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Population genetics

Candidate gene microsatellite variation is associated with parasitism in wild bighorn sheep

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The loss of genetic variation in host populations is thought to increase host susceptibility to parasites. However, few data exist to test this hypothesis in natural populations. Bighorn sheep (*Ovis canadensis*) populations occasionally suffer disease-induced population declines, allowing us to test for the associations between reduced genetic variation and parasitism in this species. Here, we show that individual mean heterozygosity for 15 microsatellite loci is associated with lungworm abundance (*Protostrongylus* spp.) in a small, recently bottlenecked population of bighorn sheep (linear regression, $r^2=0.339$, $p=0.007$). This association remains significant for seven microsatellites located in genes ($p=0.010$), but not for eight neutral microsatellites ($p=0.306$). Furthermore, heterozygotes at three of four microsatellites located within disease-related genes had lower lungworm burdens. This study corroborates theoretical findings that increased parasitism and disease may be a consequence of reduced heterozygosity in wild populations, and that certain individual loci influence parasite resistance. The results illustrate the usefulness of using genomic information, strong candidate genes and non-invasive sampling for monitoring both genetic variation and fitness-related traits, such as parasite resistance, in natural populations.

Keywords: heterozygosity–fitness correlations; ecological genomics; genetic monitoring; host–parasite interaction; conservation management; *Ovis canadensis*

1. INTRODUCTION

Evolutionary theory suggests that genetic diversity and resistance to parasites are linked (Howard & Lively 1998). Individuals with low genetic diversity may be less able to cope with parasite infection, or lack the same range of parasite resistance mechanisms carried by more heterozygous conspecifics (Coltman *et al.* 1999). Despite extensive theoretical work, few empirical studies have assessed diversity–resistance relationships in wild populations, particularly mammals. However, there is recent evidence that heterozygosity is associated with parasite infection in wild hosts (e.g. Acevedo-Whitehouse *et al.* 2003; MacDougall-Shackleton *et al.* 2005). If individuals with reduced heterozygosity are less able to resist parasite infection, then loss of genetic variation in wild populations might magnify the fitness costs of parasites and infectious diseases. Small, fragmented populations are becoming increasingly common as habitat fragmentation accelerates globally. As such, understanding interactions among genetic diversity loss, infectious disease risk and fitness in wild populations is of growing importance.

Here, we examined the association between heterozygosity and lungworm infection (*Protostrongylus* spp.) in a small, recently bottlenecked population of bighorn sheep (*Ovis canadensis*). The rapid extinction of small populations of bighorn across the western United States has garnered considerable attention over the past two decades (e.g. Berger 1990), and the search for factors contributing to these losses is ongoing. Lungworms are common nematode parasites of bighorns which, in combination with other respiratory pathogens, have been linked to epizootic pneumonia outbreaks and mortality in some populations (Spraker *et al.* 1984). Lungworms may also negatively affect sheep reproductive success (Festa-Bianchet 1989).

We used non-invasive faecal sampling to measure lungworm abundance and heterozygosity in bighorn at eight putatively 'neutral' microsatellite loci and at seven microsatellites located in genes of known function. Four of the seven markers are in genes with functions related to parasite susceptibility or lung disease and thus are strong candidate genes for parasite resistance. We predicted that individuals with lower heterozygosity in both neutral and coding genes would suffer greater parasitism, and that loci in genes, particularly the candidate genes, would disproportionately influence the association between heterozygosity and parasite abundance.

2. MATERIAL AND METHODS

(a) *Study population and sampling*

From 30 June 2006 to 15 August 2006, we collected faeces from the Mummy Range bighorn herd in Rocky Mountain National Park near Estes Park, CO. The Park's entire bighorn population has gone through several declines (electronic supplementary material). Most recently, a pneumonia outbreak occurred in the mid-1990s, which reduced the population size and lamb recruitment for several years. A mark–resight study in 2003–2004 estimated that there were only approximately 300 sheep in the park, with approximately 60 of these occurring in the Mummy Range (McClintock & White 2007).

(b) *Genetic analyses*

We used standard DNA extraction methods and microsatellite typing procedures (Maudet *et al.* 2004; electronic supplementary material). Individual heterozygosity (H) was computed as the proportion of all loci genotyped that were heterozygous (see Coltman *et al.* 1999).

(c) *Parasitological analyses*

Protostrongylid lungworms were isolated from faecal samples by the Baermann funnel technique (Foreyt 2001). Lungworm abundance (i.e. number) was estimated as the number of larvae per gram (LPG) of faeces. LPG values were $\ln(x+1)$ transformed to normalize the distribution.

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(d) *Statistical analyses*

We tested for a negative association between individual multi-locus heterozygosity and lungworm abundance using linear regression. Regressions were also conducted separately for the eight neutral loci and the seven loci in genes to assess the relative contribution of these sets of loci to the *H*–parasite association (electronic supplementary material).

We also tested for the effects of individual loci by using *t*-tests to assess whether homozygotes had higher lungworm abundance than heterozygotes, and linear regression tests to assess the degree to which removal of each locus influenced the overall *H*–lungworm relationship. We corrected the *t*-tests for multiple comparisons using the sequential Bonferroni method (electronic supplementary material). For the regressions, we removed each locus individually (from the 15-locus dataset) and re-ran the regression to test whether the removal of a locus caused the *p*-value for the *H*–parasite association to become non-significant. Non-parametric tests (not reported) revealed similar results.

3. RESULTS

Thirty faecal samples revealed distinct multi-locus genotypes representing 30 individuals. All microsatellite profiles were unambiguous, easy to score, with no failed polymerase chain reactions, suggesting that no genotyping errors occurred (McKelvey & Schwartz 2004; Luikart *et al.* 2008).

Seventeen individuals (out of the 30) had faecal samples analysed for lungworms (see electronic supplementary material). Parasite prevalence was 65% (11 out of 17 individuals), and larval counts per individual ranged from 0 to 62 LPG. The range of individual multi-locus heterozygosity was large (from 0.33 to 0.80).

Linear regression analysis revealed that individuals with lower heterozygosity had significantly higher lungworm abundance (linear regression, $r^2=0.339$, $p=0.007$; figure 1*a*). This significant relationship remained when only the seven microsatellites in genes were used to compute heterozygosity ($r^2=0.305$, $p=0.010$; figure 1*b*), but not with the eight neutral microsatellites ($r^2=0.017$, $p=0.306$; figure 1*c*).

Heterozygotes at two loci within the disease-related genes had significantly lower lungworm abundance than homozygotes (*t*-test, ADCYAP1, $p=0.046$; MMP9, $p=0.011$; table 1). However, only MMP9 remained significant after correcting for multiple tests (electronic supplementary material). In addition, two of the candidate loci (ADCYAP1 and TCRG4) when removed individually from the full dataset made the association between overall *H* and lungworm abundance non-significant (table 1). None of the eight neutral loci or three loci located in non-disease-related genes showed similar effects (table 1). Furthermore, the mean of the *p*-values for the four candidate gene loci (mean $p=0.042$, *t*-test) was far lower than that of the eight loci not in genes (mean $p=0.233$, *t*-test, table 1).

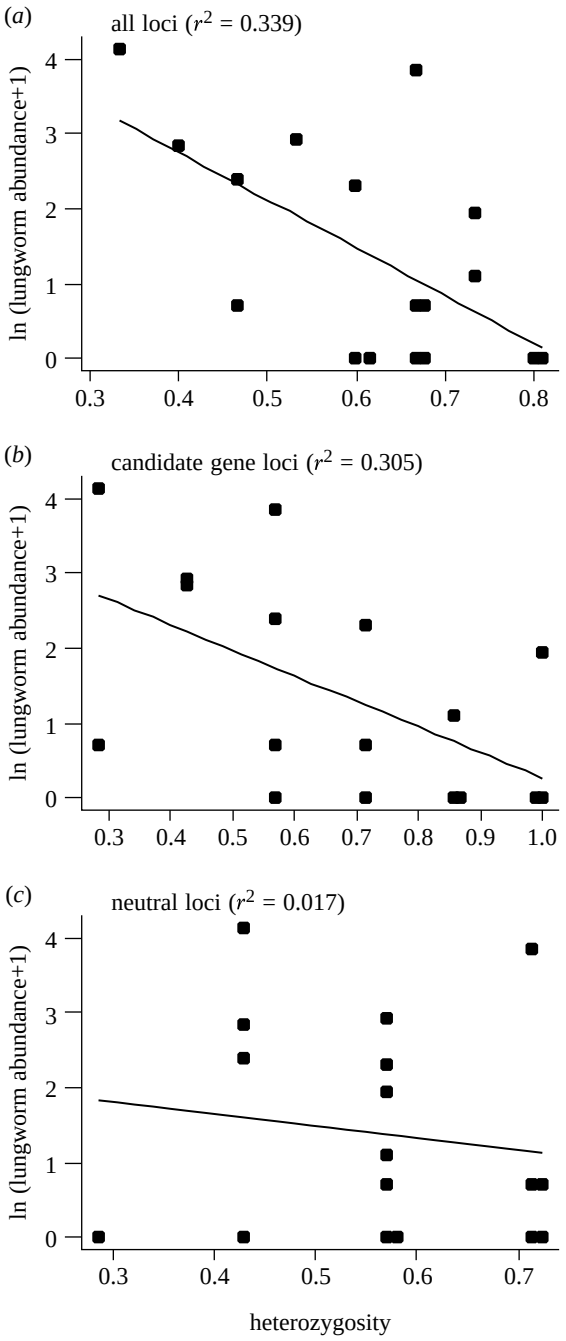

Figure 1. Regression analysis between individual heterozygosity and natural log (ln) of lungworm abundance (LPG) for (*a*) all fifteen microsatellite loci, (*b*) the seven loci in genes and (*c*) the eight loci not in genes.

4. DISCUSSION

Our results suggest that reduced heterozygosity, especially at certain loci, can increase the susceptibility to lungworm infection in bighorn sheep. More than 30% of the inter-individual variation in lungworm abundance was explained by the loci in genes, and *p*-values were far lower for the four loci in strong candidate genes than for the other loci. Previous work has shown that low heterozygosity is associated with increased nematode parasitism and lower survival in feral Soay sheep (*Ovis aries*; Coltman *et al.* 1999). Since lungworm infection may negatively influence the reproductive success, as well as survival in bighorns (Festa-Bianchet 1989; Spraker *et al.* 1984), any increase in lungworm parasitism associated with reduced genetic diversity may have important negative consequences for individual host fitness and population recovery in small, isolated or inbred populations.

Three loci (TCRG4, ADCYAP1 and MMP9) in genes with disease-related functions were associated with lungworm abundance. TCRG4 (T-cell receptor) has an immune system function involving recognition of foreign antigens. Diversity at this locus could allow the recognition of a more diverse range of parasites.

Table 1. Results of *t*-tests and linear regression tests for association between heterozygosity and lungworm abundance (LPG). The minus symbols (−) are loci at which homozygotes have fewer parasites. The regression columns show r^2 after dropping each locus one by one (from all 15 loci where p=0.007 and r^2=0.339). n.c., No change in significance level. The p-values in italics show loci that are associated with parasite load after correction for multiple comparisons (*t*-tests; see electronic supplementary material) or that when dropped make the regression become non-significant ($p>0.10$).

	t-test on each locus		regression minus one locus	
locus name	*p*-value	*t*	*p*-value	r^2
neutral loci				
MAF36	0.351	0.39	n.c.	0.356
MAF48	0.368	0.34	n.c.	0.309
FCB304	0.139	−1.13	n.c.	0.404
AE16	0.488	0.03	n.c.	0.322
HH62	0.156	−1.05	n.c.	0.303
MAF209	0.166	1.00	n.c.	0.399
MAF33	0.047	1.70	n.c.	0.251
FCB266	0.147	−1.09	n.c.	0.304
loci in genes				
KRT2	0.343	0.41	n.c.	0.686
KERA	0.235	−0.74	n.c.	0.470
SOMA	0.054	−1.70	n.c.	0.326
loci in candidate genes				
ADCYAP1	0.046	1.79	*0.112*	0.159
TCRG4	0.054	1.70	*0.109*	0.162
MMP9	*0.011*	2.53	n.c.	0.299
OLADDRbp	0.224	−0.78	n.c.	0.305

The ADCYAP1 gene (adenylate cyclase-activating polypeptide) is involved in the regulation of cytokine production including interleukin 6 that activates the production of T-helper cell 2 (Th2) cytokines involved in defence against helminths and other extracellular parasites (Mosmann & Sad 1996). Furthermore, ADCYAP1 was recently found to be associated with nematode parasite infection in domestic sheep (Crawford *et al.* 2006). The MMP9 gene (Matrix MetalloProteinase 9) plays a role in the development of chronic obstructive pulmonary disease and other diseases (e.g. O'Connor & FitzGerald 1994; Ito *et al.* 2005). MMP9 codes for an enzyme involved in lung tissue repair, and could be associated with a response to lung tissue damage by lungworms. The three genes are probable candidates for influencing lungworm resistance; however, it is also possible that selection at other genes linked to TCRG4, ADCYAP1 or MMP9 could explain the observed *H*–parasite associations. This might manifest because linkage disequilibrium could span large chromosomal segments in small or recently bottlenecked populations such as the one studied here.

Over the past few years, a growing body of evidence has emerged relating heterozygosity and parasitism in wild mammal populations. For example, heterozygosity was negatively associated with tuberculosis infection in wild boars (Acevedo-Whitehouse *et al.* 2005). Similarly, heterozygous California sea lions were less likely to be infected by a range of pathogens and show negative consequences of infection (Acevedo-Whitehouse *et al.* 2005). Our paper contributes to this work by documenting an *H*–parasite correlation in a small, recently bottlenecked wild population of bighorn sheep, and showing that diversity at specific genes is most likely driving this relationship. Our results further suggest that cases where significant *H*–parasite relationships have not been found (e.g. Côté *et al.* 2005) might benefit from a gene-targeted or candidate gene approach.

Despite the relatively small sample size used in this study (n=17), our power to detect *H*–parasite associations was probably high due to the wide range of individual heterozygosities present in our study population (0.30–0.80). This is not unlike a study with a similar sample size that reported a strong inbreeding–parasite association in a population of Cuvier's gazelles (*Gazella cuvieri*) where the range of individual inbreeding was large (F=0.06–0.31; Cassinello *et al.* 2001). Interestingly, the same association did not hold for another gazelle species (*Gazella dorcas*) where the range of inbreeding was small (F=0.00–0.08; Cassinello *et al.* 2001), which is consistent with the idea that a high range of individual heterozygosity can provide high power to detect *H*–parasite associations.

In bighorns, the mechanisms driving extinctions of small populations remain generally unknown. Although our current results are correlative, they do suggest that increased parasitism may be an important factor influencing individual fitness and population viability in small, bottlenecked bighorn populations. Our *H*–parasite associations are particularly compelling because we show links between lungworm infection and candidate genes with functions related to lung disease and parasite resistance. Understanding the phenotypes associated with heterozygosity at these candidate genes is an important next step. Future work could assess the generality and severity of lungworm effects on bighorn sheep lung tissue and individual fitness. Finally, because variation in *Protostrongylus* larval shedding rates are known to be influenced by factors such as host sex and age and time of year (Samson *et al.* 1987), additional work controlling for such variables could reveal important information on multiple factors underlying *H*–parasite relationships in this system.

In summary, results from this paper are consistent with the hypothesis that reduced genetic variation can increase host susceptibility to parasites. As a consequence, loss of genetic diversity coupled with increased disease may be a crucial mechanism driving population extinction risk (Whiteman *et al.* 2006). This study illustrates the value of genotyping strong candidate genes, which might help detect parasite or disease-related challenges in wild populations. Our results also highlight the usefulness of genomic information and gene-targeted population genomic approaches for assessing and monitoring fitness in natural populations.

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