43	0.2033	NS	0.03736
11	– 0.5953	0.6137	0.9409	1.30	0.3398	NS	0.03041
12	– 0.2488	0.8406	0.08763	1.23	0.7699	NS	0.003796

Table 8 Genetic associations between alleles of ETH10 and OMHC1 loci and FEC, horn annulus size and body mass in male Alpine ibex in the Gran Paradiso National Park. Significant values are underlined

		Dominance model			Additive model		
Term	Dependent	LogLik. Ratio	*P*	d.f.	LogLik. Ratio	*P*	d.f.
ETH10	FEC	6.91	0.03	2	12.09	0.002	2
ETH10	Body Mass	0.39	0.82	2	0.67	0.72	2
ETH10 × age	Annulus	14.24	0.007	4	38.28	< 0.001	4
OMHC1	FEC	5.22	0.16	3	7.03	0.31	6
OMHC1	Body Mass	2.63	0.45	3	9.17	0.16	6
OMHC1 × age	Annulus	10.28	0.24	8	25.91	0.03	14

heterozygotes appeared to have longer mean horn annuli than homozygotes even though these differences were not significant after applying the sequential Bonferroni method (Fig. 1).

Testing specific allele associations using dominance and additive models

In light of the significant effect of heterozygosity at ETH10 on FEC, we tested for association between specific ETH10 alleles and FEC and other traits using both dominance and additive models of genetic effects. We also tested allele–specific associations for OMHC1, a locus located in the MHC. Significant associations were found between ETH10 alleles and FEC and between ETH10 alleles and horn growth, but not body mass (Table 8). We found no significant associations of specific OMHC1 alleles with FEC or mass. However, the interaction between age and specific OMHC1 alleles on the length of horn annuli was significant (additive model only; Table 8).

MLH-inbreeding correlation and variance in inbreeding

The correlation observed between MLH and individual estimated inbreeding coefficients (f') was –0.65 ($P < 0.001$). Mean f' of the population was E(f') = 0.026. The covariance among marker loci in the estimated individual inbreeding coefficients was very low [$\sigma^2(f) = 0.00032$] and not different from 0 (SE = 0.00023) suggesting low variance in true inbreeding. The estimated correlation between MLH and true inbreeding was low [r(MLH, f) = –0.095; power($\alpha = 0.05$, $n = 159$) = 0.22]. It would require a minimum sample of $n = 870$ to demonstrate that this correlation coefficient is significantly different from 0 with a power of 0.8.

Discussion

We found no relationship between MLH or estimated individual inbreeding coefficients (f', estimated from microsatellite data) and faecal egg counts or body mass in Alpine ibex males. Moreover, trends were typically opposite to expectations, except for the relationship between f' and body mass. These results are not surprising given a recent meta-analysis of HFC in wild populations which revealed a weak effect size for MLH (mean $r = 0.0274$) (Coltman & Slate 2003) and which estimated that a minimum sample size of 600 was required for a nominal power of 80% for detecting HFC. The same study revealed an even lower mean effect size of MLH on physiological traits (including parasite resistance) only (mean $r = 0.0075$) or on morphometric traits (including body mass) only (mean $r = 0.0052$) (Coltman & Slate 2003). Alternatively, the choice of microsatellite loci used in this study might also have influenced the results. Acevedo-Whitehouse *et al*. (2005) found that heterozygosity at microsatellites linked to immunogenetic regions was an important predictor of bovine tuberculosis in wild boars *Sus scrofa*. Perhaps if we used more microsatellites linked to other immunogenetic regions local effects would have contributed more to the relationship between MLH and FEC.

Nevertheless, we suspect that we have detected local effects at two loci in our data set. Despite the lack of an overall effect of heterozygosity on FEC and body mass, heterozygotes at locus ETH10 had significantly lower FEC than homozygotes. Significant effects were not found for any other locus, and the sign of the difference between heterozygotes and homozygotes appeared to be randomly distributed among loci. We also found associations between specific ETH10 alleles and both FEC and the length of horn annuli (but not body mass). ETH10 is in a region of chromosome 5 known to be linked to growth rate and carcass traits in cattle (Li *et al*. 2004). Recent investigations have shown that ETH10 seems to be under selection in reintroduced Alpine ibex populations in Switzerland (I. Biebach and L. Keller personal communication). The positional candidate gene of ETH10 is insulin-like growth factor 1 (igf1). While an extensive literature suggests that igf1 plays an important role in immunity, other findings suggest that it is not an obligate immunoregulator (review in Dorshkind & Horseman 2000). Further studies should analyse the possibility that ETH10 is in linkage disequilibrium with this or other genes affecting parasite resistance in Alpine ibex. Associative overdominance is at least partially responsible for an association between birth weight and MLH in red deer *Cervus elaphu*s (Slate & Pemberton 2002). We did not attempt to analyse directly the relationship between igf1 and FEC in Alpine ibex because, even though surely interesting, such a study would be beyond the scopes of the present research.

The loci we analysed included OMHC1 which is linked to the MHC and has been shown to be associated with FEC in Soay sheep (Paterson *et al*. 1998). In Alpine ibex, we did not find significant association between specific OMHC1 alleles and neither FEC nor body mass. However, we found a significant interaction between age and specific OMHC1 alleles on the length of horn annuli. This result agrees with Ditchkoff *et al*. (2001) who showed that antler development of white-tailed deer was associated with variation of loci at the major histocompatibility complex (MHC) level.

Unlike FEC or body mass, MLH was positively correlated with the length of horn annuli grown at 6–13 years of age and the slope of the relationship between annulus length and MLH increased with age. The relationship between individual inbreeding coefficient (f') and the length of annuli grown at 7–13 years of age was negative as predicted. While it has been argued that HFC should decline with age, because growth and survival differences are maximal early in life (David 1998), the positive age-specific relationship between MLH and horn growth is as predicted by the mutation accumulation theory for the evolution of senescence (Charlesworth & Hughes 1996). The relative roles of mutation accumulation vs. antagonistic pleiotropy in the evolution of senescence remains controversial, and data mostly come from laboratory studies on semelparous invertebrates (for example see Hughes *et al*. 2002 and Rose *et al*. 2002).

Ibex males in GPNP do not reach an asymptote in body mass until 10–12 years (von Hardenberg 2005). Male reproductive success, which we assume to be dependent on horn and body size, should peak at about the same age. It is possible that genetic effects on horn growth start to be evident only when male ibex participate actively in the rut. As rut-related energetic expenditure peaks, only high-quality males can afford the costs of reproduction while investing in horn growth (Pelletier *et al*. in press). There is much evidence that horns and antlers reliably advertise

male quality in ungulates. Coltman *et al*. (2002) found a correlation between horn length and reproductive success in mature bighorn rams *Ovis canadensis*. Positive effects of horn length on lifetime and seasonal reproductive success have also been shown in feral sheep (Preston *et al*. 2001), and Kruuk *et al*. (2002) found a positive relationship between antler size and mating success in red deer. Antler size in red deer also correlates positively with sperm quantity and quality (Malo *et al*. 2005). In Alpine ibex, males aged 5–11 years that grew annuli shorter than the population age-specific average had reduced survival chances over the following two years (von Hardenberg *et al*. 2004). Because the survival of male Alpine ibex aged 2–8 years is extremely high (typically over 98% a year, Toïgo *et al*. 1997), our results are unlikely to be due to selective mortality of young males. The larger sample size for younger males is mostly due to the fact that we targeted young adults for capture and marking.

Our results suggest that horns are honest signals of male genetic quality in Alpine ibex (von Hardenberg *et al*. 2004). Indirect evidence for a relationship between secondary sexual traits and genetic variability in ungulates was also provided by Ditchkoff *et al*. (2001) and by Fitzsimmons *et al*. (1995) who showed that large-horned bighorn rams were more heterozygous than small-horned rams aged 6–8 years. Body weight and FEC are less accurately measured than horn growth because both may vary with season and time of day. Measurement error may therefore have limited our power to detect a similar relationship for these traits.

Correlations between MLH, based on microsatellite markers, and fitness traits have usually been interpreted as evidence of inbreeding depression (Pemberton 2004). Two recent papers, however, show that individual heterozygosity is only weakly correlated with individual inbreeding coefficients (Balloux *et al*. 2004; Slate *et al*. 2004). We found that the correlation between MLH and inbreeding was extremely low. In addition, the variance in inbreeding, estimated as the covariance among marker loci inbreeding estimates, was very low and not significantly different from 0, suggesting that HFC for horn growth may be largely due to the local effects of specific microsatellites in linkage disequilibrium with loci directly affecting fitness.

Theoretically f' should be a better measure of inbreeding than MLH because it includes information on allele frequency. Acevedo-Whitehouse *et al*. (2003) showed that an index of 'internal relatedness', estimated from microsatellite markers similarly as is f', influenced the susceptibility to pathogens in wild Californian sea lions (*Zalophus californianus*). Inbreeding coefficients (estimated from pedigree data) but not MLH detected inbreeding depression for morphological traits in domestic sheep (Slate *et al*. 2004). Contrary to predictions, however, we found that estimated individual inbreeding coefficients performed worse than MLH as an index of individual genetic variability. Indeed, while we found a significant relationship between MLH and horn annulus length grown at 9 years of age, such significant relationship could not be detected using f'. The inherent, possibly inevitable, inaccuracy of the indirect regression approach used to estimate f in the absence of pedigree information (Ritland 1996a; Lynch & Walsh 1998) may have caused too much noise around the inferred parameters, limiting their usefulness. Thomas *et al*. (2002) showed that several indirect marker-based methods were poor estimators of heritability of body weight in free-ranging Soay sheep, *Ovis aries*. Coltman (2005) showed that marker-based estimates of heritability were imprecise and downwardly biased.

In conclusion, we found evidence for a heterozygosity–fitness correlation in a secondary sexual trait (horn growth) but not in body mass or in FEC in Alpine ibex. The effect of heterozygosity on horn growth was strongly age dependent in accordance with the predictions of the mutation accumulation theory for the evolution of senescence (Charlesworth & Hughes 1996). To the best of our knowledge, this is the first study to find support for the mutation accumulation theory in a free-ranging vertebrate.

We also found lower FECs for heterozygotes compared to homozygotes at a single microsatellite locus. The possibility that this result may be due to associative overdominance with loci directly involved in parasite resistance merits further exploration. Inbreeding coefficients, estimated from microsatellite data, did not appear to be a better indicator of individual genetic variation than MLH. This is consistent with Pemberton (2004) who suggested that inbreeding coefficients in HFC studies should be used only if they can be calculated from pedigree data.

Acknowledgements

We are indebted to the park wardens of GPNP, particularly chief-warden L. Jocollé, M. Ferrando (deceased) and D. Favre for their unwavering help and support during capture operation and subsequent monitoring of tagged ibex. J.F. Aublet, E. Di Bella, C. Caux, V. Careau, F. Cattaneo, K. Gauthier-Hétu and A. Lebel, assisted with field work and analysed ibex faeces in the laboratory. We are gratefull to N. Pidancier, C. Maudet, D. Rioux, L. Gielly, P. Taberlet and E. Johnson for the genetic analyses. Financial support was provided to A.v.H. by the Gran Paradiso National Park, the natural Sciences and Engineering Research Council of Canada (Discovery grant to M.F-B), the Université de Sherbrooke and the German Academic Exchange Service (DAAD). Support for genetic analyses was granted by the Vanoise-Gran Paradiso Interreg IIA project of the European Union. A research grant from the Royal Society and the University of Sheffield provided supplemental funding for genetic analyses and supported A.v.H. visits in D.C.'s laboratory. The Québec-Italie programme provided financial support to M.F-B and P.L. Many thanks are due to François Balloux, Tim Coulson, Bill Shipley, Don Thomas and three anonymous referees for useful comments on previous versions of the manuscript.

References

Acevedo-Whitehouse K, Gulland F, Greig D, Amos W (2003) Disease susceptibility in Californian sea lions. *Nature*, **422**, 35.

Acevedo-Whitehouse K, Vincente J, Gortazar C, Höfle U, Fernandez de Mera IG, Amos W (2005) Genetic resistance to bovine tuberculosis in the Iberian wild boar. *Molecular Ecology*, **14** (10), 3209–3217.

Balloux F, Amos W, Coulson T (2004) Does heterozygosity estimate inbreeding in real populations? *Molecular Ecology*, **13**, 3021–3031.

Bassano B, von Hardenberg A, Pelletier F, Gobbi G (2003) A method to weigh free-ranging ungulates without handling. *Wildlife Society bulletin*, **31**, 1205–1209.

Charlesworth D, Charlesworth B (1987) Inbreeding depression and its evolutionary consequences. *Annual Review of Ecology and Systematics*, **18**, 237–268.

Charlesworth B, Hughes KA (1996) Age-specific inbreeding depression and components of genetic variance in relation to the evolution of senescence. *Proceedings of the National Academy of Sciences, USA*, **93**, 6140–6145.

Coltman DW (2005) Testing marker-based estimates of heritability in the wild. *Molecular Ecology*, **14** (8), 2593–2599.

Coltman DW, Slate J (2003) Microsatellite measures of inbreeding: a meta-analysis. *Evolution*, **57**, 971–983.

Coltman DW, Pilkington JG, Smith JA, Pemberton JM (1999) Parasite-mediated selection against inbred Soay sheep in a free-living, island population. *Evolution*, **53**, 1259–1267.

Coltman DW, Festa-Bianchet M, Jorgenson JT, Strobeck C (2002) Age-dependent sexual selection in bighorn rams. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **269**, 165–172.

David P (1998) Heterozygosity-fitness correlations: new perspectives on old problems. *Heredity*, **80**, 531–537.

Ditchkoff SS, Lochmiller RL, Masters RE, Hoofer SR, Van Den Bussche RA (2001) Major-histocompatibility-complex-associated variation in secondary sexual traits of white-tailed deer (*Odocoileus virginianus*): evidence for good-genes advertisement. *Evolution* **55** (3), 616–625.

Dorshkind K, Horseman ND (2000) The roles of prolactin, growth hormone, insulin-like growth factor-I, and thyroid hormones in lymphocyte development and function: insights from genetic models of hormone and hormone receptor deficiency. *Endocrine Reviews*, **21** (3), 292–312.

Festa-Bianchet M, Jorgenson JT, Bérubé CH, Portier C, Wishart WD (1997) Body mass and survival of bighorn sheep. *Canadian Journal of Zoology*, **75**, 1372–1379.

Fitzsimmons NN, Buskirk SW, Smith MH (1995) Population history, genetic variability and horn growth in bighorn sheep. *Conservation Biology*, **9** (2), 314–323.

Hansson B, Westerberg L (2002) On the correlation between heterozygosity and fitness in natural populations. *Molecular Ecology*, **11**, 2467–2474.

von Hardenberg A (2005) *Sénescence, Sélection Sexuelle et Dynamique de Population du Bouquetin des Alpes* (Capra ibex). PhD Thesis. Université de Sherbrooke, Sherbrooke, Canada.

von Hardenberg A, Bassano B, Arranz MPZ, Bogliani G (2004) Horn growth but not asymmetry heralds the onset of senescence in male alpine ibex (*Capra ibex*). *Journal of Zoology*, **263**, 425–432.

Hardy OJ, Vekemans X (2002) SPAGEDI: a versatile computer program to analyse spatial genetic structure at the individual or population levels. *Molecular Ecology Notes*, **2**, 618–620.

Hoekstra HE, Hoekstra JM, Berrigan D *et al.* (2001) Strength and tempo of directional selection in the wild. *Proceedings of the National Academy of Sciences, USA*, **98**, 9157–9160.

Hughes KA, Alipaz JA, Drnevich JM, Reynolds RM (2002) A test of evolutionary theories of aging. *Proceedings of the National Academy of Sciences, USA*, **99**, 14286–14291.

Kruuk LEB, Slate J, Pemberton JM, Brotherstone S, Guinness F, Clutton-Brock T (2002) Antler size in red deer: heritability and selection but no evolution. *Evolution*, **56** (8), 1683–1695.

Li C, Basarab J, Snelling WM *et al.* (2004) Assessment of positional candidate genes *myf*5 and *igf*1 for growth on bovine chromosome 5 in commercial lines of *Bos taurus*. *Journal of Animal Science*, **82**, 1–7.

Lynch M, Walsh B (1998) *Genetics and Analysis of Quantitative Traits*. Sinauer Associates, Sunderland, Massachusetts.

Malo AF, Roldan ERS, Garde J, Soler AJ, Montserrat G (2005) Antlers honestly advertise sperm production and quality. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **272**, 149–157.

Maudet C, Luikart G, Taberlet P (2001) Development of microsatellite multiplexes for wild goats using primers designed from domestic bovidae. *Genetics, Selection, Evolution*, **33**, S193–S203.

Maudet C, Miller C, Bassano B *et al.* (2002) Microsatellite DNA and recent statistical methods in wildlife conservation management: applications in Alpine ibex [*Capra ibex* (*ibex*)]. *Molecular Ecology*, **11** (3), 421–436.

Medawar PB (1952) *An Unsolved Problem of Biology*. H. K. Lewis, London.

Ministry of Agriculture, Fisheries, and Food (1971) *Manual of Veterinary Parasitological Laboratory Techniques*. Her Majesty's Stationery Office, London.

Paterson S, Wilson K, Pemberton JM (1998) Major histocompatibility complex variation associated with juvenile survival and parasite resistance in a large unmanaged ungulate population (*Ovis aries* L.). *Proceedings of the National Academy of Sciences, USA*, **95**, 3714–3719.

Pelletier F, Hogg JT, Festa-Bianchet M (2006) Male-mating effort in a polygynous ungulate. *Behavioral Ecology and Sociobiology*, in press.

Pemberton J (2004) Measuring inbreeding depression in the wild: the old ways are the best. *Trends in Ecology & Evolution*, **19**, 613–615.

Pinheiro JC, Bates DM (2001) *Mixed Effects Models in S and S-Plus*. Springer Verlag, New York.

Pomiankowski A, Møller AP (1995) A resolution of the lek paradox. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **260**, 21–29.

Preston BT, Stevenson IR, Pemberton JM, Wilson K (2001) Dominant males lose out by sperm depletion. *Nature*, **409**, 681–682.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics*, **155** (2), 945–959.

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.

Rice WR (1989) Analyzing tables of statistical tests. *Evolution*, **43** (1), 223–225.

Ritland K (1996a) A marker-based method for inferences about quantitative inheritance in natural populations. *Evolution*, **50**, 1062–1073.

Ritland K (1996b) Estimators for pairwise relatedness and individual inbreeding coefficients. *Genetical Research*, **67**, 175–185.

Rose MR, Drapeau MD, Yazdi PG *et al.* (2002) Evolution of late-life mortality in *Drosophila melanogaster*. *Evolution*, **56**, 1982–1991.

Scribner KT, Pearce J (2000) Microsatellites: evolutionary and methodological background and empirical applications at individual, population, and phylogenetic levels. In: *Molecular Methods in Ecology* (ed. Baker A), pp. 235–273. Blackwell Scientific, London.

Slate J, Pemberton JM (2002) Comparing molecular measures for detecting inbreeding depression. *Journal of Evolutionary Biology*, **15**, 20–31.

Slate J, David P, Dodds KG *et al.* (2004) Understanding the relationship between the inbreeding coefficient and multilocus heterozygosity: theoretical expectations and empirical data. *Heredity*, **93**, 255–265.

Thomas SC, Coltman DW, Pemberton JM (2002) The use of marker-based relationship information to estimate the heritability of body weight in a natural population: a cautionary tale. *Journal of Evolutionary Biology*, **15**, 92–99.

Toïgo C, Gaillard J-M, Michallet J (1997) Adult survival of the sexually dimorphic Alpine ibex (*Capra ibex ibex*). *Canadian Journal of Zoology*, **77**, 75–79.

Williams GC (1957) Pleiotropy, natural selection and the evolution of senescence. *Evolution*, **11**, 398–411.

Zahavi A (1975) Mate selection—a selection for a handicap. *Journal of Theoretical Biology*, **53**, 205–213.

Zahavi A, Zahavi A (1997) *The Handicap Principle*. Oxford University Press, Oxford, New York.

Zar JH (1998) *Biostatistical Analysis*, 4th edn. Prentice Hall, New York.

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