

Low Heterozygosity at Microsatellite Markers in Iberian Red Deer with Small Antlers

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Abstract

Deer antlers are costly structures subjected to directional sexual selection that may be sensitive to heterozygosity. However, a relationship between heterozygosity and antler development has only been found for select protein-coding loci and MHC genes in one deer species (the white-tailed deer *Odocoileus virginianus*). Here, we study the relationship between multilocus heterozygosity at 11 microsatellite markers and antler size (AS) in a sample of 367 Iberian red deer males (*Cervus elaphus hispanicus*) from two study areas with different ecological and genetic conditions. We found that males with very small antlers (10% of the sampled individuals with the lowest values of AS) had lower levels of heterozygosity than those with bigger antlers (significant effect in an analysis of variance, $P = 0.011$). This relationship was noticeable mainly in situations of low genetic diversity, where the differences in heterozygosity between groups of males were greater. Finally, we conducted analyses to address the hypotheses proposed by the heterozygosity–fitness correlation, and we found the local effect as the most likely hypothesis. Our findings reveal an expected but not previously detected association between low heterozygosity and reduced AS, with implications for red deer evolution and management.

Key words: *Cervus elaphus hispanicus*, fitness traits, genetic diversity, heterozygosity–fitness correlation, inbreeding

The expression of phenotypic traits subjected to directional sexual selection may depend not only on additive genetic effects but also on individual heterozygosity (Rowe and Houle 1996; Tomkins et al. 2004; Reid et al. 2005; Kempnaers 2007; Fromhage et al. 2009), and many studies have shown a link between overall heterozygosity at molecular markers and traits related to fitness (see e.g., Coulson et al. 1998; Slate and Pemberton 2002; reviews in Coltman and Slate 2003; Chapman et al. 2009).

Three main hypotheses may explain heterozygosity–fitness correlation in natural populations: direct effect, general effect, and local effect (Hansson and Westerberg 2002). The direct effect hypothesis proposes a heterozygote advantage as a result of functional overdominance at scored loci. The general effect hypothesis defends an apparent overdominance at scored loci due to its positive correlation with genome-wide heterozygosity and negative correlation with inbreeding. The local effect hypothesis proposes an apparent overdominance at scored loci due to their linkage disequilibrium with fitness loci.

Heterozygosity–fitness correlation has been widely assessed with microsatellite markers. The use of microsatellite

markers would reject the direct effect hypothesis because it is assumed that they are selectively neutral (Scribner and Pearce 2000). So, general and local effects become the main hypotheses in microsatellite heterozygosity–fitness correlations. However, the general effect hypothesis presents some drawbacks. Firstly, heterozygosity at microsatellite loci may poorly reflect genome-wide genetic diversity in natural populations (DeWoody and DeWoody 2005; Väli et al. 2008). Likewise, individual heterozygosity at microsatellite markers and actual inbreeding coefficient may be weakly correlated (Balloux et al. 2004; Slate et al. 2004), and hence, the correlation between multilocus heterozygosity (estimated with microsatellite markers) and the size of traits presumably associated with fitness (Allendorf and Leary 1986) would hardly detect inbreeding depression. In spite of these drawbacks, for heterozygosity–fitness correlation achieved with microsatellite markers, a general effect cannot be completely rejected because individual heterozygosity and actual inbreeding coefficient can be strongly correlated under certain circumstances (Hedrick et al. 2001; Balloux et al. 2004; Chapman et al. 2009; Ruiz-López et al. 2009). On the other hand, the local effect hypothesis requires

linkage disequilibrium, which occurs, for example, as a consequence of recently bottlenecked-and-expanded populations (Hansson and Westerberg 2002). Thus, the demographic history of populations has important consequences in the intensity and the genetic causes of heterozygosity–fitness correlations (Chapman et al. 2009).

In red deer (*Cervus elaphus*), as in other ungulate species like bighorn sheep (*Ovis canadensis*; Coltman et al. 2001) and Soay sheep (*Ovis aries*; Preston et al. 2001), the size of male weapons (antlers and horns) appear positively related to the fitness of individuals (Kruuk et al. 2002), as it is expected from sexual selection based on male–male competition (Darwin 1871; Andersson 1994).

Male weapons (like most sexually selected traits) can be useful traits to detect heterozygosity–trait size correlation because they should be more affected by genetic effects than other morphometric or physiological traits (Pomiankowski and Møller 1995; von Hardenberg et al. 2007). A heterozygosity–weapon size relationship has been found in bighorn sheep (*O. canadensis*; Fitzsimmons et al. 1995), Alpine Ibex (*Capra ibex*; von Hardenberg et al. 2007), and white-tailed deer (*Odocoileus virginianus*; Scribner et al. 1989; Ditchkoff et al. 2001). Although horns and antlers share very similar evolutionary functions, antlers may be particularly useful to detect heterozygosity–fitness correlations. On one hand, antlers have been described as the fastest growing tissues in the animal kingdom (Goss 1983). Their annual growth and replacement entail very high nutritional demand and mineral requirements (Meister 1956; Asleson et al. 1997). On the other hand, antler development implicates the action of androgenic hormones (Morris and Bubenik 1982), which bear immunosuppressive effects (Folstad et al. 1989). Because antlers are costly tissues to produce (Ditchkoff et al. 2001), they are expected to show condition dependence which, in turn, is likely determined by the overall genetic quality of individuals (Andersson 1982). Additionally, traits related to male–male competition can retain high levels of nonadditive genetic variance (Reid 2007). Thus, the capture of genetic diversity expected in antler development could favor the correlation between heterozygosity at neutral markers and fitness under the general (Hedrick et al. 2001; Balloux et al. 2004; Ruiz-López et al. 2009) or the local effect hypotheses (Paterson et al. 1998; Da Silva et al. 2009). On the other hand, the same feature (antlers are costly tissues with strong condition dependence) might hinder the heterozygosity–fitness correlation. Environmental or physiological factors that influence individual condition should greatly affect antler development every year. A given genotype might be associated with different values of antler size (AS) relative to age along its lifespan depending on the environmental or physiological circumstances prevailing each year. This might make antlers a poorly suited trait to find heterozygosity–fitness correlations. Under this situation, a proportional increment of AS as heterozygosity increases could not occur, although perhaps some relationship could be detected at the extreme values of the distribution range. For instance, individuals below a certain threshold of trait size or heterozygosity level may reveal some association between both variables.

Despite the expectation from theoretical grounds (Hartl et al. 1991; Kruuk et al. 2003, see above), the relationship of heterozygosity on antler development has not been found for red deer. This may be because of the high dependence of antler growth on environmental variance and covariance (Scribner et al. 1989; Harris et al. 2002; Kruuk et al. 2002; Mateos et al. 2008). Heterozygosity at select protein-coding loci and in MHC loci has been shown to relate to antler development in white-tailed deer (*O. virginianus*; Scribner et al. 1989; Ditchkoff et al. 2001). The heterozygosity–fitness correlations by using these functional loci can be due to any of the three hypotheses outlined above. However, we are unaware of any study that shows how deer antlers, as costly traits, may capture the genetic quality of individuals.

Here, we study a sample of Iberian red deer males (*C. elaphus hispanicus*) from two populations in Southwestern Spain with different environmental conditions and genetic situations. Our objective was to investigate if multilocus heterozygosity, as estimated from a set of neutral markers (11 microsatellite markers), relates to relative AS within populations.

Materials and Methods

Study Areas and Populations

The study was conducted in two study areas within the natural distribution range of Iberian red deer in Southwestern Spain: Sierra de San Pedro in Extremadura region and Sierra Morena in Andalucía region. Both areas shared similar Mediterranean habitats. These habitats typically included a part of a mountain range covered by Mediterranean scrub (*Cistus* spp., *Erica* spp., *Genista hirsuta*, *Lavandula* spp.) and forest (*Quercus* spp., *Arbutus unedo*, *Olea europaea*, *Phillyrea* spp.) and a lower and flatter land covered by open oak woodland (*Quercus* spp.) or dehesa. Despite of these similarities, Sierra Morena suffered artificial reforestation with pine trees (*Pinus* spp.) during the 1950s and 1960s. So, Sierra Morena includes a lower quality habitat than San Pedro (Pérez-González J, Carranza J, Torres-Porras J, Fernández-García JL, Castillo L, Frantz A, in preparation).

In Spain, Iberian red deer suffered an intense hunting during the 20th century. Spanish Civil War and postwar (during the mid of last century) increased hunting impact and red deer populations became relicts in certain areas of Iberian Peninsula. To our knowledge, in this period, there is no biological information about Iberian red deer populations such as number and distribution of individuals, gene flow, or generation time. After a normalization of the politic situation, Iberian red deer populations have experienced a notable expansion, and nowadays, more than 70 000 individuals are harvested every year in Spain (Carranza 2009).

From October to February, thousands of red deer stags are hunted every year in a typical Spanish commercial hunt called montería. In this type of hunt, packs of dogs are released within a shrub area to move the deer outwards to the sites where hunters are placed. Under these conditions, there is little opportunity for hunting bias to particular individuals, and montería has been shown to be the less biased procedure to obtain data from hunted red deer

(Martínez et al. 2005). This study never provoked hunters to shoot additional deer (see also Carranza et al. 2004).

Data collection

Data from monterías were collected during the hunting periods of 2004–2005 for Sierra de San Pedro and 2005–2006 for Sierra Morena. Within each study area, individuals were sampled in different hunting actions (monterías) spatially and temporarily separated (10 monterías in Sierra de San Pedro and 15 monterías in Sierra Morena). For 367 stags culled in these monterías, we recorded antler measurements, removed mandibles, and collected a sample of tissue (normally muscle). For these individuals, we made several antler measurements (see Mateos et al. 2008); however, only two measures were recorded for all these 367 males: length of right antler and number of tines longer than 2 cm in the right antler (Mateos et al. 2008). We used these two antler features as a measure of AS. We estimated stags' age by counting cementum growth marks at the interradicular pad under the first molar removed from mandibles (Mitchell 1967; Carranza et al. 2004). Tissue samples were used to obtain DNA. These samples were preserved frozen at -20°C . Genomic DNA was purified by proteinase K digestion and salting out procedure (Millar et al. 1988).

Microsatellite genotyping and genetic analyses

We typed individuals at 11 microsatellite loci: OarFCB193, OarFCB304, CelJP38, CelJP15, TGLA94, TGLA53, BM1818, CSSM22, CSSM66, ILSTS06, and CSPS115 (Coulson et al. 1998; Marshall et al. 1998; Bonnet et al. 2002; Martínez et al. 2002; Kuehn et al. 2003). After polymerase chain reaction (PCR), we used ABI3130 DNA sequencer and GENEMAPPER software (Applied Biosystems, Hammon, NJ) to determine allele sizes. We combined the markers in 4 multiplex or simplex PCRs: 1×6 , 1×3 , and 2×1 (number of PCRs \times number of markers).

We used exact test implemented by GENEPOP version 3.4 (Raymond and Rousset 1995) to assess the presence of linkage disequilibrium between loci. None of the possible pairs of loci presented linkage disequilibrium.

We quantified, for each individual, three measurements of heterozygosity (see e.g., Amos et al. 2001). We firstly used standardized heterozygosity (SH) as the ratio of the heterozygosity of an individual to the mean heterozygosity of those loci at which the individual was typed. This standardization controls for possible differences in expected heterozygosities between loci (Coltman et al. 1999). Secondly, we used internal relatedness (IR), which gives more weight to homozygotes involving rare alleles (Amos et al. 2001). Finally, we used standardized d^2 ($St\ d^2$) as a measure of heterozygosity. The parameter d^2 is a measure based on the difference (in repeated units) between microsatellite alleles (Coulson et al. 1998). $St\ d^2$ is d^2 divided by the maximum value observed at each locus and then averaged. In the main text, we show the results only for SH measurement. The results for the other heterozygosity measurements are presented in Supplementary Material.

Statistical Analyses

We built a unique variable as a measurement of AS. To do so, we firstly obtained a composite AS index including both antler length and number of tines by a principal component analysis (PCA). Secondly, we carried out a multiple regression analysis to obtain residuals from this AS index by adjusting for individual age (see Mateos et al. 2008). We assessed the heterozygosity–AS correlation by relating these residuals that quantify AS relative to age and heterozygosity.

We used three approaches in order to detect the shape of the relationship. Firstly, within a population, the slope of the heterozygosity–fitness correlation can be homogeneous over the entire range of individuals (Amos et al. 2001). To explore this possibility, we made a linear model in which we used AS as a quantitative dependent variable, and study area (Sierra de San Pedro or Sierra Morena) and heterozygosity as predictor variables. Secondly, the heterozygosity fitness correlation could be driven by those individuals with low heterozygosity (Amos et al. 2001). To assess this second possibility, the dataset was subdivided into individuals with below-mean heterozygosity and those with above-mean heterozygosity (Amos et al. 2001). We repeated the linear model like in the entire dataset but separately for both below-mean and above-mean individuals. And thirdly, the heterozygosity–fitness relationship might only occur among a small number of extremely low-quality individuals. Thus, we used AS as a qualitative variable by categorizing the individuals in those with small antlers (low-quality individuals) and those with big antlers. We used four selection criteria based on the distribution of the residuals (AS): 1) small antlers: 10% of the individuals from each study area with the lowest values of AS and big antlers: 90% of the individuals from each study area with the greatest values of AS; 2) small: 15% and big: 85%; 3) small: 20% and big: 80%; and 4) small: 50% and big: 50%. We used a linear model in which heterozygosity was included as dependent variable, and study area and categorized AS were included as factors. The model was repeated for every categorization criterion.

We compared heterozygosity between categories of AS for each locus by using nonparametric tests (Mann–Whitney U test). Statistical analyses were conducted by R package (R Development Core Team 2008).

Conditions for Heterozygosity–Fitness Correlation

The existence of null alleles might lead to an underestimate of heterozygosity. Deviations from Hardy–Weinberg equilibrium (HWE) might indicate the presence of null alleles (Pemberton et al. 1995). We assessed departures from HWE by exact tests using Markov chain as implemented by GENEPOP version 3.4 (Raymond and Rousset 1995).

Individuals culled in the same montería might share environmental, morphological, and genetic local characteristics. So a correlation between heterozygosity and AS might not be due to a direct relationship, but instead they can be consequences of unknown local properties. To check for a potential spurious correlation between heterozygosity and fitness, for both study areas, we grouped those monterías in

which at least one individual with small antlers (small-antlered individuals) was culled and those in which no small-antlered individual was culled. By using only individuals with big antlers, we made linear model in which we used AS as dependent variable and study area and montería type (with or without small-antlered individuals) as factors. This model was repeated by using SH and IR as dependent variables.

We made a heterozygosity–heterozygosity correlation (HHC) analysis (Balloux et al. 2004) to assess the power to detect inbreeding depression of our set of markers. For that, we resampled the genetic data 120 times (60 times for each study area) and randomly divided the 11 markers into 1 group of 6 and 1 of 5. We calculated heterozygosity for both sets of markers in each simulation. Finally, we correlated one estimate against the other. High r values indicate that the set of markers is informative about the inbreeding coefficient.

Results

AS Characteristics

Antler length and number of tines were strongly correlated (Pearson, $N = 367$, $r = 0.737$, $P < 0.001$). The first factor of the PCA of these two variables explained 86.85% of the variance accounted for by their correlation (eigenvalue = 1.737). Age of individuals was strongly related to this AS index under a polynomial relationship (Table 1) (Scribner et al. 1989; Mateos et al. 2008).

AS relative to age was higher in Sierra de San Pedro (mean \pm standard error [SE] = 0.280 ± 0.078) than in Sierra Morena (mean \pm SE = -0.157 ± 0.058 ; one way analysis of variance, $F_{1,365} = 20.272$, $P < 0.001$).

AS and Heterozygosity

The slope for the heterozygosity–AS correlation was not significant along the entire range of sampled individuals (Table 2). This lack of heterozygosity–fitness correlation occurred both in Sierra de San Pedro and in Sierra Morena (Table 2). In the same way, the lack of heterozygosity–fitness correlation occurred either in the below-mean individuals (Table 3 and Figure 1) or in the above-mean individuals (Table 3 and Figure 1). However, when we explored the extremely low quality individuals by using the first categorization criterion (small antlers: 10% of individuals with the lowest values of AS and big antlers: the remaining 90%), individuals with small antlers presented lower levels of SH than individuals with big antlers (Table 4 and Figure 2). Heterozygosity also differed in both study areas (Table 4 and Figure 2). Finally, although the interaction between categorized AS and study area was not significant (Table 4), the difference in heterozygosity between individuals with small and big antlers was higher in the study area with lowest mean heterozygosity (Figure 2, see also Figure S3 in Supplementary Material online). As the number of individuals within the group of small antlers increased (the remaining categorization criteria), the differ-

Table 1 Multiple regression results showing the relation between age of individuals and AS index (b : coefficient)

	b	SE	T	P
Intercept	−3.183	0.214	−14.862	<0.001
Age	1.344	0.105	12.754	<0.001
Age ²	−0.083	0.011	−7.846	<0.001

ence in heterozygosity between individuals with small and big antlers tended to disappear (Table 4 and Figure 2).

The heterozygosity–AS relationship was mainly driven by two microsatellite markers (OarFCB193 and, especially, CSSM22) and mainly in Sierra de San Pedro study area (Table 5). Additionally, the percentage of loci to which heterozygosity is lower for “small-antlered” individuals was 54.55% for Sierra de San Pedro and 72.73% for Sierra Morena (Table 5).

Conditions for Heterozygosity–Fitness Correlation

After Bonferroni correction, in Sierra de San Pedro, the following microsatellite markers showed a significant deficit of heterozygotes: CelJP15, OarFCB193, ILSTS06, and CSPA115. By contrast, in Sierra Morena, all assessed microsatellite markers were in Hardy–Weinberg equilibrium.

Individuals with big antlers from monterías, in which at least one small-antlered individual was culled, presented smaller antlers than those individuals from sampling sites in which all of them had big antlers (Table 6). In spite of AS differences, there was no difference in heterozygosity (Table 6) among individuals with big antlers from sampling sites with and without small-antlered individuals.

After 120 simulations (60 simulations per study area), HHC resulted in low r (mean \pm standard deviation [SD] = 0.014 ± 0.164). In Sierra de San Pedro, the strength of HHC was lower than in Sierra Morena (r values: mean \pm SD = -0.002 ± 0.213 in Sierra de San Pedro, mean \pm SD = 0.030 ± 0.093 in Sierra Morena).

Discussion

Our results for Iberian red deer show a relationship between multilocus heterozygosity and AS, such that males with

Table 2 Linear model results for the relationship between heterozygosity (SH) and study area on AS as a continuous variable. The table shows the estimate differences in intercepts and slopes

	Estimate (SE)	t	P
Intercept	−0.098 (0.316)	−0.309	0.757
Heterozygosity	−0.060 (0.317)	−0.190	0.849
Study area (Sierra de San Pedro)	0.090 (0.446)	0.202	0.840
Heterozygosity \times study area	0.378 (0.462)	0.817	0.414

Residual standard error = 0.894 on 363 degrees of freedom. Adjusted $R^2 = 0.047$.

Table 3 Linear model results for the relationship of heterozygosity (SH) and study area on AS as a continuous variable after dividing the dataset in below-mean individuals (individuals with heterozygosity below the mean) and above-mean individuals

	Below-mean individuals ^a			Above-mean individuals ^b		
	Estimate (SE)	<i>t</i>	<i>P</i>	Estimate (SE)	<i>t</i>	<i>P</i>
Intercept	−0.583 (0.650)	−0.897	0.371	0.126 (0.983)	0.136	0.892
Heterozygosity	0.542 (0.770)	0.704	0.482	−0.275 (0.813)	−0.339	0.735
Study area (S. S. P.)	0.628 (0.879)	0.715	0.476	1.265 (1.294)	0.978	0.329
Heterozygosity × study area	−0.389 (1.125)	−0.346	0.730	−0.646 (1.158)	−0.558	0.578

The estimate differences in intercepts and slopes are shown. S. S. P., Sierra de San Pedro.

^a Residual standard error = 0.953 on 181 degrees of freedom. Adjusted R^2 = 0.006.

^b Residual standard error = 0.831 on 178 degrees of freedom. Adjusted R^2 = 0.096.

small antlers presented lower levels of heterozygosity than males with big antlers. We found that the slope of heterozygosity–fitness correlation was significant when we used neither the entire range of individuals (Table 2) nor within the groups of individuals with low and high heterozygosity (Table 3; see Amos et al. 2001). On the contrary, the heterozygosity–fitness relationship was present in our populations through a restrictive threshold (10% categorization criterion for AS, Figure 2). Therefore, the relationship between heterozygosity and AS was not present throughout the range of individual variation, but instead the association was found in a small number of poor quality individuals with very small antlers. This relationship occurs mainly in the population with low genetic diversity and when we used SH and IR as measures of heterozygosity (it did not when using $St d^2$; see Supplementary Material). To our knowledge, this is the first study to show a relationship between multilocus heterozygosity and AS in red deer, and a heterozygosity–AS association obtained with microsatellite markers.

The presence of null alleles that tends to depart genetic frequencies from Hardy–Weinberg proportions by increasing homozygosity might bias the results of this paper. In Sierra de San Pedro, 4 microsatellite markers presented a significant deficit of heterozygotes, which might indicate the existence of null alleles (Pemberton et al. 1995). However, in Sierra Morena, as well as in other studies

with the same markers and the same genetic procedure but with different dataset (see e.g., Carranza et al. 2009; Pérez-González and Carranza 2009; Pérez-González et al. 2009), there was no evidence of departures from HWE. Thus, the deviation from Hardy–Weinberg proportions in Sierra de San Pedro should be due to other phenomena such as within-population genetic structure (Wahlund effect) rather than to the presence of null alleles. Additionally, after removing these four loci with departure from Hardy–Weinberg proportions, the results of heterozygosity–fitness association were very similar (see Supplementary Material online). In the case of within-population genetic structure, the relationship between heterozygosity and AS might be a spurious association of both (low heterozygosity and small antlers) being the consequence of some unknown local characteristic of the sampling site. In agreement with the spurious association due to a local property, AS was significantly smaller in those monterías in which at least one small-antlered individual was culled than in those monterías in which no small-antlered individuals were culled (Table 6). However, the spurious association can be rejected because heterozygosity in monterías with at least one small-antlered individual was not lower than in monterías without small-antlered individuals (Table 6).

We found low HHC, indicating that heterozygosity at the marker set we used was correlated at best weakly with inbreeding (Balloux et al. 2004). These results indicate that

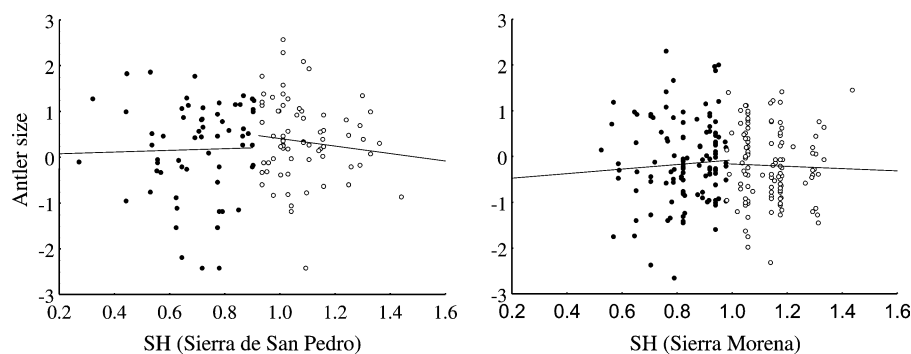


Figure 1. Scatter plot of observed AS against observed heterozygosity (SH) in both study areas. Filled circles: below-mean heterozygosity individuals and open circles: above-mean heterozygosity individuals.

Table 4 Results of the linear model to study the relation of AS and study area on individual heterozygosity (SH)

	df	(a) Small (10), big (90)		(b) Small (15), big (85)		(c) Small (20), big (80)		(d) Small (50), big (50)	
		F	P	F	P	F	P	F	P
Intercept	1	2572.5	<0.001	3662.1	<0.001	4720.4	<0.001	7262.2	0.001
Study area	1	9.067	0.003	10.675	0.001	10.879	0.001	11.345	0.001
Categorized AS	1	6.538	0.011	2.241	0.135	0.583	0.446	0.112	0.738
Study area × categorized AS	1	1.474	0.225	1.531	0.217	1.024	0.312	0.472	0.492
Error	363								

The analysis of variance results are shown. The four sections in table correspond to the four categorization criteria for AS as follows: (a) Small antlers: 10% of the individuals with the lowest values of AS, big antlers: 90% of the individuals with the greatest values of AS; (b) small: 15%, big: 85%; (c) small: 20%, big: 80%; and (d) small: 50%, big: 50%.

the local effect hypothesis of heterozygosity–fitness correlation is the most probable explanation for the relationship between heterozygosity of our set of markers and AS in Iberian red deer. Accordingly, Da Silva et al. (2009) have shown the importance of the local effect hypothesis in heterozygosity–fitness correlations in roe deer populations where a locus-specific correlation occurs between heterozygosity and juvenile survival.

For both study areas, heterozygosity–AS relationships differed for microsatellite markers (Table 5). In Sierra de San Pedro, small-antlered individuals showed lower levels of heterozygosity than “big-antlered” individuals in 54.55% of markers. However, two markers presented a strong deficit of heterozygotes in the small-antlered category: OarFCB193 and CSSM22. The heterozygosity–fitness correlation of a few markers supported the local effect hypothesis in Sierra de San Pedro (Hansson and Westerberg 2002; Pemberton 2008). According to the Australian sheep gene mapping Web site (<http://rubens.its.unimelb.edu.au>), OarFCB193 mapped at the Oar 11 (*Ovis aries*) chromosome (map position as sex average = 66.6 cM), where it is linked to insulin-like growth factor-binding protein (IGFB) 4 (map position as sex average = 67.9 cM). IGFB4 could be

implicated directly or indirectly in antler development. However, in Sierra de San Pedro, OarFCB193 presented a significant departure of HWE, so its effects should be taken with caution. CSSM22, which presented a very strong effect and no departure from HWE, mapped at Bta 5 (*Bos taurus*) chromosome (<http://locus.jouy.inra.fr>). However, we did not find information about linked genes with distances similar to that between OarFCB193 and IGFB4 (see e.g., Ihara et al. 2004). On the other hand, in Sierra Morena, small-antlered individuals showed lower levels of heterozygosity than big-antlered individuals in 72.73% of markers. A bigger proportion of markers showing lower heterozygosity in small-antlered individuals may indicate some role for a general effect.

In addition, our results illustrate the importance of the information obtained by sampling different study areas or populations (Chapman et al. 2009). Firstly, environmental conditions have crucial influence on AS. Thus, antlers were smaller in Sierra Morena than in Sierra de San Pedro, presumably because reforestations generate a poorer habitat in which antler development can suffer nutritional deficiencies (Goss 1983; Torres-Porras J, Carranza J, in preparation). This effect is found even at a local scale because AS was

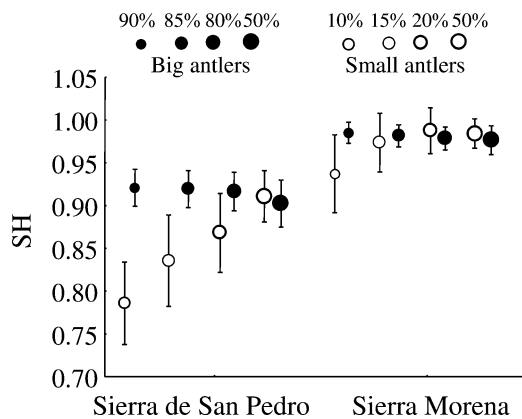


Figure 2. Observed and standard error in heterozygosity (SH) of individuals with small (open circles) and big (filled circles) antlers, grouped by study areas. The four circle sizes correspond to the four categorization criteria of AS grouping (see text for details).

Table 5 Heterozygosity and Mann–Whitney *U* results (*Z* statistic and *P* value) at each microsatellite locus for individuals with small and big antlers

Locus	Sierra de San Pedro				Sierra Morena			
	Small	Big	Z	P	Small	Big	Z	P
CelJP15	1.00	0.96	0.22	0.825	1.01	1.03	−0.07	0.945
OarFCB193	0.36	0.76	−2.05	<i>0.040</i>	0.93	0.97	−0.42	0.678
TGLA94	0.53	0.95	−1.48	0.139	0.91	1.00	−0.59	0.552
CelJP38	1.10	0.88	0.45	0.651	0.87	0.96	−0.89	0.375
OarFCB304	1.08	0.97	0.69	0.492	0.93	0.92	0.04	0.968
CSSM66	0.83	0.94	−0.75	0.451	0.93	0.96	−0.28	0.782
CSSM22	0.13	1.05	−3.58	<i><0.001</i>	1.08	1.06	0.10	0.924
BM1818	0.82	0.99	−0.94	0.348	0.98	1.03	−0.30	0.766
ILSTS06	1.03	0.86	1.02	0.308	0.73	0.90	−1.16	0.244
TGLA53	1.03	0.96	0.80	0.326	0.83	0.97	−1.26	0.208
CSPS115	0.51	0.79	−0.69	0.493	1.01	0.98	0.20	0.838
Small lower H	54.55				72.73			

Small lower H: percentage of loci to which heterozygosity was lower for small-antlered individuals. Significant *P* values are highlighted in italics.

Table 6 Results of factorial linear model which test the local dependence of AS and SH

	AS		SH	
	df	F	P	P
Intercept	1	53.887	<0.001	6429.200 <0.001
Study area	1	35.129	<0.001	5.912 0.016
Montería type	1	20.979	<0.001	1.539 0.216
Study area × montería type	1	0.085	0.770	1.081 0.299
Error	325			

The analysis of variance results is shown. AS was significantly lower for monterías in which at least one small-antlered individual was culled (mean \pm SE = 0.040 \pm 0.051) than for monterías in which no small-antlered individuals were culled (mean \pm SE = 0.433 \pm 0.067) (Montería type in table).

smaller in monterías with at least 1 small-antlered individual than in monterías without small-antlered individuals (see above). Secondly, the relationship between heterozygosity and AS was different in both study areas; it being stronger in Sierra de San Pedro than in Sierra Morena. In Sierra de San Pedro, genetic diversity was lower and presented evidence of heterozygote deficit, presumably due to a within-population genetic structure. Low levels of genetic diversity and genetic structure are both consequences of the genetic drift, which, in turn, favors the action of linkage disequilibrium and local effects (Hansson and Westerberg 2002; Chapman et al. 2009). Additionally, but taking into account the general effect hypothesis, the lower heterozygosity–fitness correlation under high genetic diversity supports the genetic diversity dependence of processes related to inbreeding depression (Charlesworth and Charlesworth 1987; Qvarnstrom 2001; Keller and Waller 2002; Coltman and Slate 2003; Mays and Hill 2004).

Heterozygosity–AS relationship was achieved when we used SH and IR as measurements of heterozygosity (see Supplementary Material). These two genetic metrics presented very similar results due to the high correlation between them (e. g. Amos et al. 2001). On the contrary, heterozygosity–AS correlation was not found when we used $St d^2$ (see Supplementary Material). This lack of significance for $St d^2$ is a common result in heterozygosity–fitness correlation studies and can be due to the loss of genetic signal because of the standardization of this measure (Chapman et al. 2009, but see Da Silva et al. 2009).

In this study, we have shown a relationship between heterozygosity at microsatellite markers and AS for red deer populations. However, our data do not allow us to infer a causal association. We suggest that the observed relationship is likely to be mainly due to linkage between some assessed microsatellite loci and loci that affect fitness. However, these fitness loci might not necessarily correspond to genes related to antler development, but they might be implicated in the expression of any other fitness traits. Because antlers are costly tissues with high condition dependence (Andersson 1982; Ditchkoff et al. 2001), the relationship between heterozygosity and fitness traits such as juvenile development or parasite resistance (see e.g., Paterson

et al. 1998 for microsatellite loci located within or adjacent to the MHC in Soay sheep) may consequently produce a decrease of AS in situations of low heterozygosity.

Other studies have also shown heterozygosity–weapon size relationships in ungulates (Fitzsimmons et al. 1995; Scribner et al. 1989; Ditchkoff et al. 2001; von Hardenberg et al. 2007). These findings are informative about the evolutionary implications of male ungulate weapons, such as the capture of genetic quality (Andersson 1982) and their possible action as honest signals of male genetic features (Ditchkoff et al. 2001; von Hardenberg et al. 2007).

If AS is related to heterozygosity, a relationship should be expected between individual heterozygosity and fitness (measured as breeding success; see Slate et al. 2000), caused by the influence of AS on fitness (Kruuk et al. 2002). Therefore, regarding heterozygosity, successful males are not expected to be a random sample of the males in the population. As a consequence, theoretically computed effective population size may be underestimated in populations where sexual selection favors competition between males on the basis of structures that, like antlers, may be related to heterozygosity. In support of this idea, Pérez-González et al. (2009), with the same microsatellite markers, have recently shown for red deer that males contribute more than females to the genetic diversity of offspring, despite the lower number of breeding males compared with females in the studied polygynous populations, presumably because of the advantage of heterozygous males in the competition for mates.

Kruuk et al. (2002) showed that AS was heritable and was subjected to selection but did not evolve in the Rum population during a period of 30 years. They argued that environmental covariance might explain their results. Heterozygosity–AS relationship suggests that heterozygote advantage may also contribute to these results. Rare alleles are more frequent in heterozygous individuals, which may increase the chances for their offspring to be heterozygotes for these loci (Mitton et al. 1993). As a consequence, some proportion of the estimated heritability of the trait may be due to the relationship between father's and son's heterozygosity. Although nonadditive genetic variance may also be subjected to directional selection (Neff and Pitcher 2008), the evolutionary response of traits with dependence on heterozygosity should be slower than those with high additive genetic components (Falconer and Mackay 1996). Thus, AS may result from the joint effects of additive genes subjected to relatively rapid directional selection along with the heterozygote advantage of alleles subjected to relatively slow directional selection, thus showing less than expected evolutionary change.

Our results have also practical implications. Red deer is an important game species all over the world, and in many areas, it is managed with the objective of producing big trophies. Hunting may tend to decrease genetic diversity of hunted species (Martínez et al. 2002; Allendorf et al. 2008). This decrease of genetic diversity may involve negative effects on trophy size and, hence, in the sustainability of game activity.

Supplementary Material

Supplementary material can be found at <http://www.jhered.oxfordjournals.org/>.

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