

# High-throughput sequencing reveals inbreeding depression in a natural population

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**Proxy measures of genome-wide heterozygosity based on approximately 10 microsatellites have been used to uncover heterozygosity fitness correlations (HFCs) for a wealth of important fitness traits in natural populations. However, effect sizes are typically very small and the underlying mechanisms remain contentious, as a handful of markers usually provides little power to detect inbreeding. We therefore used restriction site associated DNA (RAD) sequencing to accurately estimate genome-wide heterozygosity, an approach transferrable to any organism. As a proof of concept, we first RAD sequenced oldfield mice (*Peromyscus polionotus*) from a known pedigree, finding strong concordance between the inbreeding coefficient and heterozygosity measured at 13,198 single-nucleotide polymorphisms (SNPs). When applied to a natural population of harbor seals (*Phoca vitulina*), a weak HFC for parasite infection based on 27 microsatellites strengthened considerably with 14,585 SNPs, the deviance explained by heterozygosity increasing almost fivefold to a remarkable 49%. These findings arguably provide the strongest evidence to date of an HFC being due to inbreeding depression in a natural population lacking a pedigree. They also suggest that under some circumstances heterozygosity may explain far more variation in fitness than previously envisaged.**

inbreeding | genetic variability | heterozygosity fitness correlation | single nucleotide polymorphism | RAD sequencing

It has long been known that inbreeding can reduce fitness, mainly through the unmasking of recessive or partially recessive deleterious alleles (1). Such effects are well documented in the laboratory (2, 3) but until recently have remained largely unstudied in natural populations (4, 5). This is because an individual's inbreeding coefficient ( $f$ ) can be calculated directly only by using a deep pedigree, and these pedigrees are seldom available outside the laboratory (6). However, because inbreeding increases an individual's homozygosity, the heterozygosity of a panel of neutral genetic markers can in theory be used as a surrogate for  $f$ .

Initial searches for heterozygosity fitness correlations (HFCs) (7, 8) used allozymes, but the results obtained are difficult to interpret because the proteins themselves may be under selection. With the discovery of microsatellites, an abundant class of putatively neutral genetic marker, it became possible to look for HFCs without concerns about selection on the markers themselves. A burgeoning literature now shows that HFCs based on small numbers of microsatellite loci are found in many bird and mammal species for a remarkable range of fitness traits, including neonatal survival (9), parasite susceptibility (10) and lifetime reproductive success (11), and even behavioral qualities such as territory-holding ability (12), song complexity (13) and attractiveness (14). This weight of evidence suggests that HFCs are an important and widespread phenomenon in the natural world. It is therefore important to understand their basis.

Theory predicts that HFCs arise as a result of inbreeding depression, which will reduce the fitness of individuals in pro-

portion to their inbreeding coefficient  $f$  (7). Therefore, variance in inbreeding coefficients within a population is necessary to generate HFCs. However, simulation and empirical studies indicate that the variance in  $f$  in natural populations is usually low, and estimates of  $f$  based on the small numbers of markers typically deployed are often very poor (15). As a result, HFCs will usually be weak or nonsignificant even when inbreeding actually explains a large proportion of fitness. Szulkin et al. (16) summarized this by saying that HFCs allow one to see only the “tip of the iceberg” and provided two examples in which heterozygosity explains 3% and 6% of trait variation even though inbreeding is expected to account for 24% and 30% of the variance, respectively. The most favorable situations to observe HFCs are in populations where inbred individuals are not rare due to factors such as small population size, extreme reproductive skew, and natal philopatry (15), but even in these cases using only a few markers severely curtails the power to observe the actual impact of inbreeding.

To explain HFCs, authors also frequently invoke “local effects” where one or a small number of the microsatellites used as markers are by chance linked to a gene experiencing heterozygote advantage (8). Local effects are widely discussed in the literature, but their importance is unclear given that balanced polymorphisms are

## Significance

Many studies of wild populations reveal links between heterozygosity and fitness, with relatively heterozygous individuals carrying fewer parasites, living longer and being more attractive to mates. These patterns appear ubiquitous and are often highly significant, but heterozygosity usually accounts for very little of the total variation in fitness. However, most studies analyze only around 10 loci, representing a tiny fraction of the genome. We therefore used high-throughput DNA sequencing to estimate genome-wide heterozygosity based on over 10,000 loci and found it to accurately reflect inbreeding. Applied to wild harbor seals, genome-wide heterozygosity explained almost half of the variation in parasite infection. By implication, a greater proportion of fitness variation could be linked to genotype than previously thought.

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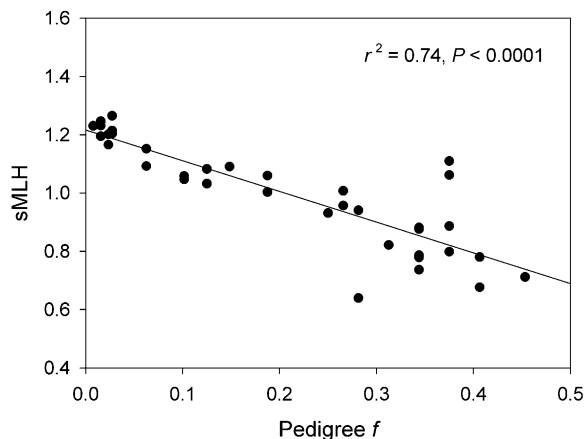
The authors declare no conflict of interest.

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Data deposition: The sequences reported in this paper have been deposited in the Short Read Archive database (accession no. [PRJEB5164](https://www.ncbi.nlm.nih.gov/sra/PRJEB5164)).

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**Fig. 1.** Correlation between pedigree-based inbreeding coefficient ( $f$ ) and RAD standardized multilocus heterozygosity (sMLH) for 36 oldfield mouse individuals. The  $r^2$  for the equivalent relationship based on 12 microsatellites is substantially lower at 0.276.

thought to be rare (17) and strong linkage between random pairs of loci is infrequent (16). It has also been argued that the contribution of local effects to HFCs may be overstated due to many studies having used an inappropriate statistical framework (16).

One means of unambiguously testing whether inbreeding alone can explain HFCs is to deploy a larger number of genetic markers (18). If an HFC is due to inbreeding depression, the use of more markers will reduce the error variance in the estimation of genome-wide heterozygosity and thereby strengthen the correlation. In contrast, if the HFC is highly dependent on one or a few marker loci being by chance linked to a fitness locus with strong effects, as in the local effect model, adding more markers located throughout the genome will reduce the strength of the HFC as the contribution of any one marker becomes progressively diluted. Until recently, however, this approach was not available to most studies because of the prohibitive costs of developing and screening large numbers of additional microsatellites.

Restriction site associated DNA (RAD) sequencing (19) has recently emerged as a rapid and economic means of genotyping thousands of single nucleotide polymorphisms (SNPs) in virtually any organism. This approach concentrates high-throughput sequencing effort around restriction enzyme cut sites that are distributed across the genome, thereby generating sufficiently deep local sequence coverage to reliably call SNPs as being either heterozygous or homozygous. Although SNPs are individually less informative than microsatellites due to their lower allelic diversity, this should be more than compensated for by the large numbers of markers screened, in principle allowing genome-wide heterozygosity to be estimated with far greater precision than previously possible.

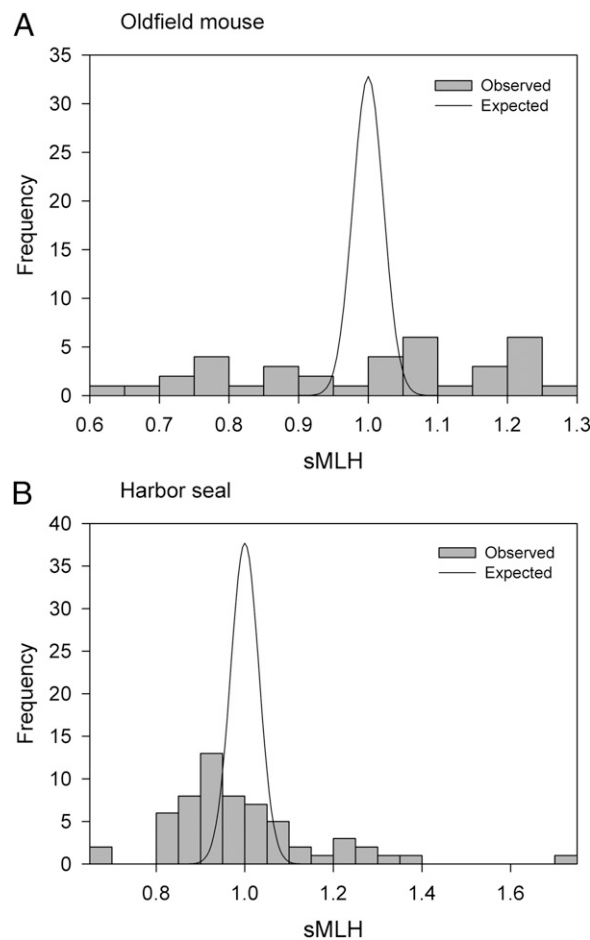
Here, we evaluated the ability of RAD sequencing to accurately estimate genome-wide heterozygosity using an experimental population of oldfield mice (*Peromyscus polionotus subgriseus*) with known pedigree-based inbreeding coefficients (20). We then applied RAD sequencing to a natural population of harbor seals (*Phoca vitulina*) to determine whether inbreeding explains previously reported HFCs for survivorship and parasite infection (21). We hypothesized that these HFCs should strengthen with the deployment of many thousands of SNPs if they are due to inbreeding.

## Results

**Relationship Between RAD Heterozygosity and  $f$ .** Illumina RAD sequencing of 36 oldfield mice yielded 265 million paired-end reads (Fig. S1) that were assembled into 63,129 RAD tags (Fig. S2A) as

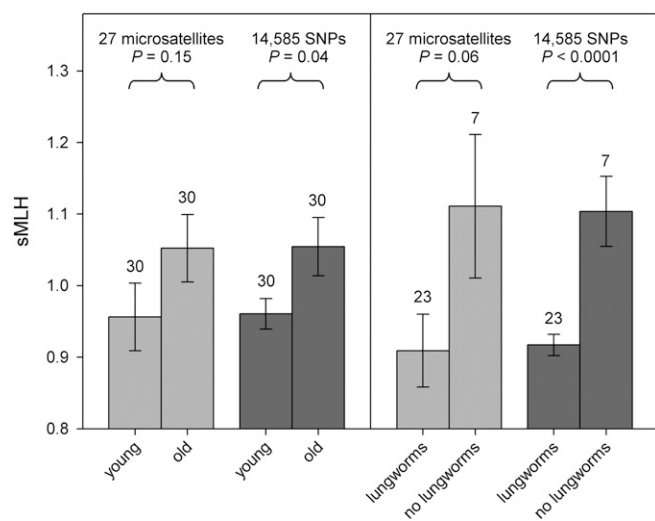
described in *SI Methods* and *SI Results*. After SNP calling and filtering (Figs. S3 and S4), 13,198 tags were retained for further analysis, each of which contained a single biallelic SNP (Fig. S5). Individual heterozygosity was expressed using the measure sMLH, which corrects for the fact that not all individuals are genotyped for the same loci by standardizing average multilocus heterozygosity in an individual by the average observed heterozygosities in the population of the subset of loci for which the focal individual was genotyped (10). RAD sMLH was strongly correlated with pedigree-based  $f$  (Fig. 1,  $r^2 = 0.74$ ,  $P < 0.0001$ ), far more so than sMLH at 12 microsatellites ( $r^2 = 0.28$ ,  $P = 0.0003$ ). A highly significant relationship was also obtained between RAD allele sharing and pedigree-based pairwise relatedness ( $r = 0.94$ ,  $P < 0.0001$ ), which was stronger than the equivalent relationship based on microsatellites ( $r = 0.68$ ,  $P < 0.0001$ ).

Variation in inbreeding within a population increases the variance in sMLH beyond that expected if heterozygosities at different loci were statistically independent. As expected, this genetic signature of inbreeding is conspicuous in a plot of the distribution of RAD sMLH in our sample of oldfield mice (Fig. 2A). The excess in variance is quantified by a parameter called  $g_2$ , which measures the extent to which heterozygosities are correlated across loci and can be computed from genotype data using the method of David et al. (22). In our sample of oldfield mice,  $g_2$  calculated from 13,198 SNPs was  $0.035 \pm 0.007$  SD, which is significantly different from



**Fig. 2.** Observed distributions of RAD sMLH (shaded bars) together with expected distributions under the null hypothesis of  $g_2 = 0$  (solid curves), meaning that there is no variance in inbreeding. See *Methods* for details. Results are shown separately for (A) oldfield mice genotyped at 13,198 SNPs and (B) harbor seals genotyped at 14,585 SNPs.





**Fig. 4.** Relationship between sMLH, estimated from microsatellites and RAD genotypes, and two fitness-related traits in harbor seals. Bars with light shading represent mean ( $\pm$  SEM) microsatellite sMLH and bars with dark shading represent mean ( $\pm$  SEM) RAD sMLH. Sample sizes are given above the bars. (Left) The mean ( $\pm$  SEM) sMLH of young and older seals, the former being classified as those that died before reaching 1 y of age (details in *Methods*). A nonsignificant tendency based on 27 microsatellites for younger seals to have lower heterozygosity than older seals strengthens to  $P = 0.04$  when 14,585 SNPs are deployed. (Right) The mean ( $\pm$  SEM) sMLH of young seals with and without lungworms. A nonsignificant trend for infected seals to have lower heterozygosity than uninfected seals based on 27 microsatellites becomes significant at  $P < 0.0001$  with 14,585 SNPs.

## Discussion

We evaluated the ability of RAD sequencing to estimate genome-wide heterozygosity using a pedigreed oldfield mouse population with substantial inbreeding (up to  $f = 0.45$ ) over six generations. We show that RAD heterozygosity is far more strongly correlated with  $f$  than microsatellite heterozygosity and that the  $r^2$  further increases to 0.93 if three outliers are removed. Additionally, the RAD data yield high het-het correlation coefficients and RAD sMLH is a marginally better predictor of body mass than  $f$ . Taken together, these findings suggest that RAD heterozygosity is a better estimate of genome-wide heterozygosity than pedigree-based estimates of  $f$  in our study. Although the exact reasons for the three outliers are unclear, in this case they are more likely the result of pedigree errors or mislabeled samples than unknown levels of relatedness among the founders. First, any effects of founder relatedness would be propagated throughout the pedigree and should therefore affect the majority of individuals. Second, although we do not have RAD genotypes of the founders, we were able to obtain relatively crude estimates of founder relatedness based on microsatellite and amplified fragment length polymorphism data reported previously (20). Adjusting the pedigree inbreeding coefficients with these estimates of founder relatedness did not improve the correlation with RAD heterozygosity. Regardless of the exact causes, our findings suggest that typing a very large number of loci may under certain circumstances allow one to estimate inbreeding even more precisely than from a pedigree. Furthermore, RAD sequencing provides a tool for error checking and otherwise refining pedigrees.

SNPs are attractive markers for correlating genome-wide heterozygosity with fitness (23). Although they are individually less variable than microsatellites, SNPs can be rapidly and economically genotyped in large numbers. Even so, it has been argued that for a large mammalian genome, a “herculean survey of 3,000 markers” will be required to produce a correlation between

heterozygosity and  $f$  of around 0.4 (24). This is supported by two recent studies that correlated sMLH with  $f$  in captive zebra finches, obtaining  $r$  values of  $-0.07$  based on 771 SNPs (25) and  $-0.46$  based on 1,359 SNPs (26). However, we show that RAD sequencing is capable of screening the heterozygosity of at least an order of magnitude more SNPs, bringing a commensurate improvement in the estimation of genome-wide heterozygosity. In our study, much of the inbreeding signal is captured by a couple of thousand markers (Figs. S6 and S7), but clearly the number of loci required in other systems will depend upon the extent to which genome-wide heterozygosity varies among individuals (15). This is currently unknown for most natural populations, although approaches such as ours make it increasingly amenable to study.

A number of factors could potentially introduce bias into our sMLH estimates, although these would be expected to affect most individuals within a sample to a similar extent. For example, in the absence of linkage maps we were unable to discriminate between SNPs on autosomes and sex chromosomes, meaning that our estimate of heterozygosity for males will be slightly lower than heterozygosity averaged over the autosomes. A second bias stems from the fact that RAD markers are affected by nonamplifying “null alleles,” which result mainly from mutations in the restriction enzyme recognition site. Based on the number of RAD tags without any SNP polymorphisms in our datasets, the level of nucleotide polymorphism,  $\pi$ , is estimated to be of the order of 0.002. Therefore, with an 8-base restriction enzyme, null alleles are expected to be found in at most only 2% of RAD markers. Null alleles are an issue for many marker types, including microsatellites, and result in a downward bias in estimated heterozygosity. Third, RAD markers paired on either side of restriction cut sites are in strong linkage and are therefore nonindependent. In our datasets, as only 10–20% of RAD tags contain a SNP (*SI Results*), the likelihood of tags on either side of a restriction cut site each carrying a SNP is relatively low. Consistent with this, based on calculations of pairwise linkage disequilibrium among all SNPs,  $\sim 10\%$  of SNPs are estimated to be in strong linkage disequilibrium with another SNP in both study systems. These factors will introduce some imprecision into estimates of heterozygosity and may be responsible for some of the unexplained variance in Fig. 1.

Despite these caveats, there are reasons to believe that RAD sequencing may produce less biased estimates of heterozygosity than many other SNP genotyping approaches. High-density genotyping arrays, for example, are usually based on SNPs identified from small “discovery panels” of individuals. This can distort the allele frequency spectrum, as SNPs with intermediate allele frequencies tend to be overrepresented and rare SNPs are often missing (27). As a result, estimates of heterozygosity based on the polymorphic SNPs tend to be inflated, whereas genome-wide heterozygosity will be underestimated because low-frequency SNPs are ignored (28). Thus, although no single approach is perfect, RAD sequencing at least eliminates ascertainment bias because the entire sample serves as the discovery panel.

Contrasting MAF distributions were obtained for the oldfield mice and the harbor seals, the latter being strongly left skewed (Fig. S5). Although we are unable to discount the possibility that at least some of the low-frequency variants in the harbor seals could be technical artifacts, we thoroughly explored the SNP calling parameter space in the oldfield mouse, using pedigree information to derive optimal parameters that were then applied equally to both species (*SI Methods* and *SI Results*). We also guarded against spurious genotypes by constructing the reference genome against which the reads were aligned using tags that were present in at least two individuals. Finally, SNPs with MAFs below 0.1 were found to contribute significantly toward the overall HFC signal, suggesting that they carry useful information about inbreeding. A plausible explanation for these contrasting distributions is therefore that they reflect species-specific differences in population size and history. Oldfield mice occur

across much of the southeastern United States and can reach densities of 18–26 animals per hectare (29). Relatively high average heterozygosity and an abundance of SNPs with intermediate allele frequencies could therefore be explained by a large and stable effective population size. In contrast, many pinniped species show evidence of strong demographic responses to historical changes in the marine environment, usually through rapid population expansion during periods of increased food or habitat availability (30, 31). Little is known about the historical demography of harbor seals, although the Waddensee population has been reported to have low heterozygosity (32), which is consistent with the effective population size having at some point in the past been very small. The excess of low-frequency polymorphisms could therefore be attributable to postglacial population expansion.

Het-het correlations (15) are often used to test for the presence of inbred individuals, but with only around 10 markers commonly deployed, this approach lacks power because the marker subsets are very small. This is exactly what we found (Fig. 3): the distribution of microsatellite-based het-het correlation coefficients in the oldfield mouse was slightly positive but with a large SD, despite the sample containing highly inbred individuals ( $f \leq 0.45$ ). In contrast, the RAD data yielded het-het correlation coefficients well in excess of 0.9 for both the oldfield mice and harbor seals. This provides clear evidence for inbred individuals being present not only in the mouse pedigree but also in the natural harbor seal population.

A second line of evidence also points toward the HFCs for survival and parasite infection being due to inbreeding depression in harbor seals. With inbreeding, the deployment of additional markers should strengthen the HFC detected, whereas with a local effect adding more markers is expected to weaken the relationship between mean heterozygosity and fitness (18). We found that the microsatellite-based HFC for lungworm infection strengthened considerably with RAD heterozygosity, the statistical significance increasing from  $P = 0.06$  to  $P < 0.0001$ . Such a dramatic strengthening of effect has not been previously reported, although one study was able to replicate an HFC for birth weight (but not one for juvenile survival) using independent panels of 10 microsatellites (33). Furthermore, the deviance in lungworm infection explained by heterozygosity steadily increases with the number of SNPs deployed (Fig. S7B), which is also strongly suggestive of genome-wide effects contributing toward inbreeding depression rather than the underlying mechanism being a local effect. Finally, an alternative explanation based on population structure seems highly unlikely as we found no evidence for distinct genetic clusters within the RAD dataset (Fig. S8).

Although many reported HFCs yield highly significant  $P$  values, the proportion of variance explained tends to be small, typically of the order of 1–5% (34). This could be interpreted as meaning that heterozygosity only weakly affects fitness. Alternatively, heterozygosity could have a large effect on fitness, but because a small panel of microsatellites provides a poor estimate of  $f$ , these studies could have limited power to detect such effects. Theory predicts that, if inbreeding is the primary mechanism and thousands of markers could be deployed, the proportion of fitness explained by HFCs would rise considerably (15, 16, 18). This is exactly what we found in wild harbor seals, where the deviance in lungworm infection status explained by heterozygosity increased from around 10% based on 27 microsatellites to nearly 50% with 14,585 SNPs. This is not only qualitatively, but also quantitatively consistent with the theoretical predictions. Under the hypothesis that inbreeding is responsible for HFCs, when switching from microsatellites to RAD SNPs, the variance in infection status explained by heterozygosity ( $r^2_{sMLH, infection}$ ) should increase in proportion to the predicted squared correlation coefficient between heterozygosity and  $f$ . Based on the  $g_2$  and variance in sMLH in microsatellites and SNPs estimated in young seals, the expected  $r^2_{sMLH, f}$  is 0.148 for microsatellites and 0.709

for SNPs. This expected 4.8-fold difference is in line with the observed 5-fold increase in deviance explained by the HFC. Thus, our results are consistent with inbreeding theory.

Our study clearly demonstrates that at least some HFCs may explain more variation in fitness than previously thought. However, we compared cases with controls, and it is possible that the former could be enriched for a small subset of unusually inbred individuals. Natal philopatry and breeding site fidelity can also be extremely strong in pinnipeds (35, 36) and might combine with polygyny to increase the risk of inbreeding. Moreover, historical changes in the structure of a population, including bottlenecks or population admixture, may also create variance in inbreeding in a broad sense (16). It therefore remains to be seen whether inbreeding depression could be responsible for HFCs more generally, as suggested by Szulkin et al. (16). This represents a fertile area for future research.

Finally, within a genome one expects that heterozygosities should be more correlated between closely linked loci than between unlinked ones (37, 38). Linkage is expected to result in stronger HFCs in some linkage groups than in others (i.e., local effects). However, in our study, models ignoring linkage strongly predict survival and parasite infection status in harbor seals, suggesting that local effects contribute very little to these HFCs, as predicted by theoretical arguments (16). The observed increase in variance explained when thousands of loci are deployed at least rules out the possibility that these relationships arise from local linkage disequilibria between microsatellites and one or a few phenotypically important loci in the absence of genome-wide inbreeding.

In conclusion, the fact that microsatellite heterozygosity is often poorly correlated with  $f$  in natural populations has been used as an argument for obtaining more and better pedigrees (39). However, we show that RAD sequencing is capable of generating enough SNP data to accurately estimate inbreeding, in this particular case even more accurately than from a pedigree of reasonable depth. Our approach is also powerful for studying heterozygosity because it can be applied to virtually any species without the need for prior genomic information.

## Methods

**Oldfield Mouse.** A population of oldfield mice was founded at Brookfield Zoo from 26 wild-caught individuals. These mice were paired to produce offspring with a range of inbreeding coefficients (0–0.453) over six generations of laboratory breeding and the resulting pedigree was recorded. Using 179 of these mice, Dasmahapatra et al. (20) reported a significantly negative correlation between pedigree-based inbreeding coefficient and heterozygosity estimated from 12 microsatellite loci. We selected 40 of these individuals at random for RAD sequencing, 36 of which yielded data of sufficient quality for analysis (details in *SI Methods*). The breeding study and the animal care protocols for the oldfield mice were approved by the Institutional Animal Care and Use Committee of the Chicago Zoological Society.

**Harbor Seal.** Rijks et al. (21) reported a negative association between heterozygosity at 27 microsatellites and lungworm burden in dead harbor seals stranded on the Dutch Wadden Sea coast. However, this was only statistically significant for young seals, defined as being less than 1 y of age, which were more likely to carry lungworms than older seals (21). We therefore RAD sequenced all 43 of the young seals from this study, together with 37 older seals selected at random from the 161 available. The sampling locations of these individuals are shown in Fig. S9. RAD sequence data of adequate quality were obtained for 30 of the young seals, of which 23 were infected with lungworms, plus 30 older seals, none of which carried lungworm infections (*SI Methods*).

**RAD Genotyping.** RAD libraries were constructed from whole genomic DNA using the protocol of Baird et al. (19) with minor modifications (details in *SI Methods*), and each library was 100-bp paired-end sequenced on an Illumina HiSeq2000 flow cell. Sequences have been deposited in the Short Read Archive (accession no. PRJEB5164). Separately for the mice and seals, Stacks (40) was used to quality filter reads, demultiplex samples, and assemble RAD stacks together with their associated paired-end contigs. After further stringent filtering steps, RAD contigs were used as a reference against which to map

back the raw reads using BWA (41), followed by maximum-likelihood genotype calling using the GATK UnifiedGenotyper (42). Individual heterozygosity and pairwise relatedness were calculated using tag sequences containing only one biallelic SNP per tag. A detailed description of the bioinformatics pipeline used is provided in *SI Methods*.

**Data Analyses.** For all analyses of the microsatellite and RAD data, individual heterozygosity was expressed as sMLH, which is defined as the total number of heterozygous loci in an individual divided by the sum of average observed heterozygosities in the population over the subset of loci successfully typed in the focal individual (10). Pairwise relatedness (RAD allele sharing) was calculated as the total number of identical alleles between individuals (zero, one, or two per tag) divided by twice the number of tags considered. The two-locus heterozygosity disequilibrium  $g_2$  was measured following David et al. (22) with the method of computation modified to analyze many thousands of loci in a reasonable computing time (details in *SI Methods*). Sensitivity of this estimate to the number of loci was explored by randomly selecting different-sized subsets of loci (between 50 and 15,000) and recalculating  $g_2$  100 times. Distributions of RAD sMLH under the hypothesis  $g_2 = 0$  were obtained by shuffling genotypes at each locus randomly

across individuals 1,000 times; as the resulting distribution is very close to normal and the mean is one according to the definition of sMLH, we extracted the variance from these 1,000 simulations and used it to represent the expected distribution as a normal curve. The mean correlation in heterozygosity across loci based on 1,000 random samples was calculated using Rhh (43).

Statistical analyses were conducted using GLMs within R (44). A linear error structure was used for oldfield mouse body mass and a binomial error structure was used for harbor seal age (coded as 0 = young and 1 = old) and lungworm burden (coded as 0 = uninfected and 1 = infected). Where models included multiple predictor variables, standard deletion testing procedures using  $F$  tests were used to sequentially remove each term unless doing so significantly reduced the amount of deviance explained (deviance is analogous to sums of squares in standard regression analysis).

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