# Divergent sex-specific effects of an IgE receptor polymorphism: from immunity to health, and ultimately, fitness

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**Abstract**

The genotype of an individual is an important predictor of their immune function, and subsequently, their ability to stay fit and healthy. However, the same genotype, subjected to different environments, can also result in divergent 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 combined data on genotype, immune gene expression, infection incidence and pedigree of individuals in a natural population of field voles (*Microtus agrestis*). We identified a polymorphism in the high-affinity Immunoglobulin E (IgE) receptor (GC and non-GC haplotypes) that has divergent effects on the immunity, health and fitness of males and females. The GC haplotype was associated with increases in the expression of both pro- and anti-inflammatory genes regardless of sex. However, we found that the signal was stronger for pro-inflammatory genes in males with the GC haplotype, who also had the same risk of infection and a lower reproductive success when compared to males with non-GC haplotypes. In contrast, females with the GC haplotype showed a stronger signal for anti-inflammatory genes, a higher risk of infection and the same reproductive success when compared to females with non-GC haplotypes. To our knowledge, this is the first time that a polymorphism with divergent sex-specific effects across all three levels (immunity, health and fitness) has been documented in a natural population.

## Introduction

In order for an individual to stay fit and healthy, 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). 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].

Furthermore, 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 sex-specific effects on long-term fitness. The study of wild animals provides us with an opportunity to (more easily and cheaply) follow large numbers of individuals, 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 fitness, but they did not consider effects on health. We know of no documented example of a polymorphism with sex-specific effects on immunity, health *and* fitness in a natural population.

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 [14], 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 [15–17], but whole networks of interacting genes [18,19]. The same genotype then, could be expressed as different immune phenotypes, and subsequently have very different consequences for the health and fitness 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 underestimating the effect of genetic variation on the health and fitness of individuals.

In humans, immunoglobulin E (IgE) mediated responses are associated with defence against helminths [20] and with allergy [21]. 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 [22]. Naturally occurring polymorphisms in FCER1 are known to affect an individual's serum IgE levels, with consequences for their susceptibility to infection [23,24] and their risk of developing inflammatory disease [25–28]. Furthermore, sex differences in serum IgE levels [29] and the incidence of IgE-mediated inflammatory disease [30] 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) [31].

In a previous study of a natural population of field voles (*Microtus 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 both males and females. In doing so, we provide the first example of a polymorphism with sex-specific effects not only on immunity, but on health, and

ultimately, on fitness in a natural population. Moreover, we show that this polymorphism has divergent outcomes for males and females.

## Results

We sampled a natural population of *M. agrestis* in Kielder Forest, Northumberland, United Kingdom, over three year (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). For each sex, 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.

### Males with the GC haplotype have a stronger pro-inflammatory response and lower reproductive success

First, we used the cross-sectional component of our study to test the consequences of the GC haplotype for inflammation in males. Differential gene expression (DGE) analysis followed by gene set enrichment analysis (GSEA) performed on 53 males assayed by RNASeq showed that males with the GC haplotype increased their expression of both pro- and anti-inflammatory genes (when compared to males without the haplotype) but with a stronger signal for pro-inflammatory genes (pro-inflammatory genes: $p < 0.01$; anti-inflammatory genes: $p = 0.03$; Fig 1A, upper panel). Males with the GC haplotype also expressed the pro-inflammatory cytokine, *Il33*, at a level 7 times higher than males without the haplotype (rank = 2/12904, log fold change (logFC) = 2.8, $p < 0.0001$, $q$ =

0.03; Table 1). In contrast, *Il33* was ranked markedly lower in females (rank = 2848/12904, logFC = 0.64, $p$ = 0.38, $q$ = 1.00). Further confirmation of the role of the GC haplotype in modulating the pro-inflammatory response in males was provided by 223 males assayed by Q-PCR (for a panel of 18 immune genes) which indicated that, on exposure to the TLR7 agonist, imiquimod, expression levels of *Gata3* (previously identified in this population as a biomarker of tolerance [11]) were significantly associated with the GC haplotype. Consistent with our previous study, males with the GC haplotype expressed *Gata3* at a level 0.92 times lower than males without the haplotype ($p$ = 0.03; Tables S1 & S2).

We then used both cross-sectional and longitudinal components of our study to test the consequences of the GC haplotype for the probability of infection with common micro- and macro-parasites, number of offspring and antioxidant superoxide dismutase 1 (SOD1) enzymatic activity in males. We found no significant effect of the GC haplotype on the probability of a male being infected with *Babesia microti* ($p$ = 0.71; Fig 1B, upper panel) or *Bartonella* spp. ($p$ = 0.38), two common micro-parasites in our population. We also found no effect of the haplotype on infection by ticks, fleas and cestode, common macro-parasites in our population, summarised by a single principal component ($p$ = 0.30). We genotyped both cross-sectional and longitudinal voles for 113 SNPs and used this dataset to construct a pedigree. According to this pedigree, males with the GC haplotype had an average of 2.6 fewer offspring than males without the haplotype ($p$ = 0.03; Fig. 1C, upper panel). Males with the GC haplotype also had 2.1 times higher SOD1 activity when compared with the AT haplotype, but this was only a borderline significant tendency ($p$ = 0.06; Table S3).

**Females with the GC haplotype have a stronger anti-inflammatory response and a higher risk of infection**

Analogous to our analysis for males, we first tested the consequences of the GC haplotype for inflammation in females. DGE analysis followed by GSEA performed on 31 females assayed by RNASeq showed that females with the GC haplotype also increased their expression of both pro- and anti-inflammatory genes (when compared to females without the haplotype) but with a stronger signal for anti-inflammatory genes (anti-inflammatory genes: $p < 0.0001$; pro-inflammatory genes: $p = 0.05$; Fig 1A, lower panel). The top-ranking gene for females was also the anti-inflammatory suppressor of cytokine signalling *Socs3* (logFC = 1.2, $p < 0.0001$, $q = 0.04$; Table 1). In contrast, *Socs3* was in the bottom 1% of genes for males (rank = 12840/12904, logFC = 0.00, $p = 0.99$, $q = 1.00$). In females, we found no effect of the GC haplotype on the expression levels of the 18 immune genes that we assayed by Q-PCR (Table S4).

We then tested the consequences of the GC haplotype for the probability of infection with micro- and macro-parasites, number of offspring and SOD1 activity in females. Females with the GC haplotype were 3.2 times more likely to be infected with *B. microti* than females without the haplotype ($p < 0.01$; Fig 1B, lower panel). However, there was no significant effect of the GC haplotype on the probability of a female being infected with *Bartonella* spp. ($p = 0.23$), and no significant effect of the haplotype on macroparasite infection ($p = 0.31$). Females with the GC haplotype did not differ significantly from females without the haplotype in their number of offspring ($p = 0.37$; Fig 1C, lower panel) or their SOD1 activity ($p = 0.90$; Table S5).

## Discussion

In this study, we describe the first example of a polymorphism with divergent sex-specific effects linking together immunity, health and ultimately fitness in a natural population.

Our results suggest opposing effects of the same 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. 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. In a previous study, Beldomenico et al. 2008 [32] established an indoor colony of *M. agrestis* from the same study population. By keeping infection to a minimum, they were able to look at underlying differences in response (rather than parasite exposure). They found that neutrophil counts (an indicator of inflammatory response) were significantly lower in breeding females than males, suggesting that females in our study population are naturally less pro-inflammatory than males. This could be because females are 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). 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 [33]. Therefore, the polymorphism appears to 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 [14], and could be driving these opposing effects.

Our results also suggest divergent effects of the same polymorphism on the immune defence strategy of males and females. In our previous study, we showed that males with the GC haplotype had a lower level of an immunological marker of tolerance to macroparasites (i.e. they were less tolerant) [3]. Here, we corroborate this and use data on infection incidence to show that 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 [34]. 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 [35]. *B. microti* may therefore represent a community of parasites, to which this haplotype affects resistance in females. Males with the GC haplotype may also be more resistant to, and have a lower incidence of, some unmeasured parasite as a result of their pro-inflammatory bias. If so, males with the haplotype would be less tolerant and more resistant, consistent with evidence for a negative genetic correlation (or trade-off) between these two defence strategies [36–39]. Disease effects on reproduction, like those observed in this study (see below), can cause cycles of both the host population and the disease prevalence [40]. Therefore, the parasite in question could be currently circulating in the population, or a recurrent parasite (currently absent from the population, or present at very low prevalence).

While effects of the polymorphism on infection risk were visible in females only, the opposite was true of effects on reproductive success, which were visible in males only. Our results therefore suggest divergent outcomes of the polymorphism for males and females. We can be confident that this reflects a true difference between the sexes, rather than reduced power to detect an effect in one sex, as sample sizes were either balanced with respect to sex (infection risk) or biased towards the sex that showed no visible effect of the polymorphism (reproductive success). However, we cannot rule out that, rather than an effect being absent in one sex, it was simply too small to be detected in our study.

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 [41]. Another realistic scenario is that the pro-inflammatory bias of males with the GC haplotype led to a heightened level of oxidative stress (as indicated by a tendency for higher SOD1 activity), which damaged sperm and negatively impacted on fertilising success. Sperm competition can be an important feature in the reproduction of Microtines e.g. meadow vole (*M. pennsylvanicus*) [42], 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) [43] which are generated at sites of inflammation [44]. In addition, the heightened level of oxidative stress experienced by these males may have reduced their investment in sexual signals e.g. major urinary proteins (MUPs) making them less attractive to females and negatively impacting on mating success [45].

Despite the immune system playing an important role in determining the health and 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. Sexually antagonistic selection, where a mutation is beneficial to one sex and harmful to the other, 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]. In the present study, conducted between 2015-2017, 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 risk, suggests that it may be under balancing selection. 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 divergent outcomes not only for the immunity, but the health and ultimate fitness 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, 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, 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, UK) for use in parasite detection (see below). On initial and subsequent captures, we administered general-use anti-helminthic (Profender, Bayer AG) and anti-ectoparasitic treatment (Frontline, Boehringer Ingelheim) to half of the trapped population (those voles with PIT tags ending in an even number, except for pregnant females).

***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), 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. For *B. microti* we used the forward primer TBCbabF and reverse primer TBCbabR targeting the 18S ribosomal RNA gene and for *Bartonella* spp. we used the forward primer TBCbartF and reverse primer TBCbartR targeting the 16S ribosomal RNA gene. 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 (diluted 1/20) and PrecisionFAST qPCR Master Mix (PrimerDesign) with low ROX and SYBR green and primers at the machine manufacturer's recommended concentrations. Alongside the pathogen assays we also ran assays for two host genes (*Ywhaz* and *Actb*) that acted as endogenous controls. 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) following manufacturer's instructions. 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 ΔΔCt

method, indexed to the calibrator sample. 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 cross-sectional voles 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, 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. 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 were recorded.

### *SOD1 measurement*

Given the well-established link between inflammation and oxidative stress [44,46], we measured antioxidant superoxide dismutase 1 (SOD1) enzymatic activity in blood samples taken from cross-sectional animals. 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), 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). Given the known

link between polymorphism in FCER1 and risk of developing inflammatory disease [25–28], we then exposed splenocytes from the remaining cross-sectional animals to inflammatory stimulation *in vitro*, namely the TLR2 agonist heat-killed, *Listeria monocytogenes* (HKLM; concentration 5 x $10^7$ cells $ml^{-1}$; Invivogen, Paisley, UK), and the TLR7 agonist, imiquimod (IMI; concentration 10 µg $ml^{-1}$; Invivogen). 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 [48]. 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 S6) in HKLM- and IMI-stimulated splenocytes 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 (23 sets, including 2 endogenous control genes) were designed *de novo* and supplied by PrimerDesign (Chandler's Ford, U.K.) (15 sets) or designed *de novo* in-house (8 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 [49]. 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 113 SNPs.

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) [50,51]. 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.

As expected from the biology of *M. agrestis*, the majority of predicted parent-offspring pairs were born in the same year (87%) and were found in similar areas (i.e. along the same transects; 90%). 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 present in a pedigree ($n = 644$), 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. Although we sampled a large proportion of the total population within clear-cuts, our sampling was not exhaustive, resulting in incomplete pedigrees. For example, half of individuals present in our pedigrees ($n = 322$) were found to have no offspring. Although some of these are true zeros, others may be false i.e. individuals that produced offspring which were not detected. We therefore consider our measure of reproductive success to be relative (rather than absolute).

**Statistical analyses**

Not all individuals appeared in all datasets, therefore sample sizes (reported in Table 2) vary between analyses. Site, being a factor with an intermediate number of levels, was included, interchangeably, as either a fixed or a random effect in models. All analyses were performed in R statistical software version 3.5.2 [52].

***Differential gene expression analysis***

DGE analysis was performed on filtered count data using the R package edgeR [53], 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. Samples from different sexes were analysed separately. 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. As all animals used to assay expression by RNASeq were collected within a short timeframe, between July and October 2015, temporal autocorrelation was deemed to be negligible for the purpose of this analysis. Only site was included as an additional covariate, in order to account for spatial autocorrelation.

Given the known link between polymorphism in FCER1 and risk of developing inflammatory disease [25–28], we tested 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? This GSEA was performed using the R package limma [54], 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

infection risk, and (iii) reproductive success to test whether the GC haplotype was associated with any fitness costs. Given the well-established link between inflammation and oxidative stress [44,46], 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 [55]. 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 [56]. 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. 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 [57] or glmmADMB [58,59].

Table 2 provides a summary of (hapassoc or regression) model specifications. All models were run separately for males and females and accounted for other biological and technical covariates (the choice of which was informed by our previous work, the literature and/or our experimental design). 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).

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

As we ran a total of 18 hapassoc models per immune agonist (one model per gene), the Benjamini and Hochberg method of correction was applied to all *p*-values [60]. Resulting *q*-values (FDR-corrected *p*-values) are reported in the Supporting information, alongside original *p*-values (Tables S1 & S4). In order to confirm these results, generalised linear mixed effects models (GLMMs) were run for those immune genes for which expression appeared to be associated with haplotype ($q < 0.1$) only and these are reported in the main text. A Gamma error distribution and log link were used for both hapassoc models and GLMMs. Other covariates considered potential drivers of immune gene expression, 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. GLMMs included random terms for site and season, as well as assay plate number (Table 2). The latter was included to account for nonindependence due to immunological assaying structure.

***Association between GC haplotype and parasite infection***

The three macroparasite measurements taken from cross-sectional animals (counts of ticks, fleas and cestodes) were summarised as a single principal component (explaining 37% of total variation). 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 binomial error distribution, 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 [3,11,61]. These included whether or not an individual was targeted for anti-parasite treatment, body condition, reproductive status,

year, season and site (Table 2). 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***

Due to our measure of reproductive success being highly zero-inflated (50% zeros), it could not be modelled using hapassoc. Instead, to test for an association between the GC haplotype and reproductive success, we ran negative binomial, zero-inflated Poisson (ZIP) and zero-inflated negative binomial (ZINB) generalised linear models (GLMs) and compared them on AIC. The negative binomial GLM (with log link) had the lowest AIC and achieved normal and homoscedastic residuals, and is therefore reported on in the Results.

We included birth month as a covariate in this model, given that autumn-born voles have been shown to have a lower chance to reproduce than spring-born voles [62]. 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 year (Table 2). Whether or not an individual was targeted for anti-parasite treatment was not included as a covariate, as this was found to have no effect on reproductive success (Mann-Whitney U = 180290; d.f. = 1; $p$ = 0.60). All covariates were included as fixed effects. A single female from the Site COL caused convergence problems in the female model and was subsequently omitted from both female and male models (for consistency).

***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 [63] and with age [64]. 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 [65]. 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 2). All covariates were included as fixed effects.

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## Figures and Tables

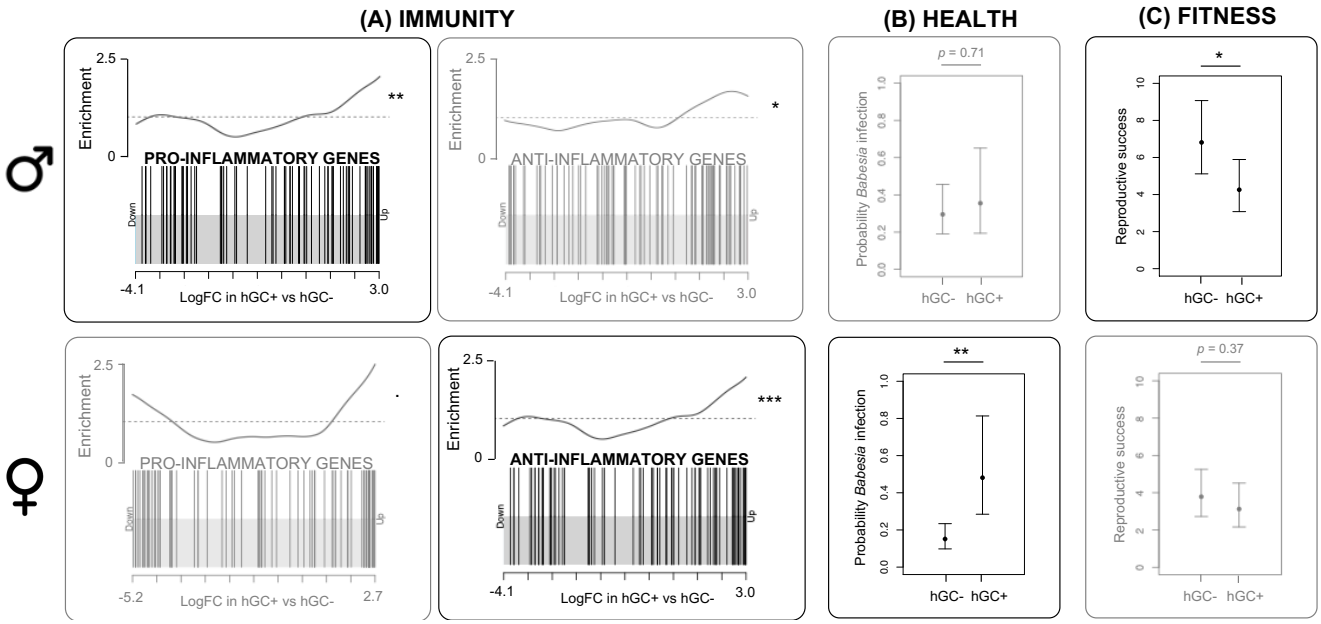


**Figure 1.** Divergent effects of GC haplotype (hGC) on males and females. Upper panel: males. Lower panel: females: (A) Immunity: Barcode plots indicating the position of pro- and anti-inflammatory genes in a ranked list of log fold changes (logFC) for individuals with (hGC+) vs. without (hGC-) the haplotype, showing that males with the haplotype have a pro-inflammatory bias, whereas females have an anti-inflammatory bias. (B) Health: Association between hGC and the probability of infection with *Babesia microti,* showing that only females with the haplotype have an increased risk of infection. (C) Association between hGC and reproductive success, showing that only males with the haplotype have lower reproductive success (error bars represent ± standard error; · indicates $p < 0.1$; * indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$).

**Table 1.** For each sex, genes which were differentially expressed between individuals with vs. without the GC haplotype, including associated log fold changes (logFC), *p*-values and *q*-values (FDR-corrected *p*-values). Genes with $q < 0.05$ were considered differentially expressed.

| **Sex** | **Gene** | **Protein** | **LogFC** | ***p*-value** | ***q*-value** |
|---|---|---|---|---|---|
| ***Males*** | *Snai3* | Zinc finger protein SNAI3 | 1.57 | < 0.0001 | 0.03 |
| | *Il33* | Interleukin-33 | 2.83 | < 0.0001 | 0.03 |
| | *Uba7* | Ubiquitin-like modifier activating enzyme 7 | 0.74 | < 0.0001 | 0.04 |
| ***Females*** | *Socs3* | Suppressor of cytokine signalling 3 | 1.18 | < 0.0001 | 0.04 |

**Table 2.** Model specifications including, for each model: covariates included, 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 |
| Treated or not | | | Y | | |
| Reproductive status | | Y | Y | | Y |
| Body condition | | Y | Y | | Y |
| Birth month | | | | Y | |
| Culled or not | | | | Y | |
| Site | Y | Y | Y | Y | Y |
| Year | | Y | Y | Y | Y |
| Season | | Y | Y | | Y |
| Assay plate | | Y (GLMM only) | | | |
| **Dataset**[a] | C | C | C (macroparasites)<br>L (microparasites) | C + L | C |
| **Sample size** | ♀31<br>♂53 | ♀59-79<br>♂223 | C: ♀82<br>♂235<br>L: ♀1247-128<br>♂1075-1104 | ♀415<br>♂229 | ♀80<br>♂227 |

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