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Contrasting effects of heterozygosity on survival and hookworm resistance in California sea lion pups

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Abstract

Low genetic heterozygosity is associated with loss of fitness in many natural populations. However, it remains unclear whether the mechanism is related to general (i.e. inbreeding) or local effects, in particular from a subset of loci lying close to genes under balancing selection. Here we analyse involving heterozygosity–fitness correlations on neonatal survival of California sea lions and on susceptibility to hookworm (*Uncinaria* spp.) infection, the single most important cause of pup mortality. We show that regardless of differences in hookworm burden, homozygosity is a key predictor of hookworm-related lesions, with no single locus contributing disproportionately. Conversely, the subsequent occurrence of anaemia due to blood loss in infected pups is overwhelmingly associated with homozygosity at one particular locus, all other loci showing no pattern. Our results suggest contrasting genetic mechanisms underlying two pathologies related to the same pathogen. First, relatively inbred pups are less able to expel hookworms and prevent their attachment to the intestinal mucosa, possibly due to a weakened immune response. In contrast, infected pups that are homozygous for a gene near to microsatellite Hg4.2 are strongly predisposed to anaemia. As yet, this gene is unknown, but could plausibly be involved in the blood-coagulation cascade. Taken together, these results suggest that pathogenic burden alone may not be the main factor regulating pathogen-related mortality in natural populations. Our study could have important implications for the conservation of small, isolated or threatened populations, particularly when they are at a risk of facing pathogenic challenges.

Keywords: California sea lion, heterosis, heterozygosity, hookworms, neonatal survival, *Uncinaria*

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Introduction

Understanding the genetic basis of fitness in natural populations has long been a central aim of evolutionary biology. As Darwin pointed out, under natural conditions populations are subjected to the influence of selective pressures that cause an incessant 'struggle for survival' where only the fittest individuals will endure. Neonatal survival in particular is thought to be a key demographic parameter under strong selection (Charlesworth 1994), especially for large-mammal populations, where the probability of surviving from birth to weaning is usually low (Baker 1984; Barlow & Boveng 1991).

One way in which genetic variation might influence neonatal survival is through inbreeding depression (O'Brien & Evermann 1998). Inbreeding depression occurs when

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two close relatives mate, thereby increasing homozygosity and allowing expression of deleterious recessive alleles (Crow 1948). It is manifest in many aspects of fitness, including survival, susceptibility to disease and reproductive success, and has been demonstrated in a wide range of species (Frankham 2003). Theory indicates that inbreeding depression will be particularly intense following a sharp population decline, making it an important concern in the conservation of threatened or endangered species where reduced health may increase the risk of extinction (Spielman *et al*. 2004). Pedigree-based studies have shown neonatal mortality to be higher in inbred than in noninbred captive animals (Ralls *et al*. 1979). However, few empirical studies have demonstrated the effects of inbreeding on neonatal survival in natural populations. This is partly due to the difficulties associated with measuring inbreeding in the field, where pedigrees are unknown and long-term observations are generally logistically unfeasible (Crnokrak & Roff 1999).

The advent of molecular techniques has allowed an indirect method for assessing parental relatedness without pedigrees, based on estimates of heterozygosity (Hansson & Westerberg 2002). Components of fitness have been found to correlate significantly with several related measures, including mean d^2 (Coulson *et al*. 1998), standardized heterozygosity (Coltman *et al*. 1999) and internal relatedness (Amos *et al*. 2001). Using these methods a growing number of studies have shown heterozygosity–fitness correlations (HFC) to be widespread and common in natural populations (Coltman & Slate 2003). In most cases, these effects have been interpreted as a reflection of inbreeding depression. However, recent thinking urges caution when interpreting these results (Hansson & Westerberg 2002). Correlations between genome-wide microsatellite heterozygosity and inbreeding coefficient (f) are generally low, even when many genetic markers are used (Slate *et al*. 2004), exceptions being small populations and/or those exhibiting extreme polygyny (Balloux *et al*. 2004). Thus, HFCs are deemed more likely to arise from a subset of loci being in linkage disequilibrium with functional loci showing heterozygote advantage (Hansson *et al*. 2004), rather than from genome-wide changes in heterozygosity (David 1998). The question of which mechanism is actually more important has begun to be addressed in natural populations (Hansson *et al*. 2004; Acevedo-Whitehouse *et al*. 2005; Overall *et al*. 2005) but the answer remains unclear.

An excellent test case for exploring the relative importance of general and local effects in HFCs is presented by neonatal survival in pinnipeds. A number of studies have examined this trait across several species, yielding highly variable results. While some have found higher heterozygosity among survivors compared with dead pups (Coltman *et al*. 1998; Coulson *et al*. 1998; Bean *et al*. 2004), explaining up to 20% of the variance in neonatal survival in one study (Coltman *et al*. 1998), some studies have been unable to find an effect (Markert *et al*. 2004; Richardson *et al*. 2004) or reported very weak correlations (Overall *et al*. 2005). While such variability in results may result from low statistical power, or from the stochastic nature of inbreeding effects, another plausible explanation for these discrepancies is that the HFCs are due mainly to local effects. If so, then the different set of loci used in different studies might well cause large variation in the reported results. However, neonatal survival is influenced by a number of genetic and environmental factors (Bowen *et al*. 1994; Boltnev *et al*. 1998; Hall *et al*. 2001), which are unlikely to be regulated by the same genes, or to be affected equally by inbreeding depression. In this sense, it is vital to determine the cause of mortality rather than simply to record neonates as dead or alive. A recent study of grey seal pup mortality attempted to distinguish between causes of death, broadly categorizing infectious and noninfectious classes (Bean *et al*. 2004). Although statistically nonsignificant, they report a general trend for pups dying from apparent infections to have lower heterozygosity than pups dying from trauma and starvation, consistent with the notion that, where protective, heterozygosity is most likely to impact on mortality that is preventable by a strong physiological response. It is therefore relevant that loci involved with immune defences, such as the major histocompatibility complex (MHC) and the killer cell immunoglobulin-like receptor (KIR) gene clusters (Trowsdale & Parham 2004), seem to depend on allele variation as a 'moving target' for rapidly evolving pathogens (Kurtz *et al*. 2004). If so, close-kin mating could potentially affect pathogen resistance by decreasing heterozygosity in immune-related regions, causing individuals to be less successful at recognizing pathogens (O'Brien & Evermann 1988).

Previously, we have studied California sea lions and shown that genetic heterozygosity is an important predictor of mortality and disease in this species (Acevedo-Whitehouse *et al*. 2003). However, this data set suffers a number of drawbacks that hamper more detailed study. Most importantly, since the study was based at a rehabilitation centre, sampling was opportunistic and many key variables cannot easily be determined, including where the animals came from, what proportion of the healthy population they represent and how many similar animals got sick but were not brought in. Consequently, to look further into the processes by which heterozygosity influences susceptibility to disease, we now focus on a system where directed sampling could be conducted, specifically on patterns of mortality and survival in California sea lion pups at San Miguel Island. Here, long-term studies have shown that hookworms (*Uncinaria* spp.) are the single most important pathogen of California sea lion pups in terms of both prevalence and virulence (Lyons *et al*. 1997), accounting for mortality rates of more than 40% (Lyons *et al*. 2001; Lyons

et al. 2005). Although the studied population is by no means small (Carretta *et al*. 2004), the highly polygynous system coupled with strong site fidelity (Reidman 1990) is likely to increase significantly the chance of consanguineous mating. This expectation is supported by tests for the presence of inbred individuals (Balloux *et al*. 2004), suggesting that inbreeding depression is the most likely mechanism underlying reported correlations between heterozygosity and disease resistance (Acevedo-Whitehouse *et al*. 2003). Consequently, this model system is ideal to explore aspects of the mechanistic basis of pup HFCs and to elucidate the role that pathogens may play in maintaining genetic variation by selection against inbred pups. Here we investigate the importance of genetic heterozygosity effects in relation to neonatal pathogen-related mortality in the California sea lion.

Materials and methods

Study site and population

Fieldwork was conducted at San Miguel Island (34°2′N, 120°44′W), the westernmost of the Channel Islands, located 46 km off the California coast. San Miguel Island has a surface of 3776 ha, containing numerous beaches and rock platforms that provide adequate breeding and hauling-out habitat for California sea lions and other pinniped species such as northern fur seals (*Callorhinus ursinus*), northern elephant seals (*Mirounga angustirostris*) and harbour seals (*Phoca vitulina*). San Miguel Island hosts the largest California sea lion breeding colony within the species' range, encompassing nearly 45% of the world population (Barlow *et al*. 1997; Carretta *et al*. 2004).

Dead pup surveys

Between June 2002 and January 2003, seven pup mortality surveys were conducted at four- to five-week intervals. Surveys consisted of two to three observers who walked along the rookeries to search for dead pups. At all times during the surveys, a distance of 10–15 m from the animals was maintained in order to minimize disturbance to the colony. When a fresh pup carcass was sighted, one observer would crawl towards it and fasten its hide to a metal hook in order to drag it away from the colony. A skin sample (approximately 32 mm^3) was collected from the interdigital margin of the fore-flipper of each pup and stored in ETOH or DMSO for genetic analyses (a total of 347 samples were collected). All sampling equipment was washed using isopropyl alcohol between uses to avoid genetic cross-contamination. At each sampling period, complete post-mortem examinations were conducted on approximately 30 of the pup carcasses that showed little or no signs of decomposition (< 36 h since death).

Live pup samples

Every year, a number of live pups are branded as part of an ongoing demographic assessment program of the National Marine Mammal Laboratory (NMML) of the USA (http://nmml.afsc.noaa.gov). Pups are branded on the left shoulder with a unique four-digit number that is easily observed from a distance of up to 200 m; skin samples are routinely obtained from the fore-flipper, stored in ETOH and archived. In order to obtain skin samples from pups that survived their first year of life, throughout June and July 2003 we conducted searches for individuals belonging to the 2002-brand cohort. During this period, four to five beaches were surveyed daily from 0800 to 1400 h. The area was repeatedly scanned from cliffs or a stationary blind until all branded animals that were visible were identified. Brands were read using 8×40 binoculars or a $25–56 \times 82$ mm spotting fieldscope. For each branded individual, two separate readings were annotated to minimize reading errors. Brand numbers belonging to the 2002 cohort were cross-referenced with the NMML database to ensure accuracy of the data. A subsample of tissue from these pups was included in our genetic analyses as representatives of 'survivor' pups (control samples). Due to having only been able to acquire samples from 23 survivors, we included a set of 22 samples from seemingly healthy pups branded during October 2002, thus increasing our live pup sample number to 45.

Pathology analyses

Necropsies were conducted systematically in all cases. Pelage, tegument, body openings, mucous membranes and internal organs were examined for evidence of pathological changes. A full set of tissue samples including lung, kidney, liver, spleen, stomach, intestines, mesenteric and mediastinic lymph nodes, cerebrum and cerebellum was collected from all carcasses, preserved in neutral buffered 10% formalin and processed for microscopical analysis by use of routine paraffin embedment techniques. Sections were cut 5 μm thick and stained with haematoxylin and eosin. Cause of death was determined following gross and histopathological examination, and was possible to ascertain for all examined (n = 181) pups. Hookworm burden was determined by direct inspection of the gastrointestinal tract (Lyons *et al*. 2001). Anaemia was determined qualitatively at necropsy by gross appearance of organs and tissues; and confirmed with histology.

Molecular genetic analyses

DNA was extracted from all samples using an adapted Chelex 100™ protocol (Walsh *et al*. 1991). Approximately 8 mg of tissue were air-dried at 55 °C. Cells were lysed by

Table 1 Polymorphisms and heterozygosity of the microsatellites used in this study. Table shows GenBank accession number, repeat unit, allele size range, number of alleles, expected heterozygosity (H_E) and the probability of deviation from Hardy–Weinberg equilibrium (HWE) at each locus. NA, not available

Locus	GenBank accession no.	Repeat unit	Size (bp)	No. alleles	H_E	HWE probability
Hg4.2	G02090	$(TG)_{17}$	144–170	9	0.66	0.261
Hg6.1	G02091	$(CA)_{11}TA(CA)_{10}$	160–182	10	0.75	0.598
Hg6.3	G02092	$(TG)_{17}$	229–245	10	0.78	0.060
Hg8.10	G02093	$(CA)_{24}$	176–188	8	0.80	0.395
Lw10	AF140592	$(GT)_{25}$	124–144	11	0.73	0.777
M11a	NA	NA	138–154	9	0.81	0.222
Pv11	U65444	$(AC)_{20}$	176–184	5	0.67	0.925
Pvc29	L40987	$(AC)_2AG(AC)_7$	130–178	21	0.79	0.499
Pvc78	L40983	$(AC)_{15}$	137–169	9	0.66	0.189
Orr1	G34933	$(TA)_{10}CAC(GT)_{15}$	191–211	10	0.76	0.150
Orr7	G34928	$(TG)_7AG(TG)_{23}$	191–203	8	0.76	0.609
Orr8	G34929	$(TG)_{11}$	179–197	6	0.67	0.307
Orr24	G34932	$(AC)_{14}$	170–194	12	0.82	0.892

adding 300 μL of 1 X low TE (10 mM Tris-HCl pH 8.3 and 0.1 mM EDTA) containing 5% Chelex 100 chelating resin, 2% SDS, 500 μg Proteinase (K). Samples were incubated for 4 h at 55 °C. 250 μg RNase were added and samples were further incubated for 12 h at 37 °C on a rotating wheel.

We genotyped each individual at a panel of 13 polymorphic microsatellite loci previously cloned from other pinniped species (Hg4.2, Hg6.10, Hg6.3 and Hg8.10 (Allen *et al*. 1995); PvC29 and PvC63 (Coltman *et al*. 1996); Pv11 (Goodman 1997); M11a (Hoelzel *et al*. 2001); Orr1, Orr7, Orr8 and Orr24 (Buchanan *et al*. 1998); Lw10 (Gelatt *et al*. 2001)). Loci were selected based on their average heterozygosity, number of alleles and ease of scoring (Table 1). Polymerase chain reactions (PCRs) were carried out in 10-μL reaction volumes containing 3 μL of 1 : 50 diluted template DNA, 1 X Thermalase buffer [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], 0.1 mM dGTP, 0.1 mM dATP, 0.1 mM dTTP, 0.01 mM dCTP, 400 nM of each primer, 0.25 U *Taq* polymerase and 0.1 μCi [α^{32}P]-dCTP. Amplification conditions were adapted from those described previously. Optimum annealing temperatures (TmA-TmB) were 54–58 °C (Pv11), 52–55 °C (Hg6.10, Hg6.3, Hg8.10, Orr7, and PvC29), 48–52 °C (Hg4.2 and M11a, Orr1, and Orr24), and 46–48 °C (PvC78, Lw10, and Orr8).

PCR products were resolved on 6% denaturing polyacrylamide gels and visualized by autoradiography. Sizing of PCR products was accomplished by using known size loci and by loading the same four samples in each gel to correct small variations in allele size assignation among runs. Success rate of amplification was 99.6%, suggesting good quality DNA. Homozygous individuals were genotyped twice to reduce error due to possible allele dropout. Allelic disequilibrium and presence of nonamplifying alleles (Pemberton *et al*. 1995) were tested by investigating deviation from Hardy–Weinberg equilibrium (HWE) for each locus using GENEPOP version 3.3 (Raymond & Rousset 1995).

We used 'Internal Relatedness' (IR) (Amos *et al*. 2001) as a derivative of multilocus heterozygosity for each pup. This measure is based on allele sharing where the frequency of every allele counts towards the final score, thereby allowing the sharing of rare alleles to be weighted more than sharing of common alleles. Noninbred individuals are expected to have IR values of 0, while relatively homozygous ('inbred') individuals will have positive IR values and heterozygous ('outbred') individuals will have negative IR values (Amos *et al*. 2001). IR was calculated for each individual as described previously, using 'IRMACRON3', an Excel macro written in VISUAL BASIC (www.zoo.cam.ac.uk/zoostaff/amos). The presence of inbred individuals may be indicated by a tendency for heterozygosity to be correlated among loci. To test for this correlation, the loci used are randomly divided into two equal subsets, each of which is used to calculate heterozygosity (in our case IR) for each individual (Balloux *et al*. 2004). Repeating this 100 times we obtained the mean and standard deviation of the correlation coefficient (r) among loci. A significantly nonzero value of r is then taken as evidence that heterozygosity is correlated among loci, and hence that the dataset includes individuals with nonzero values of f. Simulation studies show that this heterozygosity correlation test is more effective at detecting a small percentage of individuals with nonzero f than alternative approaches based on, for example, testing for shifts in mean heterozygosity or for the presence of outlying values (Balloux *et al*. 2004; W.A., unpublished).

Statistical analyses

A number of statistical analyses were conducted using routine parametric tests and generalized linear models (GLMs) within the R package (Ihaka & Gentleman 1996). All genetic data were normally distributed. Hookworm burden data were not normally distributed and consequently were normalized using a Log_{10} (parasite load + 1) transformation (Fulford 1994). For the GLMs, error structure and link were defined each time as appropriate. Variables fitted were IR and the residuals around the relationship between IR and hookworm burden (residual hookworm burden). Initially, models were constructed with all terms fitted, including two-way interactions. Using standard deletion-testing procedures, terms were dropped from the model unless doing so significantly reduced the amount of deviance explained. To compensate for overdispersion, significance testing was carried out using *F*-tests (Crawley 2002). For all models, distribution of standardized residuals around regressions was inspected to verify that they were normally distributed. To correct for multiple testing, we used Hochberg procedures on each hypothesis (Roback & Askins 2005).

Results

General heterozygosity effects on pup survival and hookworm related mortality

There were marginal differences between IR values of dead and 'survivor' pups, dead individuals being slightly more homozygous (two-tailed *t*-test, $t = -1.99$, d.f. = 390, $P = 0.05$). When testing whether relatively more homozygous pups die earlier in the season, we found no temporal variation in IR across the sampling periods (ANOVA, $F_{7,346} = 1.63$, $P = 0.13$). However, there was an apparent increase in IR of dead pups at sampling period four (October), which coincides with the peak of hookworm-related mortality (Fig. 1) and hence, was sufficiently suggestive to look further at the relationship between IR and hookworm infections.

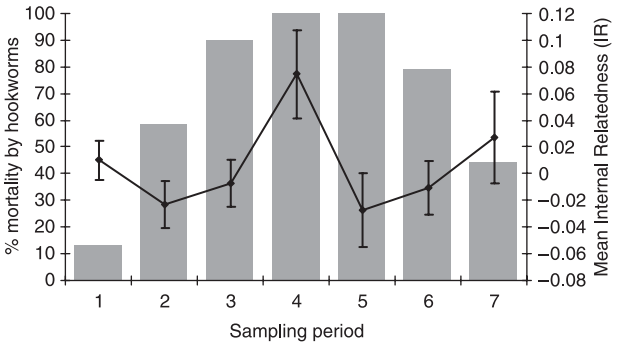

Fig. 1 Variations in the percentage of mortality caused by hookworm-related lesions (grey bars) and in the heterozygosity of dead pups (black line), measured as mean IR (high IR = low heterozygosity) across sampling periods. Black bars represent ± 1 SE. This figure shows a broad association, with the peak of hookworm-related mortality coinciding with the highest IR values.

All pups examined were infected with hookworms; individual hookworm counts ranged from 1 parasite to more than 4000. Hookworm burden was positively correlated with IR, with relatively more homozygous pups being more likely to harbour greater parasite loads ($r = 0.18$, $n = 181$, $P = 0.02$). However, although all pups included in this study were infected, not all showed evidence of hookworm-related lesions. Such lesions are associated with death due to hookworm infection and accounted for 72% of mortality ($n = 130$), with trauma or emaciation ($n = 46$) accounting for the remaining deaths. In five cases, necropsy data were not conclusive to ascertain cause of death, and these samples were excluded from subsequent analyses. If general heterozygosity increases pathogen resistance and decreases the probability of pathogen-related death, pups that died due to hookworm lesions rather than noninfectious conditions would be expected to have a higher mean IR. As predicted, the pattern observed suggests that pups that died due to trauma or emaciation had lower IR values than pups with hookworm lesions (–0.03 and 0.01, respectively) (one-tailed *t*-test, $t = -1.76$, d.f. = 174, $P = 0.04$). Surprisingly, a model that included both hookworm burden and IR as predictors of hookworm-related death revealed IR to be the most significant predictor, accounting for 7.8% of the total deviance explained in our model (GLM, $\chi^2_{1,172} = 9.07$, $P = 0.003$), while hookworm burden only explained 4.6% (GLM, $\chi^2_{1,172} = 5.5$, $P = 0.015$).

Of the 130 pups that died due to hookworm lesions, 25 were severely anaemic as a result of blood loss and 27 revealed evidence of peritonitis due to hookworm penetration through the intestinal wall. Testing both hookworm burden and IR as predictors of anaemia and peritonitis in pups with hookworm lesions revealed heterozygosity as the main predictor of anaemia (GLM, $\chi^2_{1,125} = 5.01$, $P = 0.025$), anaemia being more likely to occur in more homozygous individuals. Surprisingly, hookworm burden was removed from the model as a nonsignificant term. In contrast, hookworm burden was a significant predictor of peritonitis (GLM, $\chi^2_{1,125} = 5.77$, $P = 0.016$), with IR this time being nonsignificant (see full models for anaemia and peritonitis in Table 2).

Local heterozygosity effects

In order to test whether the observed results for pathogen-related mortality could reflect selection against inbred individuals (i.e. general effects where all microsatellites contribute equally), we conducted IR–IR correlations. This approach tests for whether homozygosity is correlated among loci as would be expected if inbred individuals are

Table 2 Tests for effects of IR and hookworm burden on both anaemia and peritonitis using generalized linear models. Explanatory variables fitted were IR and the residuals around the relationship between IR and hookworm burden (residual hookworm burden). The interaction of IR × and residual hookworm burden was not significant in either model. Because the response variables were defined as factors and the models were defined with a binomial error structure, the percentage of deviance explained was not calculated

Pathology	Term	Estimate	d.f.	*F*	*P*
Anaemia	IR	3.234	1	5.012	0.025
	Residual hookworm burden	−0.102	1	0.032	0.859
Peritonitis	IR	2.443	1	2.614	0.106
	Residual hookworm burden	−1.456	1	5.766	0.016

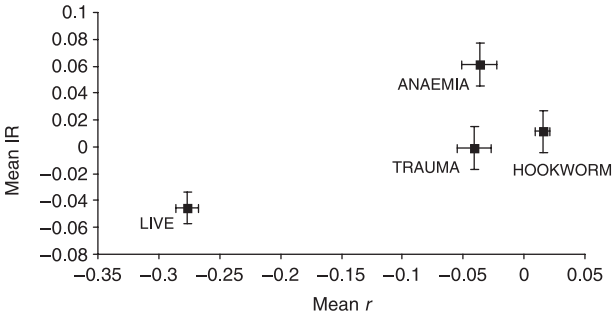

Fig. 2 Relationship between mean IR and mean IR–IR correlation coefficient (*r*) for each category. Bars represent ± SE for each parameter. Mean correlation coefficient (*r*) and standard deviation were obtained by resampling the data 100 times and randomly dividing the loci into one group of 6 and one of 7 and computing IR for both sets of loci.

present (see Methods). We found a positive relationship between mean IR and the strength of the IR–IR correlation, with pups that died from hookworm lesions having the highest correlations and live pups having the lowest. However, pups with anaemia did not appear to follow this relationship (Fig. 2), suggesting that the high IR of anaemic pups may not be due to inbreeding, but instead might be associated with a local effect. Consequently, we re-analysed our data, testing each marker independently by consecutively fitting GLMs for survival, hookworm burden, hookworm lesions and anaemia. Each model included heterozygosity at each marker as a factor, IR calculated by excluding the marker being considered, and the interaction between these two terms. Neither survival nor hookworm burden were explained by heterozygosity at any particular locus. Rather, the effects appeared to reflect overall reduced heterozygosity (Table 3). The association between heterozygosity and lesions and anaemia appeared to arise by different mechanisms. While occurrence of hookworm lesions was associated with general reduced heterozygosity (IR), with no one locus contributing disproportionately, anaemia was overwhelmingly associated with homozygosity

Table 3 Test for effects of single-locus heterozygosity and general (all loci, measured as IR) heterozygosity on neonatal survival and on hookworm burden of dead pups. Table shows *P* values. Interactions were not significant and are not presented in the table

	P values			
	Single-locus		General	
Locus	Survival	Hookworms	Survival	Hookworms
Hg4.2	0.9999	0.3189	0.0019	0.0411
M11a	0.7194	0.3806	0.0621	0.0368
Hg6.3	0.3391	0.0909	0.0333	0.0858
PvC78	0.0480	0.0704	0.1494	0.0227
Hg8.10	0.0937	0.8772	0.1754	0.0231
Hg6.10	0.7891	0.1639	0.1294	0.0649
Pv11	0.4252	0. 1833	0.0209	0.0075
Orr7	0.7263	0.1596	0.0579	0.0572
Lw10	0.0563	0.3803	0.0884	0.0103
Orr8	0.2474	0.3157	0.0262	0.0337
Orr1	0.5275	0.0439	0.0265	0.0651
PvC29	0.7429	0.4796	0.0221	0.0112
Orr24	0.1214	0.2660	0.0738	0.0776

at one particular locus, Hg4.2 ($P = 10^{-4}$), with no other locus showing a strong effect after correcting for multiple comparisons. IR calculated excluding locus Hg4.2 was also nonsignificant (Table 4).

Discussion

Our results show that the effects of heterozygosity on pup survival are dependent on the cause of death. As predicted, we find a very weak correlation between heterozygosity and survival when grouping all dead pups together. Relatively more homozygous pups were not more likely to die earlier in the pupping season. Instead, homozygosity was higher for pups that died due to hookworm infection, little or no pattern being found among pups that died from trauma or emaciation. Pups with higher IR had greater

Table 4 Test for effects of single-locus heterozygosity and general (all loci, measured as IR) heterozygosity on occurrence of hookworm-related lesions and anaemia in dead pups. Table shows *P* values. Interactions were not significant and are not presented in the table

	P values			
	Single-locus		General	
Locus	Hookworm lesions	Anaemia	Hookworm lesions	Anaemia
Hg4.2	0.0296	0.00009	0.0113	0.1355
M11a	0.0050	0.20173	0.0126	0.0514
Hg6.3	0.8244	0.23013	0.0002	0.0503
PvC78	0.0141	0.25436	0.0082	0.0073
Hg8.10	0.3681	0.33915	0.0002	0.0466
Hg6.10	0.7002	0.35880	0.0003	0.0565
Pv11	0.7247	0.47688	0.0008	0.0095
Orr7	0.0737	0.59726	0.0034	0.0292
Lw10	0.1878	0.60039	0.0016	0.0388
Orr8	0.7506	0.63439	0.0006	0.0106
Orr1	0.7506	0.70532	0.0004	0.0147
PvC29	0.5258	0.87430	0.0009	0.0185
Orr24	0.6136	0.89184	0.0005	0.0280

hookworm burdens and were more likely to die after sustaining hookworm-related lesions. When testing for general as opposed to local effects, we find that death due to lesions appears to be attributable to inbreeding (genome-wide effects), while occurrence of anaemia is associated with a single locus.

There are now many studies that report significant to highly significant correlations between heterozygosity and fitness (e.g. Coltman *et al*. 1998; Coulson *et al*. 1998; Amos *et al*. 2001; Bean *et al*. 2004), yet the proportion of fitness explained appears small, seldom rising above 3%. However, when other sources of error are taken into account, a value of 3% can appear surprisingly large. In our study, hookworms were counted only at death and the resulting value did not take into account other potentially important factors such as previous health status and secondary infections, how rapidly the hookworm burden developed and maternal health status. If such factors could be controlled, our proportion of fitness explained would almost certainly rise. Furthermore, our study deployed 13 loci, representing only a tiny fraction of the genome. If the HFC is due entirely to inbreeding, simulations indicate that the true proportion of fitness explained, were it possible to measure *f* without error (e.g. by using many hundreds of markers), could rise by 5–50 fold, depending on the true underlying variance in *f* (Balloux *et al*. 2004; W.A., unpublished). Equally, if the HFC is due to local effects, the vast majority of genes will lie too far from any of our markers to show an effect and even those few that are close enough are unlikely to be in complete linkage disequilibrium with the gene. Consequently, our small panel of markers would not only tend to underestimate the size of effect due to the genes they lie near to, but also the influence of many important genes would go undetected. Again, the 3% ($r = 0.18$, see Results section) we report will be substantially smaller than the likely true proportion of fitness that could be explained if unlimited data could be gathered.

Previous studies have demonstrated that prevalence of hookworms in both live and dead pups is 100% (Lyons *et al*. 2001, 2005). Likewise, in this study, all pups were infected with hookworms. However, not all pups died as a result of this infection. Our results show that pups with lower IR values tend to have lower hookworm loads than pups with higher values, suggesting that heterozygosity influences the efficiency of the immune response against hookworms. Since none of the loci appeared to be contributing disproportionately towards the observed effect, it seems that the effect is general and might arise from close-kin mating. We found that heterozygosity explained less of the variance than has been reported previously for other parasitic infections (Coltman *et al*. 1999; Casinello *et al*. 2001). This could be related to the particular mechanism of transmission of hookworms, where pups acquire larval parasites during nursing, via the milk (Lyons *et al*. 2001). Thus, although the number of hookworms transmitted is not likely to depend on any mechanism of natural or acquired immunity of the pup, successful resistance and expulsion of the adult parasites is likely to require a robust immune response, dependent on genetically regulated local inflammatory responses and antibody production (Behnke *et al*. 1997; Girod *et al*. 2003; Vardhani 2003; Mason *et al*. 2004). Since all pups are infected at birth, it is probable that they will develop hookworm lesions to some extent before their immune response against hookworm initiates. Only pups that are successful at eliciting an immune response capable of slowing parasite growth and promoting their elimination before the lesions become life threatening, will survive. However, infected pups may sustain severe and potentially fatal traumatic injuries, such as crushing by adult males, before developing successful immunity against hookworms. Such pups had slightly lower IR values than pups with hookworm lesions but no traumatic injuries (data not shown) suggesting that, had they not been injured, relatively more heterozygous pups could have survived hookworm infection.

The exact relationship between numbers of hookworms and clinical disease remains unclear (Lyons *et al*. 2001). After the initial infection, adult hookworms attach to the pups' intestinal wall and feed on blood, occasionally causing severe anaemia (Lyons *et al*. 2000). In some cases the parasites penetrate the intestinal wall, leading to peritonitis (Spraker *et al*. 2004). Elsewhere, damage to the

intestinal epithelium, regardless of penetration, may cause haemorrhagic enteritis leading to bacteraemia and then death. Interestingly, the relative importance of hookworm burden on the outcome of infection does not appear to be simple. For peritonitis, genetic factors were not significant and only hookworm burden had any explanatory power, suggesting either that the probability of penetration is not influenced by the host's immune responses or, more simply, that penetration is stochastic and the more worms present the more likely penetration becomes (von Allmen *et al*. 2004; Mitreva *et al*. 2005). High hookworm burdens may also increase the chance of penetration by causing generally higher level of intestinal damage. In contrast, anaemia was strongly associated with homozygosity at one particular locus (Hg4.2), regardless of hookworm burden. Here, by strong implication, the mechanism is genetic, for example with pups who are heterozygous for locus Hg4.2 having a more robust blood-coagulation response to endothelial lesion, thereby reducing blood loss and the chance of suffering severe anaemia. Potential support for this hypothesis comes from studies that show haematophagous hookworms produce potent inhibitors of coagulation and platelet function (Stanssens *et al*. 1996; Harrison *et al*. 2002). Unfortunately, the California sea lion genome is currently unmapped, so the identity of whatever gene lies near to Hg4.2 remains unknown. Candidates would include genes involved in the initiation or regulation of the blood-coagulation cascade (Preissner *et al*. 2000; Sheehan *et al*. 2001), prothrombin production (Davidson *et al*. 2003) or erythropoiesis (Mayer *et al*. 1995) and will be the focus of future immunogenetic and gene-mapping studies.

Our study supports the idea that selection against inbred individuals in natural populations is mediated by pathogens, and provides an example of both general and local mechanisms influencing a component of fitness (pathogen-driven mortality). Taken together, our results show that pathogen-induced mortality may be less stochastic than previously thought and therefore may have important implications for the conservation of small, isolated or threatened populations, particularly when they are at a risk of facing pathogenic challenges. More studies of natural populations are needed to ascertain the extent to which selective mortality occurs and what the consequences are with respect to the maintenance of genetic variation of the population.

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