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Author for correspondence:
Alice Brambilla
e-mail: alicebrambilla1@gmail.com

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Direct and indirect causal effects of heterozygosity on fitness-related traits in Alpine ibex

Alice Brambilla[1], Iris Biebach[2], Bruno Bassano[3], Giuseppe Bogliani[1] and Achaz von Hardenberg[3]

[1]DSTA-Department of Earth and Environmental Science, University of Pavia, Via A. Ferrata 9, 27100 Pavia (PV), Italy
[2]Institute of Evolutionary Biology and Environmental Studies, University of Zurich, Winterthurerstrasse 190, 8057 Zürich, Switzerland
[3]Alpine Wildlife Research Centre, Gran Paradiso National Park, Degioz 11, 11010 Valsavarenche, AO, Italy

AvH, 0000-0002-9899-1687

Heterozygosity–fitness correlations (HFCs) are a useful tool to investigate the effects of inbreeding in wild populations, but are not informative in distinguishing between direct and indirect effects of heterozygosity on fitness-related traits. We tested HFCs in male Alpine ibex (*Capra ibex*) in a free-ranging population (which suffered a severe bottleneck at the end of the eighteenth century) and used confirmatory path analysis to disentangle the causal relationships between heterozygosity and fitness-related traits. We tested HFCs in 149 male individuals born between 1985 and 2009. We found that standardized multi-locus heterozygosity (MLH), calculated from 37 microsatellite loci, was related to body mass and horn growth, which are known to be important fitness-related traits, and to faecal egg counts (FECs) of nematode eggs, a proxy of parasite resistance. Then, using confirmatory path analysis, we were able to show that the effect of MLH on horn growth was not direct but mediated by body mass and FEC. HFCs do not necessarily imply direct genetic effects on fitness-related traits, which instead can be mediated by other traits in complex and unexpected ways.

1. Introduction

Inbreeding is defined as the mating between closely related individuals and the consequent increase in homozygosity caused by such mating [1]. In small and isolated populations [2,3], and in bottlenecked populations with small effective population size [4], inbreeding cannot be avoided. The consequence of mating between relatives may be the increase in expression of deleterious recessive alleles that lead to inbreeding depression: the decrease in fitness of inbred individuals compared with outbred individuals [5]. The detrimental effects of inbreeding were first summarized by Darwin [6]. Since then, evidence of the negative impact of inbreeding on fitness and viability at both individual and population levels has accumulated [1,7–10]. The expression of recessive deleterious mutations has been indicated as the main cause of inbreeding depression [11–13]. The negative effects of inbreeding might be purged through selection against deleterious alleles [14]. A review conducted on mammals, however, revealed contrasting results, with purging not substantially reducing inbreeding depression [15]. The efficiency of purging depends on many genetic and environmental factors [1], and the time necessary to markedly weaken inbreeding depression could be highly variable and very long [12,16].

Inbreeding depression is a relative measure of the difference in fitness between inbred and outbred individuals. If the variance of the inbreeding coefficient (f) in the population is low, then all individuals may suffer from the effects of inbreeding, but they would all have similar fitness, and so inbreeding depression would not be detectable [12]. Thus, it is generally difficult to detect the effects

of inbreeding depression in small and isolated or bottlenecked populations with similar levels of inbreeding in all the individuals [13].

When complete pedigree data are available, inbreeding depression is usually measured by regressing fitness on inbreeding coefficient f [17]. Pedigree data, however, are difficult to obtain in wild populations. Moreover, pedigrees are typically relatively short and thus may miss inbreeding that accumulated prior to the beginning of the pedigree, a fact of particular importance in bottlenecked populations [10]. In the absence of pedigree data, molecular measures such as heterozygosity have been used as a proxy of inbreeding [18]. A molecular-based method widely used to test for inbreeding depression is heterozygosity–fitness correlation (HFC): the correlation between multi-locus heterozygosity (MLH) and fitness-related traits [12,13]. Whether HFCs are a signal of inbreeding depression has been a matter of debate, but in recent years a general consensus on this hypothesis has been reached [12,13,18]. There are three main hypotheses invoked to explain HFCs [19]: (i) the direct effect hypothesis, whereby the markers used to estimate heterozygosity are themselves expressed and have a direct effect on fitness; (ii) the local effect hypothesis, whereby the markers used to estimate heterozygosity are linked with loci directly affecting fitness; and (iii) the general effect hypothesis, whereby heterozygosity at microsatellite markers reflect genome-wide heterozygosity. Following the general effect hypothesis, HFCs can be considered a proxy of inbreeding depression in wild populations [12]. HFCs are usually weak [12,20], and their detection depends on sample size and on the number of available markers: an absence of HFCs is thus not necessarily an indication of absence of inbreeding depression.

While HFCs are a useful tool to investigate the effects of inbreeding in wild populations, they are not informative in distinguishing between direct and indirect effects of heterozygosity on fitness-related traits. This is due to the very nature of the multiple regression models commonly used to test HFCs, which do not allow causal inference. Controlled or randomized experiments are the straightforward method to test for causal relationships among variables [21]. Experimental manipulations, however, are very difficult (if not impossible) to conduct with large, free-living vertebrates that are part of long-term observational studies. Confirmatory path analysis, a special case of the more general structural equation models, is a causal inference method that can be used with observational data. This inference tool, introduced to biology by Shipley [22], makes it possible to formulate complex causal models represented as directed acyclic graphs (DAGs) depicting the hypothesized causal relationships among all involved variables [23]. Each hypothesized DAG can be defined by the implied conditional independencies owing to the assumed causal links among the variables, which can then be statistically tested by translating them into structural equations [21,24]. While confirmatory path analysis and structural equation models have widely been applied in general ecology [25,26], ecophysiology [27,28] and evolutionary biology [29], we are not aware of any study using them in the field of molecular ecology.

The Alpine ibex (*Capra ibex*) is a mountain ungulate currently distributed over the whole European Alps. The species owes its current distribution to reintroduction programmes, which used the Gran Paradiso population in the northwestern Italian Alps as the primary source [30]. The species almost went extinct in the eighteenth century, with fewer than 100 individuals left in the Gran Paradiso area [31,32]. Even if the current conservation status of Alpine ibex is considered as 'least concern' in the IUCN Red List of threatened species [33], its recent demographic history [34] and its generally low genetic variability [35,36] suggest this species is worth special attention. Inbreeding depression is expected to particularly affect the isolated and recently reintroduced populations founded by few individuals [37], but it probably also affects the original bottlenecked population in Gran Paradiso [38]; in fact, the main genetic bottleneck event suffered by the population at the beginning of the nineteenth century [36] is quite recent in genetic terms, considering the long generation times that characterize this species (about 7–8 years), and the reduced effective population size is expected to be enough for HFCs to arise [12,13] also in the original population. Better knowledge of the effects of inbreeding in Alpine ibex is thus important to understand how long the effects of a bottleneck can last in a long-lived mammal, and for conservation purposes.

A previous study carried out on the same population [38] only found a weak age-dependent effect of heterozygosity on horn length. The sample size, however, was probably not large enough to detect the presence of inbreeding depression. Moreover, that study [38], as well as other studies that investigated HFCs in vertebrates, limited their analysis to the finding of correlations between heterozygosity and one or more fitness-related traits, considering each trait as independently correlated to heterozygosity [10,39–42]. To the best of our knowledge, no study has tried to disentangle the possible causal relationships between heterozygosity and multiple fitness-related traits. In this study, we tested HFCs in individually tagged male Alpine ibex in the autochthonous population of Gran Paradiso National Park (Italy) using a dataset collected over 14 years. We tested the effects of standardized MLH at 37 neutral microsatellite loci on various fitness-related traits: body mass, annual horn growth (named hereafter only 'horn growth') and faecal egg counts (FECs) of nematode parasites. We used confirmatory path analysis [22,24] to test alternative models of possible direct and indirect cause–effect relationships between MLH and fitness-related traits.

2. Methods

(a) Study area and population

This study was conducted in the Levionaz study area in Gran Paradiso National Park (GPNP; Northwestern Italian Alps; 45°25′ N, 07°34′ W) within the framework of a long-term project started in 1999 on the ecology and life history of the species [38,43–45]. Most male Alpine ibex in the Levionaz study area have been captured once and individually marked with coloured ear tags. The percentage of marked males in Levionaz is around 80% (data of 2012, personal observation); the cohort of marked animals ranged between 1985 and 2009, providing 25 years of molecular data. For more details on the marking protocol, see Brambilla *et al.* [46].

(b) Genetic analysis

Samples for genetic analysis were collected as tissue or blood samples during captures. We performed microsatellite analysis on samples from $n = 149$ different male Alpine ibex. After collection, tissue samples were stored in 95% ethanol solution, and blood samples were stored in EDTA vacutainer tubes at a

temperature of −35°C or applied on absorbent paper cards for blood DNA (Whatman FTA cards) and stored at room temperature until analysis. DNA extraction was performed using QIAmp DNA mini and Biosprint 96 systems (Qiagen), applying the appropriate protocol for each type of sample. We genotyped all samples at 51 polymorphic microsatellite loci as originally described by Biebach & Keller [30]. Fragment analysis was performed on an ABI 3730 automated sequencer and electropherograms were analysed and manually checked using GeneMapper v. 4.0 (Applied Biosystems). PCR and genotyping was repeated up to three times, and a consensus genotype was built. PCR and genotyping were done twice for all blood samples that were stored on absorbent paper cards ($n = 55$) and once for all tissue and blood samples in EDTA ($n = 94$). The genotyping process was repeated if genotype quality was low (low intensity compared with other samples of the same marker) or if the two repetitions did not match. The genotype of a sample with three repetitions was considered heterozygote if at least two of the three repetitions were heterozygote, and was considered homozygote if all repetitions were homozygote. Locus-specific dropout and false allele rates were calculated using the software Gimlet [47], and then were compared with error rates found in another study of the same species [30]. Allelic dropout and false allele rates are provided as the electronic supplementary material, table S1.

(c) Fitness-related traits data collection

In Levionaz, male Alpine ibex were repeatedly weighed during summer (late May–September) with an electronic platform scale baited with salt [43]. To allow comparison between individuals measured at different times of the year, body mass on 1 August in each year was estimated [48]; $n = 391$ records of August weight were available to test the relationship between heterozygosity and body mass.

Ibex horns grow continuously throughout life. Annual growth is easily visible, thanks to the rings that form because of the lack of horn growth during winter. The annual horn growth of male ibex was measured along a central line on the external side of both horns using callipers to the nearest millimetre during captures or when the animals were found dead. In the years after captures, the annual horn growth of marked individuals (annuli) was measured from remote pictures following Bergeron [45] or Brambilla & Canedoli [49]. For each pair of annuli, horn growth was estimated as the mean value of the left and right annulus. We estimated horn growth for a total of $n = 873$ annuli. Plots showing the pattern of body mass and annual horn growth in function of age are provided in the electronic supplementary material, figures S1 and S2.

Faecal samples from marked individuals were collected once a month from May to September to determine individual FECs. A total of $n = 510$ measures of FEC were available. FECs have been used as a proxy of resistance to abomasal nematode infection (abomasal trichostrongyle: *Marshallagia marshalli*, *Teladorsagia circumcincta* and *Ostertagia occidentalis*) in ungulates, because host resistance influences parasite fecundity [50]. FECs were done following a modified McMaster technique [51] and were expressed as number of eggs per gram (EPG) of fresh faeces. We calculated the average EPG for each animal during the season. For more details, see Brambilla *et al.* [46].

(d) Data analysis

Hardy–Weinberg equilibrium (HWE) was calculated for all the microsatellites using the Microsoft Office Excel plugin GeneAlEx v. 6.5 [52]. Loci not in HWE after Bonferroni sequential correction [53] were excluded from further analysis. Standardized MLH was then calculated for each animal as the ratio of the heterozygosity of the 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 [54]. To minimize the risk of type I error from multiple tests, all probabilities were corrected with the sequential Bonferroni method [53]. Because we used the genotypes of animals born between 1985 and 2009, to exclude directional cohort effects, we fitted a linear regression to test changes in MLH over time.

We estimated g_2, a measure of the covariance in heterozygosity, using robust multi-locus estimates of selfing (RMES) freeware [55]. RMES, besides providing an estimate of g_2, also tests whether g_2 differs significantly from zero. Values of $g_2 = 0$ mean that there is no variance in inbreeding in the population, and thus HFCs are not expected to arise [13]. The analysis was performed by setting in RMES the number of populations equal to one, $n = 37$ useful microsatellites and running it for 1000 iterations.

To test the relationship between MLH and fitness-related traits, we fitted linear mixed-effect models using the 'lmer' function in package lme4 [56] in R v. 3.0.0 [57]. All the variables were standardized before analysis to obtain comparable measures of the effect size of MLH on the analysed trait. We included age and age^2 of individuals as well as the interaction between age and MLH as fixed covariates in models testing the effect of MLH on body mass and horn growth as these traits are known from previous analyses to be age-dependent following a quadratic curve [48,58]. The effect of MLH on FEC was modelled adding age and the interaction between age and MLH as fixed covariates. As we had repeated measures for individuals and measures taken in different years, individual identity and year were added as random effects in all the models. A set of models with all the possible combinations of the variables of the full model were fitted for each fitness trait by testing the relative importance of the variables using the dredge function of the R package MuMIn [59]. Coefficients were estimated model averaging among all fitted models using the model.avg function in MuMIn. Model selection was finally done comparing corrected values of Akaike's information criterion (AICc) [60]. A value of $\Delta\text{AICc} = 4$ was chosen as a threshold for the selection of the best models [61]. Pseudo-R^2-values were calculated for the four best models with the r.squaredGLMM function of MuMIn. Marginal R^2 (R^2m) represents the variance explained by fixed factors, and conditional R^2 (R^2c) is interpreted as variance explained by both fixed and random factors.

Because we hypothesized that body mass, horn growth and FEC were not independent, after testing the correlation between MLH and fitness related traits, we tested alternative hypothesized causal models of the relationship among variables using confirmatory path analysis with the d-sep method [22,24,62,63]. Following our hypothesis, we tested six different causal models represented as directed acyclic graphs in figure 1: model a, with body mass, horn growth and FEC as directly being affected by MLH; model b, with MLH having both a direct effect and an indirect effect mediated by body mass on horn growth; model c, with MLH having no direct effect on horn growth but only the indirect effect mediated by body mass; model d, with an indirect effect of MLH on horn growth mediated by body mass but no direct effect on FEC; model e, with an indirect effect of MLH on horn growth mediated both by body mass and FEC; and model f, with an indirect effect of MLH on horn growth mediated by body mass only and no direct effect of MLH on FEC, which instead affects horn growth directly. Independence claims declared to describe the hypothesized causal models were tested using linear mixed-models including individual identity and year as random factors as described by Shipley [62]. Following Shipley [22,24], the data were considered as consistent with the hypothesized causal model if the p-value was > 0.05. Competing causal models were also compared

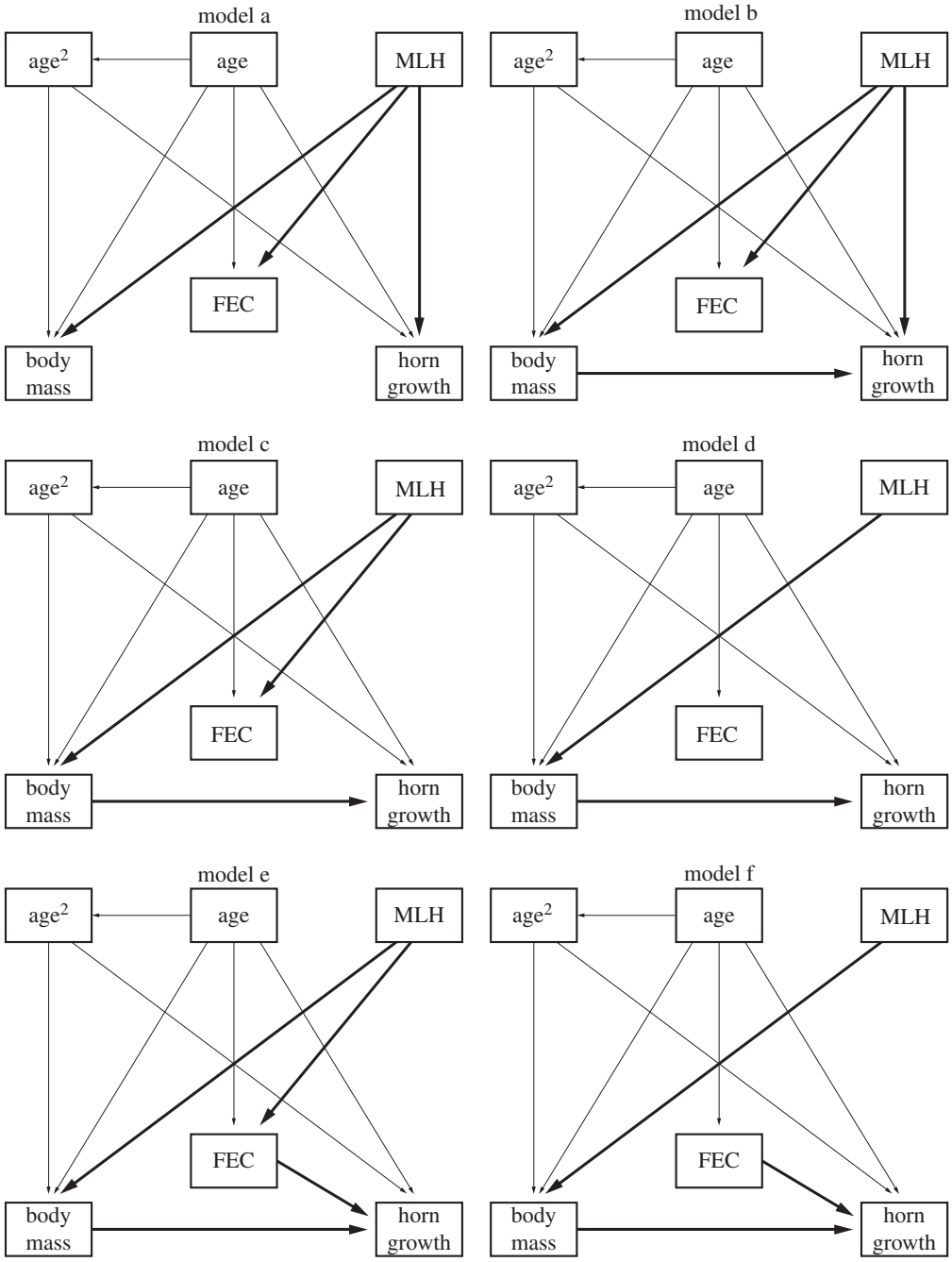

Figure 1. Representation of the six models testing causal relationships between variables in HFCs. MLH represents individual standardized multi-locus heterozygosity and FEC represents faecal egg count, a measure of individual parasite resistance and resilience. The thicker arrows highlight the causal links that change among the six models.

using AICc, following the procedure for path analysis with hierarchically structured data suggested by Shipley [62]. The number of parameters (d.f.) for each structural equation forming part of the hypothesized causal model needed to calculate AICc was extracted from the 'lmer' models using the function 'logLik' in R.

3. Results

Dropout and false allele rates (electronic supplementary material, table S1) were similar to what was found in other studies on the same species using the same microsatellite loci [30]. False allele rates were 0 for all the markers analysed, whereas dropout ranged between 0 and 0.172 (mean $\pm$ s.d. = 0.012 $\pm$ 0.028).

After Bonferroni correction, four markers were not in HWE (INRA175, OarAE54, BM2113 and SR-CRSP07). Three of these four markers (INRA175, OarAE54 and BM2113), however, were consistently in HWE in 43 populations previously analysed [30]; because an analysis of multiple populations should be more precise in finding markers not in HWE than a single population analysis such as our study, we decided to keep these three markers in our analysis and to exclude only SR-CRSP07. We also did not include 13 markers in the analysis known to be linked to loci under selection (i.e. markers within or linked to MHC or to other immune genes), markers linked to known quantitative traits loci or markers that deviated from the neutral expectation [30]. We thus calculated MLH from 37 markers that were all supposed to be neutral (electronic supplementary material, table S1).

Table 1. Model selection of mixed-effects models for testing the effect of MLH and age on body mass, $n = 391$. ID and year were fitted as random effects. Weight is the Akaike weight on which model-averaged coefficients are based. R^2m and R^2c represent marginal and conditional pseudo-R^2-values, respectively. Only the best four models are shown.

components of the model	d.f.	log-likelihood	AICc	ΔAICc	weight	R^2m	R^2c
age + age^2 + MLH	7	−227.18	468.67	0	0.87	0.61	0.92
age + age^2 + MLH + age × MLH	8	−228.06	472.52	3.85	0.13	0.62	0.91
age + age^2	6	−238.27	488.75	20.08	0	0.62	0.91
age + MLH	6	−349.88	711.99	243.32	0	0.49	0.77

Table 2. Model selection of mixed-effects models for testing the effect of MLH and age on horn growth, $n = 873$. ID and year were fitted as random effects. Weight is the Akaike weight on which model-averaged coefficients are based. R^2m and R^2c represent marginal and conditional pseudo-R^2-values, respectively. Only the best four models are shown.

components of the model	d.f.	log-likelihood	AICc	ΔAICc	weight	R^2m	R^2c
age + age^2 + MLH	7	−989.28	1992.7	0	0.81	0.34	0.43
age + age^2 + MLH + age × MLH	8	−989.71	1995.59	2.88	0.19	0.34	0.43
age + MLH	6	−1006.6	2025.29	32.59	0	0.30	0.40
age + MLH + age × MLH	7	−1008.93	2032	39.29	0	0.30	0.40

Table 3. Model selection of mixed-effects models for testing the effect of MLH and age on FEC, $n = 510$. ID and year were fitted as random effects. Weight is the Akaike model weight on which model-averaged coefficients are based. R^2m and R^2c represent marginal and conditional pseudo-R^2-values, respectively. Only the best four models are shown.

components of the model	d.f.	log-likelihood	AICc	ΔAICc	weight	R^2m	R^2c
age + MLH	6	−591.49	1195.16	0	0.95	0.10	0.47
age + MLH + age × MLH	7	−593.43	1201.1	5.94	0.05	0.10	0.47
MLH	5	−610.88	1231.89	36.73	0	0.02	0.04
age	5	−643.5	1297.12	101.95	0	0.10	0.48

There was no correlation between MLH and the cohort of individuals. This result, therefore, does not support the hypothesis of cohort effects or changes in MLH over time (linear regression: $\beta \pm$ s.e. $= 0.002 \pm 0.003$ $p = 0.420$, $n = 149$; electronic supplementary material, figure S3).

The estimate of g_2 was not significantly different from 0 ($g_2 \pm$ s.d. $= -0.0022 \pm 0.0039$, $p = 0.702$). The relatively large standard deviation of the estimate suggests that g_2 was estimated with low precision in our study.

(a) Heterozygosity–fitness correlations

(i) Body mass

The best models included MLH, age, and the interaction between MLH and age (table 1), with MLH having a weak positive relationship with body mass (model-averaged coefficient $\beta \pm$ s.e. $= 0.043 \pm 0.058$), and the interaction between age and MLH having a weak negative correlation with body mass (model-averaged coefficient $\beta \pm$ s.e. $= -0.060 \pm 0.033$). R^2m and R^2c of the best models are presented in table 1.

(ii) Horn growth

The best models included MLH, age, and the interaction between MLH and age with a weak positive relationship between MLH and annual horn growth (model-averaged coefficient $\beta \pm$ s.e. $= 0.080 \pm 0.040$), and a weak negative relationship between the interaction between MLH and age and horn growth (model-averaged coefficient $\beta \pm$ s.e. $= -0.067 \pm 0.033$; table 2). R^2m and R^2c of the best models are presented in table 2.

(iii) Faecal egg counts

The best models included both MLH and age (table 3), with MLH having a weak negative relationship with FEC (MLH: model-averaged coefficient $\beta \pm$ s.e. $= -0.087 \pm 0.053$). R^2m and R^2c of the best models are presented in table 3.

(b) Confirmatory path analysis

The only causal model consistent with the data among those we tested with confirmatory path analysis (i.e. the only with a p-value > 0.05) was model e, which implies an indirect effect of MLH on horn growth mediated by both body mass and FEC (figure 2 and table 4). The ΔAICc values confirmed that this was indeed the best-fitting causal model (table 4). Details on the independence claims describing each of the six models as well as on model testing are provided in the electronic supplementary material, table S2.

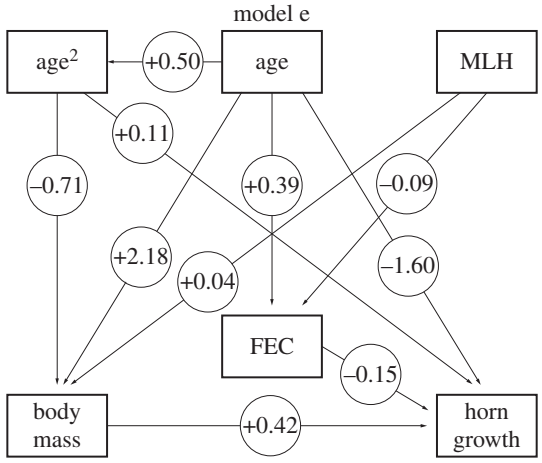

Figure 2. Representation of the causal pathways of the best-fitting model (model e) with standardized path coefficients represented within circles.

Table 4. Summary of the confirmatory path analysis results for the six hypothetical causal models. Models are presented in order of AICc. *C* represents Fisher's *C* statistic [22]. The directed acyclic graphs of all the six models are represented in figure 1. Model e, the only accepted model after model selection, is represented in figure 2 with standardized path coefficients. Basis sets with all implied conditional independence claims for all models are described in the electronic supplementary material, table S2.

model	d.f.	*C*	*p*-value	AICc	ΔAICc
e	26	17.58	0.063	69.58	0.00
c	25	26.31	0.010	76.31	6.73
b	26	25.54	0.004	77.54	7.96
f	24	45.59	<0.001	93.59	24.01
a	25	49.27	<0.001	99.26	29.68
d	24	54.29	<0.001	102.29	32.71

4. Discussion

Our results revealed HFCs in all analysed fitness-related traits in male Alpine ibex. We found a positive relationship between MLH and body mass, with more heterozygous individuals being heavier. The same relationship was found for horn growth, with more heterozygous individuals having longer annual horn growth. In the case of FECs, the relationship was negative, as expected, with less heterozygous individuals having the highest FEC.

Direct and indirect causal relationships between individual genetic variability and life-history traits are not easy to disentangle, and the effect of MLH on one trait may actually be mediated by some other trait. Indeed, the traits used to assess individual quality are often not independent: heavier males are presumably of higher quality, and they may also be able to afford to grow bigger horns [64]. Using confirmatory path analysis, we showed that the effect of MLH on horn growth was not direct, but was instead mediated by body mass and FEC: only high-quality males (i.e. with high levels of heterozygosity) become big (large body mass) and resistant to parasites (low FEC), and consequently can afford to also grow long horns. This result supports the hypothesis that horn growth in male Alpine ibex is an honest advertisement of individual quality [65], as suggested by von Hardenberg *et al.* [38]. Our final causal model contradicts the hypothesis of a direct relationship between FECs and body mass previously found in the same population by Decristophoris *et al.* [66]. However, that study [66] did not include data on MLH, whereas the causal model presented here, including this third variable, shows that the relationship originally found between FEC and body mass is actually owing to the direct causal effect of MLH on both traits. As a side result, this suggests that FECs may be a proxy of parasite resilience rather than resistance [67]: the effect of MLH on both body mass and FEC may actually be mediated by another unmeasured variable such as, for example, the ability to obtain forage of better quality (in terms of protein content) [68]. Indeed, increased individual resilience towards gastrointestinal parasites was shown to be related to the quality of the forage in domestic goats experimentally infected with abomasal parasites [69], whereas high protein diet supplementation improved both resilience and resistance to gastrointestinal parasites in domestic sheep lambs [67].

Our results are in accordance with von Hardenberg *et al.* [38], who, in the same population, found weak age-specific effects of MLH on horn growth. The broader effects that we found here are probably owing to the fact that this study relies on a considerably larger dataset of fitness-related traits compared with the von Hardenberg *et al.* [38] study, which included data only from 2000 to 2004. Moreover, the confirmatory path analysis approach that we used here allowed us to demonstrate that the effect of heterozygosity on horn growth, found also previously, was not direct but mediated by body mass. We also tested changes in MLH over time to possibly explain the different findings between our study and the previous one [38], but the increase in MLH in the Gran Paradiso population was small and non-significant over the past 25 years, suggesting that there has not been a substantial recent increase in inbreeding. This finding is in line with the relatively large population size of this population in recent years ($n = 2651$ individuals counted during the yearly total count in September 2013; data provided by GPNP Scientific Research Service).

The magnitude of the effects of MLH on fitness-related traits that we detected was weak, in accordance with the findings of many other studies [12,13,20,70]. The effect of MLH on fitness-related traits appeared to be age-dependent: the effect of MLH on body mass and annual horn growth decreased with age. This can be explained by the fact that growth differences are maximal early in life [71]. Following David *et al.* [71], there may be higher variance in fitness-related traits in young compared with old individuals, because unfit genotypes are selectively eliminated in older cohorts, and thus it may be easier to detect the effect of MLH in young compared with old individuals. Conversely, faecal egg counts decreased with increasing MLH while they increased with age, but, contrary to the other considered fitness traits, the two effects did not appear to interact.

Even if we did not use direct fitness measures, as suggested by Chapman *et al.* [12], the traits we chose to test for HFCs seem appropriate. Body mass and horn growth have an effect on the dominance status of male Alpine ibex [64], and therefore probably contribute to reproductive success [72]. Secondary sexual traits are also known to be honest signals of individual quality [58,73]. Gastrointestinal parasites, as well, have a crucial role in the life history and survival of wild herbivores, and create repercussions on

population dynamics [74–78]. Other authors found evidence of HFCs in ungulates using morphological and physiological traits; these studies, however, usually analysed the effect of MLH on single traits [10,39,54,79]. In this study, instead, we show a broad effect of MLH on several traits at once, in accordance with the general effect hypothesis for HFCs [12].

Almost 200 years have passed since the main bottleneck of this population [31]. Thus, about 25 Alpine ibex generations have passed since this bottleneck. During the Second World War, the Gran Paradiso population suffered an additional numerical reduction, with no more than 600 animals left (Gran Paradiso National Park Archives 1945, unpublished data). Since the first bottleneck, inbreeding owing to small population size has probably accumulated and heterozygosity has been lost. While we do not have direct evidence of this loss of heterozygosity in the Gran Paradiso population, this effect has been recently demonstrated in reintroduced Alpine ibex populations in Switzerland [37]. The magnitude of the increase in inbreeding and loss in heterozygosity each generation varies proportionally to the reciprocal of the effective population size [80]. The two bottlenecks might have contributed most to the inbreeding coefficient, and further inbreeding may have accumulated also in the following generations owing to the small numbers of effective individuals in the population. Heterozygosity appears to reflect inbreeding in the Gran Paradiso population: Szulkin *et al.*'s [13] test for local effects did not reveal any evidence for local effects, because the model containing specific effects for each locus did not differ from the model containing MLH ($\chi^2 = 46.087$, d.f. $= 36$, $p = 0.121$), suggesting that the HFCs we found are more likely to be due to general effects. Hence, our results are most parsimoniously interpreted as inbreeding depression, with a fitness advantage for less inbred individuals.

Contrary to our expectations, however, we found no evidence of identity disequilibrium (ID), with g_2 not significantly different from zero. As ID is considered a definite consequence of inbreeding and the proximal cause of HFCs, in the absence of ID (if $g_2 = 0$), HFCs are not expected to arise [13]. Indeed, following the formula proposed by Miller *et al.* [81], when $g_2 = 0$, the power to detect HFCs also becomes 0. However, other studies found HFCs despite having no evidence for ID [10,82]. The number of genotyped markers used in our study and other studies may in fact be too small to reliably detect ID: weak levels of inbreeding depression sometimes can be detected more easily through an analysis at phenotype level than by using genotype indexes based on a few microsatellites [13]. Our results mirror those of a recent simulation study [83], which found that only a small proportion of populations with significant HFCs exhibited significant ID when either a small number of markers was used or when the variance in inbreeding was low. Further analyses using a larger number of markers (for example using SNPs) might help to better understand HFCs in this population [81].

HFCs found in this study provide evidence for inbreeding depression in the Gran Paradiso Alpine ibex population. The genetic bottlenecks suffered by this population [36] lead to genetic drift, which in turn could lead to the fixation of deleterious alleles [1]. However, the detection of inbreeding depression indicates that there is still genetic variability in fitness-related traits in this population, and thus that deleterious alleles are not all fixed in the Alpine ibex in Gran Paradiso National Park. The persistence of inbreeding depression many years after the bottleneck is also extremely interesting from a conservation point of view. The Gran Paradiso population is the only one that survived the nineteenth century, whereas all the other populations extant today are derived from reintroduction programmes [30]; this population was therefore expected to be the least affected by inbreeding, because it experienced fewer bottlenecks during its population history. Because the autochthonous Gran Paradiso population presents signs of inbreeding depression, it would be very important to quantify inbreeding depression in the more recently founded populations, which are already known to be more inbred than the Gran Paradiso population [37]. Furthermore, we have shown the usefulness of confirmatory path analysis [22] in the field of molecular ecology to test causal models of the relationships between individual genetic variability and fitness-related traits. Specifically, we were able to show that the previously found positive relationship between MLH and horn growth in Alpine ibex [38] is not direct but is rather mediated through body mass, and parasite resistance and resilience. Disentangling correlation from causation among heterozygosity- and fitness-related traits helped us to highlight the mechanisms behind HFCs and to explain the different effect sizes of MLH on different traits [84]. We thus believe that confirmatory path analysis, and more generally causal inference methods [23], should become part of the statistical toolbox of molecular ecologists, especially of those working on long-term studies on wild populations, in which the possibilities of experimental manipulation are usually very limited.

Ethics statement. The capture and marking protocol used in this study was authorized by the Italian Ministry of Environment after review by the Italian National Institute for Environmental Protection and Research.

Data accessibility. The data used in this paper are published on Dryad: doi:10.5061/dryad.8kb87.

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References

1. Keller L, Waller P. 2002 Inbreeding effects in wild populations. *Trends Ecol. Evol.* **17**, 230–241. (doi:10.1016/S0169-5347(02)02489-8)
2. Madsen T, Stille B, Shine R. 1996 Inbreeding depression in an isolated population of adders *Vipera berus*. *Biol. Conserv.* **75**, 113–118. (doi:10.1016/0006-3207(95)00067-4)
3. Hedrick PW, Kalinowski ST. 2000 Inbreeding depression in conservation biology. *Annu. Rev. Ecol. Syst.* **31**, 139–162. (doi:10.1146/annurev.ecolsys.31.1.139)
4. Keller LF, Arcese P, Smith JNM, Hochachka WM, Stearns SC. 1994 Selection against inbred song sparrow during a natural population bottleneck. *Nature* **372**, 356–357. (doi:10.1038/372356a0)

5. Charlesworth D, Charlesworth B. 1987 Inbreeding depression and its evolutionary consequences. *Annu. Rev. Ecol. Syst.* **18**, 237–268. (doi:10.1146/annurev.es.18.110187.001321)
6. Darwin CR. 1876 *The effects of cross and self fertilization in the vegetable kingdom*. London, UK: John Murray.
7. Saccheri I, Kuussaari M, Kankare M, Vikman P, Fortelius W, Hanski I. 1998 Inbreeding and extinction in a butterfly metapopulation. *Nature* **392**, 491–494. (doi:10.1038/33136)
8. Crnokrak P, Roff DA. 1999 Inbreeding depression in the wild. *Heredity* **83**, 260–270. (doi:10.1038/sj.hdy.6885530)
9. Slate J, Kruuk LEB, Marshall TC, Pemberton JM, Clutton-Brock TH. 2000 Inbreeding depression influences lifetime breeding success in a wild population of red deer (*Cervus elaphus*). *Proc. R. Soc. Lond. B* **267**, 1657–1662. (doi:10.1098/rspb.2000.1192)
10. Ruiz-Lòpez MJ *et al*. 2012 Heterozygosity–fitness correlations and inbreeding depression in two critically endangered mammals. *Conserv. Biol.* **6**, 1121–1129. (doi:10.1111/j.1523-1739.2012.01916.x)
11. Charlesworth D, Willis JH. 2009 The genetic of inbreeding depression. *Nature* **10**, 783–796. (doi:10.1038/nrg2664)
12. Chapman JR, Nakagawa S, Coltman DW, Slate J, Sheldon BC. 2009 A quantitative review of heterozygosity–fitness correlations in animal populations. *Mol. Ecol.* **18**, 2746–2765. (doi:10.1111/j.1365-294X.2009.04247.x)
13. Szulkin M, Bierne N, David P. 2010 Heterozygosity–fitness correlations: a time for reappraisal. *Evolution* **64**, 1202–1217. (doi:10.1111/j.1558-5646.2010.00966.x)
14. Wang J. 2000 Effects of population structures and selection strategies on the purging of inbreeding depression due to deleterious mutations. *Genet. Resour.* **76**, 75–86. (doi:10.1017/S0016672399004450)
15. Boakes EH, Wang J, Amos W. 2007 An investigation of inbreeding depression and purging in captive pedigreed populations. *Heredity* **98**, 172–182. (doi:10.1038/sj.hdy.6800923)
16. Fowler K, Whitlock MC. 1999 The variance in inbreeding depression and the recovery of fitness in bottlenecked populations. *Proc. R. Soc. Lond. B* **266**, 2061–2066. (doi:10.1098/rspb.1999.0887)
17. Pemberton J. 2004 Measuring inbreeding depression in the wild: the old ways are the best. *Trends Ecol. Evol.* **19**, 613–615. (doi:10.1016/j.tree.2004.09.010)
18. Townsend SM, Jameson IG. 2013 Molecular and pedigree measures of relatedness provide similar estimates of inbreeding depression in a bottlenecked population. *J. Evol. Biol.* **26**, 889–899. (doi:10.1111/jeb.12109)
19. Hansson B, Westerberg L. 2002 On the correlation between heterozygosity and fitness in natural populations. *Mol. Ecol.* **11**, 2467–2474. (doi:10.1046/j.1365-294X.2002.01644.x)
20. Coltman DW, Slate J. 2003 Microsatellite measures of inbreeding: a meta-analysis. *Evolution* **57**, 971–983. (doi:10.1111/j.0014-3820.2003.tb00309.x)
21. Fisher RA. 1926 *The design of experiments*. Edinburgh, UK: Oliver and Boyd.
22. Shipley B. 2000 *Cause and correlation in biology: a user's guide to path analysis, structural equations, causal inference*. Oxford, UK: Oxford University Press.
23. Pearl J. 2009 *Causality: models, reasoning and inference*. Cambridge, UK: Cambridge University Press.
24. Shipley B. 2000 A new inferential test for path models based on directed acyclic graphs. *Struct. Equ. Model.* **7**, 206–218. (doi:10.1207/S15328007SEM0702_4)
25. Grace JB. 2006 *Structural equation modeling and natural systems*. Cambridge, UK: Cambridge University Press.
26. Lamb E, Mengersen KL, Stewart KJ, Attanayake U, Siciliano SD. 2014 Spatially explicit structural equation modeling. *Ecology* **95**, 2434–2442. (doi:10.1890/13-1997.1)
27. Costantini D, Ferrari C, Pasquaretta C, Carere C, von Hardenberg A, Reale D. 2012 Interplay between plasma oxidative state, cortisol and coping styles in wild Alpine marmots (*Marmota marmota*). *J. Exp. Biol.* **215**, 374–383. (doi:10.1242/jeb.062034)
28. Corlatti L, Bethaz S, von Hardenberg A, Bassano B, Palme R, Lovari S. 2012 Hormones, parasites and male mating tactics in Alpine chamois: identifying the mechanisms of life history trade-offs. *Anim. Behav.* **84**, 1061–1070. (doi:10.1016/j.anbehav.2012.08.005)
29. von Hardenberg A, Gonzalez-Voyer A. 2013 Disentangling evolutionary cause–effect relationships with phylogenetic confirmatory path analysis. *Evolution* **67**, 378–387. (doi:10.1111/j.1558-5646.2012.01790.x)
30. Biebach I, Keller LF. 2009 A strong genetic footprint of the re-introduction history of Alpine ibex (*Capra ibex ibex*). *Mol. Ecol.* **18**, 5046–5058. (doi:10.1111/j.1365-294X.2009.04420.x)
31. Grodinsky C, Stuwe M. 1987 The reintroduction of the Alpine Ibex to the Swiss Alps. *Smithsonian* **18**, 68–77.
32. Apollonio M, Bassano B, Mustoni A. 2003 Behavioral aspects of conservation and management of european mammals. In *Animal behavior and wildlife conservation* (eds M Festa-Bianchet, M Apollonio), pp. 157–170. Washington, DC: Island Press.
33. Aulagnier S, Kranz A, Lovari S, Jdeidi T, Masseti M, Nader I, de Smet K, Cuzin F. 2008 *Capra ibex*. In IUCN 2013. IUCN Red list of threatened species. Version 2013.1. See www.iucnredlist.org/details/42397/0.
34. Mignatti A, Casagrandi R, Provenzale A, von Hardenberg A, Gatto M. 2012 Sex- and age-structured models for Alpine ibex *Capra ibex ibex* population dynamics. *Wildl. Biol.* **18**, 318–332. (doi:10.2981/11-084)
35. Biebach I, Keller LF. 2012 Genetic variation depends more on admixture than number of founders in reintroduced Alpine ibex populations. *Biol. Conserv.* **147**, 197–203. (doi:10.1016/j.biocon.2011.12.034)
36. Maudet C, Miller C, Bassano B, Breitenmoser-Würsten C, Gauthier D, Obexer-Ruff G, Michallet J, Taberlet P, Luikart G. 2002 Microsatellite DNA and recent statistical methods in wildlife conservation management: applications in Alpine ibex [*Capra ibex* (*ibex*)]. *Mol. Ecol.* **11**, 421–436. (doi:10.1046/j.0962-1083.2001.01451.x)
37. Biebach I, Keller LF. 2010 Inbreeding in reintroduced populations: the effects of early reintroduction history and contemporary processes. *Conserv. Genet.* **11**, 527–538. (doi:10.1007/s10592-009-0019-6)
38. von Hardenberg A, Bassano B, Festa-Bianchet M, Luikart G, Lanfranchi P, Coltman D. 2007 Age-dependent genetic effects on a secondary sexual trait in male Alpine ibex, *Capra ibex*. *Mol. Ecol.* **16**, 1969–1980. (doi:10.1111/j.1365-294X.2006.03221.x)
39. Pérez-González J, Carranza J, Torres-Porras J, Fernández-García JL. 2010 Low heterozygosity at microsatellite markers in Iberian red deer with small antlers. *J. Hered.* **101**, 553–561. (doi:10.1093/jhered/esq049)
40. Harrison XA, Bearhop S, Inger R, Colhoun K, Gudmundssons GA, Hodgson D, McElwaine G, Tregenza T. 2011 Heterozygosity–fitness correlations in a migratory bird: an analysis of inbreeding and single-locus effects. *Mol. Ecol.* **20**, 4786–4795. (doi:10.1111/j.1365-294X.2011.05283.x)
41. Wetzel DP, Stewart IRK, Westneat DF. 2012 Heterozygosity predicts clutch and egg size but not plasticity in a house sparrow population with no evidence of inbreeding. *Mol. Ecol.* **21**, 406–420. (doi:10.1111/j.1365-294X.2011.05380.x)
42. Townsend SM, Jamieson IG. 2013 Inbreeding influences within-brood heterozygosity–fitness correlations (HFCs) in an isolated passerine population. *Evolution* **67**, 2299–2308. (doi:10.1111/evo.12113)
43. Bassano B, von Hardenberg A, Pelletier F, Gobbi G. 2003 A method to weigh free-ranging ungulates without handling. *Wildl. Soc. B* **31**, 1205–1209.
44. Grignolio S, Rossi I, Bassano B, Parrini F, Apollonio M. 2004 Seasonal variations of spatial behaviour in female Alpine ibex (*Capra ibex ibex*) in relation to climatic conditions and age. *Ethol. Ecol. Evol.* **16**, 255–264. (doi:10.1080/08927014.2004.9522636)
45. Bergeron P. 2007 Parallel lasers for remote measurements of morphological traits. *J. Wildl. Manage.* **71**, 289–292. (doi:10.2193/2006-290)
46. Brambilla A, von Hardenberg A, Kristo O, Bassano B, Bogliani G. 2013 Don't spit in the soup: faecal avoidance in foraging wild Alpine ibex (*Capra ibex*). *Anim. Behav.* **86**, 153–158. (doi:10.1016/j.anbehav.2013.05.006)
47. Valière N. 2002 GIMLET: a computer program for analysing genetic individual identification data. *Mol. Ecol.* **2**, 377–379. (doi:10.1046/j.1471-8286.2002.00228.x-i2)
48. von Hardenberg A. 2005 Sénescence, sélection sexuelle et dynamique de population du bouquetin des Alpes (*Capra ibex*). PhD thesis, Université de Sherbrooke, Quebec, Canada.
49. Brambilla A, Canedoli C. 2013 How to continue measuring horn growth after capture in long term studies. *J. Mount. Ecol.* **9**, 35–46.

50. Coltman DW, Pilkington J, Kruuk LE, Wilson K, Pemberton JM. 2001 Positive genetic correlation between parasite resistance and body size in a free-living ungulate population. *Evolution* **55**, 2116–2125. (doi:10.1111/j.0014-3820.2001.tb01326.x)
51. Ministry of Agriculture, Fisheries and Food. 1971 *Manual of veterinary parasitological laboratory techniques*. London, UK: Her Majesty's Stationery Office.
52. Peakall R, Smouse PE. 2012 GenAlEx 6.5, J. *Bioinformatics* **28**, 2537–2539. (doi:10.1093/bioinformatics/bts460)
53. Rice WR. 1989 Analyzing tables of statistical tests. *Evolution* **43**, 223–225. (doi:10.2307/2409177)
54. Coltman DW, Pilkington JG, Pemberton JM. 1999 Parasite-mediated selection against inbred Soay sheep in a free-living, island population. *Evolution* **53**, 1259–1267. (doi:10.2307/2640828)
55. David P, Pujol B, Viard F, Castella V, Goudet J. 2007 Reliable selfing rate estimates from imperfect population genetic data. *Mol. Ecol.* **16**, 2474–2487. (doi:10.1111/j.1365-294X.2007.03330.x)
56. Bates D, Maechler M, Bolker B. 2011 lme4: linear mixed-effects models using S4 classes. R package version 0.999375–42/r1414. See http://R-Forge.R-project.org/projects/lme4/.
57. R Development Core Team. 2013 *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. See http://www.R-project.org/.
58. von Hardenberg A, Bassano B, Zummel Arranz MDP, Bogliani G. 2004 Horn growth but not asymmetry heralds the onset of senescence in male Alpine ibex (*Capra ibex*). *J. Zool.* **263**, 425–432. (doi:10.1017/S0952836904005485)
59. Barton K. 2013 MuMIn: multi-model inference. R package version 1.9.0. See http://CRAN.R-project.org/package=MuMIn.
60. Burnham KP, Anderson DR. 2002 *Model selection and multimodel inference: a practical information-theoretic approach*, 2nd edn. New York, NY: Springer.
61. Burnham KP, Anderson DR, Huyvaert KP. 2011 AIC model selection and multimodel inference in behavioral ecology: some background, observations, comparisons. *Behav. Ecol. Sociobiol.* **65**, 23–35. (doi:10.1007/s00265-010-1029-6)
62. Shipley B. 2009 Confirmatory path analysis in a generalized multilevel context. *Ecology* **90**, 363–368. (doi:10.1890/08-1034.1)
63. Shipley B. 2013 The AIC model selection method applied to path analytic models compared using a d-separation test. *Ecology* **94**, 560–564. (doi:10.1890/12-0976.1)
64. Bergeron P, Grignolio S, Apollonio M, Shipley B, Festa-Bianchet M. 2010 Secondary sexual characters signal fighting ability and determine social rank in Alpine ibex (*Capra ibex*). *Behav. Ecol. Sociobiol.* **64**, 1299–1307. (doi:10.1007/s00265-010-0944-x)
65. Kokko H. 1997 Evolutionarily stable strategies of age-dependent sexual advertisement. *Behav. Ecol. Sociobiol.* **41**, 99–107. (doi:10.1007/s002650050369)
66. Decristophoris PMA, von Hardenberg A, McElligott AG. 2007 Testosterone is positively related to the output of nematode eggs in male Alpine ibex (*Capra ibex*) faeces. *Evol. Ecol. Res.* **9**, 1277–1292.
67. Louvandini H, Veloso CFM, Paludo GR, Dell'Porto A, Gennari SM, McManus CM. 2006 Influence of protein supplementation on the resistance and resilience on young hair sheep naturally infected with gastrointestinal nematodes during rainy and dry seasons. *Vet. Parasitol.* **137**, 103–111. (doi:10.1016/j.vetpar.2006.01.004)
68. Brivio F, Grignolio S, Brambilla A, Apollonio M. 2014 Intra-sexual variability in feeding behaviour of a mountain ungulate: size matters. *Behav. Ecol. Sociobiol.* **68**, 1649–1660. (doi:10.1007/s00265-014-1773-0)
69. Etter E, Hoste H, Chartier C, Pors I, Koch C, Broqua C, Coutineau H. 2000 The effect of two levels of dietary protein on resistance and resilience of dairy goats experimentally infected with *Trichostrongylus colubriformis*: comparison between high and low producers. *Vet. Res.* **31**, 247–258. (doi:10.1051/vetres:2000120)
70. David P. 1998 Heterozygosity–fitness correlations: new perspective, old problems. *Heredity* **80**, 531–537. (doi:10.1046/j.1365-2540.1998.00393.x)
71. David P, Delay B, Jarne P. 1997 Heterozygosity and growth in the marine bivalve *Spisula ovalis*: testing alternative hypotheses. *Genet. Resour.* **70**, 215–223. (doi:10.1017/S0016672397002978)
72. Willisch CS, Biebach I, Koller U, Bucher T, Marreros N, Ryser-Degiorgis M-P, Keller LF, Neuhaus P. 2012 Male reproductive pattern in a polygynous ungulate with a slow life-history: the role of age, social status and alternative mating tactics. *Evol. Ecol.* **26**, 187–206. (doi:10.1007/s10682-011-9486-6)
73. Vanpé C *et al*. 2007 Antler size provides an honest signal of male phenotypic quality in roe deer. *Am. Nat.* **169**, 481–493. (doi:10.1086/512046)
74. Gulland FMD. 1995 Impact of infectious diseases on wild animal populations: a review. In *Ecology of infectious diseases in natural populations* (eds BT Grenfell, AP Dobson), pp. 20–51. Cambridge, UK: Cambridge University Press.
75. Albon SD, Stien A, Irvine RJ, Langvatn R, Ropstad E, Halvorsen O. 2002 The role of parasites in the dynamics of a reindeer population. *Proc. R. Soc. Lond. B* **269**, 1625–1632. (doi:10.1098/rspb.2002.2064)
76. Stien A, Irvine RJ, Ropstad E, Halvorsen O, Langvatn R, Albon SD. 2002 The impact of gastrointestinal nematodes on wild reindeer: experimental and cross-sectional studies. *J. Anim. Ecol.* **71**, 937–945. (doi:10.1046/j.1365-2656.2002.00659.x)
77. Gunn A, Irvine RJ. 2003 Subclinical parasitism and ruminant foraging strategies: a review. *Wildl. Soc. B.* **31**, 117–126.
78. Irvine RJ, Corbishley H, Pilkington JG, Albon SD. 2006 Low-level parasitic worm burdens may reduce body condition in free-ranging red deer (*Cervus elaphus*). *Parasitology* **133**, 465–475. (doi:10.1017/S0031182006000606)
79. Acevedo-Whitehouse K, Vicente J, Gortazar C, Höfle U, Fernández-De-Mera IG, Amos W. 2005 Genetic resistance to bovine tuberculosis in the Iberian wild boar. *Mol. Ecol.* **14**, 3209–3217. (doi:10.1111/j.1365-294X.2005.02656.x)
80. Crow JF, Kimura M. 1970 *An introduction to population genetics theory*. New York, NY: Harper & Row.
81. Miller JM, Malenfant RM, David P, Davis CS, Poissant J, Hogg JT, Festa-Bianchet M, Coltman DW. 2014 Estimating genome-wide heterozygosity: effects of demographic history and marker type. *Heredity* **112**, 240–247. (doi:10.1038/hdy.2013.99)
82. Grueber CE, Waters JM, Jamieson IG. 2011 The imprecision of heterozygosity–fitness correlations hinders the detection of inbreeding and inbreeding depression in a threatened species. *Mol. Ecol.* **20**, 67–79. (doi:10.1111/j.1365-294X.2010.04930.x)
83. Kardos M, Allendorf FW, Luikart G. 2014 Evaluating the role of inbreeding depression in heterozygosity–fitness correlations: how useful are tests for identity disequilibrium? *Mol. Ecol. Res.* **14**, 519–530. (doi:10.1111/1755-0998.12193)
84. Reed DH, Frankham R. 2001 How closely correlated are molecular and quantitative measures of genetic variation? A meta-analysis. *Evolution* **55**, 1095–1103. (doi:10.1111/j.0014-3820.2001.tb00629.x)