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Getting Long in the Tooth: A Strong Positive Correlation between Canine Size and Heterozygosity in Antarctic Fur Seals *Arctocephalus gazella*

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Abstract

Most studies of heterozygosity–fitness correlations (HFCs) in natural populations relate to fitness traits expressed early in life, whereas traits that are often more difficult to measure such as longevity and adult body size remain elusive. Teeth provide a window on an individual's life history, allowing the reliable estimation of both age and body size. Consequently, we collected paired upper canine teeth and tissue samples from 84 adult male Antarctic fur seals *Arctocephalus gazella* that died of natural causes at Bird Island, South Georgia. Tooth size is a good predictor of skull and body size both within and across taxa, and we similarly find a strong relationship with skull size in our species. In turn, tooth size is itself predicted strongly by genetic heterozygosity estimated using 9 microsatellites. With only 9 loci, the exact mechanisms involved remain unclear, although the observed pattern appears largely attributable to a small subset of loci, suggesting that associative overdominance rather than inbreeding depression provides the proximate mechanism. In addition, locating these markers in the dog genome reveals proximity to genes involved with fat metabolism and growth. Our study illustrates how canine teeth, and potentially other structures such as tympano-periotic bone, waxy inner earplugs, or otoliths, may be used to explore links between genetic variation and important life-history traits in free-ranging vertebrate populations.

Key words: *body size, canine tooth, heterozygosity–fitness correlation, marine mammal, otariid, pinniped, skull size*

Increasing numbers of studies report correlations between heterozygosity, as measured by a handful of neutral genetic markers, and some aspect of fitness in natural populations (heterozygosity–fitness correlations, HFCs). Initially, these correlations tended to be ascribed to inbreeding depression, with heterozygosity providing a surrogate measure of an individual's inbreeding coefficient, *f*. However, both theoretical and empirical studies suggest that the number of markers used is unlikely, except under extreme circumstances, to be able to capture useful information about *f*, and hence that in most cases the mechanism is more likely to involve chance linkage between one or more of the markers and a gene experiencing balancing selection (e.g., Mitton and Pierce 1980; Balloux et al. 2004; Slate et al. 2004).

The number of fitness traits reported to be involved with HFCs is remarkable, ranging from direct measures such as birth weight (Coltman et al. 1998), parasite load (Rijks et al. 2008), and longevity (Coltman et al. 1999) through lifetime reproductive success (Slate et al. 2000) even to behavioral qualities such as territory-holding ability (Seddon et al. 2004), aggressiveness (Tiira et al. 2003), song complexity (Marshall et al. 2003), and attractiveness (Hoffman et al. 2007). However, because most studies focus on only 1 or 2 aspects of fitness, the proximate mechanisms remain unclear. In other words, are there genes involved directly with each of the many traits or could it be that, for example, heterozygosity at immune-related genes confers greater resistance to disease, thereby allowing improved performance in many other life-history traits such as growth, dominance, and longevity?

The Antarctic fur seal *Arctocephalus gazella* provides an interesting case in question. A long-term genetic study of a colony at Bird Island, South Georgia, has allowed large numbers of identified individuals to be tracked over much or all of their lives (Hoffman, Trathan, and Amos 2006). At

the study colony, an aerial walkway allows unprecedented access both to observe and to sample individual seals. Consequently, we have been able to sample almost every breeding male as well as large numbers of females and their offspring over a period of almost a decade (Hoffman and Amos 2005a). Genetic parentage testing was used to quantify male reproductive success, while daily observations allowed us to link these results to individual behavior (Hoffman et al. 2003). We found that almost every aspect of male reproductive success could be linked to heterozygosity measured at just 9 highly polymorphic microsatellite markers (Hoffman et al. 2004). Thus, the most heterozygous males tend to arrive earliest in the breeding colony, hold territories in tough El Niño years and are also actively chosen by females (Hoffman et al. 2007). However, we have been unable to examine how heterozygosity impacts on fitness beyond neonatal mortality, where no link is found, possibly because most pups die either from being trampled by adults (Hoffman, Forcada, and Amos 2006) or through starvation when environmental conditions are adverse for their mothers (Reid and Forcada 2005).

One of the key traits we would ideally like to measure is male longevity because longer lived males are likely both to be healthier and to enjoy greater reproductive success (Hoffman et al. 2003). Unfortunately, fur seals are long lived to the extent that a study would need to cover 2 decades in order to track a reasonable sample of individuals. However, every year around the large breeding colonies on South Georgia, fresh carcasses can be found of animals which have died from natural causes (Baker and Doidge 1984) and from which it is possible to sample teeth. Teeth have been used in a variety of ecological and evolutionary studies (e.g., Evans et al. 1995; Hoppe et al. 2006; Jay et al. 2008) and have been used extensively in carnivores to determine age (e.g., Laws 1953), sex (Huber 1994), and even diet (Hobson and Sease 1998; Newsome et al. 2007). In addition, canine size also correlates strongly with body size both across (Gould 1975; Wood 1979; Creighton 1980) and within taxa (Lavelle 1973; Baker and Fowler 1990; Boyd and Roberts 1993). Given the high levels of sexual dimorphism for body size seen in many pinnipeds, and particularly the Otariids and other species where strong polygyny occurs (Lindenfors et al. 2002), body size seems likely to be a key male trait that will correlate with dominance, territory holding, and, ultimately, reproductive success. Consequently, by extracting canine teeth from dead males, it should be possible simultaneously to assess the impact of HFCs on the key life-history traits of body size and longevity.

The morphology of Antarctic fur seal teeth has already been thoroughly described and growth is known to occur via the annual deposition of successive layers of dentine in a way analogous to a series of stacked cones (Figure 1) providing a means of accurately estimating age from the number of growth layers (Payne 1978; Arnbom et al. 1992; Boyd and Roberts 1993). Upper canine size has also been shown to correlate strongly with body size in this species (Boyd and Roberts 1993). We therefore collected paired upper canine teeth and genetic samples from 84 adult male Antarctic fur seals that died of natural causes at Bird Island, South Georgia, and examined relationships between heterozygosity and both longevity and tooth size.

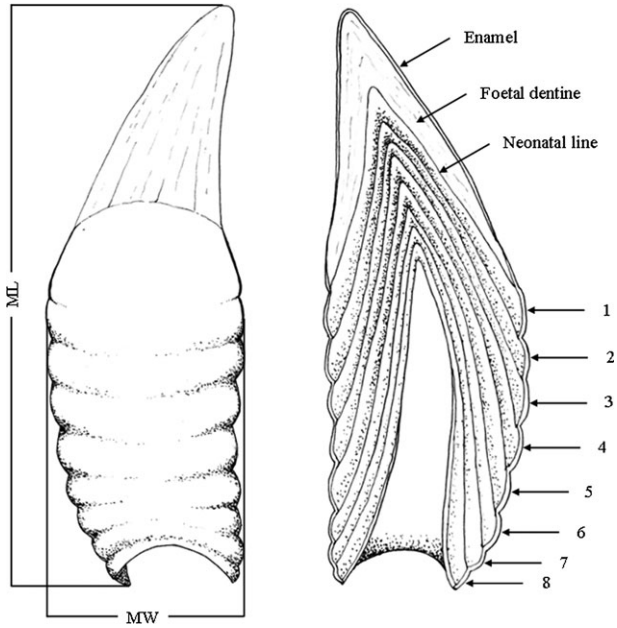

Figure 1. External surface and longitudinal section of the upper canine tooth of an 8-year-old male Antarctic fur seal. Note the external ridges on the tooth corresponding to the annual growth layers, which are numbered in order of deposition. ML, maximum length; MW, maximum width.

Materials and Methods

Collection of Canine Teeth and Tissue Samples

Paired tissue samples and upper canine teeth were collected from adult male fur seals that died of natural causes during November through December 2002 within approximately 500 m of the British Antarctic Survey base at Bird Island, South Georgia. Only animals that were deemed to have died recently (within the previous 2 days) were sampled to avoid any potential difficulties associated with in situ DNA degradation. Tissue samples were taken from the interdigital margin of the foreflipper using piglet ear-notching pliers and stored individually in 95% ethanol at –20 °C. Because canine teeth were very difficult to remove from fresh carcasses, we fastened a numbered plastic tag to each male via a cable tie threaded through the lower jaw. This enabled us to return after a prolonged period of decomposition (ca. 10 weeks) to more easily extract the teeth. Each canine tooth was then cleaned and stored individually in 95% ethanol at room temperature.

Canine Tooth Measurements and Age Determination

Maximum canine length and canine width (Figure 1) were measured to the nearest mm following Boyd and Roberts (1993). We also recorded the mass of each tooth to the nearest 0.001 g. Canine teeth were then sectioned longitudinally from the root to the crown using a Cutrock (Croft) diamond burred

circular saw just off-center of the pulp cavity in order to expose the center of the tooth. These sections were etched in 10% formic acid for 24 h, thoroughly rinsed with water, air-dried and then rubbed with graphite powder (Dickie and Dawson 2003). Light was projected across the surface of the sections under ×6.7 magnifications using a Leica Wild M3C Sterozoom microscope, and the anterior edge of the section was photographed using a Carl Zeiss Axiocam and Axiovision software. Age at death was determined following Boyd and Roberts (1993) by counting the number of annual growth layers, each comprising a raised light and a hollow darker layer.

Generation of Genetic Data

Total genomic DNA was extracted from tissue samples using a Qiagen DNeasy tissue extraction kit following the manufacturer's recommended protocols and quantified using a Nanodrop ND-1000 spectrophotometer. Each sample was then genotyped at 9 polymorphic dinucleotide microsatellite loci previously isolated from a variety of different pinniped species (Table 1) following Hoffman and Amos (2005b). Briefly, polymerase chain reactions (PCRs) were carried out in 10 μl reaction volumes containing approximately 10 ng template DNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Tween 20, 0.1% gelatine, 0.1% IGEPAL, 60 mM tetramethylammonium chloride, 2.5% formamide, 0.1 mM dGTP, 0.1 mM dATP, 0.1 mM dTTP, 0.02 mM dCTP, 4 pmol of each primer, 0.25 units of *Taq* polymerase, and 0.01 μCi [α^{32}P]-dCTP. Loci were amplified using the following PCR profile: one cycle of 120 s at 94 °C, 45 s at T_1, 50 s at 72 °C; 10 cycles of 30 s at 94 °C, 45 s at T_1, 50 s at 72 °C; 25 cycles of 30 s at 89 °C, 45 s at T_2, 50 s at 72 °C; and one final cycle of 5 min at 72 °C (see Table 1 for T_1 and T_2). PCR products were resolved by electrophoresis on standard 6% polyacrylamide sequencing gels, detected by autoradiography and scored manually. Microsatellite genotypes were then tested for deviations from Hardy–Weinberg equilibrium and linkage disequilibrium using GENEPOP (http://wbiomed.curtin.edu.au/genepop/; Raymond 1995 Raymond and Rousset 2005). For each test, the dememorization number was set to 10 000, the number of batches to 1000, and the number of iterations per batch to 10 000. GENEPOP was also used to quantify the number of alleles at each locus and to calculate observed and expected heterozygosities. 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

Heterozygosity can be calculated using microsatellite data in a number of different ways. These include standardized heterozygosity (SH, Coltman et al. 1999), internal relatedness (IR, Amos et al. 2001), and heterozygosity weighted by locus (HL, Aparicio et al. 2006). SH estimates the proportion of loci that are heterozygous while weighting the contribution of each locus by the expected heterozygosity at that locus. IR instead estimates the relatedness of an individual's parents using the extent of allele sharing

Table 1 Details of the 9 microsatellite loci employed in this study and their polymorphism characteristics in 84 dead adult male Antarctic fur seals

Locus	Isolated from species	Reference	Genbank accession number	T_1 (°C)	T_2 (°C)	Number of alleles	H_O	H_E	Hardy–Weinberg equilibrium *P* value	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)	AF055864	42	46	12	0.849	0.843	0.050	0.0005
Hg6.3	Gray seal Halichoerus grypus	Allen et al. (1995)	G02092	46	48	12	0.861	0.893	0.676	−0.0209
Hg8.10	Gray seal Halichoerus grypus	Allen et al. (1995)	G02093	42	46	4	0.444	0.440	1.000	0.0009
Lw10	Weddell seal Leptonychotes weddellii	Davis et al. (2002)	AF40592	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 Halichoerus grypus	Allen et al. (1995)	G02096	48	52	10	0.779	0.738	0.148	0.0238
PvcA	Harbour seal Phoca vitulina	Coltman et al. (1996)	L40983	46	48	8	0.836	0.857	0.403	−0.0155
PvcE	Harbour seal Phoca vitulina	Coltman et al. (1996)	L40987	45	50	14	0.856	0.843	0.635	0.0044

Genbank accession numbers are not available for all the loci. H_E, expected heterozygosity; H_O, observed heterozygosity.

relative to random expectations. This measure tends to perform marginally better than SH across a range of scenarios (Amos et al. 2001). HL is a recently developed measure that weights heterozygosity by the variability of each locus at which an individual is homozygous. HL has yet to be used widely but can outperform IR, particularly at loci with high allelic diversity (Aparicio et al. 2006; Rijks et al. 2008). However, in practice when SH, IR, and HL were calculated over all 9 loci for our data set, the resulting values were strongly intercorrelated (r^2 values ranged from 0.91 to 0.95) as also found by Araya-Ajoy et al. (2009). Moreover, virtually identical results were obtained using the 3 measures (data not shown). Consequently, to facilitate comparison with previous studies of this and other pinniped species (e.g., Amos et al. 2001; Bean et al. 2004; Hoffman et al. 2004), we present analyses using IR.

Elsewhere, microsatellite heterozygosity has been shown to correlate negatively with the number of loci that fail to amplify (Campbell et al. 2007). Such a relationship could be attributed to variation in sample quality with, for example, allelic dropout downwardly biasing the apparent heterozygosity of poor-quality samples (Walsh et al. 1992). This is unlikely to be the case for our data set because clear PCR profiles were obtained for all of the samples and the rate of genotyping success was high, with only 2 of the samples failing to amplify at a single locus each (success rate = 754/756 reactions, 99.74%). Nevertheless, because our samples were derived from dead animals and some degree of in situ DNA degradation could conceivably have occurred, we explored this possibility by correlating DNA extract concentrations with IR values. DNA concentrations averaged 36.6 ng/μl and did not correlate significantly with IR ($r = 0.031$, $n = 84$) indicating that DNA quantity had no effect on IR.

Bayesian Cluster Analysis

Because population structure has the potential to generate artificial links between heterozygosity and fitness (Slate and Pemberton 2006), we conducted a Bayesian cluster analysis of the microsatellite genotype data set using the program STRUCTURE 2.2.3 (Pritchard et al. 2000). This program uses a maximum likelihood approach to determine the most likely number of genetically distinct groups in a sample (K) by subdividing the data set in a way that maximizes Hardy–Weinberg equilibrium and linkage equilibrium within the resulting clusters. The membership of each individual in a population is then estimated as q, which varies between 0 and 1 with the latter indicating full population membership. We ran 5 independent runs each for $K = 1$–10 using 1×10^6 Markov chain Monte Carlo iterations after a burn-in of 1×10^5, specifying the correlated allele frequencies model and assuming admixture.

Statistical Analyses

To analyze the relationship between canine size and heterozygosity, we constructed general linear models (GLMs) within the software package R (Ihaka and Gentleman 1996). Canine tooth length, mass, and width were fitted as response variables and modeled using a Gaussian error structure because they were approximately normally distributed. Initially, each GLM was constructed fitting age at death, heterozygosity, and the interaction between these 2 as explanatory variables. Models were then simplified by sequentially removing terms that did not cause a significant reduction in deviance explained (deviance is analogous to sums of squares in standard regression analysis) by their removal (Crawley 2002). 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.

To explore effects at individual loci, we next constructed separate GLMs of canine tooth length, mass, and width in which each locus was fitted individually, following Hoffman et al. (2004). To compensate for nonnormality in single-locus IR values, we instead fitted heterozygosity (as a categorical variable with heterozygotes coded as one and homozygotes coded as zero). The same models were also repeated including age as a covariate.

In addition to using a direct test for the impact of marker heterozygosity, we also used a new method (Amos and Acevedo-Whitehouse 2009) that aims to detect any form of association between genotype and a fitness trait. This may be appropriate because, even with close linkage between a marker and a gene under balancing selection, recombination and mutation will tend to create an imperfect correlation between heterozygosity at the gene and heterozygosity at the marker. The new method is based on arranging the data to maximize the strength of association between genotype and fitness. At each locus, genotypes with above average fitness scores are classified as "low risk" and below average fitness as "high risk," creating a highly significant test for "risk" against fitness. The size of the resulting test statistic is then assessed by randomizing the genotypes and repeating the process many times, the argument being that if a genuine genotype–fitness association is present, a stronger effect should be achieved with the real as opposed to 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.

Finally, because we conduct a large number of statistical tests of association, it would be desirable to control formally for type I errors through either Bonferroni (Hochberg 1988) or false discovery rate (FDR) correction (Benjamin and Hochberg 1995). However, it is difficult to implement table-wide correction because both methods assume that all the tests are independent, whereas many of our tests show clear nonindependence (e.g., canine length and canine length controlling for age). At the same time, including results for canine width that show lower significance will skew the overall distribution of P values. In view of this, we elected to control type I errors per group of tests (i.e., each set of 9 tests for a given trait). 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).

BLAST Alignments

Where associations were found between specific microsatellite loci and canine size measurements, putative homology between the flanking sequences of these loci and the dog genome was investigated using basic local alignment search tool (BLAST) searches (Altschul et al. 1990). Where available, clone sequences were downloaded from GenBank (www.ncbi.nih.gov) and BLAST searched against the dog genome (http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=9615) using default parameters. Where more than one BLAST match was recovered, the one with the highest score and lowest *E*-value was recorded.

Relationships between Canine Tooth Measures and Skull Size

We collected 3 measures of tooth size: length, width, and mass. Ideally, we would like to determine which of these is the best predictor of body size, but the high density of scavengers like giant petrels (*Macronectes giganteus* and *M. halli*) means that fur seal carcasses are rapidly degraded, even before death. To circumvent this difficulty, we assumed that skull size was a good proxy for body size and collected 80 naturally weathered adult male skulls found within the study area. For each, we removed an upper canine tooth and measured it as described above and then recorded skull mass in g plus 18 different skull morphometric measurements (Figure 2) to the nearest mm following Daneri et al. (2005).

Results

To determine whether heterozygosity is associated with age and/or tooth size in a natural population of Antarctic fur seals, we genotyped 84 dead adult males at 9 microsatellite loci and measured and sectioned an upper canine from each. The distribution of age at death (Figure 3) reveals a strong peak at ages 7 and 8, consistent with a previous observation that most adult males begin to establish territories for the first time at around 8 years of age (Payne 1979). Age at death and canine length, mass, and width were all positively intercorrelated, with the majority of coefficients being highly significant (Table 2) and the strongest relationship being found between canine length and mass ($r = 0.794$, $P < 0.0001$). As shown previously (Hoffman, Forcada, and Amos 2006), none of the microsatellite loci deviated significantly from Hardy–Weinberg equilibrium (Table 1) and no pairs of loci exhibited significant linkage disequilibrium.

Bayesian Cluster Analysis

Cryptic population structure has the potential to generate spurious links between heterozygosity and fitness (Slate and Pemberton 2006). Consequently, we sought to eliminate this possibility by conducting a Bayesian cluster analysis of the microsatellite genotype data set using STRUCTURE (Pritchard et al. 2000), a program that uses an iterative approach to cluster the genotypes into K populations without knowledge of the population membership of individuals. The replicate runs for each value of K were highly concordant for their output log-likelihood values, with the highest values being consistently associated with $K = 1$ (data not shown), indicating an absence of any genetic structure in our sample of dead adult male fur seals.

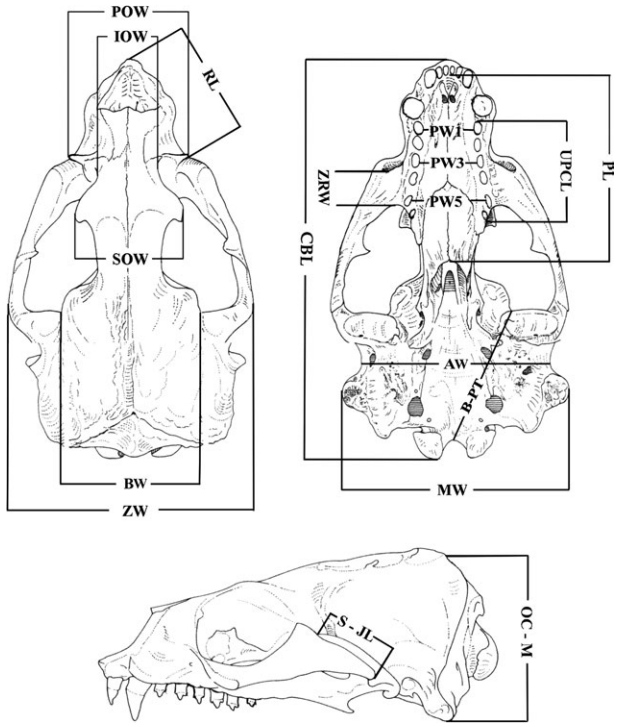

Figure 2. Skull measurements used in this study, reproduced from Daneri et al. (2005) with the permission of the authors. AW, auditory width; B-PT, distance between basion and bend of pterigoyd; BW, braincase width; CBL, condylobasal length; IOW, interorbital width; MW, mastoid width; OC-M, occipital crest-mastoid; PL, palatal length; POW, preorbital width; PW1, palate width at postcanine 1; PW3, palate width at postcanine 3; PW5, palate width at postcanine 5; RL, rostral length; S-JL, squamosal-jugal suture length; SOW, supraorbital width; UPCL, upper postcanine length; ZRW, zygomatic root width; ZW, zygomatic width.

HFCs for Longevity and Canine Size

To test for an effect of heterozygosity on male longevity, we first regressed age at death, measured from the number of annual growth layers visible in the dentine, against IR. No significant relationship was found ($\chi^2 = 0.83$, df = 1, $P = 0.361$). Consequently, we next explored possible links between heterozygosity and 3 measures of tooth size: canine length, canine mass, and canine width. Strong and significant regressions were obtained between IR and both length (Figure 4a, $\chi^2 = 13.18$, df = 1, $P = 0.0003$) and mass

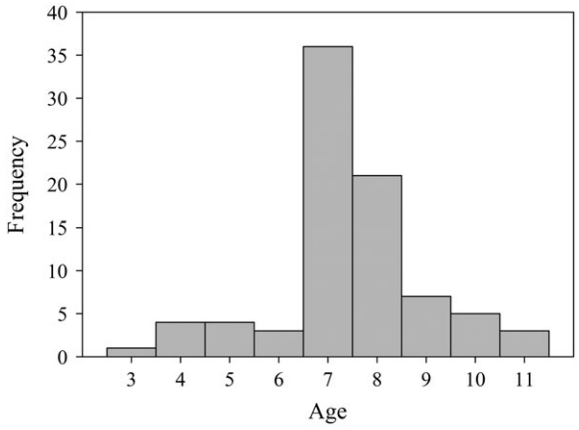

Figure 3. Frequency distribution of age at death for 84 adult male Antarctic fur seals.

(Figure 4b, $\chi^2 = 9.12$, df = 1, $P = 0.003$), whereas the one for width was much weaker but still significant (Figure 4c, $\chi^2 = 4.09$, df = 1, $P = 0.043$). To compensate for any potential confounding effects of age, we also constructed GLMs of canine length, mass, and width in which IR, age at death, and the IR:age interaction were fitted as predictor variables. Both IR and age were retained in the reduced models of canine length and mass, with IR being highly significant in both cases ($P = 0.0003$ and 0.0024, respectively, Table 3). Only IR was retained in the GLM of canine width (Table 3c).

Relationships between Canine Tooth and Skull Size

The relatively weak correlation between canine width and IR raises questions as to how the 3 different measures of canine size relate to body size. Consequently, we collected 80 naturally weathered adult male skulls from the study area and tested the relationships between each measure of tooth size and skull mass plus 18 different skull morphometric measurements (Figure 2). Broadly in line with our results for IR, skull mass was found to correlate strongly with both canine length ($r = 0.398$, $P = 0.0003$) and canine mass ($r = 0.416$, $P = 0.0001$) but not significantly with canine width ($r = 0.189$, $P = 0.093$). A similar pattern was obtained for skull morphometric measurements (Table 4), with 12/18 measurements correlating significantly with canine length, 16 with canine mass and only 8 with canine width at $P < 0.05$. This suggests that the stronger HFCs for canine length and mass probably reflect differences in skull size and explains why only a very weak HFC was found for tooth width.

Table 2 Interrelationships among canine tooth parameters for 84 dead Antarctic fur seal males

	Length	Mass	Width	Age
Length	—	0.794	0.440	0.569
Mass	<0.0001	—	0.699	0.635
Width	<0.0001	<0.0001	—	0.210
Age	<0.0001	<0.0001	0.055	—

Correlation coefficients are shown in the top half of the matrix and corresponding *P* values are given below. *'s correspond to the diagonal of the matrix - i.e. where each variable is compared against itself, and therefore there are no *P-values*

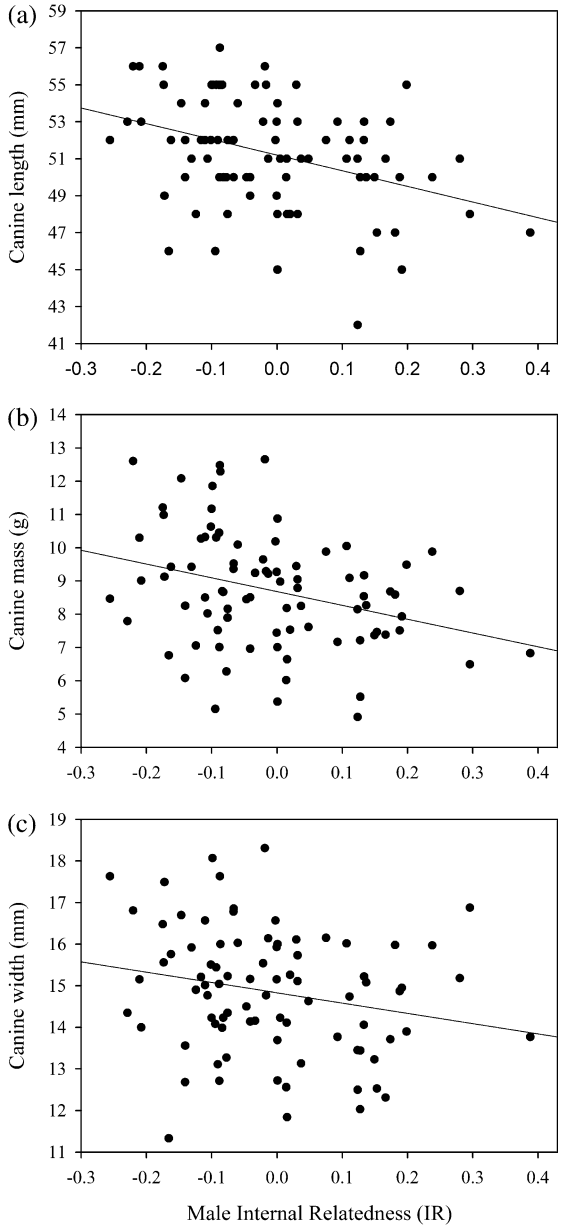

Figure 4. Relationships between male IR and (**a**) canine length ($y = -8.447x + 51.203$, $r^2 = 0.139$); (**b**) canine mass ($y = -4.1494x + 8.682$, $r^2 = 0.100$); and (**c**) canine width ($y = -2.471x + 14.832$, $r^2 = 0.048$) for 84 dead adult male Antarctic fur seals.

Exploring the Possible Underlying Mechanisms

HFCs may arise either through general effects (inbreeding depression) or through local effects caused by chance linkage between one or more markers and a gene exhibiting

Table 3 Results of GLMs of canine length, canine mass, and canine width

Term	Estimate	χ^2	df	*P*
Canine length				
$N = 84$, total deviance = 750.70, total explained deviance = 42.34%				
IR	−7.22	14.09	1	0.0003
Age	1.04	40.05	1	<0.0001
Canine mass				
$N = 84$, total deviance = 250.39, total explained deviance = 46.77%				
IR	−3.35	9.81	1	0.0020
Age	0.68	55.93	1	<0.0001
Canine width				
$N = 84$, total deviance = 186.69, total explained deviance = 4.75%				
IR	−2.47	4.09	1	0.0430

Explanatory variables fitted in the full model were IR, age at death, and their interaction. Only significant terms remaining in the reduced models are shown. 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.

balancing selection (Hansson and Westerberg 2002). The former explanation predicts a genome-wide effect with the relationship between heterozygosity and fitness being more or less similar across microsatellite loci. In contrast, if the relationship was driven by local effects, the impact of one or a small number of loci should dominate. Consequently, we fitted each of the loci, coded as 0 = homozygote and 1 = heterozygote, in separate GLMs of canine length, width, and mass, both alone and with age included as a covariate. Loci Pv9 and PvcA were retained as significant terms in GLMs of canine length regardless of whether age was also fitted (Table 5) and these remained significant following FDR correction for multiple tests. Moreover, when IR was calculated excluding these markers, the relationship between IR and canine length became nonsignificant ($\chi^2 = 3.11$, df = 1, $P = 0.082$). Loci Pv9 and PvcA were also retained as significant predictors of canine mass, but only when age was not fitted as a covariate, and this time significance was lost after FDR correction. None of the loci were retained as significant predictors of canine width. When the same data were analyzed using the more general test for genotype–fitness association proposed by Amos and Acevedo-Whitehouse (2009), PvcA again revealed an association with both canine length and residual canine length (Table 6). Locus Pv9 was no longer significant and instead Hg6.3 revealed a weak association with both canine mass and canine width but not with the residuals of either of these variables on age. However, none of these tests remained significant following FDR correction.

An alternative approach to test for general as opposed to local effects is to look for consistency of direction of effect across loci, with the general effect model predicting that most loci will exhibit similar trends and the local effect model predicting that trends will be in random directions for all but 1 or 2 loci (Hoffman et al. 2004). We find that while positive associations form the majority for canine length, mass, and width (7/9, 6/9, and 7/9 respectively), this trend fails in all cases to reach statistical significance (sign tests, $P = 0.18$, 0.51, and 0.18 respectively). A last test for the presence of inbred individuals was also conducted by repeatedly dividing the loci randomly into 2 subsets and using these to calculate paired heterozygosity estimates for each individual. When inbred individuals are rare or absent, these paired estimates will be uncorrelated, while the presence of inbred individuals will tend to create a positive correlation. In our data set, the heterozygosity–heterozygosity

Table 4 Correlation coefficients and associated *P* values for relationships between 3 different canine size measurements and skull mass and 18 skull morphometric measurements (see Figure 2 for details)

	Canine length		Canine mass		Canine width	
Morphological measurement	*r*	*P*	*r*	*P*	*r*	*P*
Skull mass	0.398	0.0003	0.416	0.0001	0.189	0.0931
Skull size						
CBL	0.402	0.0002	0.524	<0.0001	0.288	0.0096
ZW	0.377	0.0006	0.524	<0.0001	0.220	0.0499
OC-M	0.361	0.0010	0.596	<0.0001	0.340	0.0020
BW	0.216	0.0543	0.100	0.3775	0.270	0.0154
RL	0.330	0.0028	0.435	0.0001	0.213	0.0578
POW	0.334	0.0025	0.376	0.0006	0.176	0.1184
PL	0.302	0.0065	0.383	0.0005	0.144	0.2025
AW	0.329	0.0029	0.463	<0.0001	0.301	0.0067
MW	0.292	0.0086	0.475	<0.0001	0.186	0.0986
SOW	0.127	0.2616	0.352	0.0014	0.123	0.2771
IOW	0.146	0.1963	0.381	0.0005	0.119	0.2931
UPCL	0.319	0.0039	0.273	0.0143	0.086	0.4481
ZRW	0.338	0.0022	0.293	0.0083	0.096	0.3969
B-PT	0.487	<0.0001	0.469	<0.0001	0.308	0.0054
S-JL	0.171	0.1294	0.321	0.0037	0.074	0.5142
PW1	0.319	0.0039	0.429	0.0001	0.277	0.0129
PW3	0.124	0.2731	0.287	0.0098	0.271	0.0150
PWS	0.134	0.2360	0.207	0.0654	0.139	0.2188

Table 5 Results of GLMs of canine length, mass, and width, fitting heterozygosity at each of the 9 microsatellite loci separately as explanatory variables

		Canine length			Canine length (age fitted as a covariate)			Canine mass			Canine mass (age fitted as a covariate)			Canine width			Canine width (age fitted as a covariate)		
Locus	*n*	Estimate	χ^2	*P*	Estimate	χ^2	*P*	Estimate	χ^2	*P*	Estimate	χ^2	*P*	Estimate	χ^2	*P*	Estimate	χ^2	*P*
Aa4	84	1.23	2.30	0.130	0.42	0.37	0.540	0.71	2.32	0.128	0.19	0.25	0.615	0.48	1.37	0.241	0.34	0.68	0.410
Hg1.3	83	−0.51	0.31	0.580	−0.04	0.00	0.960	−0.18	0.12	0.730	0.12	0.09	0.766	−0.26	0.34	0.562	−0.18	0.00	0.960
Hg6.3	84	1.30	1.52	0.220	1.20	1.89	0.170	0.53	0.74	0.390	0.46	0.94	0.333	0.81	2.38	0.123	0.79	2.34	0.126
Hg8.10	84	0.43	0.42	0.520	0.61	1.25	0.260	0.29	0.56	0.454	0.41	1.88	0.170	0.29	0.76	0.384	0.32	0.98	0.321
Lw10	84	1.26	1.68	0.190	1.30	2.68	0.100	0.61	1.20	0.273	0.65	2.22	0.136	0.54	1.24	0.266	0.55	1.32	0.251
M11a	84	−0.94	0.78	0.380	−0.36	0.16	0.690	−0.20	0.10	0.748	0.18	0.14	0.707	0.16	0.09	0.767	0.27	0.26	0.609
Pv9	84	1.97	7.53	**0.010***	1.57	6.85	**0.010***	0.86	4.18	**0.041**	0.60	3.27	0.710	−0.15	0.15	0.695	−0.23	0.38	0.540
PvcA	84	2.75	9.48	**0.002***	2.05	7.31	**0.010***	1.16	4.77	**0.029**	0.69	2.66	0.103	0.82	3.17	0.075	0.70	2.29	0.130
PvcE	83	0.27	0.09	0.770	−0.08	0.01	0.910	−0.04	0.01	0.941	−0.27	0.42	0.517	0.02	0.00	0.958	−0.04	0.01	0.910

N differs among loci due to small numbers of missing single-locus genotypes. 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. Statistically significant *P* values at $\alpha = 0.05$, uncorrected for multiple tests, are highlighted in bold. Asterisks denote *P* values that remained significant after controlling for the FDR (see methods for details).

correlation is weakly and nonsignificantly negative, suggesting that there may be little variation in the inbreeding coefficient of the individuals in our sample. Consequently, a genome-wide effect seems unlikely, although with so few markers this test is very weak.

BLAST Search Results

To explore the possibility of local effects further, we used BLAST searches to locate Pv9, PvcA, and Hg6.3 in the dog genome. The resulting matches are summarized in Table 7. The most convincing BLAST match was obtained for locus Pv9, which aligns to a genomic region in the dog lying just more than 19 kb from a glycerol kinase 2-like gene. Matches were also obtained for loci PvcA and Hg6.3, with the closest features in the dog being an ubiquitin-conjugating–like enzyme and a gene resembling isocitrate dehydrogenase [NAD] subunit gamma, respectively.

Discussion

We have found associations between heterozygosity and overall tooth size, regardless of whether one controls for age. By implication, in this species, teeth provide a good surrogate source of information about an animal's ability to attain a large body size and confirm that heterozygosity is an important predictor of this key component of fitness. Just how such a link may operate remains largely unclear. One possibility is that high heterozygosity increases resistance to one or, more likely, a variety of pathogens that would otherwise compromise growth. An alternative mechanism might be that heterozygosity impacts directly an individual's metabolism, perhaps by allowing it to perform better across a wide range of environmental conditions. To elucidate the mechanism would require a better understanding of whether one, several, or many loci are involved, an aspect that we endeavor to unpick in the accompanying sister paper (Hoffman et al. 2010).

Although heterozygosity has been linked to growth rates and metabolic efficiency in numerous organisms (reviewed by Mitton and Grant 1984), studies of body size are rare. In one such study, heterozygosity predicted size at metamorphosis in frogs (Lesbarres et al. 2007). Conversely, heterozygosity was not found to predict body size in either brown bears (Dahle et al. 2006) or ibex (Von Hardenberg et al. 2007), even though the latter did reveal a relationship between horn growth and heterozygosity. Part of the reason for there being so few studies of body size is that appropriate measurements are difficult to collect from large free-ranging animals, particularly if the species is aquatic. In many cases, individuals cannot be captured and, even when they can be, statistics such as body weight are liable to vary greatly for many reasons other than innate metabolic efficiency. However, tooth size appears to present a reliable index of body size in many species (e.g., Baker and Fowler 1990), suggesting that where such measurements can be collected, they may provide a good proxy for individual

Table 6 Results of single-locus association tests using the approach of Amos and Acevedo-Whitehouse (2009) for canine length, mass, and width

	Canine length	Residual canine length	Canine mass	Residual canine mass	Canine width	Residual canine width
Aa4	0.057	0.170	0.078	0.270	0.764	0.876
Hg1.3	0.075	0.570	0.421	0.944	0.188	0.310
Hg6.3	0.248	0.496	**0.033**	0.071	**0.032**	**0.042**
Hg8.10	0.765	0.480	0.741	0.402	0.333	0.290
Lw10	0.207	0.591	0.090	0.152	0.521	0.468
M11a	0.738	0.686	0.745	0.320	0.803	0.750
Pv9	0.157	0.186	0.633	0.719	0.513	0.493
PvcA	**0.049**	**0.018**	0.677	0.399	0.342	0.225
PvcE	0.116	0.252	0.538	0.457	0.739	0.826

To compensate for the effect of age on these measurements, we also analyzed the residuals of canine length, mass, and width on age. Significant P values at $\alpha = 0.05$ are highlighted in bold. None of these remained significant following correction for multiple tests using the FDR method.

"quality." Having said this, although in many male mammals, larger individuals are more dominant, acquire larger territories, and generally enjoy enhanced reproductive success, this may not be true for all species. Elsewhere, alternative strategies such as sneaking may be important (e.g., Young et al. 2007), while even in strongly polygynous species there may be costs associated with rapid growth and extreme body size, for example, through greater risk of mortality in tougher years (Clutton-Brock et al. 1985).

Exploring 3 different measures of tooth size, we find that width performs less well than either length or mass, both in terms of correlation with skull size and with the chance of showing a relationship with genotype. Interestingly, however, the strongest HFC is found for tooth length, whereas tooth mass actually correlates more strongly with skull size. This may be due simply to chance, though it is also possible that canine mass and length capture subtly different aspects of size even though they are strongly intercorrelated.

The use of teeth as indicators of skull size in particular and body size in general has a long history and embraces many mammalian taxa from humans and primates (Lavelle 1973; Wood 1979) to rodents and artiodactyls (Gould 1975). Moreover, teeth are also informative about other aspects of life including diet (Hobson and Sease 1998; Newsome et al. 2007) and the impact of environmental variation (Baker and Fowler 1990; Boyd and Roberts 1993). In pinnipeds, teeth have been extensively used to determine age (e.g., Laws 1953; Payne 1978; Arnbom et al. 1992; Boyd and Roberts 1993), and tooth size has been shown to correlate well with body size for both Northern fur seals (Baker and Fowler 1990) and Antarctic fur seals (Boyd and Roberts 1993). By adding in genetics, it now seems possible to open up a new window onto otherwise elusive life-history traits in long-lived species where life spans far exceed the tenure of most research grants. How much this approach can be applied to other species is unclear at present because fur seals may be unusually amenable, dying at highly predictable breeding colonies. Nevertheless, teeth are available from many other species and it may also be possible to use other tissues including tympano-periotic (auditory) bone, waxy inner earplugs, or otoliths (Lockyer 1984; Marmotel et al. 2006; Zuykova et al. 2009) in a parallel way.

With a strong relationship between tooth size and overall heterozygosity, we were able to ask whether the mechanism was likely to involve inbreeding depression or associative overdominance, or even a mixture of the 2. On balance, our evidence points toward associative overdominance probably being the primary mechanism. For example, although there is a general tendency for an excess of loci showing positive associations with canine size, these trends fail to reach significance. Moreover, given that a trend in average heterozygosity is known to be present, the null hypothesis is not simply equal trend directions among all loci but instead should be equal trend directions among loci not involved in associative overdominance. Furthermore, we find no evidence that heterozygosity is correlated among markers, as it should be if our data set included appreciably inbred individuals. This is perhaps surprising given that inbreeding may not be unusual in Otariids due to the relatively high skew in male reproductive success (Hoffman et al. 2003; Kiyota et al. 2008) combined with strong natal site fidelity (Campbell et al. 2008). One possible explanation might be that our samples contain few or no inbred individuals because such animals die younger and away from the breeding colony. Alternatively, with only 9 loci this test has rather little power, so the failure to achieve a positive result may be due simply to a type II error. For this reason, it would be desirable to deploy many more genetic markers as we do in our companion paper (Hoffman et al. 2010).

Table 7 Results of BLAST searches of microsatellite clone sequences to the dog genome for loci Pv9, PvcA, and Hg6.3

Locus	Genbank accession number	Length of clone sequence (bp)	Bp overlap (identity)	Score	*E*-value	Chromosome	Identity of nearest gene[a]	Distance from nearest gene (bp)
Pv9	G02096	292	90/97 (92%)	139	6×10^{-31}	32	Glycerol kinase 2	19 026 (5′)
PvcA	L40983	148	47/49 (95%)	78.7	6×10^{-13}	36	Ubiquitin-conjugating enzyme E2 E3	379 613 (5′)
Hg6.3	G02092	288	58/64 (90%)	84.2	6×10^{-14}	21	Isocitrate dehydrogenase [NAD] subunit gamma	320 692 (5′)

[a] Derived by automated computational analysis using a gene prediction method.

We were surprised by the strength of the correlation between IR and tooth length. In a meta-analysis of HFC studies spanning 24 different animal taxa, the mean *r* value reported using multilocus heterozygosity was 0.0274 (Coltman and Slate 2003) compared with our values which reached as high as 0.373 for canine length. However, because small sample sizes tend either to yield large effect sizes, which are required to achieve statistical significance, or go unpublished (Coltman and Slate 2003), a study of our size is more likely to find a large value. Nonetheless, 0.373 is one of the largest values reported and appears much larger than might be expected to arise through inbreeding depression in a real population (Balloux et al. 2004) thereby supporting our conclusion that the effect is probably due to associative overdominance.

Adding weight to this conclusion, we find evidence of a small number of loci being individually associated with tooth size. Thus, testing for a simple correlation with heterozygosity, Pv9 and PvcA are significantly associated with tooth length, whereas when tested for a more general genotype–phenotype association, we again find evidence for PvcA but not Pv9 affecting tooth length and for Hg6.3 impacting on tooth mass and width, although the latter tests did not remain significant following FDR correction. Interestingly, the 2 loci that we identify as being associated with canine length, Pv9 and PvcA, have been implicated in HFCs in other pinniped species. Thus, heterozygosity at Pv9 significantly predicts pup survival in gray seals (Bean et al. 2004) and PvcA predicts sea lion pup survival but not resistance to hookworm infestation (Acevedo-Whitehouse et al. 2006). Whether the difference between the 2 methods reflects our relatively small sample size or a genuine difference in the nature of the underlying association is unclear and would require the collection of additional samples in the future.

Given some tendency for the same loci to appear significant in related but independent studies, it is interesting to ask whether there is evidence that they lie near plausible candidate genes. We therefore used BLAST searches to locate Pv9, PvcA, and Hg.6.3 in the dog genome. We found convincing single hits on chromosomes 32, 36, and 21 respectively, and recorded for each the nearest "local feature." In dogs, Pv9 matches to a genomic region that lies just over 19 kb from a glycerol kinase 2-like gene. Glycerol kinase 2 is a phosphotransferase enzyme involved in lipolysis and represents a juncture between sugar and fat metabolism in mammals (Kida et al. 1973). The closest gene to PvcA in the dog is a ubiquitin-conjugating–like enzyme described by Universal Protein Resource as being "involved with growth regulation" (Glickman and Ciechanover 2002). Similarly, the closest gene to Hg6.3 in the dog appears to be one resembling isocitrate dehydrogenase [NAD] subunit gamma, a mitochondrially bound enzyme that features in the Krebs cycle and is therefore involved in carbohydrate metabolism (Huang and Colman 1990). Further work is needed to establish whether polymorphism at these genes does impact on growth, using first fine-mapping to confirm the identity of the gene at each site, followed by searches for polymorphisms within the candidate genes that reveal a much stronger association with phenotype. Without these steps, the candidate gene status should be viewed as speculative at best.

Another possible explanation for the unusually strong relationships that we document could relate to the importance of body size in a highly polygynous mammal with strong male–male competition. Large body size may confer an advantage in combat, reducing the risk of fatal injury, and males lose approximately 5 kg of body mass for each day that they are able to hold tenure among females (Boyd and Duck 1991), suggesting that only the largest individuals are able to extend their tenure through the prime periods of the season. Body size also influences mate choice decisions in many animal species, with large mates frequently being preferred by both males and females. Consequently, our findings may help to explain 2 previous findings in this species that relatively heterozygous males have greater reproductive success (Hoffman et al. 2004) and that females appear to choose males that have high heterozygosity (Hoffman et al. 2007).

Finally, in the current study, we focus exclusively on males. This is for a variety of reasons. Most obviously, our previous work has focused on male reproductive success, and hence we knew already that male success was strongly linked to heterozygosity. In contrast, female success is more difficult to quantify and requires many more years of study because while most males have a reproductive life span totaling less than 5 years, females may carry on producing pups for up to 20 years (Lunn et al. 1994). More generally, given the large sexual size dimorphism in this species, there is good reason for believing that body size will play a much stronger role in predicting reproductive success in males than in females. Having said this, as our study continues and our data on core females extends to cover complete reproductive histories, it should become possible to explore the role of heterozygosity and body size in females as well as males.

Conclusion

Our study demonstrates how canine teeth can be used to explore HFCs for important life-history traits in free-ranging vertebrate populations. We show that heterozygosity strongly predicts canine size and, by implication, body size in Antarctic fur seals. The mechanism appears to depend at least in part on chance linkage to genes experiencing balancing selection, and by crossing to the dog genome, we were able to identify plausible candidates worthy of further study. However, conclusive evidence for the exact mechanism must await the deployment of further markers, work that is described in our companion paper (Hoffman et al. 2010).

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