

No relationship between microsatellite variation and neonatal fitness in Antarctic fur seals, *Arctocephalus gazella*

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Abstract

Published studies of wild vertebrate populations have almost universally reported positive associations between genetic variation measured at microsatellite loci and fitness, creating the impression of ubiquity both in terms of the species and the traits involved. However, there is concern that this picture may be misleading because negative results frequently go unpublished. Here, we analyse the relationship between genotypic variation at nine highly variable microsatellite loci and neonatal fitness in 1070 Antarctic fur seal pups born at Bird Island, South Georgia. Despite our relatively large sample size, we find no significant association between three different measures of heterozygosity and two fitness traits, birth weight and survival. Furthermore, increasing genetic resolution by calculating parental relatedness also yields no association between genetic variation and fitness. Our findings are consistent with necropsy data showing that most pups die from starvation or trauma, conditions that are unlikely to be influenced strongly by genetic factors, particularly if the benefits of high heterozygosity are linked to immune-related genes.

Keywords: birth weight, heterozygosity–fitness correlation (HFC), inbreeding depression, internal relatedness, maternal effect, necropsy, parental relatedness, pinniped, survival

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Introduction

Numerous studies of wild vertebrate populations have reported associations between presumed neutral genetic variation and key components of fitness, including juvenile survival (Coulson *et al.* 1998), disease susceptibility (Acevedo-Whitehouse *et al.* 2003), parasite burden (Coltman *et al.* 1999) and reproductive success (Slate *et al.* 2000). Where documented, such heterozygosity–fitness correlations (HFCs) have a clear potential to influence the evolution of mate choice and the interaction between pathogens and their hosts. However, important questions remain about the exact nature of HFCs. In particular, we still do not know which of two possible genetic mechanisms is most important, nor the extent of the generality of this phenomenon.

HFCs at presumed neutral genetic markers and a fitness trait may arise in two main ways (reviewed by Hansson & Westerberg 2002). First, the observed variation in fitness could be due to inbreeding depression. In this model,

heterozygosity at the loci sampled estimates genome-wide heterozygosity that in turn correlates with f , the inbreeding coefficient of an individual. Low heterozygosity then indicates inbreeding and fitness may be lost through increased expression of deleterious recessive alleles. The alternative ‘local effect’ model involves chance linkage to individual genes that are themselves experiencing some form of balancing selection. Both models have their problems. Inbreeding depression appears unlikely in most species because the number of individuals with detectably non-zero inbreeding coefficients is generally too low (Balloux *et al.* 2004; Slate *et al.* 2004; DeWoody & DeWoody 2005), possible exceptions being small isolated populations and those exhibiting strong polygyny (Balloux *et al.* 2004). Unfortunately, the local effect model is also somewhat implausible because studies typically use only between 5 and 10 markers, and chance linkage would only occur if large numbers of genes were to be under balancing selection, far more than most people currently believe.

Faced by these uncertainties, researchers have sought new ways to obtain better estimates of genome-wide heterozygosity. One such approach, developed by Duarte *et al.* (2003)

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is to calculate the relatedness of an individual's parents. This approach is interesting because it potentially separates the two mechanisms: parental relatedness should be effective at estimating inbreeding coefficients but will be only weakly correlated with offspring heterozygosity at any particular locus. Unfortunately, as with studies that attempt to reconstruct pedigrees, there is a strict requirement for many unambiguous paternities to be assigned.

A further problem is publication bias (Coltman & Slate 2003). While the number of studies reporting HFCs is large and increasing, there is concern that negative results are going unreported (for exceptions see Duarte *et al.* 2003 and Overall *et al.* 2005). Indeed, it is noticeable that many published studies involve either polygynous species or small/isolated populations. These are just the conditions that have been identified as likely to generate sufficient numbers of inbred individuals for genome-wide effects to become important. Publication bias therefore may influence both our perception of how common HFCs are, and the most likely mechanisms that underpin them.

Pinnipeds provide an interesting model for exploring the relationship between genetic variation and fitness because many species exhibit a combination of highly polygynous mating and breeding-site fidelity that is expected to favour incestuous matings. Furthermore, they are ideal for studying neonatal fitness because females usually give birth to only a single pup each season (Spotte 1982) effectively eliminating any influence of confounding factors such as unequal parental provisioning and/or sibling competition. Consequently it is perhaps unsurprising that studies of grey and harbour seals have reported significant HFCs (Coltman *et al.* 1998; Bean *et al.* 2004), although in neither study was the effect consistent across fitness traits and across alternative heterozygosity metrics. Interestingly, Coltman *et al.* (1998) found an effect with mean d^2 , a metric thought to be a relatively poor estimator of genome-wide heterozygosity, and not with straight heterozygosity, perhaps suggesting local rather than general effects.

A colony of Antarctic fur seals *Arctocephalus gazella* at Bird Island, South Georgia, is particularly amenable to study, with a scaffold walkway allowing unprecedented ease of access for observation, marking and tissue sampling. Breeding behaviour in this species is believed to be strongly polygynous and although one genetic study called this into question (Gemmell *et al.* 2001), a subsequent and more comprehensive study of the same population confirmed that territorial males indeed father the majority of pups (Hoffman *et al.* 2003). Male–male competition is intense, and relatively heterozygous territorial males achieve enhanced reproductive success (Hoffman *et al.* 2004). Consequently, we might also expect heterozygosity to be implicated in neonatal fitness.

Here, we analyse a data set comprising 1070 neonatal Antarctic fur seals genotyped at nine highly polymorphic

microsatellite loci. As with similar studies of wild vertebrates (e.g. Coltman *et al.* 1998; Overall *et al.* 2005), we analysed both neonatal survival and birth weight, although the extent to which birth weight influences lifetime fitness in Antarctic fur seals is currently unknown. In addition to calculating individual heterozygosity values for each pup, we also used genetic parentage assignments to calculate parental heterozygosity and parental relatedness. However, despite our sample size being large, we find no relationship between any of these measures of genetic variation and neonatal fitness.

Materials and methods

Study site, data collection and tissue sampling

This study was conducted at Bird Island, South Georgia (54°00'S, 38°02'W) during the austral summers of 1994/1995–2001/2002 (hereafter breeding seasons are referred to by the year in which they began). The study population was located at a small cobblestone breeding beach that was separated from adjacent breeding sites by a cliff on the east side, open sea on the west and rocky outcrops to the north and south. The beach covers an area of approximately 440 m² at high tide and on average 649 pups were born there annually during the study period. An elevated scaffold walkway (Doidge *et al.* 1984) provided access to all parts of the beach and minimized disturbance to the animals.

Approximately 550 adult females were randomly selected and tagged using plastic cattle ear tags (Dalton Supplies) placed in the trailing edge of the foreflipper (Lunn & Boyd 1993). Pups born to tagged females were captured on the day of birth, sexed and weighed to the nearest 100 g in a nylon bag suspended from a 10-kg capacity spring scale (Pesola® Model 220, Oskar Lüdi and Company). They were then marked with temporary serial numbers by bleaching the fur on their backs before returning them to their mothers. Territorial adult males were identified using distinctive natural markings (e.g. scars) and by applying small patches of gloss paint to the pelage (Arnould & Duck 1997). Piglet ear notching pliers were used to collect a small skin sample from the interdigital margin of the foreflipper of tagged females and their pups (Majluf & Goebel 1992). Adult males that held territories on the study beach were remotely sampled using a biopsy dart system. Sampling equipment was washed with ethanol between uses. Skin samples were stored individually in the preservative buffer 20% dimethyl sulphoxide (DMSO) saturated with salt (Amos & Hoelzel 1991) and stored at –20 °C.

Necropsy protocol

Each season, twice-daily surveys were made of pups born to tagged females from November 1 until the end of

the pupping period (early January). Following a simplified protocol developed by a veterinarian specializing in pinniped pathology (Doidge *et al.* 1984) pups that died were examined both internally and externally to determine the most likely cause of death. Taking all necropsy observations into account, each dead pup was assigned to one of the following categories:

- 1 Starvation: characterized by a thin or absent layer of subcutaneous blubber and absence of milk in the stomach
- 2 Trauma: pups exhibiting traumatic injuries (e.g. crushed skull or ribs) and associated haematomas
- 3 Stillbirth: when the pup's umbilicus was still attached, suggesting that the individual was younger than 24 h at death, a piece of lung tissue was removed and placed into water. Sinking tissue indicated that the pup had not taken a breath
- 4 Possible infection: in the absence of any obvious signs of trauma or starvation, the presence of lesions, pus and/or liver discoloration indicated that infection was the most likely cause of death
- 5 Unknown: pups that were scavenged by birds or could not be assigned to any of the above categories. It is likely that many of these deaths were caused by starvation or trauma, given that these are the predominant causes of mortality on the study beach

DNA extraction and genotyping

Genomic DNA was extracted from skin biopsy samples using an adapted Chelex 100 protocol (Walsh *et al.* 1991). Samples were then genotyped using a panel of nine dinucleotide-repeat microsatellite loci as described elsewhere (Hoffman & Amos 2005a). Reactions yielding uncertain genotypes (e.g. with faint or unclear bands) were repeated. Multilocus genotypes were generated for a total of 1070 pups born on

the beach during the study period (Table 1). These genotypes were tested for deviations from Hardy–Weinberg equilibrium and linkage disequilibrium using GENEPOP (<http://wbiomed.curtin.edu.au/genepop/>; Raymond & Rousset 1995). For each test, we set the dememorization number to 10 000, the number of batches to 1000 and the number of iterations per batch to 10 000.

Individual heterozygosity

Heterozygosity at nine microsatellite loci was calculated for all pups ($n = 1070$) using three different measures that have each been found to correlate with neonatal fitness in previous studies of pinnipeds. The simplest of these, standardized heterozygosity (SH), estimates the proportion of loci that are heterozygous while weighting the contribution of each locus by the expected heterozygosity at that locus (Coltman *et al.* 1999). The second approach, mean d^2 (Coulson *et al.* 1999) exploits the fact that microsatellites tend to mutate in a way such that the squared length difference between two alleles provides an estimate of the time to their most recent common ancestor (Goldstein *et al.* 1995). Consequently, the squared difference in repeat units between the two alleles at a locus, averaged over all loci at which an individual is typed, is expected to reflect the evolutionary distance between the maternal and paternal genomes. In this case, mean d^2 was standardized by dividing each value by the maximum observed at that locus, thereby ensuring that each locus contributed equally (Hedrick *et al.* 2001). A third approach attempts to estimate the relatedness of an individual's parents using the extent of allele sharing relative to random expectations. This has been achieved in the measure known as internal relatedness (IR, Amos *et al.* 2001) by adapting the methods developed by Queller & Goodnight (1989) to the special case of one allele per parent per locus. When calculated across multiple loci,

Table 1 Summary of Antarctic fur seal pups analysed, including annual mortality rates for our sample of pups. For mortality rates calculated over all pups born on the study beach, see Reid & Forcada (2005)

Year	Total number of pups born on study beach	Genotyped pups				
		Number	Number that died	Mortality rate %	Number with genetically assigned mothers	Number with both parents genetically assigned
1994	584	176	16	9.09	109	25
1995	797	162	20	12.35	102	74
1996	732	173	17	9.83	126	95
1997	501	109	24	22.02	82	57
1998	693	106	16	15.09	72	27
1999	444	68	4	5.88	56	38
2000	696	139	23	16.55	113	72
2001	747	137	19	13.87	105	73
Total	5194	1070	139	12.99	765	461

IR values are approximately normally distributed around zero for offspring born to random 'unrelated' parents. Here, IR was calculated using population allele frequencies. Elsewhere, Hoffman *et al.* (2004) have shown that any differences in allele frequency distributions among adult males, females and pups are so small as to have a negligible impact on the calculation of IR.

Parentage assignment and calculation of parental relatedness

First, we checked the genotypes of putative mother–offspring pairs identified in the field ($n = 832$) for mismatches (e.g. genotypes that do not share a common allele). Given that even a very low rate of genotyping error can generate mismatches between mothers and their true offspring (Hoffman & Amos 2005b), we double-checked autoradiographs for scoring errors whenever a pair mismatched at a single locus. After correcting 15 genotyping errors, the majority of pups ($n = 765$, 92%) were found to match their putative mothers at all nine loci and were consequently classified as filial. The remaining 67 mother–offspring pairs mismatched, indicating the probable occurrence of fostering and/or milk stealing behaviours in the study colony.

Pups were assigned paternity as described by Hoffman *et al.* (2003) using a strict exclusion approach implemented in the program NEWPAT XL (Worthington Wilmer *et al.* 1999). To maximize confidence in our paternity assignments, only pups that genetically matched their putative mothers ($n = 765$) were analysed. Stringent match criteria were set, allowing a maximum of one unscored locus and no mismatches. To guard against false exclusions resulting from genotyping errors, the analysis was then repeated allowing up to two mismatches, and all mismatching genotypes were checked for scoring errors. A total of 461 paternities were assigned, of which a small number ($n = 23$) invoked single-locus scoring errors. Elsewhere using a reduced data set from the same colony (Hoffman *et al.* 2003) we examined the extent to which paternities assigned by NEWPAT XL were independently assigned using a likelihood approach implemented in the program CERVUS version 1.0 (Marshall *et al.* 1998). Strong concordance was found between the two programs with 386 out of 388 paternities assigned by NEWPAT XL also being identified as matches by CERVUS. However, because CERVUS accepted additional paternities that invoked multilocus mismatches and/or incompatible mother–pup–father triads, we prefer to conservatively restrict our analyses to paternities assigned by NEWPAT XL.

Unfortunately, despite the large numbers of parentage assignments we have been able to make, fur seals are too long-lived for our study to embrace more than a single generation, precluding the direct calculation of inbreeding coefficients. Instead, following the approach of Duarte

et al. (2003), we estimated pairwise relatedness between each pup's parents using Queller and Goodnight's statistic r (1989). Relatedness calculations were implemented using population allele frequencies within the program KINSHIP version 1.3.1 (Goodnight & Queller 1999).

Statistical analyses

Statistical analyses were conducted using generalized linear models (GLMs) within the software package R (R Development Core Team 2005). Pups with missing data were excluded from models. Birth weight and survival were fitted as response variables and modelled using Gaussian and binary error structures respectively. Genetic variables were not transformed because they were approximately normally distributed and GLMs are robust to modest departures from normality (Genstat 5 Committee 1995). Furthermore, distributions of standardized residuals between the genetic variables and birth weight were approximately normal. Initially, each GLM was constructed with all explanatory terms fitted, including first-order interactions wherever possible (see the Results section for details of the explanatory variables fitted in each model). Using standard deletion-testing procedures (Crawley 2002), each term was then dropped from the model 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 equal to the difference in degrees of freedom between the models with and without the term in question. Since strong correlations among explanatory variables can lead to unstable parameter estimates, we also checked for correlations among our predictor variables. In the only instance where two explanatory variables were nonindependent (sex and birth weight in GLMs of pup survival), we compensated for this problem by constructing separate models in which we varied the relative order in which these terms were fitted.

Results

We genotyped 1070 Antarctic fur seal pups (Table 1) at nine highly polymorphic microsatellite loci and used GLMs to analyse the extent to which heterozygosity, together with a number of potentially confounding non-genetic terms, explained variation in neonatal weight and survival. After applying sequential Bonferroni corrections to compensate for multiple statistical tests, none of the microsatellite loci showed significant deviation from Hardy–Weinberg equilibrium (Table 2) and no pairs of loci exhibited significant linkage disequilibrium (Table 3). Pup birth weights were approximately normally distributed around a mean of 5.1 kg (Fig. 1) and the overall rate of

Table 2 Summary of the microsatellite loci used in this study, including literature sources and polymorphism characteristics for 1070 Antarctic fur seal pups. None of the Hardy–Weinberg equilibrium P values remained significant following sequential Bonferroni correction for multiple tests with $\alpha = 0.05$

	Isolated from species	Reference	Number of alleles	Expected heterozygosity (H_E)	Hardy–Weinberg equilibrium probability
Aa4	South American fur seal <i>Arctocephalus australis</i>	Gemmell <i>et al.</i> (1997)	7	0.749	0.481
Hg1.3	Grey seal <i>Halichoerus grypus</i>	Gemmell <i>et al.</i> (1997)	12	0.866	0.150
Hg6.3	Grey seal <i>Halichoerus grypus</i>	Allen <i>et al.</i> (1995)	12	0.858	0.046
Hg8.10	Grey seal <i>Halichoerus grypus</i>	Allen <i>et al.</i> (1995)	5	0.440	0.627
Lw10	Weddell seal <i>Leptonychotes weddellii</i>	Davis <i>et al.</i> (2002)	19	0.907	0.778
M11a	Southern elephant seal <i>Mirounga leonina</i>	Hoelzel <i>et al.</i> (1999)	18	0.924	0.743
Pv9	Grey seal <i>Halichoerus grypus</i>	Allen <i>et al.</i> (1995)	10	0.776	0.512
PvcA	Harbour seal <i>Phoca vitulina</i>	Coltman <i>et al.</i> (1996)	8	0.774	0.961
PvcE	Harbour seal <i>Phoca vitulina</i>	Coltman <i>et al.</i> (1996)	15	0.876	0.180

Table 3 Tests for linkage disequilibrium implemented using the Fisher exact test in GENEPOP for 1070 Antarctic fur seal pup multilocus genotypes. χ^2 values are given above the diagonal and P values below. None of the P values remained significant following sequential Bonferroni correction for multiple tests with $\alpha = 0.05$

Locus	Aa4	Hg1.3	Hg6.3	Hg8.10	Lw10	M11a	Pv9	PvcA	PvcE
Aa4	*	1.592	3.044	2.716	1.406	0.359	0.778	4.317	12.100
Hg1.3	0.451	*	5.521	0.668	6.613	0.337	5.450	2.513	2.392
Hg6.3	0.218	0.063	*	0.668	1.658	1.356	0.073	0.477	9.790
Hg8.10	0.257	0.716	0.716	*	1.821	0.951	0.086	2.796	1.779
Lw10	0.495	0.037	0.437	0.402	*	6.065	2.000	10.921	0.191
M11a	0.836	0.845	0.508	0.621	0.048	*	2.826	1.186	3.271
Pv9	0.678	0.066	0.964	0.958	0.368	0.243	*	0.216	1.307
PvcA	0.115	0.285	0.788	0.247	0.004	0.553	0.898	*	4.521
PvcE	0.002	0.302	0.007	0.411	0.909	0.195	0.520	0.104	*

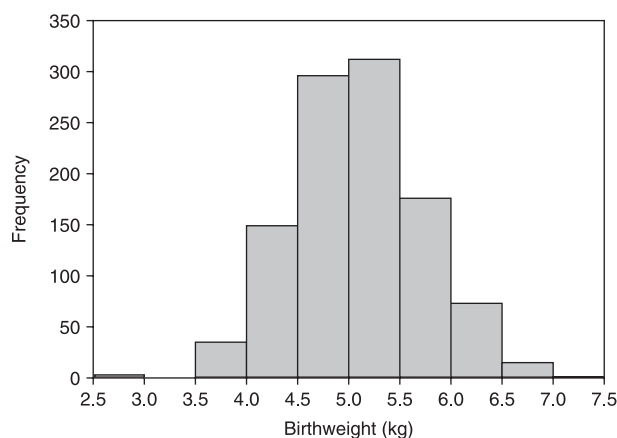


Fig. 1 Frequency distribution of the birth weights of 1062 Antarctic fur seal pups born on the study beach (eight birth weight records were missing).

mortality among our sample of pups was 13% ($n = 139$ dead individuals, Table 1). The sex ratio of pups did not differ significantly from one (526 males to 544 females, $\chi^2 = 0.15$, d.f. = 1, $P = \text{NS}$).

Table 4 Pearson correlation coefficients (r) for the relationships among IR, SH and standardized mean d^2 values calculated for 1070 Antarctic fur seal pups. All correlations are significant at $P < 0.0001$

Variable	IR	SH	Standardized mean d^2
IR	*	−0.980	−0.259
SH	*	*	0.279
Standardized mean d^2	*	*	*

Individual heterozygosity

Heterozygosity was quantified for each individual using the measures internal relatedness (IR) and standardized heterozygosity (SH). For comparison, we also included standardized mean d^2 , a measure that is increasingly regarded as less informative. The frequency distributions of these three variables are shown in panels a to c of Fig. 2. To determine the extent to which these variables were measuring similar properties, IR, SH and standardized mean d^2 were correlated against each other (Table 4). All of

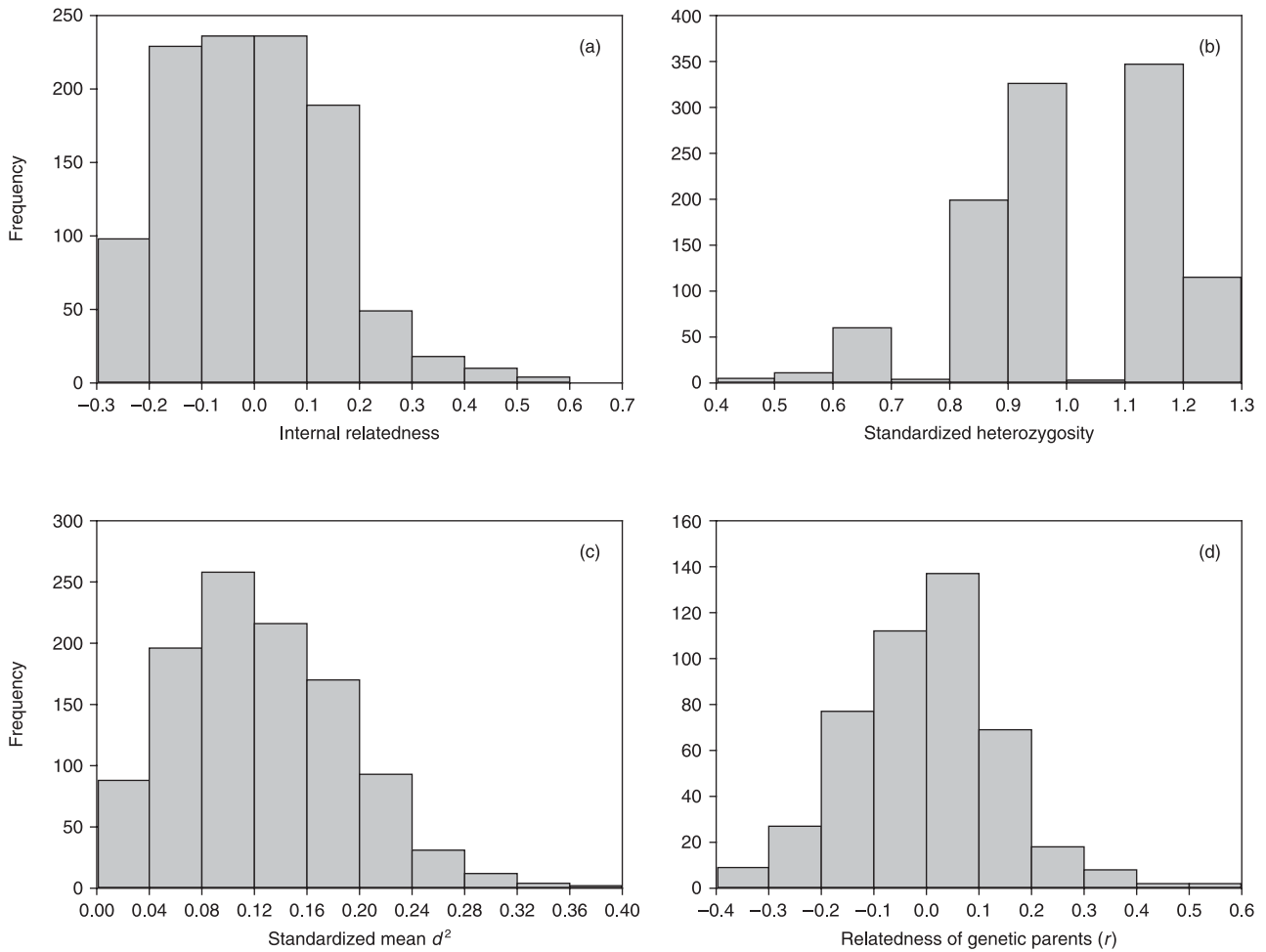


Fig. 2 Frequency distributions of (a) internal relatedness (IR); (b) standardized heterozygosity (SH); and (c) standardized mean d^2 for 1070 Antarctic fur seal pups; (d) pairwise relatedness values (r) of the genetic parents of pups assigned both maternity and paternity ($n = 461$).

the correlations were statistically significant ($P < 0.0001$), with IR and SH being the most strongly correlated ($r^2 = 0.96$).

Offspring heterozygosity and fitness

To determine whether heterozygosity explained a significant proportion of variation in birth weight, we constructed separate GLMs of pup weight, fitting pup IR, SH or standardized mean d^2 together with sex (categorical variable, male or female), day of birth (the number of days after November 1 that a pup was born) and year of birth (categorical variable, 1–8). Due to a small number of missing records of birth weight ($n = 8$) and birth date ($n = 7$), analyses were restricted to 1055 individuals. In the resulting models, neither IR, SH nor standardized mean d^2 explained significant variation in pup birth weight. Instead, the only significant explanatory factor (accounting for 21.0% of the total deviance) was sex ($F_{1,1054} = 280.65, P < 0.0001$) with males being born heavier than females. To examine whether heterozygosity impacts

Table 5 Logistic regression of pup survival. See text for details of explanatory variables that were fitted. Only significant terms in the model are shown. The χ^2 values for each term represent the change in deviance after removing that term from the model

$n = 1055$, total deviance = 810.89, residual deviance = 760.98

Term	Estimate	d.f.	χ^2	P
Birth weight	0.763	1	26.03	< 0.0001
Year of birth	—	7	19.04	0.020
Day of birth	-0.030	1	6.18	0.013

neonatal survival, we then constructed GLMs of survival using a binary error structure and fitting the same explanatory variables as before plus birth weight. Once again, none of the genetic variables approached significance when fitted as main or interaction terms (see Table 5). Instead, survival

was significantly associated with birth weight (heavier pups were more likely to survive), the timing of birth within seasons (pups born earlier were more likely to survive), and the year of birth (see Table 1 for annual mortality rates).

Because more than half of our focal females gave birth to more than one pup during the course of the study, it is possible that the most prolific mothers could disproportionately influence our analyses. To compensate for this, it is in principle possible to fit a generalized linear mixed model (GLMM), adding 'mother' as a random factor to allow for some mothers being more successful at rearing pups than others. However, mixed models are unlikely to be reliable when many mothers have only one or two offspring as in our study because estimates of within-family variance are either inaccurate (two pups) or not possible (one pup). An alternative is to fit 'mother' as a fixed factor, but this would result in a heavily over-specified model. Consequently, for simplicity we repeated the previous GLMs after randomly selecting a single pup from each mother. As found before, only sex explained significant variation in birth weight ($F_{1,376} = 110.65$, $P < 0.0001$). This time however, only birth weight was retained as a significant term in the model of pup survival ($F_{1,376} = 8.94$, $P = 0.003$). Comparable results were obtained for two additional randomly selected subsets of pups.

Maternal heterozygosity

Antarctic fur seal females that are large, heavy and in good condition tend to give birth to heavier pups (Boyd & McCann 1989), suggesting a potential link between maternal heterozygosity and offspring fitness. Consequently we examined a subset of 765 pups whose mothers were genotyped at nine microsatellite loci. Controlling once more for pseudoreplication, we randomly selected one pup from each female, restricting the data set to 274 pups with complete observational data. We then constructed separate GLMs of pup weight and survival fitting the same terms as before, but including the mother's IR, SH or standardized mean d^2 as an additional genetic variable. Neither pup nor mother's heterozygosity were retained as significant terms in the resulting GLMs. Instead only sex explained significant variation in birth weight ($F_{1,273} = 41.22$, $P < 0.0001$), and only birth weight was significantly associated with survival ($F_{1,273} = 4.98$, $P = 0.026$).

Parental relatedness

When the genotypes of both parents are known, it becomes possible to directly calculate an offspring's parental relatedness following the approach of Duarte *et al.* (2003). We used a strict paternal exclusion approach to assign 461 paternities with high confidence (Hoffman *et al.* 2003, 2004). Relatedness

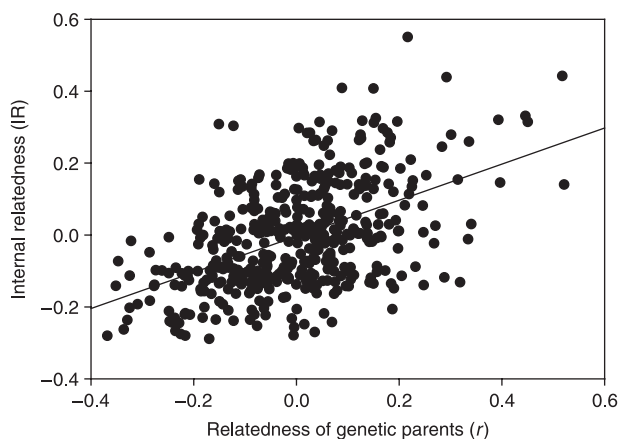


Fig. 3 The relationship between offspring internal relatedness (IR) and parents' pairwise relatedness (r) for 461 Antarctic fur seal pups.

between each pup's parents was then calculated using Queller and Goodnight's statistic r (1989). The resulting r values were approximately normally distributed around a mean of -0.002 (Fig. 2d) and correlated strongly with IR (Fig. 3, Pearson correlation coefficient $r = 0.48$, $n = 461$, $P < 0.0001$), SH ($r = -0.48$, $n = 461$, $P < 0.0001$) and to a lesser extent standardized mean d^2 ($r = -0.17$, $n = 461$, $P < 0.0001$). The mean r was not significantly different from that expected from random pairings (unpaired t -test; $t = 0.70$, d.f. = 920, $P = \text{NS}$). Selecting one pup per mother or father (no parent represented more than once) yielded a data set of $n = 98$ pups. In the multiple regression models of birth weight, we fitted parental relatedness plus mother and father's IR, SH or standardized mean d^2 as the genetic variables, together with sex, day of birth, and year of birth. Once more only sex remained significant in the final model ($F_{1,97} = 28.49$, $P < 0.0001$). In the logistic regression models of survival, we fitted parental relatedness plus mother and father's IR, SH or standardized mean d^2 as the genetic variables, together with birth weight, sex, day of birth, and year of birth. This time, none of these terms were found to be significantly associated with survival.

Causes of mortality

Elsewhere, the strength of a HFC varies between classes of mortality (e.g. Acevedo-Whitehouse *et al.* 2003). Consequently we examined post-mortem data from 139 pups to determine the most likely causes of death. Figure 4 shows that while many of our necropsies were inconclusive ($n = 46$), the most commonly assigned causes of mortality among our sample of pups were starvation ($n = 43$), trauma ($n = 36$) and stillbirth ($n = 13$), conditions that are unlikely to be influenced by a pup's heterozygosity. Only a single pup exhibited obvious signs of infectious disease. Finally, we

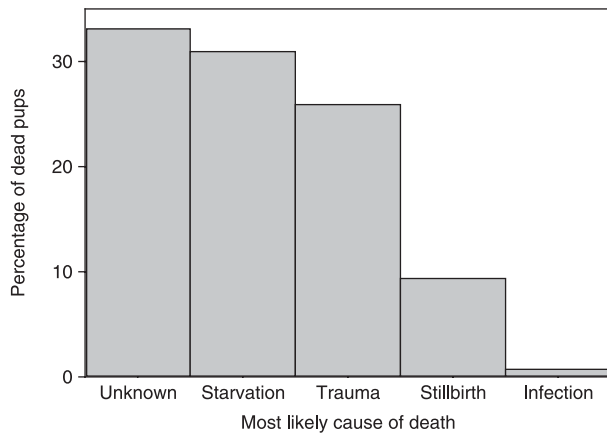


Fig. 4 Histogram showing the most likely cause of death of 139 Antarctic fur seal pups. Necropsies were inconclusive for 46 pups.

also examined temporal variation for each mortality category by classifying pups according to their birth dates into four groups, each containing 25% of pup births. The resulting profiles (Fig. 5) indicate that mortality rates peaked mid-season for pups assigned to the categories trauma and unknown. This reflects the temporal pattern of crowding on the beach (data not shown) and supports the suggestion that the majority of those unknown deaths were likely to be caused by starvation or trauma. There was also a clear trend for increased starvation rates among pups born later in seasons ($\chi^2 = 17.65$, d.f. = 3, $P < 0.001$).

Discussion

Here we analyse the relationship between genetic variation at nine highly variable microsatellite loci and fitness in 1070 Antarctic fur seal pups born at Bird Island, South Georgia. Despite this relatively large sample size, we find no significant associations between measures of heterozygosity and fitness. In addition, we attempted to increase genetic resolution by calculating parental relatedness (Duarte *et al.* 2003) rather than relying on measures of heterozygosity alone. However,

once again we find no relationship between genetic variation and fitness. This is in contrast with previous findings in great reed and Seychelles warblers (Hansson 2004; Richardson *et al.* 2004) but is consistent with Duarte *et al.* (2003), the only study that we are aware of that reported no relationship between parental relatedness measured using microsatellites and a fitness trait, fecundity.

Previous studies of HFCs in pinnipeds include work on harbour seal pups by Coltman *et al.* (1998) that documented a remarkably strong relationship between mean d^2 and birth mass explaining almost 20% of the trait variance. A significant relationship between IR and preweaning survival was also shown for grey seal pups although, in contrast to Coltman *et al.*'s study but in line with most other HFC studies, the proportion of trait deviance explained was small, being less than 5% (Bean *et al.* 2004). Similarly, in Antarctic fur seals we previously found that a variety of traits related to territorial male fitness correlate with heterozygosity at the same panel of microsatellite loci (Hoffman *et al.* 2004). Consequently, we were initially surprised to find no HFC for survival in fur seal pups.

One reason for not detecting HFCs could lie with the fact that the Bird Island population is large and growing. Such a population structure may generate very few inbred individuals, thereby reducing or eliminating general-effect HFCs (Balloux *et al.* 2004). However, the large overall population probably gives a misleading impression because individual breeding beaches can be quite small and fur seals show both strong polygyny (Hoffman *et al.* 2003) and fidelity to their natal beaches (Lunn & Boyd 1991). We estimate that at a breeding colony with an average pup production of approximately 650 individuals, top males may father in excess of 70 pups in their lifetime. In such a system, the potential for inbreeding is obvious, although whether it actually occurs is unknown. To test for the presence of inbred individuals, we applied the method of Balloux *et al.* (2004), repeatedly dividing the markers into two groups and looking for a correlation in heterozygosity between the groups, and obtained a negative result. However, this is to be expected since nine markers is a very small panel, particularly when divided in half. Alternative

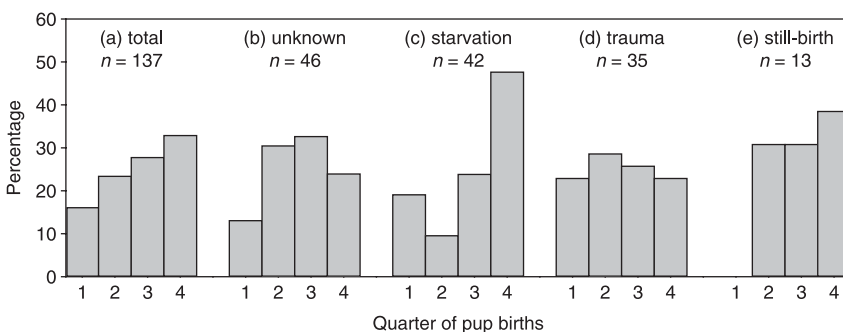


Fig. 5 Histogram showing the most likely causes of death in relation to timing of birth for 137 Antarctic fur seal pups. Note that this figure does not include two pups whose birth dates were unknown. Also, a further pup dying from infection is included in the 'total' but not shown separately.

approaches such as simply looking for an excess of unusually high IR values similarly lack the statistical power to detect anything but strong inbreeding in a few individuals or moderate numbers of individuals with small to medium inbreeding coefficients.

A second possible explanation for the lack of HFCs in pups is that by chance none of the nine loci we chose lie close enough to a gene experiencing balancing selection to exhibit associative overdominance. Although plausible, this argument is somewhat at odds with the finding of strong HFCs relating to many diverse aspects of adult male reproductive success in the same colony (Hoffman *et al.* 2004). Whether HFCs found in adults are due to inbreeding or to single-locus effects, their overall strength and the fact that they affect several traits including reproductive longevity together indicate that there is nothing inherent in this system that precludes the formation of HFCs.

If one assumes that HFCs do exist in pups but that these were simply not detected in our study, the fact that we found widespread HFCs among adult males using the same markers suggests two possible mechanisms. Most obviously, the genes influencing pup survival may have little in common with those that dictate aspects of adult reproductive behaviour. For example, with previous studies emphasizing a link between heterozygosity and resistance to pathogens (Coltman *et al.* 1999; Acevedo-Whitehouse *et al.* 2003, 2005; Hawley *et al.* 2005; MacDougall-Shackleton *et al.* 2005), a key component of pup survival may involve immune-related genes acting to prevent disease. Conversely, immune-related genes might play a smaller role in relation to fitness traits in adult males such as competitive ability. Alternatively, it is possible that adult fitness is dominated by inbreeding effects while pup fitness depends more on local effects, or vice versa. Why this should be true is unclear, but it is a possibility that should be tested in the future using a larger panel of markers.

In practice, our limited necropsy data indicate that disease is not an important cause of Antarctic fur seal pup mortality, in contrast to other pinniped species such as the grey seal. Instead, extrinsic factors such as starvation and trauma seem to be the dominant causes of death. This is consistent with previous studies conducted at the same colony (Doidge *et al.* 1984; Reid & Forcada 2005) including a detailed bacteriological survey that revealed a very low incidence of infections among pups (Baker & Doidge 1984). Also, as found previously, a small number of additional non-genetic factors impact pup fitness, with heavier-born pups being more likely to survive (Lunn *et al.* 1994) as well as pups born in good years when local food availability may be high. Within seasons, survivorship is also higher among pups born earlier, perhaps reflecting a tendency for smaller, younger mothers to give birth towards the end of seasons (Boyd & McCann 1989; Lunn *et al.* 1994).

However, these temporal patterns are relatively weak, failing to reach significance after restricting analyses to only one pup per mother (entailing an almost threefold reduction in n).

In conclusion, despite large sample sizes and relatively high-quality field data, we find no relationship between genetic variation at nine microsatellite loci and neonatal fitness in Antarctic fur seals. Instead, only a small number of non-genetic factors such as sex and the timing of birth appear to be influential. This may be because the primary mortality agents are starvation and trauma, conditions that are unlikely to be influenced much, if at all, by a pup's heterozygosity.

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