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Exploring the Mechanisms Underlying a Heterozygosity–Fitness Correlation for Canine Size in the Antarctic Fur Seal *Arctocephalus gazella*

Joseph I. Hoffman, Jaume Forcada, and William Amos

From the Department of Zoology, University of Cambridge, Cambridge, CB2 3EJ, UK (Hoffman and Amos); and the British Antarctic Survey, Natural Environment Research Council, Cambridge, UK (Forcada).

Address correspondence to Joseph I. Hoffman at the address above, or e-mail: jih24@cam.ac.uk.

Abstract

Although heterozygosity–fitness correlations (HFCs) are widely reported in the literature, most studies use too few markers to allow the proximate mechanisms to be convincingly resolved. Two competing hypotheses have been proposed: the general effect hypothesis, in which marker heterozygosity correlates with genome-wide heterozygosity and hence the inbreeding coefficient *f*, and the local effect hypothesis, in which one or more of the markers by chance exhibit associative overdominance. To explore the relative contributions of general and local effects in a free-ranging marine mammal population, we revisited a strong HFC found using 9 microsatellite loci for canine tooth size in 84 male Antarctic fur seals *Arctocephalus gazella* (Hoffman JI, Hanson N, Forcada J, Trathan PN, Amos W. 2010. Getting long in the tooth: a strong positive correlation between canine size and heterozygosity in the Antarctic fur seal *Arctocephalus gazella*. J Hered.). Increasing the number of markers to 76, we find that heterozygosity is uncorrelated across loci, indicating that inbred individuals are rare or absent. Similarly, while the HFC based on overall heterozygosity is lost, stochastic simulations indicate that when an HFC is due to inbreeding depression, increasing marker number invariably strengthens the HFC. Together these observations argue strongly that the original HFC was not due to inbreeding depression. In contrast, a subset of markers show individually significant effects, and these are nonrandomly distributed across the marker panel, being preferentially associated with markers cloned from other species. Using basic alignment search tool searches, we were able to locate 94% of loci to unique locations in the dog genome, but the local genes are functionally diverse, and the majority cannot be linked directly to growth. Our results suggest that inbreeding depression contributes little if at all to the relationship between heterozygosity and tooth size but that instead the primary mechanism involves associative overdominance. These findings contribute to a growing body of evidence suggesting that general effects are likely to be uncommon in natural populations.

Key words: *associative overdominance, cross-amplification, heterozygosity–fitness correlation, local effect, otariid, pinniped*

Recent years have seen a wealth of papers describing heterozygosity–fitness correlations (HFCs) in which heterozygosity, usually measured at around 10 microsatellite markers, is shown to predict some aspect of an individual's fitness. The direction of the relationship seems almost invariably to be in the direction of higher heterozygosity indicating higher quality, and the traits studied embrace almost all aspects of life, from birth weight (Coulson et al. 1999) and parasite resistance (Rijks et al. 2008) though recruitment and reproductive and success (Amos et al. 2001; Cohas et al. 2009) to plumage coloration (Foerster et al. 2003), song pitch (Araya-Ajoy et al. 2009), attractiveness (Hoffman, Forada, et al. 2007), dominance status (Tiira et al. 2006) and territory holding ability (Höglund et al. 2002). As such, HFCs appear to represent an important component of fitness in many or perhaps even most systems.

Despite the number of studies reporting HFCs, the proximate mechanism or mechanisms remain open to debate (reviewed by Hansson and Westerberg 2002). Two main possibilities exist. First, heterozygosity could be a proxy for genome-wide heterozygosity, which in turn correlates with an individual's inbreeding coefficient. If so, HFCs would be interpreted as reflecting inbreeding depression, with the more homozygous individuals being

relatively inbred and of low quality. This is termed the general effect hypothesis. The alternative has been termed associative overdominance. Here, one or a few of the microsatellites used as markers by chance lie near genes experiencing some form of balancing selection, either through simple heterozygote advantage or perhaps through a more complicated model such as repulsion phase disequilibrium (Ohta 1971) where 2 linked genes are segregating for deleterious alleles that are in negative phase with each other. Under this scenario, linkage disequilibrium between the marker and genes tends to cause a correlation between heterozygosity at the nearby microsatellite and higher than average fitness. This is termed the local effect hypothesis.

Both the general and the local effect hypotheses suffer from criticisms on theoretical grounds. In the general effect hypothesis, there is a requirement for heterozygosity at a few markers to correlate strongly with f, the inbreeding coefficient. Unfortunately, individuals with detectably non-zero inbreeding coefficients are generally thought to be rare in most real populations (Balloux et al. 2004; Slate et al. 2004). Only in very small populations where there are high levels of reproductive skew does it become likely that as few as 10 markers will be able to detect an HFC (Balloux et al. 2004). On the other hand, the local effect model is also not straightforward. Here, the requirement is for a randomly selected microsatellite to lie near enough to a gene experiencing balancing selection for heterozygosity at the marker to reflect heterozygosity at the gene. However, the genome is a big place and balancing selection is generally thought to be rather rare (Gemmell and Slate 2006), making it ostensibly unlikely that such a marker-gene linkage would occur by chance. Put another way, if most HFCs reflect a local effect, balancing selection would have to be far more common than is currently believed.

Although it is true that a number of studies have attempted to distinguish between general and local effects, the problem is by no means easy, making firm conclusions hard to come by. As we ourselves found in our companion paper, the use of only around 10 markers affords rather little statistical power. On the one hand, it is difficult to demonstrate convincingly the correlation in heterozygosity across markers that would indicate a general effect. On the other hand, occasional single-locus associations, while suggestive of balancing selection, are also expected under the general effect model due to the background level of association that affects all markers in the presence of inbreeding; the most extreme of these potentially appearing as associations that remain significant even after correction for multiple tests. The most elegant approach to bypass these problems is perhaps to use individuals where f is known from deep pedigree data (Jensen et al. 2007) or to control for f by comparing heterozygosity and fitness among individuals which have the same f because they are full siblings (Hansson et al. 2001). Because these approaches are not open to most studies, a variety of other methods have been tried, including testing each locus individually, testing the effect of dropping each locus in turn, and using heterozygosity–heterozygosity (het–het) correlations (Balloux et al. 2004) to test for the presence of inbred individuals. The latter involves testing the prediction that, under the general effect model, heterozygosity should be correlated among markers and is based on repeatedly calculating the correlation across individuals of heterozygosity calculated on 2 random but equal subsets of markers. However, with only around 10 markers commonly deployed, these approaches tend to lack power. For het–het correlations, the marker subsets are very small, while single-locus tests suffer the problem that, if a general effect is present, all markers will tend to exhibit a weak effect, raising the chance that 1 or 2 markers individually show a significant effect above the level expected after correction for multiple tests.

Perhaps the most generally applicable test of local versus general effects is to deploy a much larger number of markers (e.g., Slate and Pemberton 2002; Campbell et al. 2007). Although this represents more experimental effort, it offers the chance of unambiguously resolving the question. With general effects, the more markers that are used, the stronger should be the HFC detected because the use of more markers will reduce the error variance in estimation of genome-wide heterozygosity, that is, f. In contrast, the result of using more markers to resolve HFCs due to local effects is unclear. If only a single gene is involved across the whole genome, adding more markers should weaken the relationship between mean heterozygosity and fitness. However, if many genes are involved, adding more markers will pick up further associations, potentially strengthening the trend, though probably not as much as for a general effect. This is because, as long as the markers are mostly unlinked, individuals which are heterozygous at one locus will often be homozygous at another and vice versa, thereby to some extent canceling each other out.

A long-term study of Antarctic fur seals, *Arctocephalus gazella*, provides an exceptional opportunity to explore the mechanisms underlying HFCs in a natural population, partly because HFCs have already been documented for several traits (Hoffman et al. 2004; Hoffman, Forada, et al. 2007) but also because both mechanisms have ample opportunity to operate. Local effects are likely to play at least a limited role, having previously been identified for both male reproductive success (Hoffman et al. 2004) and canine size (Hoffman et al. 2010). However, fur seals are highly polygynous (Hoffman et al. 2003) and show strong site fidelity (Hoffman et al. 2006), conditions that could potentially generate inbreeding and hence favor general effects. Against this, behavioral mechanisms including female choice (Hoffman, Forada, et al. 2007) appear to have evolved specifically to minimize inbreeding. A further issue is that our standard panel of microsatellite markers was derived by selecting the some of the most variable loci found in other species. This might conceivably bias them in favor of loci exhibiting associative overdominance because loci near genes under balancing selection should tend to show persistently high levels of polymorphism across species. Finally, heavy exploitation of fur seals during the

C18th and C19th could also favor local effects because strong population bottlenecks tend to increase linkage disequilibrium, thereby increasing the distance over which an association can be detected between a marker and a gene.

In this study, we take a relatively strong HFC based on male fur seal canine tooth size at death (see our companion paper for details, Hoffman et al. 2010) and raise the number of markers used from 9 to 76 in order to dissect the likely underlying mechanism(s).

Materials and Methods

The Microsatellite Data Set

The methods used are essentially identical to those in our companion paper. To our previous data set, comprising 84 fur seal tissue samples genotyped at our standard panel of 9 microsatellite loci (Hoffman et al. 2010), we added a further 67 loci isolated from a variety of different pinniped species and one locus from the black bear (see Table 1 for details). Microsatellite genotypes were generated following Hoffman and Amos (2005) and we tested for deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium using the program GENEPOP (Raymond and Rousset 1995; http://genepop.curtin.edu.au/). Null allele frequencies were calculated using the program MICRO-CHECKER (Van Oosterhout et al. 2004) following the equation of Chakraborty et al. (1992).

Calculation of Individual Heterozygosity and Data Analyses

Individual heterozygosity was calculated as internal relatedness (IR, Amos et al. 2001) for reasons described in our companion paper. Standardized heterozygosity (SH, Coltman et al. 1999) and heterozygosity weighted by locus (HL, Aparicio et al. 2006) were also calculated, but the 3 measures were strongly intercorrelated and generated virtually identical results (data not shown). Data analyses focused on canine length and mass, 2 measures of canine size that were found to be strongly correlated with IR when calculated at 9 loci. We did not analyze canine width because this was previously found to be only marginally significantly correlated with IR. Analyses were carried out using generalized linear models (GLMs) in R (R Development Team 2005), fitting either heterozygosity alone or including age at death and the age:heterozygosity interaction. Using standard deletion-testing procedures (Crawley 2002), each term was then dropped from models unless doing so significantly reduced the amount of deviance explained (deviance is analogous to sums of squares in standard regression analysis). The change in deviance between full and reduced models was distributed as χ^2 with degrees of freedom (df) equal to the difference in df between the models with and without the term in question.

We also analyzed each locus using the randomization approach detailed by Amos and Acevedo-Whitehouse (2009) and used in our companion paper. In this analysis, data are arranged such that the association between genotype and phenotype is maximized; in our case, all genotypes with above average tooth length are placed in one group and all those with below average length in another, making 2 groups. The strength of the resulting association is recorded as a test statistic, here a *t*-test. To interpret this statistic, we then repeatedly scramble the relationship between genotype and phenotype, each time repeating the process of finding the strongest possible association. When a genuine association is present, the expectation is that the original *t*-value will be higher than those obtained from the randomized data. Significance was assessed nonparametrically, expressed as the proportion of times the randomized data yielded a test statistic as large, or larger, than the one obtained with the raw data.

Controlling for Multiple Statistical Tests

During this study, we conduct a large number of statistical tests of association such that formal control of Type I errors through either Bonferroni (Hochberg 1988) or false discovery rate (FDR) correction (Benjamini and Hochberg 1995) would be desirable. However, table-wide correction is inappropriate because both methods assume that all the tests are independent, whereas many of our tests show clear nonindependence. We therefore elected to control Type I errors per group of tests (i.e., each set of 72 or 76 tests for a given trait, the exact number of tests depending on whether X-linked markers were included in the analysis). *P* values were corrected using the FDR method implemented in the program Q-VALUE (http://genomics.princeton.edu/storeylab/qvalue/index.html; Storey and Tibshirani 2003).

Locating Markers in the Dog Genome

We conducted basic local alignment search tool (BLAST) searches (Altschul et al. 1990) to explore putative homology between microsatellite clone sequences, where available, and the dog genome (http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=9615). BLAST searches with default match parameters were conducted using full-length clone sequences downloaded from GenBank (www.ncbi.nih.gov). For the majority of resulting matches, the query sequence revealed significant similarity to a single genomic location in the dog. However, in the few cases where multiple matches were obtained, we recorded the top-scoring match (e.g., the one with the lowest *E*-value). For the remaining loci, we assumed that local rearrangements at or near the microsatellite made alignment difficult. In such cases, we searched using BLASTN with the longest, least repetitive sequence from one side of the microsatellite, repeating with other subsequences where necessary. A match was judged to have been found if there was a single low *E*-value (typically $\leq 10^{-10}$) and if visual inspection of the matching sequence revealed evidence both of the expected microsatellite and of homology with the other flanking sequence.

Simulations

To test the prediction that adding more loci should strengthen an HFC under the general effect hypothesis

Table 1 Details of the 76 microsatellite loci employed in this study and their polymorphism characteristics in 84 dead adult male Antarctic fur seals

Locus	Isolated from species	Reference	T_1 (°C)	T_2 (°C)	Number of alleles	H_E	H_O	HWE probability	Null allele frequency
Aa4	South American fur seal *Arctocephalus australis*	Gemmell et al. (1997)	46	48	6	0.737	0.798	0.229	−0.0428
Hg1.3	Gray seal *Halichoerus grypus*	Gemmell et al. (1997)	42	46	12	0.849	0.843	0.050	0.0005
Hg6.3	Gray seal *H. grypus*	Allen et al. (1995)	46	48	12	0.861	0.893	0.676	−0.0209
Hg8.10	Gray seal *H. grypus*	Allen et al. (1995)	42	46	4	0.444	0.440	1.000	0.0009
Lw10	Weddell seal *Leptonychotes weddellii*	Davis et al. (2002)	46	48	14	0.867	0.869	0.160	−0.0039
M11a	Southern elephant seal *Mirounga leonina*	Hoelzel et al. (1999)	46	48	18	0.928	0.893	0.781	0.0162
Pv9	Gray seal *H. grypus*	Allen et al. (1995)	48	52	10	0.779	0.738	0.148	0.0238
PvcA	Western Atlantic harbor seal *Phoca vitulina concolor*	Coltman et al. (1996)	46	48	8	0.836	0.857	0.403	−0.0155
PvcE	Western Atlantic harbor seal *P. vitulina concolor*	Coltman et al. (1996)	45	50	14	0.856	0.843	0.635	0.0044
Ag1 (1)	Antarctic fur seal *Arctocephalus gazella*	Hoffman et al. (2008)	46	48	11	0.872	0.905	0.849	−0.0213
Ag1 (2)	Antarctic fur seal *A. gazella*	Hoffman et al. (2008)	46	48	3	0.545	0.422	**0.009**	0.1245
Ag2	Antarctic fur seal *A. gazella*	Hoffman et al. (2008)	48	52	7	0.800	0.831	0.723	−0.0222
Ag3	Antarctic fur seal *A. gazella*	Hoffman et al. (2008)	46	48	2	0.310	0.333	0.724	−0.0389
Ag4	Antarctic fur seal *A. gazella*	Hoffman et al. (2008)	46	48	15	0.851	0.833	0.202	0.0075
Ag6	Antarctic fur seal *A. gazella*	Hoffman et al. (2008)	48	52	8	0.723	0.726	0.558	−0.005
Ag7	Antarctic fur seal *A. gazella*	Hoffman et al. (2008)	46	48	6	0.739	0.810	0.727	−0.0483
Ag8	Antarctic fur seal *A. gazella*	Hoffman et al. (2008)	48	52	16	0.877	0.720	**0.028**	0.0954
Ag9	Antarctic fur seal *A. gazella*	Hoffman et al. (2008)	48	52	2	0.451	0.440	1.000	0.0088
Ag10	Antarctic fur seal *A. gazella*	Hoffman et al. (2008)	46	48	7	0.721	0.687	0.163	0.0215
Agaz1	Antarctic fur seal *A. gazella*	Hoffman (2009)	50	54	11	0.871	0.881	0.524	−0.0085
Agaz2	Antarctic fur seal *A. gazella*	Hoffman (2009)	46	48	9	0.815	0.786	0.256	0.0153
Agaz3	Antarctic fur seal *A. gazella*	Hoffman (2009)	46	48	4	0.604	0.631	0.451	−0.0249
Agaz4	Antarctic fur seal *A. gazella*	Hoffman (2009)	48	52	6	0.770	0.361	**<0.0001**	0.3587
Agaz5	Antarctic fur seal *A. gazella*	Hoffman (2009)	48	52	3	0.511	0.530	0.652	−0.0212
Agaz6	Antarctic fur seal *A. gazella*	Hoffman (2009)	46	48	4	0.688	0.790	0.230	−0.0622
Agaz7	Antarctic fur seal *A. gazella*	Hoffman (2009)	46	48	5	0.735	0.756	0.315	−0.0232
Agaz8	Antarctic fur seal *A. gazella*	Hoffman (2009)	48	52	14	0.850	0.783	0.237	0.0381
Agaz9	Antarctic fur seal *A. gazella*	Hoffman (2009)	60	65	8	0.797	0.714	0.601	0.0515
Agaz10	Antarctic fur seal *A. gazella*	Hoffman JI, unpublished data	55	60	9	0.785	0.679	**0.001**	0.0699
Agaz11	Antarctic fur seal *A. gazella*	Hoffman JI, unpublished data	55	60	10	0.772	0.699	0.093	0.047
Agaz12	Antarctic fur seal *A. gazella*	Hoffman JI, unpublished data	55	60	7	0.783	0.759	0.679	0.0124

Table 1 Continued

Locus	Isolated from species	Reference	T_1 (°C)	T_2 (°C)	Number of alleles	H_E	H_O	HWE probability	Null allele frequency
G1A	Black bear *Ursus americanus*	Paetkau et al. (1995)	55	60	12	0.842	0.714	0.129	0.079
Hg1.4[a]	Gray seal *H. grypus*	Gemmell et al. (1997)	48	52	7	0.747	0.000	**<0.0001**	1
Hg4.2	Gray seal *H. grypus*	Allen et al. (1995)	46	48	19	0.914	0.506	**<0.0001**	0.284
Hg6.10	Gray seal *H. grypus*	Allen et al. (1995)	55	60	12	0.878	0.869	0.449	0.0022
Hl4	Leopard seal *Hydrurga leptonyx*	Davis et al. (2002)	46	48	5	0.575	0.605	0.223	−0.0287
Hl16	Leopard seal *H. leptonyx*	Davis et al. (2002)	46	48	11	0.843	0.750	0.131	0.0551
Lc5	Crabeater seal *Lobodon carcinophagus*	Davis et al. (2002)	46	48	3	0.308	0.310	0.690	−0.006
Lc28	Crabeater seal *L. carcinophagus*	Davis et al. (2002)	48	52	10	0.858	0.819	0.900	0.0198
Lw8	Weddell seal *L. weddellii*	Davis et al. (2002)	42	46	12	0.909	0.929	0.716	−0.0135
Lw15(1)	Weddell seal *L. weddellii*	Davis et al. (2002)	48	52	12	0.877	0.805	0.050	0.0398
Lw15(2)	Weddell seal *L. weddellii*	Davis et al. (2002)	48	52	10	0.855	0.889	0.144	−0.0228
Lw18[a]	Weddell seal *L. weddellii*	Davis et al. (2002)	46	48	4	0.179	0.000	**<0.0001**	1
Ms15	Hawaiian monk seal *Monachus schauinslandi*	Schultz et al. (2009)	48	52	7	0.789	0.793	0.655	−0.036
Ms23	Hawaiian monk seal *M. schauinslandi*	Schultz et al. (2009)	48	52	8	0.832	0.586	**<0.0001**	0.203
Ms265	Hawaiian monk seal *M. schauinslandi*	Schultz et al. (2009)	54	58	5	0.712	0.699	0.090	−0.0072
Ms647	Hawaiian monk seal *M. schauinslandi*	Schultz et al. (2009)	55	60	15	0.895	0.940	0.916	−0.0598
OrrFCB1	Atlantic walrus *Odobenus rosmarus rosmarus*	Buchanan et al. (1998)	45	50	9	0.833	0.802	0.320	0.0154
OrrFCB2	Atlantic walrus *O. rosmarus rosmarus*	Buchanan et al. (1998)	48	52	11	0.873	0.881	0.666	−0.0077
OrrFCB3	Atlantic walrus *O. rosmarus rosmarus*	Buchanan et al. (1998)	46	48	17	0.745	0.691	0.799	0.0342
OrrFCB7	Atlantic walrus *O. rosmarus rosmarus*	Buchanan et al. (1998)	55	60	10	0.855	0.821	0.299	0.0171
OrrFCB8	Atlantic walrus *O. rosmarus rosmarus*	Buchanan et al. (1998)	55	60	6	0.789	0.738	0.095	0.0301
OrrFCB16	Atlantic walrus *O. rosmarus rosmarus*	Buchanan et al. (1998)	55	60	4	0.598	0.643	0.250	−0.0388
SGPv17[a]	Eastern Atlantic harbour seal *P. vitulina vitulina*	Goodman (1997)	46	48	6	0.748	0.000	**<0.0001**	1
Ssl2x	Steller sea lion *Eumetopias jubatus*	Huebinger et al. (2007)	46	48	7	0.632	0.571	0.231	0.0477
Ssl5x	Steller sea lion *E. jubatus*	Huebinger et al. (2007)	46	48	11	0.893	0.929	0.529	−0.0227
Ssl39	Steller sea lion *E. jubatus*	Bickham J, unpublished data	48	52	7	0.805	0.798	0.801	0.0016
Ssl301	Steller sea lion *E. jubatus*	Huebinger et al. (2007)	46	48	14	0.885	0.867	0.803	0.0068
Ssl441	Steller sea lion *E. jubatus*	Huebinger et al. (2007)	48	52	5	0.473	0.494	0.856	−0.0244

Table 1 Continued

Locus	Isolated from species	Reference	T_1 (°C)	T_2 (°C)	Number of alleles	H_E	H_O	HWE probability	Null allele frequency
Zcwa05	Galapagos sea lion *Zalophus californianus wollebaeki*	Hoffman, Steinfartz, and Wolf (2007)	46	48	15	0.894	0.893	0.657	−0.0026
Zcwa12	Galapagos sea lion *Z. californianus wollebaeki*	Hoffman, Steinfartz, and Wolf (2007)	48	52	18	0.858	0.833	0.873	0.0114
Zcwb03[a]	Galapagos sea lion *Z. californianus wollebaeki*	Hoffman, Steinfartz, and Wolf (2007)	48	52	7	0.789	0.000	**<0.0001**	1
Zcwb07	Galapagos sea lion *Z. californianus wollebaeki*	Hoffman, Steinfartz, and Wolf (2007)	48	52	10	0.873	0.892	0.262	−0.0138
Zcwb09	Galapagos sea lion *Z. californianus wollebaeki*	Wolf et al. (2005)	46	48	12	0.870	0.831	0.449	0.0196
Zcwc01	Galapagos sea lion *Z. californianus wollebaeki*	Hoffman, Steinfartz, and Wolf (2007)	48	52	12	0.860	0.845	0.281	0.0098
Zcwe04	Galapagos sea lion *Z. californianus wollebaeki*	Hoffman, Steinfartz, and Wolf (2007)	46	48	11	0.869	0.802	0.088	0.0369
Zcwe12	Galapagos sea lion *Z. californianus wollebaeki*	Hoffman, Steinfartz, and Wolf (2007)	46	48	8	0.816	0.807	0.475	0.0026
Zcwf07	Galapagos sea lion *Z. californianus wollebaeki*	Hoffman, Steinfartz, and Wolf (2007)	46	48	8	0.786	0.928	0.092	−0.0858
Zcwf09	Galapagos sea lion *Z. californianus wollebaeki*	Hoffman, Steinfartz, and Wolf (2007)	50	54	9	0.779	0.827	**0.025**	−0.0026
Zcwg04	Galapagos sea lion *Z. californianus wollebaeki*	Hoffman, Steinfartz, and Wolf (2007)	46	48	14	0.895	0.893	0.931	−0.0017
ZcwCgDh1.8	California sea lion *Z. californianus*	Hernandez-Velazquez et al. (2005)	46	48	7	0.797	0.843	0.891	−0.0312
ZcwCgDh5.16	California sea lion *Z. californianus*	Hernandez-Velazquez et al. (2005)	46	48	6	0.795	0.795	0.583	−0.0028
ZcwCgDh48	California sea lion *Z. californianus*	Hernandez-Velazquez et al. (2005)	46	48	8	0.545	0.571	0.546	−0.0263
ZcwCgDh7tg	California sea lion *Z. californianus*	Hernandez-Velazquez et al. (2005)	46	48	18	0.902	0.878	0.823	0.0103
ZcwCgDh4.7	California sea lion *Z. californianus*	Hernandez-Velazquez et al. (2005)	46	48	13	0.877	0.928	0.327	−0.0313
ZcwCgDhB.14	California sea lion *Z. californianus*	Hernandez-Velazquez et al. (2005)	46	48	6	0.753	0.867	0.134	−0.0734

Our standard panel of 9 loci is shown at the top of the table, with the newly added markers being listed afterward in alphabetical order. Significant deviations from HWE without correction for multiple tests are highlighted in bold. T_1 and T_2 denote polymerase chain reaction annealing temperatures. H_E, expected heterozygosity; H_O observed heterozygosity.

[a] These have allele frequencies consistent with X linkage.

but have a weaker or even negative impact under the local effect hypothesis, we ran a series of stochastic simulations, based on varying numbers of microsatellite loci, each with 4 alleles at population frequencies of 0.4, 0.3, 0.2, and 0.1. In each simulation, 100 individuals were generated, each with a multilocus genotype from which IR was calculated, and a fitness score drawn from a normal distribution and then modified according to the genotype–phenotype link in the model being tested. For both the general effect and the local effect models, a range of parameter values were used in order to generate a spread of HFC strengths. For the general effect model, the parameters varied were 1) frequency of inbred individuals (N individuals with $f = 0.25$ [range of N explored = 5–35 in steps of 5], plus an equal number of individuals with $f = 0.125$, all other individuals have $f = 0$); 2) impact of inbreeding on fitness [$\text{fit}' = \text{fit}\,(1 - F)^x$, where fit′ = adjusted fitness score, fit = original fitness score, and x = 1, 2, 3, or 4]; 3) variance in fitness, determined as the sum of either 5 or 25 random numbers between zero and one, thus yielding Gaussian distributions with the former having approximately twice the variance of the latter. For the local effect model, parameters varied were 1) the impact of being homozygous [$\text{fit}' = \text{fit} \times Z$, where Z is the impact factor = 0.25, 0.5, or 0.75]; 2) the frequency of loci contributing to the effect (one locus every x loci, x = 3–10 inclusive]; 3) variance in fitness, determined as for the general effect model.

Results

To explore the likely mechanisms underlying an HFC for canine size in the Antarctic fur seal (Hoffman et al. 2010), we increased the numbers of markers genotyped from 9 to 76 (Table 1). The number of alleles at each locus varied between 2 and 19 and expected heterozygosity ranged from 0.18 to 0.93. Four loci were homozygous in every one of our male tooth samples, even though they are polymorphic in control samples from females, and hence were probably X linked. A further 7 loci exhibited significant deviations from HWE, mostly in the direction of homozygote excess and probably due to null alleles.

Relationship between Canine Size and IR

In our companion paper (Hoffman et al. 2010), strong relationships are documented between IR when calculated at 9 microsatellite loci and 2 measures of canine size, length, and mass. We extended this analysis by constructing GLMs of canine length and mass, this time fitting IR calculated using 72 loci (the 4 putatively X-linked loci were excluded from the analysis). IR was found to be no longer significant (canine length, $\chi^2 = 0.29$, df = 1, $P = 0.590$; canine mass, $\chi^2 = 0.34$, df = 1, $P = 0.559$). To compensate for any potential confounding effects of age, we also built GLMs of canine length and mass in which IR, age at death and the IR:age interaction were fitted as predictor variables. Again, IR was not retained as a significant predictor variable in either of these models. Finally, the 7 loci that deviated significantly from HWE were excluded and the analyses repeated, but again none of the regressions approached significance.

Heterozygosity–Heterozygosity Correlations

To test for the presence of inbred individuals, we repeatedly divided the 72 loci into 2 equal subsets, calculated IR separately for both and obtained correlation coefficients between the 2. The distribution of resulting het–het correlation coefficients was centered around −0.07 suggesting an absence of appreciably inbred individuals from the data set. Being somewhat puzzled by the negative correlation we investigated further, replacing all genotypes with randomly chosen alleles and finding the expected average correlation of zero. We next sequentially removed all 7 loci exhibiting significant deviations from HWE. As each was deleted the average correlation increased, reaching zero when the last one was removed. It thus seems that null alleles exert a disproportionate influence on the apparent level of correlation in heterozygosity across loci and account for the slightly negative overall correlation.

Simulated General and Local Effects

The results of our simulations are presented in Figure 2. For each set of parameter values, a single data point was generated, plotted as the HFC using 10 loci (x axis) and the equivalent value based on 70 loci expressed as a ratio relative to the first value (y axis). As expected, the general effect model tends to strengthen as more loci are added, the 70-locus correlation tending toward being 3–4 times higher than the correlation obtained with 10 loci for all but the weakest HFCs. For the local effect model, the results were more variable, with the correlation sometimes strengthening (y values above one) and sometimes weakening (y values below one), depending on the input parameters. However, overall it is clear that general effects always lead to a strengthening of the correlation with increased numbers of loci and almost invariably the strengthening is greater than seen when the mechanism involves local effects. Since in our data the HFC initially observed for 9 loci disappeared when locus number was increased to 70, this provides strong support that our results are due to local effects.

Associations between Individual Loci and Canine Size

To attempt to identify which loci are involved in any local effects, we next constructed separate GLMs of canine length and mass, fitting heterozygosity at each of the loci as predictor variables, both alone and with age fitted as a covariate. We also applied the recently developed approach of Amos and Acevedo-Whitehouse (2009) as an alternative means of detecting genotype–phenotype associations. The results of these analyses are summarized in Table 2. A total of 16 loci (21.1%) yielded at least one significant test statistic. We previously noted that tooth length yielded a more significant HFC compared with tooth

Table 2 Results of single-locus GLMs of canine size for 84 dead adult male Antarctic fur seals genotyped at 76 microsatellite loci

		Canine length				Canine length (compensating for age)				Canine mass				Canine mass (compensating for age)			
Locus	*n*	Estimate	χ^2	*P*	P_{rand}	Estimate	χ^2	*P*	P_{rand}	Estimate	χ^2	*P*	P_{rand}	Estimate	χ^2	*P*	P_{rand}
Aa4	84	1.23	2.30	0.130	0.057	0.42	0.37	0.540	0.170	0.71	2.32	0.128	0.078	0.19	0.25	0.615	0.270
Hg1.3	83	−0.51	0.31	0.580	0.075	−0.04	0.00	0.960	0.570	−0.18	0.12	0.730	0.421	0.12	0.09	0.766	0.944
Hg6.3	84	1.30	1.52	0.220	0.248	1.20	1.89	0.170	0.496	0.53	0.74	0.390	**0.033**	0.46	0.94	0.333	0.071
Hg8.10	84	0.43	0.42	0.520	0.765	0.61	1.25	0.260	0.480	0.29	0.56	0.454	0.741	0.41	1.88	0.170	0.402
Lw10	84	1.26	1.68	0.190	0.207	1.30	2.68	0.100	0.591	0.61	1.20	0.273	0.090	0.65	2.22	0.136	0.152
M11a	84	−0.94	0.78	0.380	0.738	−0.36	0.16	0.690	0.686	−0.20	0.10	0.748	0.745	0.18	0.14	0.707	0.320
Pv9	84	1.97	7.53	**0.010**	0.157	1.57	6.85	**0.010**	0.186	0.86	4.18	**0.041**	0.633	0.60	3.27	0.710	0.719
PvcA	84	2.75	9.48	**0.002**	**0.049**	2.05	7.31	**0.010**	**0.018**	1.16	4.77	**0.029**	0.677	0.69	2.66	0.103	0.399
PvcE	83	0.27	0.09	0.770	0.116	−0.08	0.01	0.910	0.252	−0.04	0.01	0.941	0.538	−0.27	0.42	0.517	0.457
Ag1 (1)	84	0.86	0.58	0.450	0.176	0.96	1.07	0.300	0.138	0.20	0.09	0.759	0.202	0.26	0.28	0.600	0.931
Ag1 (2)	83	−0.25	0.14	0.710	0.216	−0.10	0.03	0.800	0.269	−0.17	0.19	0.666	0.531	−0.07	0.06	0.814	0.578
Ag2	83	0.63	0.52	0.470	0.352	−0.74	0.95	0.330	0.752	0.19	0.14	0.713	0.516	−0.72	3.11	0.078	0.626
Ag3	84	0.50	0.51	0.470	0.649	0.36	0.39	0.530	0.681	−0.11	0.08	0.778	0.819	−0.20	0.42	0.517	0.517
Ag4	84	−0.27	0.09	0.760	0.672	0.52	0.49	0.490	0.742	−0.41	0.65	0.419	0.541	0.09	0.05	0.823	0.882
Ag6	84	0.02	0.00	0.980	0.654	0.52	0.71	0.400	0.557	−0.19	0.20	0.656	0.474	0.13	0.15	0.701	0.592
Ag7	84	−0.67	0.63	0.430	0.182	−0.81	1.37	0.240	0.367	0.13	0.07	0.791	0.431	0.04	0.01	0.922	0.757
Ag8	82	−1.21	2.78	0.100	0.063	−0.60	0.96	0.330	0.379	−0.41	0.95	0.331	0.209	−0.01	0.00	0.985	0.089
Ag9	84	−0.54	0.66	0.420	0.459	−0.09	0.03	0.870	0.720	−0.56	2.17	0.141	0.115	−0.27	0.83	0.362	0.200
Ag10	83	−0.45	0.40	0.530	0.511	−0.24	0.16	0.690	0.669	−0.30	0.51	0.477	0.268	−0.16	0.24	0.628	0.655
Agaz1	84	−0.82	0.66	0.420	0.679	−0.97	1.35	0.250	0.494	−0.61	1.08	0.298	0.893	−0.70	2.43	0.119	0.690
Agaz2	84	−0.78	0.96	0.330	0.152	−0.74	1.27	0.260	0.501	−0.23	0.25	0.615	0.493	−0.21	0.33	0.564	0.641
Agaz3	84	0.69	1.03	0.310	0.611	0.13	0.05	0.820	0.140	0.32	0.66	0.418	0.454	−0.04	0.02	0.887	0.153
Agaz4	83	0.57	0.69	0.410	0.869	0.74	1.67	0.200	0.613	0.45	1.27	0.259	0.952	0.55	3.31	0.069	0.106
Agaz5	83	0.18	0.07	0.790	0.998	0.13	0.06	0.810	0.896	−0.07	0.04	0.850	0.924	−0.10	0.12	0.730	0.647
Agaz6	81	0.59	0.58	0.440	**0.038**	0.73	1.26	0.260	0.079	−0.19	0.17	0.683	0.113	−0.09	0.06	0.808	**0.039**
Agaz7	82	0.81	1.05	0.310	0.627	0.63	0.91	0.340	0.421	0.54	1.41	0.235	0.784	0.42	1.44	0.230	0.538
Agaz8	83	0.59	0.56	0.450	0.057	0.80	1.45	0.230	0.295	−0.10	0.04	0.834	0.437	0.04	0.01	0.912	0.846
Agaz9	84	0.38	0.28	0.600	0.980	0.45	0.55	0.460	0.891	0.35	0.68	0.410	0.620	0.39	1.42	0.233	0.097
Agaz10	84	0.02	0.00	0.980	0.953	−0.30	0.26	0.610	0.986	−0.14	0.12	0.734	0.734	−0.35	1.20	0.274	0.943
Agaz11	83	0.07	0.01	0.820	0.857	0.57	0.91	0.340	0.592	−0.18	0.19	0.664	0.991	0.14	0.20	0.653	0.957
Agaz12	83	−0.90	1.36	0.240	0.156	−1.05	2.78	0.100	0.472	−0.25	0.31	0.578	0.077	−0.34	0.98	0.321	0.654
G1A	77	0.24	0.09	0.760	0.113	0.35	0.29	0.590	0.158	0.32	0.52	0.471	0.102	0.39	1.41	0.236	0.142
Hg1.4	82			—	**0.007**	—	—	—	**0.006**	—	—	—	**0.029**	—	—	—	0.059
Hg4.2	77	−0.19	0.07	0.790	0.335	−0.17	0.09	0.760	0.659	0.18	0.21	0.649	0.088	0.19	0.38	0.539	0.277
Hg6.10	84	−0.52	0.29	0.590	0.295	−0.94	1.37	0.240	0.257	−0.36	0.42	0.518	0.768	−0.64	2.14	0.144	0.834
Hl4	81	0.75	1.21	0.270	**0.014**	0.49	0.75	0.390	0.161	0.29	0.56	0.455	**0.033**	0.12	0.17	0.683	0.217
Hl16	84	−0.14	0.04	0.850	0.559	−0.13	0.04	0.840	0.338	−0.32	0.54	0.460	0.302	−0.31	0.84	0.359	0.202
Lc5	84	−0.90	1.61	0.200	0.466	−0.97	2.79	0.100	0.238	−0.22	0.27	0.600	0.472	−0.26	0.67	0.412	0.105
Lc28	83	−0.83	0.93	0.340	**0.040**	−1.31	3.41	0.060	0.077	−0.10	0.04	0.844	**0.021**	−0.40	1.06	0.302	0.341
Lw8	84	−2.40	3.65	0.060	0.169	−1.53	2.10	0.150	0.070	−1.52	4.44	0.035	0.296	−0.96	2.84	0.092	0.052
Lw15(1)	82	1.08	1.64	0.200	0.135	0.02	0.00	0.980	0.760	0.73	2.29	0.130	0.139	0.06	0.02	0.878	0.734

Table 2 Continued

		Canine length				Canine length (compensating for age)				Canine mass				Canine mass (compensating for age)			
Locus	*n*	Estimate	χ^2	*P*	P_{rand}	Estimate	χ^2	*P*	P_{rand}	Estimate	χ^2	*P*	P_{rand}	Estimate	χ^2	*P*	P_{rand}
Lw15(2)	81	−1.11	1.06	0.300	0.852	−0.93	1.08	0.300	0.206	−0.55	0.78	0.377	0.967	−0.43	0.80	0.370	0.685
Lw18	84			—	0.683	—	—	—	0.920	—	—	—	0.476	—	—	—	0.827
Ms15	82	0.93	1.28	0.260	0.664	0.86	1.60	0.210	0.904	0.66	1.91	0.167	0.263	0.61	2.78	0.095	0.456
Ms23	70	−1.56	4.64	**0.030**	0.652	−1.69	8.59	**0.003**	0.227	−0.69	2.43	0.119	0.531	-0.77	5.44	**0.020**	0.267
Ms265	83	0.48	0.43	0.510	0.624	0.74	1.52	0.220	0.334	0.42	1.03	0.510	0.518	0.59	3.44	0.063	0.408
Ms647	83	0.06	0.00	0.970	0.461	0.06	0.00	0.960	0.252	0.43	0.29	0.592	0.838	0.44	0.48	0.488	0.768
OrrFCB1	81	0.43	0.26	0.610	0.564	0.77	1.26	0.260	0.276	0.35	0.50	0.479	0.696	0.56	2.29	0.130	0.461
OrrFCB2	84	−1.16	1.33	0.250	0.698	−1.06	1.61	0.200	0.637	−0.44	0.57	0.449	0.322	−0.38	0.68	0.409	0.425
OrrFCB3	81	−0.19	0.06	0.800	0.262	0.37	0.36	0.550	0.545	−0.43	1.05	0.306	0.503	−0.08	0.06	0.809	0.840
OrrFCB7	84	−0.23	0.07	0.790	0.855	−0.53	0.55	0.460	0.591	−0.22	0.20	0.657	0.957	−0.41	1.14	0.286	0.302
OrrFCB8	84	−0.37	0.24	0.620	0.429	−0.38	0.37	0.540	0.360	−0.45	1.08	0.299	0.153	−0.45	1.86	0.172	**0.040**
OrrFCB16	84	−0.20	0.08	0.780	0.972	0.00	0.00	0.990	0.429	−0.09	0.06	0.815	0.671	0.04	0.01	0.990	0.758
SGPv17	73			—	0.349	—	—	—	0.604	—	—	—	0.974	—	—	—	0.983
Ssl2x	84	0.72	1.19	0.280	0.223	0.40	0.53	0.470	0.523	0.42	1.23	0.268	0.209	0.22	0.53	0.469	0.446
Ssl5x	84	1.73	1.86	0.170	0.657	1.83	3.09	0.080	0.116	1.01	1.90	0.168	0.615	1.07	3.63	0.057	0.635
Ssl39	84	−1.13	1.94	0.160	**0.006**	−1.14	2.90	0.090	**0.032**	−0.13	0.08	0.782	0.613	−0.14	0.14	0.712	0.754
Ssl301	83	−0.25	0.07	0.790	0.569	0.04	0.00	0.960	0.365	−0.62	1.24	0.266	0.573	−0.44	1.02	0.312	0.760
Ssl441	83	−0.74	1.27	0.260	0.546	−0.38	0.48	0.490	0.789	−0.19	0.25	0.616	0.480	0.04	0.02	0.881	0.703
Zcwa05	84	−0.07	0.00	0.950	0.663	0.67	0.56	0.450	0.387	0.21	0.11	0.737	0.875	0.69	2.06	0.151	0.868
Zcwa12	84	−0.53	0.36	0.550	0.673	−0.13	0.03	0.850	0.979	−0.31	0.37	0.543	0.535	−0.06	0.02	0.889	0.663
Zcwb03	84			—	0.664	—	—	—	0.193	—	—	—	0.559	—	—	—	0.124
Zcwb07	83	0.87	0.68	0.410	0.753	0.79	0.81	0.370	0.766	0.73	1.46	0.227	0.824	0.68	2.09	0.149	0.125
Zcwb09	83	−0.57	0.35	0.560	**0.013**	−0.63	0.62	0.430	**0.005**	−0.42	0.56	0.455	0.362	−0.45	1.12	0.291	0.214
Zcwc01	76	0.66	0.45	0.500	0.549	−0.09	0.01	0.910	0.508	0.43	0.61	0.437	0.497	−0.03	0.00	0.952	0.091
Zcwe04	81	0.53	0.38	0.540	0.896	0.57	0.69	0.410	0.903	0.12	0.06	0.806	0.822	0.15	0.15	0.696	0.983
Zcwe12	83	0.33	0.15	0.700	**0.032**	0.11	0.02	0.880	0.513	0.26	0.27	0.601	0.076	0.11	0.09	0.764	0.838
Zcwf07	83	0.54	0.18	0.670	0.448	0.40	0.14	0.710	0.315	0.67	0.85	0.357	0.370	0.57	1.00	0.317	0.538
Zcwf09	81	−0.61	0.47	0.490	0.777	−0.05	0.01	0.940	0.092	−0.17	0.10	0.749	0.900	0.19	0.23	0.630	0.396
Zcwg04	84	1.05	0.99	0.320	0.426	0.41	0.21	0.650	0.751	0.75	1.49	0.222	0.760	0.33	0.47	0.494	0.720
ZcwCgDh1.8	83	2.23	6.35	**0.010**	0.289	0.95	1.44	0.230	0.633	1.11	4.70	**0.030**	0.346	0.25	0.36	0.551	0.211
ZcwCgDh5.16	83	−1.03	1.58	0.210	0.248	−0.32	0.21	0.650	0.110	−0.85	3.30	0.069	0.475	−0.39	1.12	0.289	0.434
ZcwCgDh48	84	−0.74	1.24	0.270	0.872	−0.90	2.74	0.100	0.198	−0.31	0.65	0.422	0.883	−0.41	1.94	0.163	0.687
ZcwCgDh7tg	82	1.26	1.53	0.220	0.745	0.38	0.20	0.660	0.992	1.50	7.09	**0.008**	0.435	0.97	4.55	**0.033**	0.816
ZcwCgDh4.7	83	−1.37	1.17	0.280	0.184	−1.24	1.36	0.240	**0.034**	−1.09	2.27	0.132	0.420	−1.01	3.15	0.076	0.182
ZcwCgDhB.14	83	0.33	0.11	0.730	0.313	0.03	0.00	0.970	**0.017**	0.21	0.14	0.704	0.327	0.02	0.00	0.967	0.109

Analyses were conducted separately for canine length, canine mass, and for both of these measures after compensating for age (see Materials and Methods for details). For each GLM, the estimate (slope), χ^2 and *P* value, uncorrected for multiple tests, are given. The χ^2 values for each term represent the change in deviance after removing that term and all interactions involving that term from the model. GLMs could not be conducted for the 4 X-linked loci because these were homozygous for all individuals. *P* values derived using the approach of Amos and Acevedo-Whitehouse (2009) are also given for every locus. Significant *P* values are highlighted in bold, none of which remained significant following FDR correction (see Materials and Methods for details).

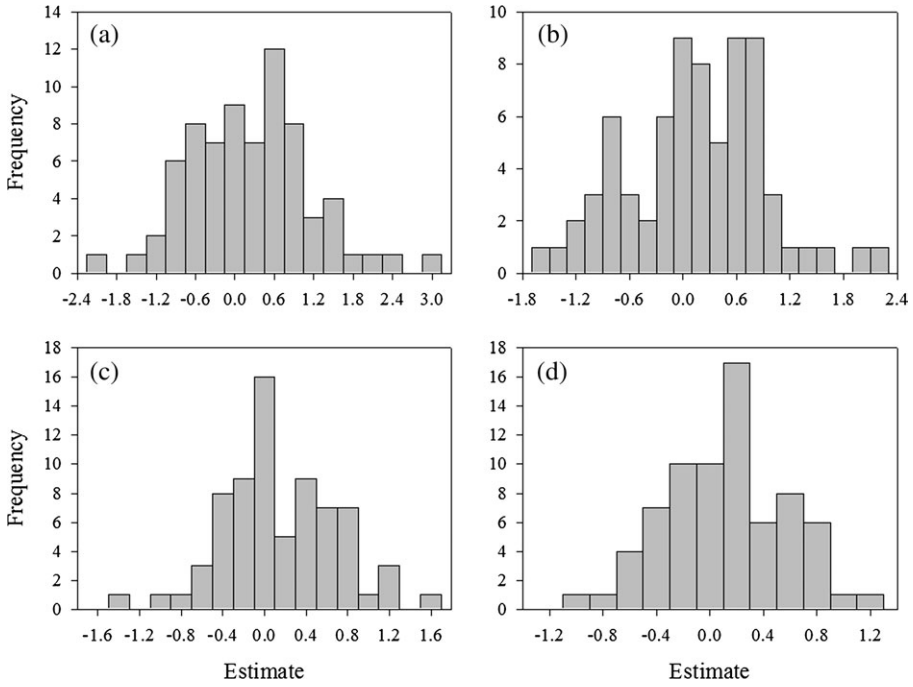

Figure 1. Distribution of estimates (slopes) of GLMs of (**a**) canine length; (**b**) canine length with age fitted as a covariate; (**c**) canine mass; and (**d**) canine mass with age fitted as a covariate.

mass (Hoffman et al. 2010). This trend continues, with almost twice as many significant tests at $P < 0.05$ for length ($n = 21$) compared with mass ($n = 12$). This imbalance is even more marked at $P \leq 0.01$, where length gives 10 tests and mass only one. Following FDR correction for multiple tests, significance is lost table wide. However, the significant excess of low P values for canine length over canine width provides weak support for local effects impacting on canine length. Moreover, although Type I errors alone should be distributed randomly across traits and markers, the low P values are also highly nonrandomly distributed across the markers (see below).

Elsewhere, tests in this species for consistency of the direction of effect across loci have yielded contrasting results, with heterozygosity at 8/9 loci being positively associated (sign test, $P = 0.04$) with both male reproductive success (Hoffman et al. 2004) and attractiveness (Hoffman, Forada, et al. 2007), but a less clear picture emerging for canine size, with 7 of the 9 loci showing the same tendency with tooth length and width ($P = 0.18$) and only 6 loci with tooth mass ($P = 0.51$, Hoffman et al. 2010). Consequently, we analyzed the distribution of the estimates (slopes) obtained from GLMs of canine length and mass for each of the 72 loci, fitting heterozygosity either alone or together with age (Figure 1). Canine length showed a slight but nonsignificant tendency toward an excess of positive slopes regardless of whether age was fitted as a covariate (fitted alone, 38 +ve vs. 34 –ve, sign test, $P = 0.724$; fitted with age, 39 +ve vs. 33 –ve sign test, $P = 0.556$). Canine width showed a nonsignificant excess of negative slopes when fitted alone (33 +ve, 39 –ve sign test, $P = 0.556$) but a slight excess of positive slopes when age was included as a covariate (39 +ve vs. 33 –ve sign test, $P = 0.556$). Thus, in none of the sets of models was a significant excess of positive slopes obtained, again suggesting that inbreeding depression is not involved.

To explore factors influencing whether a given locus yielded a local effect, we constructed a final GLM. The strength of each local effect was expressed as the number of

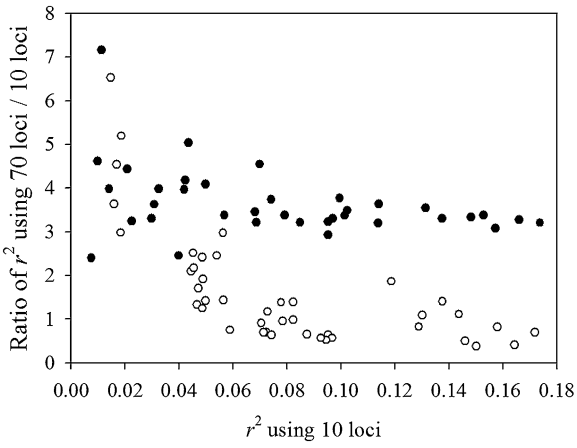

Figure 2. Results of stochastic simulations (see Materials and Methods for details) exploring how the r^2 value for an HFC measured using 10 loci changes as the number of loci is increased to 70 under the general (filled circles) and local effect models (open circles).

tests yielding a significant *P* value relative to the total number of tests conducted for that locus and was modeled using a binomial error structure. We fitted as predictor variables the number of alleles at each locus, chosen to reflect levels of variability, the source of the locus (as a factor with 1 = isolated in the focal species and 0 = derived from a different species), and the number of alleles:locus interaction. The only term retained in the final model was the source of the locus ($\chi^2 = 12.18$, df = 1, $P = 0.0005$), with those developed in fur seals being significantly less likely to reveal evidence of a local effect. This trend remained statistically significant even using a far more conservative chi-squared test on the number of loci yielding at least one significant *P* value ($\chi^2 = 5.08$, df = 1, $P = 0.024$).

BLAST Search Results

Finally, to attempt to shed light on the genomic distribution of the microsatellite loci used in this study and to identify nearby genes that could be candidates for local effects, we conducted BLAST searches of full-length clone sequences, wherever available (n = 72), against the dog genome. Half of these sequences revealed highly significant similarity (75.0% of *E*-values were $<1\times 10^{-20}$) to genomic regions within the dog (Supplementary Table 1). Assuming that local rearrangements, insertions or deletions may have compromised our ability to obtain matches for the remaining loci, we next carried out searches using the longest, least repetitive sequence from one side of the microsatellite, repeating with other subsequences where necessary. This recovered a further 32 matches to bring the total to 68/72 sequences matching (94.4%). These alignments also obtained strong statistical support, with 62.5% of *E*-values being less than 1×10^{-20} and all 4 putatively X-linked loci correctly assigned to the X chromosome. The loci were distributed across 33 different chromosomes in the dog, with a maximum of 5 locating to any one chromosome.

Wherever BLAST matches were obtained, the algorithm returned either a gene within which the query sequence appears to be located (25/68, 37%) or the nearest genes in both downstream and upstream positions, all of which were within 2 Mb of the microsatellite locus. Previously, we identified 3 markers associated with canine size that all lay next to genes plausibly involved directly in promoting growth. In our larger data set, this trend does not appear to hold up: The microsatellites lie near to genes with a wide range of functions and loci showing a significant association with tooth size do not appear to lie preferentially close to genes that can be obviously linked to growth (Supplementary Table 1).

Discussion

We have revisited a strong HFC in which 9 loci revealed both an overall effect of heterozygosity and loci with significant individual effects (Hoffman et al. 2010). Adding a further 67 loci, we find that evidence of an overall effect is lost, being replaced by evidence that a small number of individual loci are important. Microsatellites showing individual effects appear to be a nonrandom subset, tending to have been originally characterized in other species. Thus, inbreeding depression seems to contribute little if at all to the relationship between heterozygosity and tooth size in adult male Antarctic fur seals.

Several recent theoretical studies have concluded that local effects probably represent the dominant mechanism underlying HFCs (Balloux et al. 2004; Slate et al. 2004; DeWoody and DeWoody 2005). However, the interpretation of any individual study based on around 10 markers is often difficult. A key reason why the general effect model is seen as unlikely is the theoretical rarity of detectably inbred individuals in natural populations (Balloux et al. 2004). Although this statement may be true for an "average" population, 2 factors undermine its validity for many actual studies. First, study systems are often based either on small, isolated populations where a high proportion of individuals can be identified, or on highly polygynous species where questions revolve around determinants of male success. In both cases, the rate of inbreeding may be unusually high. Second, some studies compare cases with controls, for example diseased/dead with healthy individuals (e.g., Acevedo-Whitehouse et al. 2003). Here, the cases may be a small subset of all individuals and these may be highly enriched for those that are most inbred. All these scenarios potentially bypass the argument that inbred individuals are too rare to contribute to HFCs. In our current study, we do not compare cases with controls, but the species is polygynous and highly site faithful (Hoffman et al. 2003, 2006), so the presence of some inbred individuals cannot be ruled out.

In our study, we find several lines of evidence that general effects are weak or nonexistent. First, the HFC based on overall heterozygosity which is strong when only 9 markers are used is lost when marker number is increased. This result parallels the findings of an earlier study on red deer, where increasing marker number from 9 to 71 resulted in the loss of the HFC for juvenile survival (Slate and Pemberton 2002). Our simulations indicate, across a wide range of scenarios, that when an HFC due to inbreeding depression explains around 10% of the variation in fitness using 9 markers, increasing to 70 markers invariably strengthens that correlation, on average raising the r^2 value to around 0.3. In contrast, local effects have a more variable impact on the r^2, sometimes increasing it but often reducing it. Second, we used het–het correlations to ask whether there was evidence that our data included any genuinely inbred individuals. We found no evidence that heterozygosity is correlated across markers, even when over 70 loci are deployed, indicating that inbred individuals are rare or absent in our sample of dead adult males.

When testing for local effects, our results are perhaps not as clear-cut as one might hope. Nonetheless, we believe several strands of evidence point to our data including several markers lying near to genes that influence tooth size. The biggest problem is that, with 4 traits tested, each in 2 different ways and for over 70 different markers, the total number of tests conducted is large such that correction for

multiple tests, even using FDR, causes loss of significance. This is a well-known problem in disease association studies and was identified as a problem for HFCs by Slate and Pemberton (2002), who found that 2 individually significant loci lost significance following Bonferroni correction for 71 tests, despite these both being linked to quantitative trait loci previously identified for the same trait. However, if most of the significant tests were truly attributable to Type I errors, they should be distributed randomly, and in our study they are not. Thus, tests based on tooth length attract almost twice as many significant results compared with tooth mass and, among these, length achieves $P \leq 0.01$ ten times compared with tooth mass achieving this level just once. Nonrandomness is also seen in the way individually significant associations are preferentially associated with markers cloned from other species. This trend is important because it helps to explain why the first 9 loci, all cloned from other species, gave a highly significant HFC while the addition of more loci, many of which were cloned from fur seals, if anything weakens the net effect. Finally, in our original study, we obtained a strong result that we can now say with confidence was not due to a general effect. Put together therefore, our results suggest that single-locus associations are present, but that they are rather weak with current sample sizes, to the extent that they lie close to the limit of what can be detected given the number of tests being conducted. Clearly, future work should aim to increase sample sizes appreciably.

Our study also allows an interesting comparison to be made between specific tests for a link between heterozygosity and fitness, and a more general test for an association between any genotypes and fitness (Amos and Acevedo-Whitehouse 2009). We find that although the more general test yields more significant results, suggesting greater power, there are several instances in which the direct test of an effect of heterozygosity is significant where the more general test is not. Such instances could be due to Type I errors, though it might be expected that a direct test should sometimes prove more powerful. Against this, it should be remembered that the association between heterozygosity at a microsatellite and heterozygosity at a neighboring gene is almost invariably imperfect and probably needs to be unusually strong for the direct test to prevail.

With encouraging results from our initial study based on 9 loci (Hoffman et al. 2010), we attempted to go a step further and ask whether there was any obvious pattern in the genes found to lie next to putative BLAST matches to the dog genome. On the one hand, this was extraordinarily successful, in the sense that in only 4 instances did we fail to find a convincing single location in the dog genome, and the distribution across the genome was reassuringly even. This has positive implications for future studies. On the other hand, we did not find a clear excess of genes that could in some obvious way be linked directly to growth. Instead, the closest genes were found to be diverse, for instance, linked to cell adhesion (Ms23), protein folding (Agaz 6), cell osmoregulation (Ssl39), messenger RNA processing (ZcwCgDh7tg), transcriptional regulation (ZcwCgDh1.8), and photoreceptor function (Hg1.4). Having said this, locus Lc28 lies <0.5 Mb away from Cbi-interacting protein Sts-1, which promotes the accumulation of activated target receptors (such as T-cell receptors) on the cell surface, and locus ZcwCgDhB.14 lies <0.25 Mb from a gene encoding for a receptor for interleukin 17, a cytokine with numerous immune regulatory functions.

There are several possible explanations for why our larger study reveals a diversity of genes, many of which cannot be intuitively linked directly to growth. First, growth is a diffuse concept and while some genes are obvious, under the right circumstances almost any gene should contribute to an animal's ability to thrive, including elements of the immune system for fighting disease, sensory system for finding food, and most metabolic pathways. Second, although we believe several of our associations are genuine, we also inevitably include a number of Type I errors. A clearer picture might emerge if these could be reduced by increasing considerably our sample of teeth. Third, although we focus on the nearest gene, any association could easily involve any gene within 2 Mb, and possibly further given the fur seals' breeding system and population history. To identify key genes, future studies need to consider both increasing sample sizes and adding further markers nearby putative hits. In the current study, we did attempt to fine-map local effects for loci Pv9 and PvcA by identifying dinucleotide repeat sequences flanking these genes in the dog genome, designing primers and testing for amplification in seals. Unfortunately, none of the 30 primer pairs tested amplified an interpretable product.

Finally, it is worth comparing our original study (Hoffman et al. 2010) with the current, much larger analysis. Our first study is typical of large numbers of studies across many species that use around 10 markers to explore HFCs in natural populations. We have deliberately left this study as it was originally written before expanding the marker database in order to provide an informative comparison between the sorts of conclusions one is likely to draw and those that are justified when large numbers of markers can be deployed. In our case, the original study suggested both a general effect and 2 or possibly 3 local effects. Although the general effect was lost after excluding the 2 strongest effect loci, such a pattern might have occurred if the genuine general effect had been present because eliminating the 2 strongest effects will always tend to weaken the overall *P* value. In the current study, we are able convincingly to reject the general effect model. By implication, the highly significant pattern seen in out initial study is indicative of 2 loci of large effect whose impact is heightened by the relatively small number of nonsignificant loci tested at the same time. In the larger study, local effects were hinted at rather than demonstrated unequivocally, and hence, it is only be taking the 2 studies together that a good case can be made that a small number of loci are linked to genes influencing aspects of growth in the Antarctic fur seal. In other words, loci employing small panels of markers lose power due to the small number of loci involved, whereas

those using far larger panels of markers tend to lose power due to the need to correct for multiple tests.

Conclusion

In conclusion, by greatly increasing marker numbers, we have been able to show convincingly that inbreeding depression is unlikely to account for HFCs relating to body size in fur seals. By implication, the HFCs are due to chance linkage between individual markers and specific genes. Through cross-mapping to the dog genome, we have identified a number of candidate regions, but the large number of marker-trait tests prevents us at this stage from pinpointing specific genes. The fact that general effects are not found even in a strongly polygynous species with natal site fidelity suggests that HFCs due to inbreeding depression are likely to be rare in natural populations.

Supplementary Material

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

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