Sex-specific effects of an IgE polymorphism on immunity, susceptibility to infection and reproduction in a wild rodent

Klara M Wanelik[1*], Mike Begon[1], Janette E Bradley[2], Ida M Friberg[3], Joseph A Jackson[3], Christopher H Taylor[2], Steve Paterson[1]

[1] Institute of Infection, Veterinary and Ecological Sciences, University of Liverpool, Liverpool, UK

[2] School of Life Sciences, University of Nottingham, Nottingham, UK

[3] School of Environment and Life Sciences, University of Salford, Salford, UK

* Corresponding author

Current address: Department of Zoology, University of Oxford, Oxford, UK

Email: klara.wanelik@zoo.ox.ac.uk

Abstract

The genotype of an individual is an important predictor of their immune function, and subsequently, their ability to control or avoid infection and ultimately contribute offspring to the next generation. However, the same genotype, subjected to different environments, can also result in different outcomes. The sexes represent two such different environments. Sexual dimorphism is widespread across the animal kingdom. Despite this, very little is known about the importance of sex for the expression of genotype in the context of health and disease, particularly in natural populations. We identified a synonymous polymorphism in the high-affinity Immunoglobulin E (IgE) receptor (GC and non-GC haplotypes) that has sex-specific effects on immune gene expression, susceptibility to infection and reproductive success of individuals in a natural population of field voles (*Microtus agrestis*). We found that the effect of the GC haplotype on the expression of genes affecting inflammation displayed a significant interaction with sex. While males with the GC haplotype had upregulated pro-inflammatory genes, in particular the pro-inflammatory cytokine *Il33,* females had upregulated anti-inflammatory genes, in particular the anti-inflammatory cytokine inhibitor *Socs3.* Furthermore we found that the effect of the GC haplotype on the probability of infection with a common microparasite*, Babesia microti,* displayed a significant interaction with sex. While males with the GC haplotype did not differ significantly in their susceptibility to infection, females with the GC haplotype were more likely to be infected. Finally, we found that the effect of the GC haplotype on reproductive success also displayed a significant interaction with sex. While males with the GC haplotype had a lower reproductive success, females with the GC haplotype did not differ in the number of offspring they produced. To our knowledge, this is the first time that a polymorphism with sex-specific

effects across all three levels (immune gene expression, susceptibility to infection and reproductive success) has been documented in a natural population.

Introduction

In order for an individual to control or avoid infection and ultimately contribute offspring to the next generation, they must have a well-functioning immune system [1]. An individual's immune function is in part determined by their genotype (e.g. [2–4]). However, the effect of any genotype will depend on the context in which it occurs. Sexual dimorphism is widespread in the animal kingdom, with males and females representing different contexts or environments within which genes must act. Sex can therefore have an important effect on the phenotypic expression of genotype (genotype by sex interactions), with the same genotype having either (i) effects in the same direction but of different magnitude in the sexes, (ii) an effect in one sex but not the other, or in the most extreme case (iii) opposing effects in the sexes. However, the majority of evidence for genotype by sex interactions, in the context of health and disease, comes from laboratory experiments conducted on model organisms [5,6].

Evidence for genotype by sex interactions in natural populations is limited. Studies of natural populations have explored the effects of genotype on immune phenotype, and have observed consequences for susceptibility to infection. Most notably, variability in the genes of the Major Histocompatibility Complex (MHC) has been associated with resistance to intestinal nematodes in domestic sheep [2] and with resistance to malaria, hepatitis, and AIDS in humans [7–9]. The role of variability elsewhere in the genome, for shaping immune phenotype, has also been studied [3,4]. However, in the majority of

these studies, genotype by sex interactions are not considered. In some studies, this is because their low sample sizes make it difficult to test for data-hungry genotype by sex interactions [10]. In some studies, sex is considered a confounding variable which is controlled for, in order not to hide any subtle genetic associations [2]. Other studies focus on a single sex for the sake of simplicity [11].

The few studies that do consider genotype by sex interactions do not tell the whole story. While there are examples of gene variants with sex-specific effects on human immunity and/or health in the medical literature (reviewed in [12]), longitudinal studies involving large numbers of participants are still rare, making it difficult to explore long-term sex-specific effects. The study of wild animals provides us with an opportunity to (more easily and cheaply) follow large numbers of individuals, exposed to a challenging environment and with varying genetic backgrounds, throughout their lives. This allows us to make a more complete assessment of the impacts of genotype throughout the life of an individual, whether male or female. For example, Graham et al 2010 [13] found evidence for heritable variation in immunity associated with sex-specific effects on Soay sheep reproduction. However, we know of no documented example of a polymorphism with sex-specific effects on immunity, susceptibility to infection *and* reproduction in a natural population. Here, we use a wild rodent, the field vole (*Microtus agrestis*), as a model in which to do this. Wild rodents, in particular, offer an opportunity to quickly follow large numbers of individuals throughout their lives, given their short lifespans. They also offer the opportunity to draw on the immunological and genetics resources developed for laboratory rodents, while providing a much more realistic ecological model of human populations [14,15].

The sexes differ not only in their behaviour and morphology, but less conspicuously, in their physiology, biochemistry and in their gene expression. Driven in part by sex hormones [16], sex-biases in the expression and coexpression of many autosomal genes have now been documented, suggesting that males and females differ not only in their regulation of single genes [17–19], but whole networks of interacting genes [20,21]. The same genotype then, could be expressed as different immune phenotypes, and subsequently have very different consequences for the susceptibility to infection and reproductive success of males and females. Furthermore, males and females differ in the environments they inhabit and the selective pressures they face, all of which may also interact with genotype. One might therefore expect genotype by sex interactions to be even more important in natural populations. We argue then that by neglecting to consider sex, particularly in studies of natural populations, there is a danger of diluting the effect of genetic variation if for example an effect is present in only one sex, or worse, overlooking the effect of genetic variation completely if opposing effects in males and females cancel each other out.

In humans, immunoglobulin E (IgE) mediated responses are associated with defence against helminths [22] and with allergy [23]. They are controlled by the high-affinity IgE receptor, FCER1, which is found on the surface of various immune effector cells e.g. mast cells, basophils and eosinophils [24]. Naturally occurring polymorphisms in FCER1 are known to affect an individual's serum IgE levels, with consequences for their susceptibility to infection [25,26] and their risk of developing inflammatory disease [27–30]. Furthermore, sex differences in serum IgE levels [31] and the incidence of IgE-mediated inflammatory disease [32] have been documented in humans, suggesting that any polymorphism in this pathway is likely to experience different contexts in males and

females. Indeed, one study found evidence for a sex-specific effect of a polymorphism in the *Fcer1a* gene (the alpha chain of FCER1) on susceptibility to systematic lupus erythematosus (a chronic inflammatory disease) [33].

In a previous study of a natural population *M. agrestis*, we found that males carrying the GC haplotype of the *Fcer1a* gene expressed the transcription factor GATA3 at a lower level than males carrying non-GC haplotypes [3]. GATA3 is a biomarker of tolerance to macroparasites in mature males in our population (macroparasite infection gives rise to increased expression of GATA3, which gives rise to improved body condition and survival [11]). Here, we explore the effects of this GC haplotype further in males, as well as extending our analysis to females. In doing so, we provide the first example of a polymorphism with sex-specific effects not only on immune gene expression, but susceptibility to infection, and ultimately, on reproductive success in a natural population.

Results

We sampled a natural population of *M. agrestis* in Kielder Forest, Northumberland, United Kingdom, over three years (2015-2017) and across seven different sites. Our study involved a cross-sectional component (n = 317 destructively sampled voles) and a longitudinal component (n = 850 marked individuals monitored through time, with n = 2,387 sampling points). We tested the consequences of the GC haplotype of the *Fcer1a* gene using both cross-sectional and longitudinal components of our study. Two other major haplotypes were present in our study population: AC haplotype and AT haplotype.

The GC haplotype has sex-specific effects on inflammation

In humans, naturally occurring polymorphisms in the *Fcer1a* gene have previously been linked to inflammatory disease [27–30]. Therefore, we used the cross-sectional component of our study to test the effects of the GC haplotype on inflammation in males and females. Differential gene expression (DGE) analysis performed on 53 males and 31 females assayed by RNASeq, showed that the identity of top-responding inflammation-associated (pro- and anti-inflammatory) genes differed between the sexes. In males, the top-responding gene was the pro-inflammatory cytokine, *Il33* (log fold change (logFC) = 2.76, p = 0.00000015, q = 0.00048; Table S1) while in females it was the anti-inflammatory suppressor of cytokine signalling *Socs3* (logFC = 1.07, p = 0.000061, q = 0.05; Table S2). Looking at the ranking of each top-responding gene in the opposite sex strengthens the case for distinct effects in males and females, with *Il33* ranked markedly lower in females (rank = 8224/12904, logFC = 0.29, p = 0.70, q = 1.00) and *Socs3* markedly lower in males (rank = 10886/12904, logFC = -0.05, p = 0.84, q = 1.00).

To test whether these sex-specific effects on inflammation were limited to these genes or were more widespread, we performed a gene set enrichment analysis (GSEA) which looked at the rankings of all pro-/anti-inflammatory genes. This analysis showed that both males and females upregulated their expression of both pro- and anti-inflammatory genes. However, males showed a stronger signal for pro-inflammatory genes (pro-inflammatory genes: p = 0.007; anti-inflammatory genes: p = 0.04; Fig 1A, upper panel) while females showed a stronger signal for anti-inflammatory genes (anti-inflammatory genes: p = 0.001; pro-inflammatory genes: p = 0.04; Fig 1A, lower panel).

Finally, to confirm these sex-specific effects on inflammation, we used an independent dataset for males and females whose spleens were stimulated with an immune agonist and assayed by Q-PCR (for a panel of 18 immune genes). From this *ex vivo* assay, one can gain insight into the types of immune response that could be made to a pathogen *in vivo* (see Methods for details). We found that the effect of the GC haplotype on the stimulated expression levels of *Il17a*, a pro-inflammatory cytokine, displayed a significant interaction with sex (genotype by sex interaction in follow-up LMM, LRT $p = 0.001$). While females with the GC haplotype expressed lower levels of *Il17a* (estimate = -0.12, 95% CI = -0.19 – -0.04), males with the GC haplotype did not differ in their expressed levels of *Il17a* (estimate = 0.03, 95% CI includes zero = -0.02 – 0.08; Table S3 & S4).

The GC haplotype has the same effects on oxidative stress in both sexes

Inflammation and oxidative stress are closely related and often lead to pathology [34]. To test whether the effects of the GC haplotype on inflammation may be having knock-on effects on oxidative stress, we again used the cross-sectional component of our study. We tested the effect of the GC haplotype on antioxidant superoxide dismutase 1 (SOD1) enzymatic activity in males and females. SOD1 is one of the main antioxidative enzymes within the antioxidative enzyme system [35]. We found that (irrespective of sex) individuals with the AT haplotype had lower SOD1 activity when compared with other haplotypes, including the GC haplotype ($p = 0.04$; Table S5).

The GC haplotype has sex-specific effects on the susceptibility to infection

We then used the longitudinal component of our study to test the effects of the GC haplotype for the probability of infection with common parasites known to infect our study population: two micro-parasites *Babesia microti* and *Bartonella* spp. and a range of

macro-parasites (ticks, fleas and cestodes). We found an effect of the GC haplotype on the probability of infection with *B. microti* which displayed a significant interaction with sex (genotype by sex interaction, LRT $p = 0.048$; Table S6). While females with the GC haplotype were more likely to be infected with *B. microti* (odds ratio = 1.25, 95% CI = 0.44 – 2.07 ; Fig 1B, lower panel), males with the GC haplotype did not differ in their susceptibility to infection (odds ratio = 0.14, 95% CI includes zero = -0.76 – 1.03; Fig 1B, upper panel). Howevere, we found no effect of the haplotype (interactive or not) on the probability of infection with the other parasites in our population (Tables S7, S8 & S12)

The GC haplotype has sex-specific effects on reproductive success

We genotyped both cross-sectional and longitudinal voles for 114 SNPs and used this dataset to construct a pedigree. We estimated each individuals' reproductive success by counting the number offspring it produced according to this pedigree. We then tested the effects of the GC haplotype on this measure of reproductive success. We tried to run a single model with both sexes (as above) but resulting residual variances differed significantly between the sexes, which reflects the fact that male and female reproduction are inherently different traits. This made it impossible to formally test for a genotype by sex interaction and so instead we ran a separate model for each sex. We found that males with the GC haplotype had an average of 2.2 fewer offspring than males without the haplotype ($p = 0.04$; Table S9; Fig. 1C, upper panel). Despite a larger sample size and lower variability in reproductive success, females with the GC haplotype did not differ significantly in their number of offspring (genotype term did not appear in best model; Table S10; Fig 1C, lower panel). This is suggestive of a significant genotype by sex interaction.

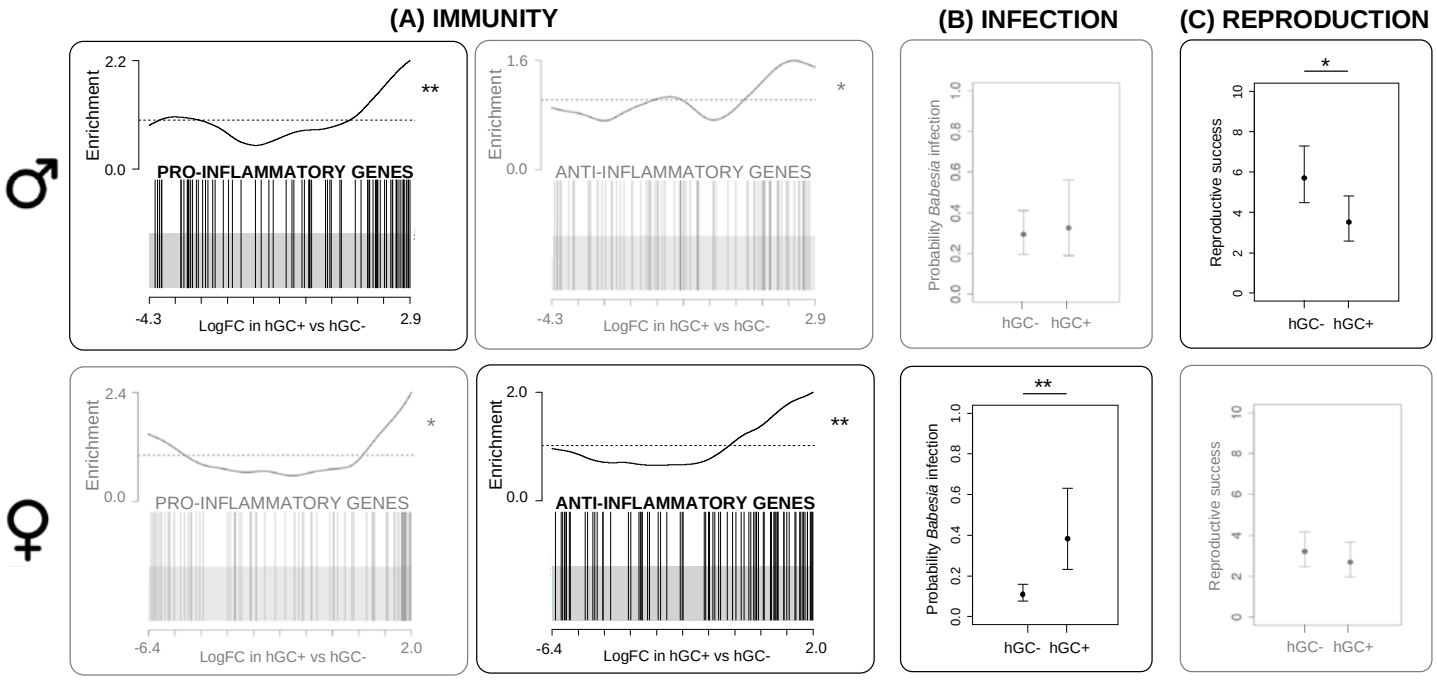

Figure 1 Sex-specific effects of GC haplotype (hGC). Upper panel: males. Lower panel: females: (A) Immune gene expression: Barcode plots indicating the position of pro- and anti-inflammatory genes in a ranked list of log fold changes (logFC) for individuals with (hGC+) vs. without (hGC-) the haplotype, showing that males with the haplotype have a pro-inflammatory bias, whereas females have an anti-inflammatory bias. (B) Susceptibility to infection: Association between hGC and the probability of infection with *Babesia microti,* showing that only females with the haplotype have an increased susceptibility to infection. (C) Reproduction: Association between hGC and reproductive success, showing that only males with the haplotype have lower reproductive success (error bars represent ± standard error; * indicates $p < 0.05$; ** indicates $p < 0.01$).

Discussion

In this study, we describe the first example of a polymorphism with sex-specific effects linking together immune gene expression, susceptibility to infection and ultimately reproductive success in a natural population. Here, we have found evidence for opposing effects in the sexes (unstimulated immune gene expression), and an effect present in one sex but not the other (stimulated immune gene expression, susceptibility to infection and reproductive success), what could be called qualitative rather than quantitative sex differences. However, we cannot rule out that, in the case of susceptibility to infection and reproductive success, there was a smaller effect present in the opposite sex (in the same or opposite direction) which our analysis did not have sufficient power to detect.

The polymorphism in question lies in the high-affinity receptor for IgE. In humans, naturally occurring polymorphisms in this receptor have previously been linked to inflammatory disease [27–30]. Our work is consistent with this. More specifically though, our results suggest opposing effects of this polymorphism on the inflammatory phenotype of males and females. While males with the GC haplotype showed a pro-inflammatory bias, females with the haplotype showed an anti-inflammatory bias. Again, this is consistent with previous work. A previous study on another polymorphism in the same gene, *Fcer1a*, also found evidence for sex-specific effects on inflammatory disease [33]. In order to fully understand this genotype by sex interaction, it is important to consider background differences in the inflammatory phenotypes of males and females. Females in this study were pregnant for a large proportion of their lives (57% of post-juvenile females were pregnant or lactating at the time of capture in the longitudinal

component of our study). In mammals, including humans, pregnancy has been shown to be a largely anti-inflammatory state, with pregnant females suppressing inflammation. This is to protect the foetus from attack by the mother's immune system [36]. While testosterone in males has an anti-inflammatory effect [37], it also drives males to use more space [38] and to be more aggressive [39]. These behaviours increase their rates of contact with other individuals, and with their environment, and hence their likelihood of infection and subsequent immune stimulation. We suggest that the polymorphism appears may be exaggerating background sex differences in inflammatory activity. Sex hormones have been implicated in the differential expression of some autosomal genes in males and females [16], and could be driving these opposing effects.

Consistent with the association between IgE-mediated responses and defence against helminths [22], in our previous study, we showed that males with the GC haplotype had a lower level of an immunological marker of tolerance to ticks, fleas and adult cestodes (i.e., they were less tolerant) [3]. Here, we use data on infection incidence to show that, in a different context, the same haplotype is also associated with resistance to a microparasite, *B. microti*, in females. Although observational studies of this kind are not able to identify causal relationships, a realistic scenario is that females with the GC haplotype are more likely to be infected with *B. microti* (i.e., they are less resistant) as a result of a lower pro-inflammatory to anti-inflammatory cytokine ratio. Pro-inflammatory cytokines (e.g., IL-6, IFN-γ, and TNF-α) may help to resist *B. microti* infection [40]. A lack or imbalance of these cytokines may hamper this resistance. The panel of parasites that we have measured is not exhaustive and previous studies have highlighted the important role of species interactions in the parasite community in driving infection risk in

this study population [41]. *B. microti* may therefore represent a community of parasites, to which this haplotype affects resistance in females.

There are major challenges associated with modelling the predictors of reproductive success. As well as being non-normal and zero-inflated, distributions of reproductive success are highly skewed – with an uneven distribution of success among individuals. In polygynous species, like *M. agrestis*, reproductive skew is higher in males due to intense competition for females. While we were able to account for non-normality and zero-inflation using a quasi-Poisson error distribution, it is perhaps not surprising that we were unable to fit a single model for reproductive success (and to formally test for a genotype by sex interaction) due to unequal residual variances for males and females (heteroscedasticity). Instead we ran a separate model for each sex. Nevertheless, we argue that our data are suggestive of a significant genotype by sex interaction. While we found a significant negative effect of the GC haplotype on reproductive success in males, no significant effect was found in females despite a larger sample size and lower reproductive skew (or variance) which should equate to higher statistical power.

A realistic scenario is that the larger cost incurred by males with the GC haplotype due to inflammation reduced their reproductive value, as reproducing and mounting a pro-inflammatory response are both costly activities [42]. Another realistic scenario is that the positive effect of the GC haplotype on levels of oxidative stress (as indicated by a tendency for higher SOD1 activity) may have had a more detrimental effect in males, damaging sperm and reducing fertilising success. Sperm competition can be an important feature in the reproduction of microtines, e.g., meadow vole (*M. pennsylvanicus*) [43], with males having to produce many sperm of high quality in order

to successfully outcompete other males. Sperm are also highly susceptible to damage by reactive oxygen species (ROS) [44]. In addition, the heightened level of oxidative stress may have impaired olfactory sexual signals, e.g., excretion of major urinary proteins (MUPs) making males less attractive to females and negatively impacting on mating success [45].

Despite the immune system playing an important role in determining the health and reproductive fitness of an individual, natural selection has not converged on a single immune optimum. Instead, individuals in natural populations vary widely in their response to infection. Understanding why immunoheterogeneity is maintained is a key question in eco-immunology. Antagonistic selection, where a mutation is beneficial in some environmental contexts and harmful in others, is thought to be one mechanism by which balancing selection is generated, and genetic variation in immunity can be maintained within natural populations [13]. In samples collected between 2008-2010, we previously identified the three major haplotypes at this locus, GC, AC and AT, at frequencies of 0.12, 0.76 and 0.07 respectively [3]. Seven years on, these frequencies have remained relatively unchanged (0.08, 0.81 and 0.10). The fact that the GC haplotype remains in the population, despite its detrimental effects on male reproductive success and female infection susceptibility, suggests that it may be under balancing selection.

In order to be under balancing selection though, the GC haplotype would need to confer some advantage. We were not able to find evidence for any such advantage in this study, but we can speculate on one. Microtines show cyclical population dynamics, alternating between a “peak” phase (associated with good conditions and high vole

densities) and a “crash” phase (poor conditions and low vole densities). Selection pressures acting during these two phases will be very different. We found that GC haplotype was associated with a pro-inflammatory bias in males, indicating higher investment in promoting inflammation. Thus, one possible scenario is that higher investment in promoting inflammation is disadvantageous in the “peak” phase when the risk of parasitism is low due to dilution effects, and it pays not to waste energy mounting a strong immune response that is unnecessary. However, it might be advantageous in the “crash” phase when conditions are poor, and the risk of parasitism is high due to the time lagged transmission of parasite infectious stages that have accumulated during the peak phase. Vole numbers were extremely low in our study area between 2012 and 2013 (indicating a “crash phase”). In 2014, vole numbers began to increase and by the beginning of 2015 (the start of our study) had reached a peak. Although vole numbers declined throughout our study period, they never reached the extremely low numbers recorded in 2012-2013 (X. Lambin, personal communication, 2016). This suggests that we sampled in the region of a “peak” phase, when this investment was disadvantageous, and that if we sampled during a true “crash” phase we might detect an advantage to the GC haplotype in males. Our future work will investigate this possibility further.

There is a growing interest in human genomic (or precision) medicine, with the potential to use a patient’s genotypic information to personalise their treatment. What we have shown here demonstrates that considering genotype in isolation can be misleading, as the same polymorphism can have different outcomes not only for the immune gene expression, but the susceptibility to infection and ultimate reproductive success of males and females.

Materials and Methods

We studied *M. agrestis* in Kielder Forest, Northumberland, UK, using live-trapping of individual animals from natural populations. Trapping was performed from 2015-2017 across seven different sites, each a forest clear-cut. Access to the sites was provided by the Forestry Commission. At each site, 150-197 Ugglan small mammal traps (Grahnab, Gnosjo, Sweden) were laid out in a grid spaced approximately 3-5 m apart. Our study was divided into longitudinal and cross-sectional components.

Ethics statement

All animal procedures were performed with approval from the University of Liverpool Animal Welfare Committee and under a UK Home Office licence (PPL 70/8210 to S.P.).

Longitudinal data

Every other week, traps were checked every day, once in the morning and once in the evening. Newly trapped field voles were injected with a Passive Integrated Transponder (PIT) tag (AVID, Uckfield, UK) for unique identification. This approach allowed us to build up a longitudinal record for voles that were caught on multiple occasions. A total of 850 voles were individually marked in this way. We also took a small tissue sample from the tail, for genotyping of individuals and a drop of blood from the tail which we put into 500 ml of RNAlater (Fisher Scientific, Loughborough, UK) for use in parasite detection (see below).

Parasite detection

We quantified infections by microparasites (*Babesia microti* and *Bartonella* spp.) in blood samples taken from longitudinal animals using SYBR green based two-step reverse transcription quantitative PCR (Q-PCR) targeted at pathogen ribosomal RNA genes. Expression values were normalized to two host endogenous control genes. Blood samples were derived from tail bleeds. RNA was extracted from blood samples stored in RNAlater at -70°C using the Mouse RiboPure Blood RNA Isolation Kit (ThermoFisher, Waltham, Massachusetts), according to manufacturer's instructions, and DNAse treated. It was then converted to cDNA using the High-Capacity RNA-to-cDNA™ Kit (ThermoFisher), according to manufacturer's instructions. *B. microti* primer sequences targeting the 18S ribosomal RNA gene were as follows CTACGTCCCTGCCCTTTGTA (forward primer sequence) and CCACGTTTCTTGGTCCGAAT (reverse primer sequence). *Bartonella* spp. primer sequences targeting the 16S ribosomal RNA gene were as follows GATGAATGTTAGCCGTCGGG (forward primer sequence) and TCCCCAGGCGGAATGTTTAA (reverse primer sequences). Assays were pipetted onto 384 well plates by a robot (Pipetmax; Gilson, Middleton, Wisconsin) using a custom programme and run on a QuantStudio 6-flex Real-Time PCR System (ThermoFisher) at the machine manufacturers default real-time PCR cycling conditions. Reaction size was 10 µl, incorporating 1 µl of template (diluted 1/20) and PrecisionFAST qPCR Master Mix (PrimerDesign, Chandler's Ford, UK) with low ROX and SYBR green and primers at the machine manufacturer's recommended concentrations. Alongside the pathogen assays we ran assays for two host genes (*Ywhaz* and *Actb*) that acted as endogenous controls. For the pathogen assays we used as a calibrator sample a pool of DNA extracted from 154 blood samples from different *M. agrestis* at our study sites in 2015 and 2016; these DNA extractions were carried out using the QIAamp UCP DNA Micro Kit (Qiagen,

Hilden, Germany) following manufacturer's instructions. For the *Ywhaz* and *Actb* assays the calibrator sample was the same cDNA calibrator sample as described below for other host gene expression assays. Relative expression values (normalised to the two host endogenous control genes and presumed to relate to the expression of pathogen ribosomal RNA genes and in turn to the force of infection) used in analyses below are RQ values calculated by the QuantStudio 6-flex machine software according to the $\Delta\Delta$Ct method, indexed to the calibrator samples. Melting curves and amplification plots were individually inspected for each well replicate to confirm specific amplification. We validated our diagnostic results by comparing our PCR RQ values to independent data for a subset of voles from the cross-sectional component of our study with mapped genus-level pathogen reads from RNASeq analysis of blood samples (n = 44), finding the two data sets strongly corroborated each other (*Bartonella* spp., r^2 = 0.81, p < 0.001; *B. microti*, r^2 = 0.81, p < 0.001).

Cross-sectional data

For the cross-sectional component of the study (which ran from 2015-2016 only), animals were captured and returned to the laboratory where they were killed by a rising concentration of CO_2, followed by exsanguination. As our UK Home Office licence did not allow us to sample overtly pregnant females in this way, there are fewer females than males present in this dataset. This component of the study allowed us to take a more comprehensive set of measurements, and to culture cells and perform stimulatory assays on them. A small tissue sample was taken from the ear of cross-sectional animals for genotyping (see below).

Parasite detection

After culling, the fur of cross-sectional animals was examined thoroughly under a binocular dissecting microscope to check for ectoparasites, which were recorded as direct counts of ticks and fleas. Guts of cross-sectional animals were transferred to 70% ethanol, dissected and examined under a microscope for gastrointestinal parasites. Direct counts of cestodes (the dominant endohelminths) were recorded.

SOD1 measurement

Given the well-established link between inflammation, oxidative stress and pathology [34], we measured antioxidant enzymatic activity in blood samples taken from cross-sectional animals. We chose to measure superoxide dismutase 1 (SOD1) because it is one of the main antioxidative enzymes within the antioxidative enzyme system [35], and is clearly linked to changes in immune gene expression in laboratory mice [46]. Assays were carried out using the Cayman Superoxide Dismutase kit and following the manufacturer's instructions except where otherwise indicated below. Blood pellets from centrifuged cardiac bleeds were stored at -70°C and thawed on ice prior to assay. A 20 µl aliquot from each pellet was lysed in 80 µl of ultrapure water and centrifuged (10000 G at 4°C for 15 minutes) and 40 µl of the supernatant added to a 1.6 × volume of ice-cold chloroform/ethanol (37.5/62.5 (v/v)) (inactivating superoxide dismutase 2). This mixture was then centrifuged (2500 G at 4°C for 10 minutes) and the supernatant removed and immediately diluted 1:200 in kit sample buffer. A seven-point SOD activity standard was prepared (0, 0.005, 0.010, 0.020, 0.030, 0.040, 0.050 U/ml) and the assay conducted on a 96 well microplate with 10 µl of standard or sample, 200 µl of kit radical detector solution and 20 µl of xanthine oxidase (diluted as per manufacturer's instructions) per well. Plates were then covered with a protective film, incubated at room temperature for

30 mins on an orbital shaker and read at 450 nm on a VERSAmax tuneable absorbance plate reader (Molecular Devices, San Jose, California), subtracting background and fitting a linear relationship to the standard curve in order to estimate SOD activity in unknown samples.

Splenocyte cultures

Spleens of cross-sectional animals were removed, disaggregated, and splenocytes cultured under cell culture conditions equivalent to those used in [47]. Unstimulated splenocytes, taken from 84 cross-sectional animals collected between July and October 2015, were initially used to assay expression by RNASeq (see below). We exposed splenocytes from the remaining cross-sectional animals to stimulation with anti-CD3 antibodies (Hamster Anti-Mouse CD3e, Clone 500A2 from BD Pharmingen, San Diego, California) and anti-CD28 antibodies (Hamster Anti-Mouse CD28, Clone 37.51 from Tombo Biosciences, Kobe, Japan) at concentrations of 2 µg/ml and of 1 µg/ml respectively for 24 hr. Costimulation with anti-CD3 and anti-CD28 antibodies was used to selectively promote the proliferation of T cells [48]. We assumed that this would reflect the potential response of T-cell populations *in vivo*. Stimulated splenocytes were used to assay expression by Q-PCR.

RNASeq

Full details of the methods used for RNA preparation and sequencing can be found in [3]. Briefly, samples were sequenced on an Illumina HiSeq4000 platform. High-quality reads were mapped against a draft genome for *M. agrestis* (GenBank Accession no. LIQJ00000000) and counted using featureCounts [49]. Genes with average log counts per million, across all samples, of less than one were considered unexpressed and

removed from the data (*n* = 8,410). Following filtering, library sizes were recalculated, data were normalized and MDS plots were generated to check for any unusual patterns in the data.

Q-PCR

We used SYBR green based Q-PCR to measure the expression levels of a panel of 18 genes (Table S11) in splenocytes stimulated with anti-CD3 and anti-CD28 antibodies from our cross-sectional animals. We did this, in part, to validate our RNASeq results in an independent dataset. We used the observed expression profile as a measure of the potential responsiveness of the immune system to an inflammatory stimulation *in vivo*. The choice of our panel of genes was informed by (i) known immune-associated functions in mice, combined with (ii) significant sensitivity to environmental or intrinsic host variables in our previous studies [11,47] or in a recent DGE analysis of RNASeq data (not reported here).

Primers (20 sets, including 2 endogenous control genes) were designed *de novo* and supplied by PrimerDesign (16 sets) or designed *de novo* in-house (4 sets) and validated (to confirm specific amplification and 100 ± 10% PCR efficiency under assay conditions). All PrimerDesign primer sets were validated under our assay conditions before use. The endogenous control genes (*Ywhaz* and *Sdha*) were selected as a stable pairing from our previous stability analysis of candidate control genes in *M. agrestis* splenocytes [47]. We extracted RNA from splenocytes conserved in RNAlater using the RNAqueous Micro Total RNA Isolation Kit (ThermoFisher), following the manufacturer's instructions. RNA extracts were DNAse treated and converted to cDNA using the High-Capacity RNA-to-cDNA™ Kit (ThermoFisher), according to manufacturer's instructions, including reverse

transcription negative (RT-) controls for a subsample. SYBR green-based assays were pipetted onto 384 well plates by a robot (Pipetmax, Gilson) using a custom programme and run on a QuantStudio 6-flex Real-Time PCR System (ThermoFisher) at the machine manufacturers default real-time PCR cycling conditions. Reaction size was 10 µl, incorporating 1 µl of template and PrecisionFAST qPCR Master Mix with low ROX and SYBR green (PrimerDesign) and primers at the machine manufacturer's recommended concentrations. We used two standard plate layouts for assaying, each of which contained a fixed set of target gene expression assays and the two endogenous control gene assays (the same sets of animals being assayed on matched pairs of the standard plate layouts). Unknown samples were assayed in duplicate wells and calibrator samples in triplicate wells and no template controls for each gene were included on each plate. Template cDNA (see above) was diluted 1/20 prior to assay. The calibrator sample (identical on each plate) was created by pooling cDNA derived from across all splenocyte samples. Samples from different sampling groups were dispersed across plate pairs, avoiding confounding of plate with the sampling structure. Gene relative expression values used in analyses are RQ values calculated by the QuantStudio 6-flex machine software according to the $\Delta\Delta$Ct method, indexed to the calibrator sample. Melting curves and amplification plots were individually inspected for each well replicate to confirm specific amplification.

Longitudinal and cross-sectional data

Genotyping

We genotyped both cross-sectional and longitudinal animals for 346 single nucleotide polymorphisms (SNPs) in 127 genes. See [3] for details of the approach used to select

these SNPs. Our list included two synonymous and tightly linked (r^2 = 0.50) SNPs in the gene *Fcer1a* (the alpha chain of the high-affinity receptor for IgE; see Fig S1 for location) which we had previously identified as a candidate tolerance gene in a natural population of *M. agrestis.* In this previous work, we had identified three haplotypes at this locus present in our population: GC, AC and AT, at frequencies of 0.12, 0.76 and 0.07 respectively. We had also identified the GC haplotype as being of particular interest, given its significantly lower expression level of the transcription factor GATA3 (a biomarker of tolerance to macroparasites in our population) compared to the other haplotypes [3]. We concluded that this haplotype marked a causal (non-synonymous) mutation. DNA was extracted from a tail sample (longitudinal component) or an ear sample (cross-sectional component) taken from the animal using DNeasy Blood and Tissue Kit (Qiagen). Genotyping was then performed by LGC Biosearch Technologies (Hoddesdon, UK; http://www.biosearchtech.com) using the KASP SNP genotyping system. This included negative controls (water) and duplicate samples for validation purposes. The resulting SNP dataset was used for two purposes (i) genotyping individuals within the locus of interest, and (ii) pedigree reconstruction (see below).

Pedigree reconstruction

We used a subset of our SNP dataset to reconstruct a pedigree for both cross-sectional and longitudinal animals using the R package *Sequoia* [50]. SNPs which violated the assumptions of Hardy-Weinberg equilibrium were removed from the dataset. For pairs of SNPs in high linkage disequilibrium (most commonly within the same gene), the SNP with the highest minor allele frequency (MAF) was chosen. A minimum MAF cut-off of 0.1 and call rate of > 0.7 was then applied, and any samples for which more than 50% of

SNPs were missing were removed. This resulted in a final dataset including 114 SNPs - a sufficient number for very good performance of parentage assignment [50].

Life history information, namely sex and month of birth, was inputted into *Sequoia* where possible. Juvenile voles weighing less than 12 g on first capture were assigned a birth date 2 weeks prior to capture. Juvenile voles weighing between 12 and 15 g on first capture were assigned a birth date 4 weeks prior to capture. Finally, adult voles breeding on first capture, were assigned a birth date 6 weeks prior to capture (minimum age at first breeding) [51,52]. Adult voles not breeding on first capture could not be assigned a birth date, as it was not known whether they had previously bred or not. Virtually all samples (99%) were assigned a sex, and approximately half (54%) were assigned a birth month. As we sampled individuals from across seven different clear-cut areas of the forest, each several kilometres apart, these were assumed to be independent, closed populations with negligible dispersal. Site-specific pedigrees were therefore generated.

We assessed the accuracy of our reconstructed pedigrees by checking whether predicted parent-offspring pairs met expectations given the biology of *M. agrestis*. As expected, the majority of predicted parent-offspring pairs (87%) were born in the same year. We also expected parents and offspring to overlap in space. Again, as expected, the majority of predicted parent-offspring pairs (92%) were, at some point, found along the same transects (horizontal or vertical). We also inspected log10 likelihood ratios (LLRs) for parent pairs as recommended in the user manual for *Sequoia*. Almost all LLRs were positive (n = 698/720 or 97% of LLRs) indicating confidence in our assignments. Individuals with vs. without our haplotype of interest did not differ in their probability of appearing in a pedigree ($\chi^2 = 0.09$; d.f. = 1; $p = 0.76$). For each individual

that ended up in a pedigree i.e. with one or more relatives recorded (*n* = 652; Site COL = 3; Site BLB = 125 ; Site GRD = 204; Site CHE = 137; Site RAV = 16; Site SCP = 90; Site HAM = 77), the number of offspring was counted to provide a measure of their reproductive success. Few individuals were first trapped as juveniles, with the majority trapped as adults which had already recruited into the population. Our measure of reproductive success then, more closely resembles the number of recruited (rather than newborn) offspring per individual. Half of individuals present in our pedigrees (*n* = 325) were found to have no offspring. We expect the majority of these to be true zeros (representing actual reproductive failure) as we generally sampled a large proportion of the total population within clear-cuts. We also minimised the chance of false zeros by excluding those individuals (e.g. at the periphery of a study grid) which did not end up in a pedigree because we identified no relatives (including offspring), likely because we had not sampled in the right place.

Statistical analyses

Not all individuals appeared in all datasets, therefore sample sizes (reported in Table 1) vary between analyses. All analyses were performed in R statistical software version 3.5.2 [53].

Differential gene expression analysis

DGE analysis was performed on filtered and normalized count data using the R package edgeR [54], the aim being to identify individual genes which were differentially expressed between those individuals with and without a copy of the GC haplotype. Only those individuals for which haplotype could be inferred with certainty could be included (*n* = 53

males and *n* = 31 females). Samples from different sexes were analysed together. To test whether the top-responding genes differed between the sexes, we included a dummy variable with four levels (males with haplotype, males without haplotype, females with haplotype, females without haplotypes) and inspected the contrasts of interest (males with versus without haplotypes; females with versus without haplotype). As this was an exploratory analysis, used in conjunction with more targeted measurements of immune gene expression (see below), model specification was kept as simple as possible.

Having confirmed a sex-specific effect of the GC haplotype on the expression of some inflammatory genes (see Results) in the initial DGE analysis, we ran separate DGE analyses for males and females and tested more broadly, for enrichment of pro- and anti-inflammatory genes in our results; more specifically, the gene ontology terms, 'positive regulation of inflammatory response' (GO:0050729; *n* = 143) and 'negative regulation of inflammatory response' (GO:0050728; *n* = 149). The aim here was to answer the question: are pro- or anti-inflammatory genes more highly ranked relative to other genes, when we compare individuals with and without the GC haplotype, and does this also differ between the sexes? This GSEA was performed using the R package limma [55], and genes were ranked on log fold change.

Haplotype association analyses

Following the exploratory DGE analysis, the GC haplotype was tested for associations with (i) gene expression assayed by Q-PCR to validate these results, (ii) macro- and microparasite infection to test whether the GC haplotype predicted an individual's susceptibility to infection, and (iii) reproductive success to test whether the GC haplotype

was associated with any reproductive costs. Given the well-established link between inflammation and oxidative stress [56,57], we also tested for an association between the GC haplotype and SOD1 activity.

For all analyses, this was initially attempted using the R package hapassoc [58] in order to maximise sample size (see below). Hapassoc infers haplotypes on the basis of data from single SNPs, and allows likelihood inference of trait associations with resulting SNP haplotypes and other attributes. It adopts a generalized linear model framework and estimates parameters using an expectation–maximization algorithm. Hapassoc models assumed an additive genetic model. If the haplotype combination of an individual cannot, with certainty, be inferred from its genotyping data (i) because it is heterozygous at two or more markers or (ii) because it has missing data for a single marker, the approach implemented in hapassoc is to consider all possible haplotype combinations for that individual. Standard errors accounting for this added uncertainty are calculated using the Louis' method [59]. We compared the GC haplotype against the other two major haplotypes (AC and AT). Another haplotype, the GT haplotype, was identified in the population but this was present at such low frequencies (frequency < 0.01 among individuals for which haplotype could be inferred with certainty) that it was omitted from all analyses. Results reported in the text for macroparasites and SOD1 activity come from these hapassoc models.

However, there are some restrictions on model specification within hapassoc (e.g. random terms cannot be included, limited choice of error distributions), so this was followed up with regression models for some analyses. These regression models only included those individuals whose haplotype combination could be inferred with certainty.

Results reported in the text for gene expression assayed by Q-PCR, microparasites and reproductive success come from these regression models. As in the DGE analysis, genotype was coded as the presence or absence of the GC haplotype. Regression models were run using the R package lme4 [60] or glmmADMB [61,62].

Table 1 provides a summary of (hapassoc or regression) model specifications. As we found evidence for sex-specific effect in the initial DGE analysis, with the exception of the model for reproductive success (see below), all models included a genotype by sex interaction which was retained if it improved model fit. All models also accounted for other biological and technical covariates (the choice of which was informed by our previous work, the literature and/or our experimental design). Full regression models (including all covariates and interactions of interest) were reduced using backward stepwise deletion of non-significant terms to minimize Akaike's information criterion (AIC), following the drop1 function in the lme4 package. This was not possible for hapassoc models. Summary tables including all estimates, standard errors and *z*-statistics are included in the Supplementary information. Associated significance values are also included in these tables, except for LMMs and GLMMs, for which significance values were based on likelihood ratio tests (LRTs) and are reported in the main text. Throughout, residuals from regression models were checked for approximate normality and homoscedasticity, and all covariates were tested for independence using variance inflation factors (all VIFs < 3).

Table 1. Model specifications including, for each model: covariates included in the full model, datasets used and sample sizes.

	DGE analysis	Haplotype association analyses			
		Response variable			
		Immune gene expression assayed by Q-PCR	Parasite infection	Reproductive success	SOD1 activity
Covariates					
Snout-vent length		Y			Y
Eye lens weight		Y			Y
Reproductive status		Y	Y		Y
Body condition		Y	Y		Y
Birth month				Y	
Culled or not				Y	
Site		Y	Y	Y	Y
Year		Y	Y	Y	Y
Season		Y	Y		Y
Assay plate		Y (*Il17a* LMM)			
Dataset[a]	C	C	C (Macroparasites) L (Microparasites)	C + L	C
Sample size	♀31 ♂53	*Il17a* LMM: ♀73 ♂220	Macroparasites: ♀82 ♂235 *B. microti*: ♀1075 ♂1247 *Bartonella* spp.: ♀1283 ♂1104	♀419 ♂232	♀81 ♂227

[a] C, cross-sectional; L, longitudinal.

Association between GC haplotype and immune gene expression assayed by Q-PCR

As we ran a total of 18 hapassoc models (one model per gene), the Benjamini and Hochberg method of correction was applied to all *p*-values [63]. Resulting *q*-values (FDR-corrected *p*-values) are reported, alongside original *p*-values (Table S3). A hapassoc model including a genotype by sex interaction was tested against a null model without an interaction term. A Gamma error distribution and log link were used. Other covariates considered potential drivers of immune gene expression and included in both models, were informed by our previous work [47]. These included snout-vent length (SVL), eye lens weight (categorised into seven intervals; SVL and eye lens weight

represent the combined influence of age and historical growth trajectory), reproductive status (males were considered to be reproductively active if they had descended testes; females if they were pregnant or had perforate vaginas) and body condition (estimated by regressing body weight against life history stage, SVL and its quadratic term). Site, year and season (four levels, designated as spring [March & April], early summer [May and June], late summer [July and August) and autumn [September and October]) were included to account for any spatial and/or temporal autocorrelation. All covariates were included as fixed effects.

In order to confirm these results, a linear mixed effects model (LMM) was run for a single immune gene for which expression appeared to be associated with genotype (q = 0.037). The LMM included random terms for site and season, as well as assay plate number (Table 1). The latter was included to account for nonindependence due to immunological assaying structure. All other covariates were the same as the hapassoc model. A Yeo-Johnson transformation (with $\lambda = -2$) was used to achieve more normal and homoscedastic residuals [64].

Association between GC haplotype and parasite infection

The three macroparasite measurements taken from cross-sectional animals (counts of ticks, fleas and cestodes) were log-transformed ($\log_{10}[x+1]$) and summarised as a single principal component (explaining 39% of total variation; Table S12) to avoid difficulties in interpretation due to multiple testing. See [3] and [11] for full details of this approach. This combined measure of macroparasite burden was modelled using a hapassoc model with a Gaussian error distribution.

Microparasite infection status was assessed multiple times for the majority of individuals in the longitudinal component of the study (mean = 2.8; range = 1-11). Due to these repeated measures, microparasite infection could not be modelled using hapassoc. Instead, to test for an association between the GC haplotype and the probability of an individual being infected with a microparasite, we ran a GLMM with a a binary response (infected or not), log link and a random term for individual. Other covariates, considered potential drivers of both macro and microparasite infection were, again, informed by our previous work [3,11,65]. These included body condition, reproductive status, year, season and site (Table 1). Season and site were included as random terms in the GLMM for microparasites, as was individual identity, to account for nonindependence due to repeat sampling.

Association between GC haplotype and reproductive success

Our measure of reproductive success was zero-inflated (50% zeros). This is consistent with a previous study of the closely-related common vole (*Microtus arvalis*) [66]. A Poisson error distribution was therefore deemed inappropriate and it could not be modelled using hapassoc. Instead, to test for an association between the GC haplotype and reproductive success, we ran a GLM with a quasi-Poisson error distribution. This distribution has been previously used to model predictors of reproductive success in other organisms, and accounts for the overdispersion caused by excess zeros.

We only recorded a reproductive failure (i.e. zero reproductive success) for those individuals with some other familial relationship in our pedigree, purposefully omitting those individuals (e.g. at the periphery of a study grid) for which we may have recorded no relatives (including offspring) simply because we had not sampled in the right place.

Therefore, we expect all (or most) of our zeros to represent actual reproductive failure. For this reason, zero-inflated models were deemed inappropriate.

As detailed in the Results, we tried to run a single quasi-Poisson GLM with both sexes but resulting residual variances differed significantly between the sexes (F test to compare variances of two samples; $p = 0.02$), making it impossible to formally test for a genotype by sex interaction. Instead, we ran a separate model for each sex. We included birth month as a covariate in this model, given that autumn-born voles (of the closely-related common vole) have been shown to have a lower chance to reproduce than spring-born voles [66]. Other covariates included in this model were, whether or not an individual was culled for the cross-sectional component of this study (again, reducing the opportunity to reproduce), site and the year in which an individual was most frequently captured (Table 1). All covariates were included as fixed effects. Only a single female was trapped in one of the sites (COL) and consequently caused convergence problems in the female model. This female was therefore omitted.

Association between GC haplotype and SOD1 activity

SOD1 activity was modelled using a hapassoc model with a Gaussian error distribution. Other covariates, considered potential drivers of SOD1 activity, were informed by the literature. Previous studies on wild rodents have shown that antioxidant levels increase with both reproductive effort [67] and with age [68]. Studies on birds have also shown that improvements in body condition are often accompanied by increases in antioxidant activity, for example in response to supplemental feeding [69]. We therefore included SVL, eye lens weight, reproductive status and body condition as covariates in our model. As in other models, we included site, year and season to account for spatial and/or

temporal autocorrelation in our data (Table 1). All covariates were included as fixed effects.

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Competing interests

The authors declare no competing interests.

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