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MHC heterozygosity and survival in red junglefowl

KIRSTY WORLEY,*† JULIE COLLET,† LEWIS G. SPURGIN,* CHARLIE CORNWALLIS,† TOMMASO PIZZARI† and DAVID S. RICHARDSON*

**School of Biological Sciences, University of East Anglia, Norwich, Norfolk, NR4 7TJ, UK, †Department of Zoology, Edward Grey Institute, University of Oxford, Oxford, OX1 3PS, UK*

Abstract

Genes of the major histocompatibility complex (MHC) form a vital part of the vertebrate immune system and play a major role in pathogen resistance. The extremely high levels of polymorphism observed at the MHC are hypothesised to be driven by pathogen-mediated selection. Although the exact nature of selection remains unclear, three main hypotheses have been put forward; heterozygote advantage, negative frequency-dependence and fluctuating selection. Here, we report the effects of MHC genotype on survival in a cohort of semi-natural red junglefowl (*Gallus gallus*) that suffered severe mortality as a result of an outbreak of the disease coccidiosis. The cohort was followed from hatching until 250 days of age, approximately the age of sexual maturity in this species, during which time over 80% of the birds died. We show that on average birds with MHC heterozygote genotypes survived infection longer than homozygotes and that this effect was independent of genome-wide heterozygosity, estimated across microsatellite loci. This MHC effect appeared to be caused by a single susceptible haplotype (CD_c) the effect of which was masked in all heterozygote genotypes by other dominant haplotypes. The CD_c homozygous genotype had lower survival than all other genotypes, but CD_c heterozygous genotypes had survival probabilities equal to the most resistant homozygote genotype. Importantly, no heterozygotes conferred greater resistance than the most resistant homozygote genotype, indicating that the observed survival advantage of MHC heterozygotes was the product of dominant, rather than overdominant processes. This pattern and effect of MHC diversity in our population could reflect the processes ongoing in similarly small, fragmented natural populations.

Keywords: heterozygote advantage, junglefowl, major histocompatibility complex, overdominance, survival

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Introduction

Genes of the major histocompatibility complex (MHC) are integral components of the vertebrate immune system. MHC genes encode molecules that present peptide antigens to T lymphocytes, thereby initiating the pathogen immune response (Klein 1986). In the majority of species MHC genes are highly polymorphic, with highly divergent gene sequences encoding different forms of the molecules' peptide binding region. Each MHC allele may therefore bind and confer resistance to, different pathogen-derived peptides. Correlations between specific MHC alleles and disease resistance have been shown in a range of species (e.g. Hill *et al.* 1991; Paterson *et al.* 1998; Langefors *et al.* 2001; Quinnell *et al.* 2003; Croisetiere *et al.* 2008).

Three main, non-exclusive, mechanisms of pathogen-mediated selection have been put forward to explain the high levels of polymorphism in MHC genes; negative frequency-dependence (rare-allele advantage), fluctuating selection and heterozygote advantage (see reviews by Apanius *et al.* 1997; Meyer & Thomson 2001; Spurgin & Richardson 2010). Negative frequency-dependent selection results from co-evolutionary interactions between the pathogen and host; rare alleles

Correspondence: David S. Richardson,
E-mail: david.richardson@uea.ac.uk

conferring pathogen resistance in the host increase in frequency, introducing selection pressure on the pathogen to respond, ultimately leading to the maintenance of genetic variation in the population (Bodmer 1972; Jeffery & Bangham 2000). Fluctuating selection may occur if pathogen load varies and as such the fitness advantage of possessing a resistant allele also varies, maintaining variation across time or space (Hedrick *et al.* 1987). Finally, individuals that are heterozygous at MHC genes may have increased fitness compared to homozygous individuals, as they are potentially able to recognize and resist a larger variety of pathogen-derived peptides (Doherty & Zinkernagel 1975). Many studies have provided evidence that is consistent with one or more of these mechanisms (e.g. Thursz *et al.* 1997; Carrington *et al.* 1999; Penn *et al.* 2002; Meyer-Lucht & Sommer 2005; Bryja *et al.* 2007; Croisetiere *et al.* 2008; Dionne *et al.* 2009). However, as the three hypotheses are not mutually exclusive and can lead to identical observed allele frequency distributions (Takahata & Nei 1990) it is difficult to identify the relative importance of each mechanism in the maintenance of MHC diversity (reviewed in Spurgin & Richardson 2010).

Of the mechanisms suggested to explain MHC variation, heterozygote advantage is the simplest to explore empirically. However, the term heterozygote advantage, as used in this field of study (e.g. Penn *et al.* 2002; Oliver *et al.* 2009), actually encompasses two different processes; heterozygote advantage through dominance and heterozygote advantage through overdominance (sometimes referred to as 'heterozygote superiority'). Dominance, in this context, occurs where a dominant pathogen resistance trait in the heterozygote form masks any susceptibility conferred by the second allele. Dominance has been reported by several studies into MHC heterozygote advantage, revealed when the fitness of MHC heterozygotes is greater than the *mean* fitness of homozygote individuals, but not greater than the *best* of the homozygote genotypes (e.g. Thursz *et al.* 1997; Carrington *et al.* 1999; Penn *et al.* 2002). Importantly, theoretical studies suggest that heterozygote advantage through dominance alone cannot maintain the levels of polymorphism present at the MHC, given the unrealistic assumption that all alleles have equal fitness (De Boer *et al.* 2004). Heterozygote advantage though overdominance is based on the idea that the combined presence of two alleles confers pathogen resistance 'over and above' that provided by any of the homozygote forms and this process is thought to be able to maintain high levels of MHC polymorphism (Takahata & Nei 1990; McClelland *et al.* 2003b). As yet, there is relatively little unambiguous evidence for heterozygote advantage through overdominance at the MHC (but see McClelland *et al.* 2003b; Oliver *et al.* 2009). This lack of evidence for overdominance may arise as many experimental infections consist of single strain pathogens and overdominance is best explored in systems with multiple pathogens (Penn & Potts 1999). In natural conditions, where multiple simultaneous infections can take place (Hughes & Nei 1992), overdominance may be more frequent. This is because individuals possessing two divergent alleles are likely to have resistance to different pathogen derived peptides. Therefore, selection will act against homozygotes that may be susceptible to one of the additional pathogens in the population (Penn & Potts 1999).

The domestic chicken (*Gallus gallus domesticus*) provides some of the best evidence for direct associations between MHC haplotype and resistance to infectious diseases. For example, Marek's disease, Rous sarcoma virus and lymphoid leukosis virus are all associated with different MHC (class I) alleles (Collins *et al.* 1977; Schierman *et al.* 1977; Bacon *et al.* 1981; Briles *et al.* 1983). This evidence supports the idea that MHC polymorphism has arisen as a result of benefits associated with resistance to multiple pathogen resistance (Kaufman *et al.* 1995; Wallny *et al.* 2006). Furthermore, in contrast to the MHC regions of many species, the domestic chicken MHC B locus is particularly well characterized (Guillemot *et al.* 1988; Kaufman *et al.* 1999a) and relatively simple (Kaufman *et al.* 1995, 1999b; Jacob *et al.* 2000; Shaw *et al.* 2007). No incidents of recombination have been recorded between the class I and class II B genes in experimental matings (Hala *et al.* 1979, 1988; Koch *et al.* 1983; Skjødt *et al.* 1985), therefore loci appear to be linked in stable B haplotypes. As such, the chicken represents a relatively simple system in which to investigate the MHC. In particular, populations of red junglefowl (*Gallus gallus* ssp.), the wild ancestor of the domestic chicken (Fumihito *et al.* 1996), provide a promising opportunity to test the relationship between MHC polymorphism and survival under natural or semi-natural conditions. Our recent development of an efficient and highly repeatable method with which to screen locus specific MHC variation in junglefowl has further enhanced the suitability of this system for studies into the causes and consequences of MHC variation (Worley *et al.* 2008; Gillingham *et al.* 2009).

We conducted survival analysis on a cohort of red junglefowl that were reared in semi-natural conditions as part of an ongoing project investigating the role of the MHC in this species. These birds were derived from a small, genetically bottlenecked population in which we had previously detected significant MHC diversity and evidence of selection (Worley *et al.* 2008). There are, however, relatively few MHC alleles in this

population compared to that observed in other fowl populations (Worley *et al.* 2008), thus providing a simpler and more tractable system in which to analyse the effects of individual MHC haplotypes. This cohort suffered severe levels of mortality (over 80% before sexual maturity) associated with an outbreak of coccidiosis, a parasitic disease of the intestinal tract of animals caused by the protozoan parasite *Eimeria* spp. This disease spreads from one animal to another by contact with infected faeces or ingestion of infected tissue and so elevated levels of infection may have occurred because of the contained conditions of the fowl population. We investigated the relationship between MHC variation and survival in this population during this outbreak of disease. First, we investigated whether, as predicted, MHC heterozygotes had an overall survival advantage over MHC homozygote birds. Secondly, we aimed to determine how specific MHC genotypes and haplotypes affected survival. Thirdly, we examined whether birds that were homozygous for different haplotypes had differential survival when compared to heterozygote individuals. Patterns of survival between homozygotes and heterozygotes were then examined to test for evidence of overdominance vs. dominance.

Materials and methods

Experimental birds

We studied a small (i.e. ranging approximately between 40 and 150 adults) population of red junglefowl, originating from Thailand, which has been bred in captivity for the past seven generations and is currently housed at the John Krebs Field Station of the University of Oxford. The birds of the study cohort were the offspring of 18 females and 16 males (mean 4.3 offspring per female). Reproductive pairs were constituted blind with respect to genetic relatedness and MHC similarity, assigning a male as the sperm donor of a female that was currently in laying condition. We artificially inseminated each female which removes the possibility that male phenotype might influence female differential maternal investment or sperm selection. Following insemination, females were housed in individual pens for egg laying. Eggs were collected from each pen, labelled by female and incubated until hatching in five different batches between October–November 2007: batch 1, 7–10 October; batch 2, 14–15 October; batch 3, 21–23 October; batch 4, 29–31 October and batch 5, 5–6 November. Upon hatching, chicks were weighed and housed indoors. Tissue samples were also taken from the 19 embryos that failed to hatch. Estimates of the developmental stage of embryos ranged from 1 to 20 days from the start of incubation. All hatched birds from each batch were housed indoors in groups until 50 days of age, when birds were transferred to five outdoor pens, of approximately 3.3 m × 3.3 m. This gave an initial average bird density of 1.7 birds/m^2, which declined further with increasing mortality. In line with our policy of keeping intervention within this semi-natural population to a minimum, none of the birds were vaccinated against coccidiosis during the period of study.

In total 98 birds were hatched in the year 2007, 83 of which died within 250 days of hatching, by which time both males and females had reached sexual maturity (Etches 1996). This mortality was associated with symptoms such as reduced body-weight gain and blood loss, which are typical of coccidian infections, a common source of juvenile mortality in fowl (McDougald 2008; McDonald & Shirley 2009). Microbiological analyses of faecal samples by the Oxford University Veterinary Services indicated a high prevalence of coccidiosis in the pens where juvenile birds were housed (mean ± SE = 68% ± 0.16 of samples per pen were positive) and high levels of infection (up to 314 400 oocysts/g). In addition, one of the 83 birds that died was examined post-mortem by the Oxford University Veterinary Services confirming the presence of coccidia oocysts in the intestine, mixed inflammatory cell infiltrate with lymphoid predominance in the intestine and multifocal inflammatory infiltrates with lymphoid predominance in the liver and lack of other pathogens. Two more dead birds were tested for sources of mortality alternative to coccidiosis, including Marek's disease virus and these necropsies provided negative results. Birds were monitored daily and once a bird was seen to be suffering from coccidiosis symptoms, it was moved out of the main enclosures and housed in isolation. At 250 days, the surviving birds underwent systematic treatment through the administration of oral drugs to reduce coccidiosis-related mortality (coccidiostat). This treatment was successful and the remaining birds recovered and survived. The pathological, microbiological and epidemiological evidence, when combined, strongly suggests that coccidiosis was the main cause of the mortality recorded in the young generation of the study population.

Molecular methods

Blood samples (approximately 100 μL) were taken by brachial venipuncture, diluted in 800 μL of 100% ethanol in a screw-cap microfuge tube and stored at 4 °C until use. Genomic DNA was extracted using a standard salt-extraction method (Sunnocks & Hales 1996). Extractions were normalised to concentrations of 10 ng/μL and stored at −20 °C. Birds that died before

blood was taken were sampled by collecting tissue samples shortly after death.

All samples were genotyped at 27 variable microsatellite loci (Cheng & Crittendon 1994; Crooijmans *et al.* 1996, 1997; Gibbs *et al.* 1997; Dawson *et al.* 1998, 2010; McConnell *et al.* 1999; Navarro *et al.* 2005; see Table S1, Supporting information). Microsatellite loci were amplified in 2 μL reactions as described in Kenta *et al.* (2008). Amplified products were genotyped using an ABI 3730 sequencer and analysed using GeneMarker software (Softgenetics). Microsatellite allelic frequencies and deviations from Hardy–Weinberg equilibrium were calculated using Cervus 3.0 (Marshall *et al.* 1998). Linkage disequilibrium was estimated using GenePop 3.4 (Raymond & Rousset 1995). Individual multilocus heterozygosity was estimated from microsatellite genotypes using a method based on the contribution of each locus, *hL*, which was calculated using the excel macro Cernicalin (Aparicio *et al.* 2006). This measure has been shown to provide a better correlation with genome-wide heterozygosity than locus heterozygosity, particularly when the number of markers used is low (Aparicio *et al.* 2006).

The MHC genotypes at the B locus (comprising two class I and two class II loci) of all offspring were determined by reference strand conformation analysis (RSCA) following Worley *et al.* (2008).

Statistical analyses

Survival analyses were conducted on birds that died before 250 days of age in the 2007 population. We were able to successfully MHC genotype 78 individuals. MHC heterozygosity and the presence of specific MHC genotypes and haplotypes were examined for their effect on survival. Rare haplotypes and/or genotypes (identified in two or fewer individuals; $N = 7$) were excluded from the specific analyses of haplotypes/genotypes. Many factors unrelated to the MHC may influence survival of the birds. In order to control for some of these we initially considered mothers' identity, fathers' identity, hatching batch, sex, weight at hatching and multilocus heterozygosity in the survival analyses. However, correlations may exist between some explanatory variables, so these were explored before a final survival model was assembled. MHC heterozygous birds did not have higher individual multilocus heterozygosities than MHC homozygous birds ($N = 57$ vs. 21 respectively; $F_{1,76} = 0.010$, $P = 0.920$). Therefore MHC heterozygosity and multilocus heterozgosity could be entered into models as independent factors. The mothers' and fathers' identity could not both be entered into the model due to colinearity in the dataset. Therefore, only the mothers' identity was entered as a stratum to all models to control for any family effect. Data relating to the sex of birds was missing for individuals that died in the first few weeks of life. Therefore, sex was not entered into the survival models and instead we analysed the effect of sex on survival using a smaller subset of birds (31 females and 37 males). The remaining variables (hatch batch, hatch weight, MHC genotype, haplotype or heterozygosity and multilocus heterozygosity) were entered into the models as covariates. For all survival analyses, we used Cox regression, with stepwise deletion of terms $P \geq 0.1$.

Results

Microsatellite variation

Observed and expected heterozygosity, allele sizes and allelic frequencies of the 27 microsatellite loci surveyed are shown in Table S1 (Supporting information). No departures from Hardy–Weinberg equilibrium were observed at any loci. Linkage disequilibrium was detected in 18 out of 351 locus pairs, but none remained significant after sequential Bonferroni correction. Considering all microsatellite loci, mean and SE *hL* were 0.455 and 0.012, respectively.

Major histocompatibility complex variation

There were seven non-recombining MHC haplotypes in the study cohort, two of which were only found in one or two individuals and so were excluded from further analysis. The most common haplotypes AB_ab and CD_c occurred in 65% and 64% of birds, respectively (while the rarer haplotypes occurred in less than 20% of birds) (Table S2, Supporting information). These haplotypes combined to produce 10 genotypes, seven of which were found in more than two individuals: two homozygotes and five heterozygotes, all with varying frequencies (ranging from 4% to 42% of individuals; Table S2, Supporting information). Given the parental pairings used to produce this cohort, the maximum number of possible homozygote genotypes was three and heterozygote genotypes 11. An excess of MHC heterozygotes, such as is expected under balancing selection, cannot be tested in our study population due to the non-random mating of parental birds. The lack of the final (and rarest) homozygote genotype (E_dg) in the offspring was likely due to the small number of offspring ($N = 2$) produced by the only pair of parents where the genotype could possibly have been produced.

Survival analyses

The first regression model revealed that MHC heterozygous birds lived significantly longer on average

than MHC homozygous birds (117.7 ± 14.8 days vs. 165.5 ± 14.8 days, respectively; Table 1A, Fig. 1). No significant effect of multilocus heterozygosity on survival was found (Table 1A). An analysis of a smaller subset of samples for which the sex of the birds was known, revealed no effect of gender on survival (Female $N = 31$, Male $N = 37$; Wald = 0.681, d.f. = 1, $P = 0.409$).

We then examined the effect of specific MHC genotypes on survival in the cohort by replacing 'MHC heterozygosity' with 'genotype' in the survival analysis. There was an overall effect of MHC genotype on survival (Table 1B). This effect was due to the CD_c homozygous genotype having reduced survival compared to birds with any of the other genotypes (Table 1B; Fig. 2). There was no differential survival effect between the remaining genotypes. The only other homozygous genotype in the cohort (AB_ab) did not show a lower survival rate than any of the heterozygote genotypes (Table 1B; Fig. 2). These results indicate that the lower survival of the CD_c homozygote genotype is due to the deleterious effect of the CD_c haplotype and not to an effect of homozygosity *per se*.

We further explored the relative effects of heterozygosity, the presence/absence of the CD_c haplotype and their interaction in a third model (Table 1C). The other haplotypes were not included in the same model because none of the genotypes in which they were found had a significant effect on survival (Fig. 2). The model indicated that, in addition to the effect of MHC heterozygosity, there was an effect of the haplotype CD_c and, importantly, a significant interaction between haplotype CD_c and MHC heterozygosity on survival (Table 1C).

To better visualize the interaction between MHC haplotype and MHC heterozygosity we compared the survival of each of the 'AB_ab' and 'CD_c' haplotypes in their homozygous form (the only two homozygotes) with the survival of heterozygote genotypes that contained these haplotypes (Fig. 3). Birds with the homozygote genotype AB_ab did not have decreased survival compared to those that were AB*XX*_ab*xx* heterozygous (homozygote AB_ab = 150.6 ± 13.8 days, heterozygote AB*XX*_ab*xx* = 169.4 ± 7.1 days; $\chi^2 = 2.23$, d.f = 1, $P = 0.136$; Fig. 3A and B). There was also no significant decrease in survival for birds with the homozygote genotype AB_ab compared to all other heterozygotes, regardless of their genotype ($\chi^2 = 1.51$, d.f. = 1, $P = 0.220$). In contrast, birds with the homozygote genotype CD_c had significantly lower survival than individuals with heterozygote genotypes containing the haplotype CD_c (homozygote = 93.8 ± 21.5 days, heterozygote = 168.4 ± 7.9; $\chi^2 = 8.03$, d.f = 1, $P = 0.005$; Fig. 3C and D). This result shows that the deleterious effect of the CD_c haplotype on survival is negated when the haplotype is in the heterozygous form with any other haplotype.

Table 1 Regression models for predictors of survival in the junglefowl population. In model (A) all variables entered into the model are shown but those that remained in the final model are given in bold. In models B and C the same variables as in A were entered but only those that remained in the final model are shown. In model (B), MHC heterozygosity was replaced with 'MHC genotype'. In model (C) the presence/absence of the CD_c haplotype was entered into the model alongside MHC heterozgosity and the interaction between haplotype CD_c and heterozygosity

	B	SE	Wald	d.f.	P
A					
MHC heterozygosity	**2.344**	**0.644**	**13.229**	**1**	**<0.001**
Hatch batch			**8.694**	**4**	**0.039**
Hatch weight	0.029	0.108	0.072	1	0.788
Multilocus heterozygosity	−0.276	1.771	0.024	1	0.878
B					
MHC genotype			**17.555**	**7**	**0.014**
AB_ab	1.672	1.746	0.918	1	0.338
ABCD_abc	−1.787	1.552	1.326	1	0.249
ABE_abdg	−0.310	1.745	0.032	1	0.859
ABFI_abef	1.162	1.735	0.449	1	0.503
CD_c	**3.357**	**1.866**	**3.235**	**1**	**0.072**
CDFI_cef	2.188	2.611	0.702	1	0.402
EFI_defg	−2.126	1.797	1.399	1	0.237
Hatch batch			**11.368**	**4**	**0.023**
C					
MHC heterozygosity	**2.823**	**0.801**	**12.435**	**1**	**0.000**
CD_c	**1.459**	**0.666**	**4.795**	**1**	**0.029**
CD_c*MHC heterozygosity	**−2.075**	**0.877**	**5.596**	**1**	**0.018**
Hatch batch			**8.398**	**4**	**0.078**

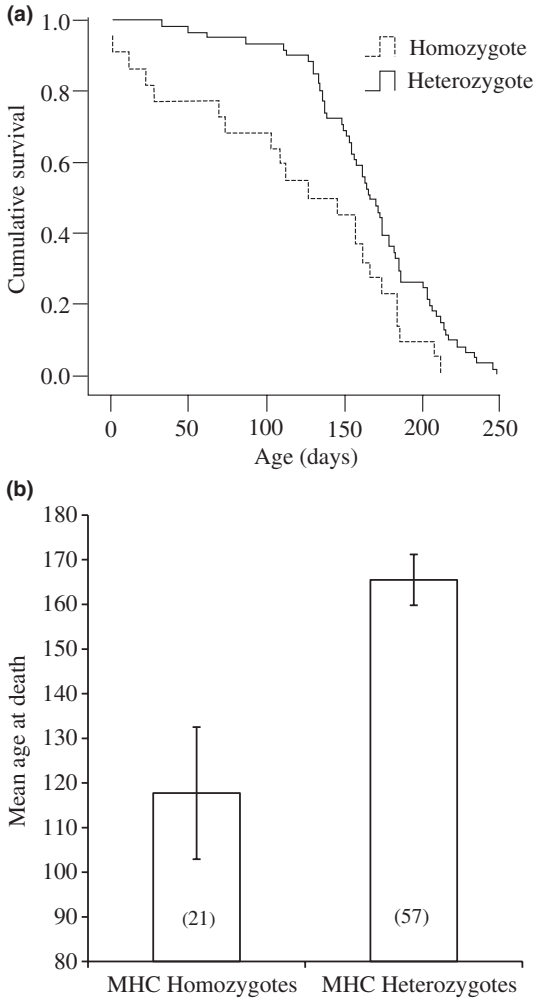

Fig. 1 Kaplan-Meier curves comparing the survival of birds with MHC heterozygotes ($N = 57$) to those with MHC homozygote genotypes ($N = 21$). (a) MHC heterozygotes (solid line) have increased survival when compared to MHC homozygotes (dashed line) across all days from 0 to 250 days of age. (b) This is also reflected in the mean survival age of birds. Error bars on plot B represent SE of the mean survival. Sample sizes are shown in brackets.

When comparing the 78 'hatched' birds with the 19 'non-hatched' embryos (out of the total of 98 birds monitored) there was no significant difference in the proportion of MHC heterozygotes ($\chi^2 = 0.24$, d.f. = 1, $P = 0.77$), or in the proportion of each MHC haplotype ($\chi^2 = 3.34$, d.f. = 4, $P = 0.50$). Therefore, there was no evidence of selective viability before hatching between MHC heterozygote and MHC homozygote embryos in our limited sample.

Discussion

Our captive population of red junglefowl was kept in semi-natural conditions in large aviaries where multiple bacterial and viral challenges are likely to occur. For example, we have recorded bacterial (e.g. *Campylobacter jejuni*) and viral infections (e.g. Marek's disease virus) in some cohorts of the study population. It is worth noting however, that the cohort studied here did not show clinical evidence of infection by lethal pathogens other than *Eimeria*. Pathological, microbiological and epidemiological evidence consistently suggest that coccidiosis was the likely main cause of mortality for this cohort. Our survival analysis showed that, in the face of this coccidiosis outbreak, survival was on average higher for MHC heterozygous individuals and, therefore, that a form of heterozygote advantage was occurring.

The hypothesised advantage of MHC heterozygosity is based on the idea that heterozygote individuals are resistant to a wider array of pathogen-derived peptides than MHC homozygotes (Doherty & Zinkernagel 1975). Several studies have been carried out in laboratory populations, where environmental variables are eliminated, showing significant effects of MHC heterozygosity on fitness (Hedrick *et al.* 2001; McClelland *et al.* 2003a; Wedekind *et al.* 2005). For example, MHC heterozygote mice are better able to clear *Salmonella* infection than homozygotes (Penn *et al.* 2002). The number of studies that directly compare the fitness of MHC heterozygotes with homozygotes in more natural populations is limited (Arkush *et al.* 2002; Froeschke & Sommer 2005; Ilmonen *et al.* 2007). MHC heterozygotes were shown to have a fitness advantage in striped mice (*Rhabdomys pumilio*) (Froeschke & Sommer 2005) and salmon populations (*Oncorhynchus tshawytscha*) (Arkush 2002), although there was not a heterozygote advantage in experimentally infected mice (Ilmonen *et al.* 2007). Our study population is comparable to the latter of these examples, as like the mice the junglefowl were housed in semi-natural conditions. However, our study on junglefowl was not a controlled single-infection experiment – the *Eimeria* infection occurred 'naturally' and other unidentified infections may have been occurring alongside and interacted with, the *Eimeria* (though we have no evidence that this did occur – see above). The final result of our study also differed from what was found in the mouse (Ilmonen *et al.* 2007), as in our study MHC heterozygote advantage following infection was observed.

Importantly, heterozygote advantage can result from two mechanisms; overdominance (heterozygote superiority) and dominance (e.g. Penn *et al.* 2002; Oliver *et al.* 2009) Dominance, where a dominant pathogen resistance trait in the heterozygote form masks any susceptibility conferred by the second allele, is revealed when the fitness of MHC heterozygotes is greater than the 'mean' fitness of homozygote individuals, but not greater than the 'best' of the homozygote genotypes. On the other hand, overdominance is based on the idea

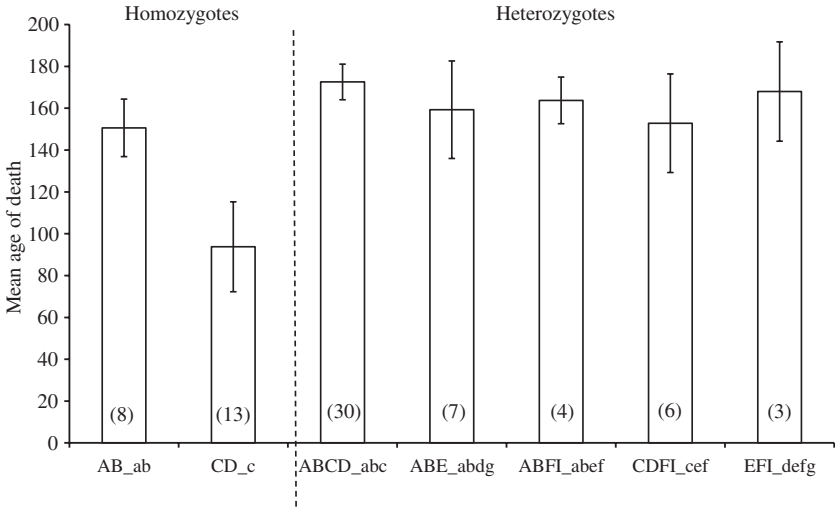

Fig. 2 A comparison of the mean length of survival of birds with different genotypes in the junglefowl cohort. Error bars represent SE of the mean survival. Sample sizes are shown in brackets. Three genotypes are not included as they were present in two or fewer individuals each.

that the combined presence of two alleles confers pathogen resistance 'over and above' that provided by any of the homozygote forms. In this case heterozygotes would confer greater resistance than the most resistant homozygote genotype. Various studies have reported evidence for heterozygote advantage through dominance (e.g. Thursz *et al.* 1997; Carrington *et al.* 1999; Penn *et al.* 2002 Wedekind *et al.* 2005). However, there are relatively few studies that provide unambiguous evidence to support the idea of MHC overdominant heterozygote advantage (McClelland *et al.* 2003b; Kekäläinen *et al.* 2009; Oliver *et al.* 2009). In the present study the effect of MHC heterozygosity appeared to be caused by a single susceptible haplotype (CD_c), the effect of which was masked in heterozygote genotypes by other dominant haplotypes. The CD_c homozygous genotype had lower survival than all other genotypes (Fig. 2), but CD_c heterozygous genotypes survived as well as the most resistant homozygote genotype. As no heterozygotes conferred greater resistance than the most resistant homozygote genotype there was no evidence of any overdominant effects. Instead, the evidence from this study therefore indicates that heterozygote advantage through dominance occurred in this junglefowl population during the coccidiosis outbreak.

In contrast to the relatively few studies revealing heterozygote advantage in natural populations, there is a large body of evidence showing relationships between specific MHC alleles and either disease resistance or susceptibility (e.g. Hill *et al.* 1991; Paterson *et al.* 1998; Barribeau *et al.* 2008; Loiseau *et al.* 2008), including those associated with fowl coccidiosis infection (Caron *et al.* 1997; Kim *et al.* 2008). Our results now suggest that there is an association between susceptibility to coccidiosis and the CD_c haplotype in this junglefowl population. In the face of the coccidiosis outbreak, the CD_c haplotype had a detrimental effect on survival, but this effect was cancelled out by the presence of a second haplotype in heterozygous individuals, thus generating the heterozygote advantage. This relationship was also demonstrated by a significant interaction between the presence of the CD_c haplotype and MHC heterozygosity. The CD_c haplotype is unusual in the population as it only contains one class II allele across the two class II loci (allele 'c'; Worley *et al.* 2008). It may be that in the CD_c homozygote genotype the possession of only one class II allele reduces the ability of junglefowl to respond to the coccidiosis infection. Interestingly, while the CD_c haplotype may decrease in frequency in the population because of the selection pressure against it in the homozygous form, because its effect is masked in the heterozygote genotype it is never likely to be purged from the population.

The relationship between genome-wide heterozygosity and fitness has been widely examined, largely in relation to inbreeding (e.g. Coltman *et al.* 1998; Reed & Frankham 2003; Richardson *et al.* 2005; Brouwer *et al.* 2007). Inbreeding results in increased homozygosity and, subsequently, the expression of recessive deleterious alleles and loss of any heterozygote advantage across the genome in general (including the MHC), thus leading to a decrease in fitness (see reviews by Hansson & Westerberg 2002; Keller & Waller 2002). Consequently MHC heterozygosity may be correlated to genome-wide heterozygosity and any benefit associated with MHC heterozygosity could result from either MHC specific effects, overall genome-wide heterozygosity, or both. To investigate the specific effect of MHC heterozygosity on survival it is therefore important to control for genome-wide heterozygosity. One way to do this has been to include a measure of multilocus heterozygosity across neutral loci (e.g. Boyce *et al.* 1997; Westerdahl *et al.* 2004; Bryja *et al.*

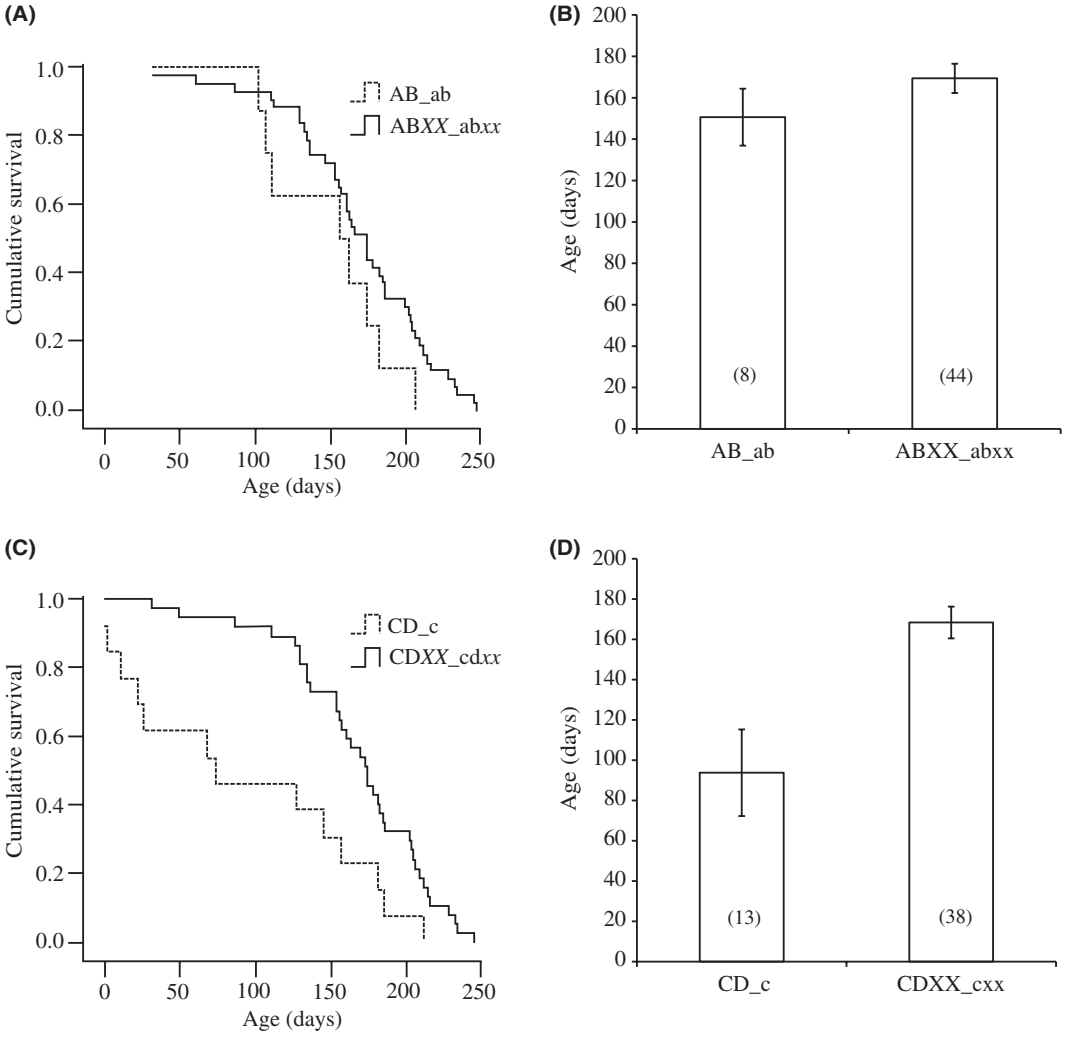

Fig. 3 (A) and (C) Kaplan–Meier curves comparing survival of birds with homozygote AB_ab and CD_c haplotypes with that of heterozygotes containing each haplotype. (B) Birds with heterozygote genotypes containing the haplotype AB_ab (AB*XX*_ab*xx*) do not have significantly increased survival compared to those with the homozygote genotype AB_ab. (D) Birds with the homozygote genotype CD_c had significant lower survival than individuals with heterozygote genotypes containing the haplotype CD_c (CD*XX*_c*xx*). Error bars on plots B and D represent SE of the mean survival. Sample sizes are shown in brackets.

2007). However recent studies have pointed out that multilocus heterozygosity estimates based on a handful of markers will not be accurately measure genome-wide heterozygosity (Balloux *et al.* 2004; Slate *et al.* 2004). Indeed, Slate *et al.* (2004) showed that in most populations large numbers of loci (hundreds) would have to be screened to get an accurate measure, and only in populations with very high variance in inbreeding coefficient amongst individuals may lower numbers of loci suffice. The general conclusion now is that where possible direct pedigree based estimates of relatedness will provide a much better measure of the effects of genome-wide heterozygosity on survival than estimates calculated from microsatellite genotypes (Balloux *et al.* 2004; Slate *et al.* 2004).

Unfortunately, in the present study direct pedigree based estimates of relatedness were not available. Consequently we had to use a measure of multilocus heterozygosity to estimate genome-wide heterozgosity. On the positive side, the small, genetically bottlenecked, junglefowl population studied is likely to have high variance in inbreeding, thus reducing the number of loci required to get an accurate estimate (Balloux *et al.* 2004; Slate *et al.* 2004). Based on the analysis by Slate *et al.* (2004) we estimate that the 27 microsatellite loci we screened provided a measure that correlates approximately 60% to genome-wide heterozygosity/inbreeding. While clearly not perfect we think this provides a reasonable attempt to control for such effects.

We found no correlation between MHC heterozygosity and multilocus heterozygosity, suggesting that the two factors can be analysed independently. Furthermore, while there was strong evidence that MHC heterozygosity influenced survival there was no evidence that multilocus heterozygosity did. Perhaps most importantly, the argument that genome-wide heterozygosity may be responsible for the heterozygote advantage effect we see here may be null anyway. The heterozygote advantage observed appeared to be due to dominance NOT overdominance: the haplotype CD-c conferred susceptibility but its effect was masked in heterozygotes by the second haplotype. While it is clear that a heterozygote advantage due to overdominance could be an artefact of correlated genome-wide heterozygosity, there is no reason why a heterozygote advantage that arises from an allelic effect will be. Overall therefore, we are confident that the effect of MHC on survival observed in our study is a genuine one and not an artefact of genome-wide processes.

It is unusual for such high proportions of fowl to die from coccidiosis, a disease which is generally present in all poultry (McDougald 2008; McDonald & Shirley 2009). These parasites develop and reproduce inside the host intestine causing bleeding and swelling, resulting in the bird being unable to absorb nutrients from its food. Birds in constant contact with small numbers of parasite oocysts usually develop immunity without showing any signs of disease. However, where large numbers of birds are kept together, large numbers of oocysts can be present, leading to outward signs of disease (McDougald 2008). We think that the semi-natural rearing conditions and/or stress factors may have led to the increased mortality in our junglefowl population. This may in turn affect the interaction between MHC heterozygosity and survival in this study, as the benefits of heterozygosity may be greater in stressful conditions (Kempenaers 2007). Our junglefowl population is maintained for use in extensive studies of the role of the MHC in pre- and post-copulatory sexual selection. Investigating the causes and outcome of disease is therefore important in order that we can limit any future mortality in the population and avoid any problems caused by the selective survival of specific MHC genotypes.

It may be that the adverse survival effects resulting from MHC homozygosity in the present study may not be obvious in large, outbred populations. In such populations, where a greater number of MHC haplotypes are present, the vast majority of birds are likely to be MHC heterozygotes, thereby masking the negative effects of susceptible haplotypes. However, in small, inbred or genetically bottlenecked populations the effects of MHC homozygosity on survival may become increasingly important as these populations may contain low levels of MHC variation (Mainguy *et al.* 2007; Biedrzycka & Radwan 2008; Miller *et al.* 2008; Oliver *et al.* 2009; but see Richardson & Westerdahl 2003; Hansson & Richardson 2005; van Oosterhout *et al.* 2006). As inbred and bottlenecked populations regularly occur in nature, the results from small, semi-natural populations such as in this study could reflect processes ongoing in the small, natural populations typical of rare and vulnerable species.

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Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Expected and observed heterozygosities (H_e and H_o), allele sizes and allelic frequencies of 27 microsatellite loci in a captive population of red jungle fowl

Table S2 MHC haplotype and genotype frequencies in the captive population of red junglefowl. Values represent the percentage of individuals in which each allele or genotype is found

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