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Early-life immune expression profiles predict later-life health and fitness in a wild rodent

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Abstract

Individuals differ in the nature of the immune responses they produce, affecting disease susceptibility and ultimately health and fitness. These differences have been hypothesized to have an origin in events experienced early in life that then affect trajectories of immune development and responsiveness. Here, we investigate how early-life immune expression profiles influence life history outcomes in a natural population of field voles, *Microtus agrestis*, in which we are able to monitor variation between and within individuals through time by repeat sampling of individually marked animals. We analysed the co-expression of 20 immune genes in early life to create a correlation network consisting of three main clusters, one of which (containing *Gata3*, *Il10* and *Il17*) was associated with later-life reproductive success and susceptibility to chronic bacterial (*Bartonella*) infection. More detailed analyses supported associations between early-life expression of *Il17* and reproductive success later in life, and of *Il10* expression early in life and later infection with *Bartonella*. We also found significant association between an *Il17* genotype and the early-life expression of *Il10*. Our results demonstrate that immune expression profiles can be manifested during early life with effects that persist through adulthood and that shape the variability among individuals in susceptibility to infection and fitness widely seen in natural populations.

KEYWORDS
Bartonella, early life, Ecoimmunology, interleukin 10, interleukin 17, *Microtus agrestis*

1 | INTRODUCTION

Differences at birth, or experienced during neonatal or juvenile development, are well understood to affect an individual's later immune competence and health. Nonetheless, the relative contributions of different immunological pathways to such predetermined ontogenetic trajectories are less well understood. Here, through monitoring of multivariate immunological phenotypes and life history outcomes in cohorts of wild field voles (*Microtus agrestis*), we begin to disentangle what types of immune response are involved in the fixation and subsequent unfolding of early-life influences.

In the field of human health, the importance of early life effects involving immunity is well established (Dowling & Levy, 2014). It is embodied in influential ideas such as the developmental origins theory (Barker, 2007; Wadhwa et al., 2009), that traces the aetiology of chronic inflammatory and allergic disorders to early-life events, or the hygiene hypothesis (Ober et al., 2017; Von Mutius & Vercelli, 2010),

that links early-life microbial exposure to the development of a well-regulated immune system. Indeed, the well-known training of the immune system, by maternal influences (Apostol et al., 2020) and by environmental exposures to immunogens and microbes (Dowling & Levy, 2014), provides an obvious mechanism through which early-life events can determine later phenotypes. Coupled with the effects of genetic polymorphisms (Brodin et al., 2015), early-life environmental exposures can give rise to substantial variation among individuals in their immune responses (Khafipour & Ghia, 2013; Shao et al., 2019; Stewart, Ajami, et al., 2018).

Studies of natural vertebrate systems, such as we carry out here, are an important counterpoint to studies in anthropogenic contexts (i.e. in humans, in laboratory models or in domesticated animals), as any observed responses are expected to be adaptive to a natural environmental setting and honed by natural selection. Results from natural systems may provide valuable external reference points for studies in anthropogenic systems, allowing conserved patterns to be identified, or, on the other hand, highlighting unappreciated phenotypic possibilities that may not be easily observable in the laboratory or in humans or captive animals subject to limited environmental variation or standardized conditions. Moreover, the high degree of functional and genetic conservation apparent in the immune system of the mammalian lineage, or, indeed, even in the broader jawed vertebrate lineage (Flajnik & Kasahara, 2001; Redmond et al., 2018), makes such comparative study potentially highly informative.

Previous studies of early-life effects on immunity in natural populations have typically considered few immune parameters. For example, a number of avian studies have shown associations between broad-brush measures of nestling immune function, such as responsiveness to phytohemagglutinin (PHA) injection, and recruitment (Bowers et al., 2014; Cichoń & Dubiec, 2005; López-Rull et al., 2011; Moreno et al., 2005). However, immunity involves a complex network of interacting molecular and cellular pathways—which drive a spectrum of distinctive effector and regulatory responses with different roles in protection and immunopathology. To better represent this, multivariate measurements, of the expression of different pathway components, are required. The existence of genome and transcriptome sequences makes this possible in non-model organisms, such as the field vole, via mRNA transcript measurements of genes with known roles in conserved pathways. Here, we use high throughput Q-PCR (quantitative real-time polymerase chain reaction) to measure the transcriptional expression of key immune regulators such as cytokines that are indicative of activity within different conserved immune pathways (Jackson et al., 2011; Turner & Paterson, 2013).

Our study sites are located in the Kielder Forest area of north-eastern England, where the ecology of *M. agrestis* is well studied (reviewed in Turner et al., 2014). Populations undergo locally synchronous multi-annual density fluctuations tending towards a period of 3–4 years, and range in density from 5–770 voles ha^{-1} on individual grassy patches. The voles have high fecundity but high population turnover. *M. agrestis* are a short-lived species (typical lifespan of 1 month, with survival rate driven primarily by seasonal effects and only varying between 0.5–0.8 within our trapping period; Burthe et al., 2008; see below). Body size is sufficiently large that even juvenile specimens can be unobtrusively fitted with a Passive Integrated Transponder (PIT tag), allowing unambiguous individual identification thereafter. This study system thus allows us to sample tagged individuals repeatedly (at 2-weekly intervals) through their lives, measuring multivariate immunophenotypes which can be related to subsequent variations in measures of health, including responses to infection determined through molecular diagnostics, and fitness, estimated from a SNP pedigree. By integrating such data, we asked what components of immune expression early in life predict: (1) the reproductive success of mature individuals (a measure of fitness), and (2) the response to infection of mature individuals (a measure of health).

2 | MATERIALS AND METHODS

2.1 | Field design and animals

M. agrestis were live-trapped from natural populations in Kielder Forest, Northumberland, UK, from 2015–2017 across seven different sites. All sites were grassy forest clear-cuts that represent an optimal habitat for field voles (Turner et al., 2014). Trapping was carried out year-round, except during the winter months, with trapping sessions taking place at approximately 2-week intervals. At each trapping session, voles were trapped at 4 different sites. During the study, 3 sites were reassigned due to practical constraints, giving a total of 7 different sampling sites. At each site, 150–197 Ugglan small mammal traps (Grahnab) were laid out in a grid spaced 3–5 m apart. Traps were placed in permanent (pegged) stations in runs and feeding sites used by voles (as indicated by field signs). Trapping sessions lasted 3 days, with traps checked morning and afternoon.

On first capture, voles were injected with a Passive Integrated Transponder (PIT tag; AVID) for unique identification. On first and subsequent captures, a blood sample was taken whenever possible from the tail tip for immune gene expression assays and parasite detection, and on first capture a tail sample was taken for genotyping for pedigree reconstruction (see below). Other basic information was recorded including the snout–vent length, weight and pelage (for ageing). Some of these tagged voles were culled later in life for more detailed measurements to be taken (e.g. lens weight for ageing purposes; see below). Those voles which were sampled on the final day of each 2-weekly trapping session, which had been previously captured on multiple occasions, and which were not pregnant or lactating females were prioritized for culling (up to 20 per session). The majority of culled voles were adults ($n = 432/510$ or 85%). 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.)

The whole study population comprised 2881 tagged individuals from which a total of 6288 blood samples were collected. In this study, we primarily focussed on 223 tagged individuals which were first blood sampled in early life, that is, prior to breeding (at 6 weeks or less) and from which a total of 616 blood samples were collected. However, we also used the whole study population to reconstruct the

pedigree (see below). We used trapping records and known relationships between vole biometric data and age to estimate age for each individual. Based on previous work on the same study population (Begon et al., 2009; Gebert, 2008), voles weighing 11 g or less were assumed to be juveniles aged 2.5 ± 0.5 weeks old (using the halfway point between the minimum age of 2 weeks and the maximum age of 3 weeks) and voles weighing more than 11 g but no more than 15 g were assumed to be juveniles aged 4.5 ± 1.5 weeks (using the halfway point between the minimum age of 3 weeks and the maximum age of 6 weeks). Those voles weighing more than 15 g but aged as juvenile on pelage, and those voles aged as subadult on pelage were assumed to be 6 weeks old. Finally, those voles aged as juvenile on pelage but with no weight measurement were assumed to be 4 ± 2 weeks old (using the halfway point between the minimum age of 2 weeks and the maximum age of 6 weeks). According to these criteria, a total of 195 voles were first blood sampled prior to breeding.

Some individuals may have first been caught as subadults but not recorded as such due to their adult-like pelage. In order to identify these individuals (and to increase our sample size) we drew on the 510 tagged voles which were culled in later life for more detailed measurements to be taken (e.g. lens weight; see below). We regressed age against eye lens weight for those tagged voles first sampled prior to breeding (for which we had an estimated birth date) that were culled in later life ($n = 99$). The relationship between age and eye lens weight has been previously described (Rowe et al., 1985). A quasipoisson GLM with quadratic and cubic terms for eye lens weight, and log link, provided a good model fit for our data ($r^2 = .56$; Figure S1). We used this regression to predict the ages for all other tagged voles which were culled ($n = 411$). Of these, 28 were inferred to have also been first blood sampled prior to breeding. Therefore, the final number of tagged voles first sampled prior to breeding was 223. These voles were captured a total of 1–30 times throughout their lives (median = 5, SD = 5.89 times) at time intervals of 0–179 days (median = 1, SD = 10.14 days), and blood sampled a total of 1–10 times (median = 2, SD = 1.89 times) at time intervals of 1–179 days (median = 16, SD = 14.64 days).

2.2 | Immune gene expression assays

As in many previous studies, by ourselves and others, we assumed that relative mRNA concentrations would reflect the functional status of the immune system (see e.g., Stewart, Hablützel, et al., 2018). We used SYBR green-based Q-PCR to measure the mRNA expression levels of a panel of 20 immune-associated genes (Table 1) in blood. The choice of our panel of genes was informed by (1) known immune-associated functions in mice, combined with (2) significant sensitivity of gene expression to environmental or intrinsic host variables in our previous studies (Jackson et al., 2011, 2014) or in a recent differential expression analysis of RNASeq data (not reported here).

All primer sets were designed de novo in-house and validated (to confirm specific amplification and 100 ± 10% PCR efficiency under assay conditions). *Ywhaz* and *Actb* were employed as endogenous control genes. We extracted RNA from blood conserved in RNAlater using the Mouse RiboPure Blood RNA Isolation Kit (ThermoFisher), according to manufacturer's instructions. RNA extracts were DNAse treated and converted to cDNA using the High-Capacity RNA-to-cDNA™ Kit (ThermoFisher), according to the 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 manufacturer's 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 three 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 triplets of the standard plate layouts). Individual vole (unknown) samples were assayed in duplicate wells, calibrator samples in triplicate wells and no template controls for each gene were included on each plate. Template cDNA

TABLE 1 Panel of 20 immune-associated genes for which expression levels in blood samples were measured using Q-PCR.

Gene	Protein
Cd8a	T-cell surface glycoprotein CD8 alpha chain
Cd86	T-lymphocyte activation antigen CD86
Cd99l2	CD99 antigen-like protein 2
Foxp3	Forkhead box protein P3
Gata3	GATA binding protein 3
Ifng	Interferon gamma
Irf2	Interferon regulatory factor 2
Irf9	Interferon regulatory factor 9
Tollip	Toll-interacting protein
Retnlg	Resistin-like gamma
Tgfb1	Transforming growth factor beta 1
Il4	Interleukin-4
Il10	Interleukin-10
Il17	Interleukin-17
Il1b	Interleukin-1 beta
Il1rap	Interleukin-1 receptor accessory protein
Il1rn	Interleukin-1 receptor antagonist protein
Apobr	Apolipoprotein B receptor
Orai1	Calcium release-activated calcium channel protein 1
ccn2 (ctgf)	Cellular communication network factor 2

(see above) was diluted 1/20 prior to assay. A main calibrator sample (identical on each plate) was created by pooling cDNA from blood samples taken from many different voles from the study site. As *Tollip*, *Il1rap* and *Irf2* were relatively poorly represented in this main calibrator sample, a synthesized 478 bp gene fragment containing the amplification target for each of these genes was used as an additional calibrator sample (at 10×10^5 copies μL^{-1}) in these cases. Samples from different field sampling groups were dispersed across plate triplets, 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 ΔΔCt method, indexed to the appropriate calibrator samples. Melting curves and amplification plots were individually inspected for each well replicate to confirm specific amplification.

2.3 | Parasite detection

To provide measures of infection susceptibility (herein defined as the probability of being infected) we focussed on the microparasites *Babesia microti* (vectored by ticks) and *Bartonella* spp. (vectored by fleas) in blood. We chose these due to their relatively high prevalence of detectable active infection over the study period in our blood samples. For quantification of infection, we used a strategy analogous to the host gene expression assays above, targeting pathogen ribosomal RNA gene expression, normalizing to the host endogenous control gene expression and indexing to a calibrator sample. We included two extra sets of primers in the blood Q-PCR assays described above (in one of the standard plate layouts for each plate triplet above). For *B. microti* we used the forward primer CTACGTCCCTGCCC TTTGTA and reverse primer CCACGTTTCTTGGTCCGAAT targeting the 18S ribosomal RNA gene and for *Bartonella* spp. we used the forward primer GATGAATGTTAGCCGTCGGG and reverse primer TCCCCAGGCGGAATGTTTAA targeting the 16S ribosomal RNA gene. The primers for *Bartonella* spp. were designed to amplify all nominal species (Telfer et al., 2007) previously recorded in field voles at Kielder. As a calibrator sample we employed the main calibrator sample used for host gene expression in blood (above) in addition to 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 the manufacturer's instructions.

Babesia and *Bartonella* ribosomal RNA relative expression values (calculated by the ΔΔCt method, as for host gene expression) are presumed to relate to the expression of their ribosomal RNA genes and in turn to the intensity of infection. More information on the validation of these assays is provided in the Supplementary Materials & Methods. Briefly, we validated the assays with subsamples of cardiac bloods from culled voles. For one set of samples, we made direct microscopic counts of microparasite stages in blood smears. For another set, we enumerated aligned *Babesia* and *Bartonella* reads from an RNAseq analysis (see Supplementary Materials & Methods; Figure S2; Table S1). We found that Q-PCR relative expression values were highly predictive of both blood smear counts and RNAseq read counts. We recognize that in limiting our diagnostics to *Babesia* and *Bartonella* we may have overlooked the effects of other pathogen and parasite species.

2.4 | Genotyping

We genotyped voles from their tail samples for 346 single nucleotide polymorphisms (SNPs) in 127 genes. See Wanelik et al. (2018) for details of the approach used to select these SNPs. Briefly, we combined information from an external database of human immune genes (Immunome database; http://structure.bmc.lu.se/idbase/Immunome/index.php) with our own existing knowledge of the study system. DNA was extracted from a tail sample taken from the animal using DNeasy Blood and Tissue Kit (Qiagen). Genotyping was then performed by LGC Biosearch Technologies (http://www.biosearchtech.com) using the KASP SNP genotyping system. This included negative controls (water) and duplicate samples for validation purposes.

2.5 | Statistical methods

2.5.1 | Pedigree reconstruction

All analyses were performed in R statistical software version 3.5.2 (R Core Team, 2018). We used a subset of our SNP data set ($n = 114$ SNPs) to reconstruct a pedigree using the R package *Sequoia* (Huisman, 2017). Full details can be found in Wanelik et al. (2019). Briefly, we inputted life history information into *Sequoia* where possible (99% of samples were assigned a sex; 54% were assigned a birth month), and generated site-specific pedigrees (assuming no dispersal between sites, each several kilometres apart). We inspected log10 likelihood ratios (LLRs) for parent pairs, as recommended in the user manual for *Sequoia*. Almost all LLRs were positive (97% of LLRs) indicating confidence in our assignments. For each individual present in a pedigree ($n = 652$; see below), the number of surviving offspring was counted to provide a measure of their reproductive success. Half of individuals present in our pedigrees ($n = 325$) were found to have no surviving offspring. We expect the majority of these to be true zeros (representing actual lack of surviving offspring rather than incomplete sampling) as we sampled a large proportion of the total population within clear-cuts, and we minimized the chance of false zeros by excluding from the pedigree those individuals (e.g. at the periphery of a study grid) for which we recorded no relatives (including offspring) likely because we had not sampled in the right place and, for example, offspring may have dispersed beyond the study grid.

2.5.2 | Correlation networks and cluster analysis

Although the genes we studied were varied and selected from different pathways, nonetheless some redundancy might still be expected in their pattern of expression. Such redundancy is informative as it might reflect co-regulation, or the tendency of different genes to work in functional units. We thus employed a network approach to identify co-expression (correlation) clusters among gene expression variables and to guide onwards analyses. For this, we constructed an expression correlation matrix for all immune genes measured prior to breeding, in early life. We used Spearman Rank correlation coefficients (1) in case of any nonlinear relationships between genes, and (2) because our expression data were not normally distributed. There were some missing data, due to sample degradation, in our expression data set (640/4460 values = 14%). In order to maximize the sample size used to generate each correlation coefficient, we used pairwise complete observations. This resulted in 72% of correlations based on at least 75% of the total samples. For each correlation coefficient, we randomly permuted the data 1000 times to calculate a *p*-value. We also adjusted all *p*-values for multiple testing using the Benjamini–Hochberg method (Benjamini & Hochberg, 1995). We thresholded our correlation network using these corrected *p*-values—only keeping those edges with a significant corrected *p*-value ($p \leq .05$). Genes were clustered on edge betweenness, using the edge betweenness algorithm in igraph (Csardi & Nepusz, 2006). The edge betweenness score of an edge is a measure of the number of shortest paths that go through it. The algorithm identifies densely connected modules by gradually removing edges with the highest edge betweenness scores (Newman & Girvan, 2004). We used these gene clusters in onward analyses.

We then ran an exploratory analysis, repeating the steps above to construct a correlation network for all immune genes measured in early life (now assigned into clusters; see above) and six additional measures of later life or lifetime success (hereafter 'life history outcomes'). The life history outcomes included were: (1) whether or not an individual was recaptured (a proxy for survival), (2) reproductive success in later life (number of surviving offspring in the pedigree), the proportion of later life infected with two major microparasites in our population, namely (3) the bacterium *Bartonella* spp. and (4) the protozoan *Babesia microti*, (5) mean scaled mass index (SMI; a measure of an individual's average condition across their lifetime; Peig & Green, 2009) and (6) coefficient of variation in SMI (a measure of an individual's resilience in maintaining their condition across their lifetime; with the caveat that this life history outcome will be less reliable when based on fewer captures; see Figure S3). There was a lack of variation in the first of these measures, whether or not an individual was recaptured (total of 191 recaptured and 32 not recaptured) which led to zero variance warnings and missing correlation coefficients for some pairwise comparisons. This measure was therefore omitted from the network and included in follow-up analyses only (using a Fisher's exact test; see below). There were slightly more missing data in our final combined expression and life history outcome data set, again due to sample degradation (1078/5575 values = 19%). As before, we used pairwise complete observations. This time, with 45% of correlations based on at least 75% of the total samples.

2.5.3 | Confirming associations between immune clusters and life history outcomes

We identified one immune cluster as containing a counter-intuitive set of genes with mixed functionality (see Section 4: Discussion), while also being enriched with associations between immune expression and life history outcomes (containing two such associations). We considered confirming these associations particularly important for generating new insights and therefore ran follow-up analyses to do so. They were: (1) a significant positive correlation between *Il17* expression in early life and reproductive success, and (2) a significant positive correlation between *Il10* expression in early life and the proportion of later life infected with *Bartonella* spp. (see Section 3: Results).

We had information about both *Il17* expression in early life and reproductive success for 131 voles (see Figure S4 for plots showing raw data). The median number of offspring produced by these voles was 0 (range = 0–6). Because our measure of reproductive success was zero-inflated (107/131 or 82% zero values), it was simply coded as either reproduced or not. We then ran a logistic regression to model the probability of reproducing. *Il17* expression in early life was also found to be zero-inflated (117/131 or 89% zero values) and was simply coded as either expressed or not. We included birth month as a (continuous) covariate in the model, given that autumn-born voles have a lower chance of reproducing than spring-born voles (Wang et al., 2019). Other (categorical) covariates included in the model as fixed effects were: sex, whether or not an individual was culled (again, reducing the opportunity to reproduce) and the year in which an individual was born. We also included a random effect for the site in which an individual was born.

We had information about *Il10* expression in early life and future *Bartonella* infection for 104 voles (see Figure S5 for plots showing raw data). *Bartonella* infection status was assessed multiple times for the majority of individuals (mean = 2.3; range = 1–6). Therefore, we ran a logistic regression, with a random effect for individual, to model the probability of an individual being infected throughout their life. The response variable was whether (1) or not (0) an individual tested positive at a particular time. As was the case for *Il17*, *Il10* expression in early life was found to be zero-inflated (89/104 or 86% zero values) and was simply coded as either being expressed or not. Other individual-specific covariates, considered potential drivers of future *Bartonella* infection and included as fixed effects were: the season in which *Bartonella* infection status was assessed (three levels, designated as spring [March to May], summer [June to August] and winter [September to November]), the sex of the individual, whether or not the individual was targeted for anti-macroparasite treatment (which may have affected the flea vectors of *Bartonella*), whether or not the individual was already infected with *Bartonella* in early life (only

17% of individuals are known to clear infection, so the majority of individuals infected in early life are likely to remain infected for the rest of their life), and birth year. We also included a random effect for birth site. The anti-macroparasite treatments were part of a wider study design, but are not the focus here, and (as for other individual-specific covariates) are included in models merely to increase the accuracy of the estimated *Il10* effect.

Logistic regressions were run using the R package glmmADMB (Fournier et al., 2012; Skaug et al., 2016). All covariates were tested for independence using variance inflation factors (all VIFs < 3). Full submodel sets were generated from each global model, including all fixed and random terms of interest, using the MuMIn package (Bartoń, 2019). All candidate models were then evaluated and ranked on relative fit using the Akaike information criterion, AIC. The best model, with the lowest AIC, is reported in the text and figures.

2.5.4 | Testing the age specificity of these associations

To test whether these associations are strictly early life or also present in adult (breeding) samples, we re-ran the same best models for reproductive success and the proportion of later life infected with *Bartonella* on adult blood samples. We maximized the sample size for these analyses by including all individuals sampled as adults, whether or not they were first sampled prior to breeding and therefore had an estimated birth date. For this reason, we were unable to account for birth month (reproductive success) and birth year (reproductive success and *Bartonella* infection). The best models were otherwise unchanged. After omitting those samples with missing data, we had information about both *Il17* expression in adult life and reproductive success for 357 voles, and information about both *Il10* expression in adult life and *Bartonella* infection for 798 voles. For individuals with more than one adult sample, we selected one adult sample at random from which to measure expression. To ensure that our results were robust to different random draws, we repeated this process 1000 times, re-running the best models on 1000 random draws. We report the number of random draws for which each association was significant ($p \leq .05$) compared to that expected by chance (which is 50/1000). We also tested for an association between whether or not an individual expressed each immune parameter (*Il10* or *Il17*) early in life, and whether or not they expressed it in later life using a Fisher's exact test.

2.5.5 | Testing for associations between immune clusters and genetic polymorphisms

We looked for associations between *Il10* and *Il17* expression in early life and genetic polymorphisms. In order to avoid difficulties in interpretation due to multiple testing, we looked only at polymorphisms in the immune genes found within immune cluster 3 (see Section 3: Results and Figure 1; *Il17* = 1 SNP, *Il10* = 2 SNPs, *Gata3* = 2 SNPs). We used the R package hapassoc (for *Il10* and *Gata3*, for which we had information about more than one SNP; Burkett et al., 2004, 2006) and the package SNPassoc (for *Il17*, for which we had information about a single SNP; Gonzales et al., 2014). Both hapassoc and SNPassoc models assumed an additive genetic model.

3 | RESULTS

3.1 | Three main clusters of immune genes are visible in early life

We constructed a correlation network for 20 immune genes whose expression was measured in our voles prior to breeding. Following thresholding, one immune gene, *Il4*, was dropped from the network. Using clustering analysis we identified three main immune clusters in early life: (1) immune cluster 1 (*Il1rap*, *Il1rn*, *Il1b*, *Orai1*, *Retnlg*, *Apobr*, *Cd8a*, *Ifng*, *Tgfb1*, *Cd86*), (2) immune cluster 2 (*Irf2*, *Irf9*, *Foxp3*, *Tollip*) and (3) immune cluster 3 (*Gata3*, *Il10*, *Il17*; Figure 1a).

3.2 | Exploratory analysis points to all three immune clusters being associated with life history outcomes

We then ran an exploratory analysis, repeating the same process as above, but adding six life history outcomes. Two life history outcomes (coefficient of variation in SMI and the proportion of later life infected with *Babesia*) were not correlated with any other variables and dropped out of the network. All three immune clusters were associated with life history outcomes. In cluster 1, this included a significant negative correlation between *Orai1* expression in early life and mean SMI ($\rho = -0.33$; corrected $p < .01$). In cluster 2, this included a significant positive correlation between *Foxp3* expression in early life and mean SMI ($\rho = 0.23$; corrected $p = .04$), and a significant positive correlation between *Tollip* expression in early life and the proportion of later life infected with *Bartonella* spp. ($\rho = 0.27$; corrected $p = .02$). In cluster 3, this included a significant positive correlation between *Il17* expression in early life and reproductive success ($\rho = 0.22$; corrected $p = .04$), and a significant positive correlation between *Il10* expression in early life and the proportion of later life infected with *Bartonella* spp. ($\rho = 0.22$; corrected $p = .05$).

3.3 | Follow-up analyses confirm association between immune cluster 3 and life history outcomes

Given the counter-intuitive set of genes with mixed functionality in cluster 3 (cluster highlighted in green in Figure 1b), we ran follow-up analyses to confirm the two correlations involving immune genes in this cluster (*Il10* and *Il17*; edges highlighted in red in Figure 1b). A logistic regression confirmed that whether or not an individual expressed *Il17* in early life was significantly associated with their

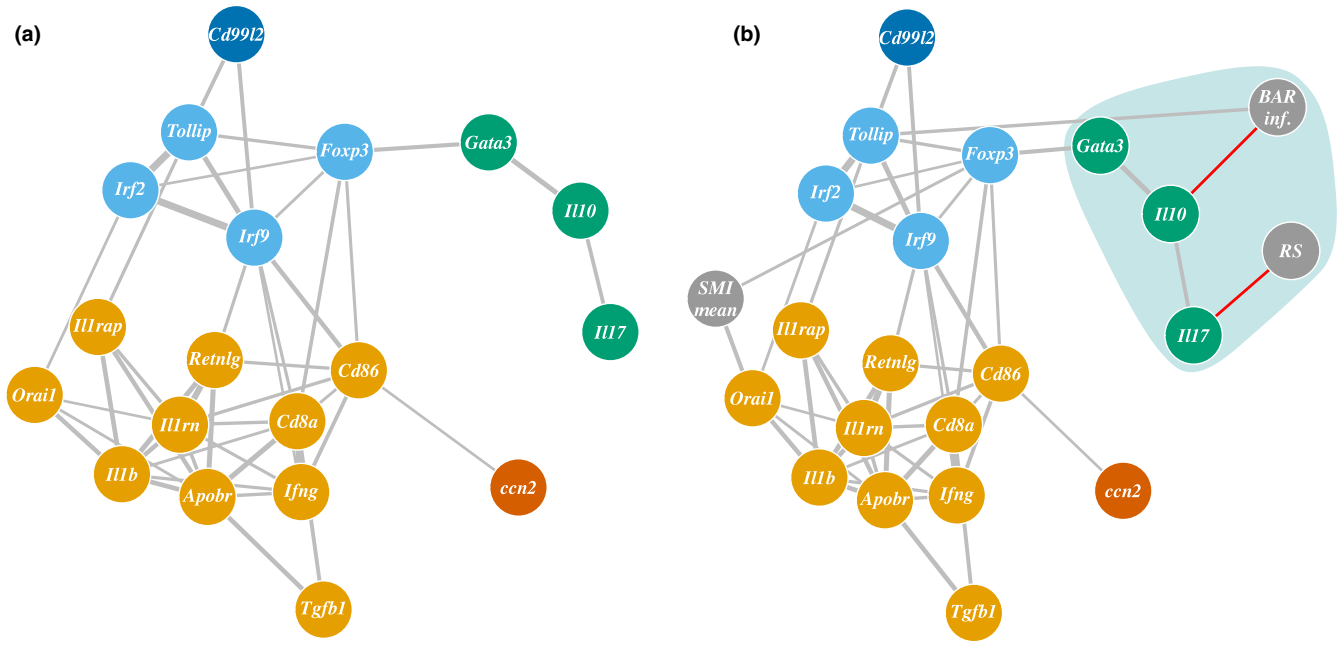

FIGURE 1 Network showing significant correlations (and associated nodes) of magnitude ≥0.2 between (a) immune parameters in early life (coloured nodes) and (b) immune parameters in early life and measures of later life or lifetime success (life history outcomes; grey nodes). Nodes without any significant correlations of magnitude ≥0.2 are not shown in either (a) or (b). All edges represent positive correlations except for a single edge between *Orai1* and mean SMI. The width of an edge represents the exact magnitude of the Spearman rank correlation coefficient. Nodes coloured in grey represent life history outcomes. All other nodes represent immune parameters, coloured by cluster. Three main clusters of immune parameters emerge based on edge betweenness (indicated by three of these colours in (a): yellow, light blue and green). One of these (the cluster with green nodes and also highlighted in green) includes a counter-intuitive set of genes with mixed functionality while also being associated with two life history outcomes (*Bartonella* infection and reproductive success) and was investigated further, in particular the edges highlighted in red in (b).

probability of reproducing in later life (reproductive success coded as a binary variable in the follow-up analysis rather than the number of surviving offspring in the pedigree as in the exploratory analysis; odds ratio = 5.10; 95% CI = 1.28–20.32; $p = .02$). Other fixed effects which appeared in the best model for, and were significantly associated with, the probability of reproducing in later life were the year in which an individual was born, and the month in which it was born (Tables S2 and S3; Figure 2a). There was no significant association between *Il17* expression in later life and reproductive success (robust to sample selection; $n = 7/1000$ random samples for which $p \leq .05$) indicating that this association was specific to early-life expression of *Il17*. Consistent with this result, we found no association between whether or not an individual expressed *Il17* early in life, and whether or not they expressed *Il17* later in life (Fisher's exact test; odds ratio = 0.64; 95% CI = 0.18–2.03; $p = .45$). We found no association between *Il17* expression in early life and probability of recapture (a proxy for survival; Fisher's exact test, odds ratio = 3.92; 95% CI = 0.58–168.17; $p = .21$).

Another logistic regression tested whether or not an individual expressed *Il10* in early life was significantly associated with their probability of being infected with *Bartonella* in later life (repeated sampling accounted for with a random effect for individual in the follow-up analysis rather than calculating the proportion of positive samples as in the exploratory analysis; odds ratio = 9.98; 95% CI = 1.29–77.08; $p < .01$). The year in which an individual was born was the only other fixed effect present in the best model for, and was also significantly associated with, *Bartonella* infection (Tables S4 and S5; Figure 2b). The association between *Il10* expression in later life and probability of infection was also significant (robust to sample selection; $n = 993/1000$ random samples for which $p \leq .05$) indicating that this association was not specific to early life expression. We found no association between whether or not an individual expressed *Il10* early in life, and whether or not they expressed *Il10* later in life (Fisher's exact test; odds ratio = 1.05; 95% CI = 0.25–3.90; $p = 1.00$). *Bartonella* infection status in early life did not appear in the best model for explaining the probability of infection in later life, but there was a significant association between this variable and *Il10* expression in early life (Fisher's exact test; odds ratio = 5.90; 95% CI = 1.38–53. 41; $p = .01$). In addition, we found a significant negative association between *Il10* expression in early life and probability of recapture (a proxy for survival; Fisher's exact test, odds ratio = 0.39; 95% CI = 0.14–1.15; $p = .05$).

3.4 | The association between immune cluster 3 and life history outcomes is partly driven by genetics

We looked for associations between *Il10* and *Il17* expression in early life and genetic polymorphisms in the immune genes *Il17*, *Il10* and

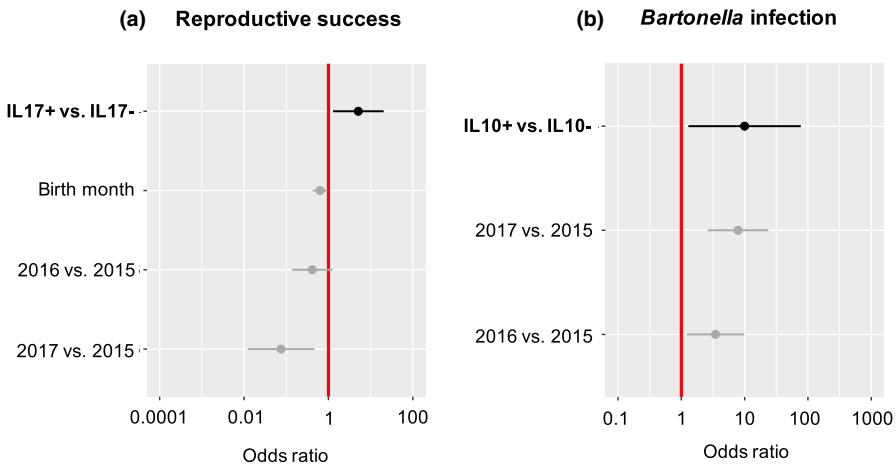

FIGURE 2 Odds of later-life success given immune parameters in early life. In bold: (a) odds of reproduction for individuals expressing *Il17* in early life (IL17+) compared to individuals not expressing *Il17* (IL17-), and (b) odds of *Bartonella* infection for individuals expressing *Il10* in early life (IL10+) compared to individuals not expressing *Il10* (IL10-). Not in bold: odds of reproduction or infection associated with other fixed effects which also appeared in the best models: the year in which an individual was born (2015, 2016 or 2017) and the month in which an individual was born (treated as a continuous variable). All estimates are taken from the best models (see text). Error bars represent 95% confidence intervals.

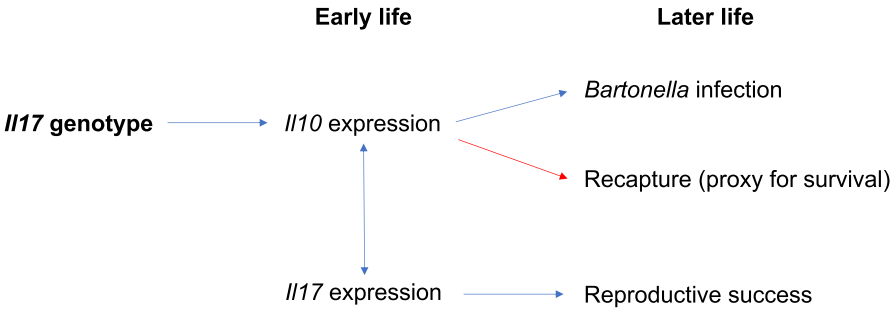

FIGURE 3 Summary diagram of confirmed associations. Blue arrows indicate positive associations, red arrows indicate negative associations. One-way arrows indicate a possible causal association, two-way arrows indicate a correlation only.

Gata3. Among these, we found only one significant association between a single SNP in the *Il17* gene and *Il10* expression in early life under an additive model, with the probability of expressing *Il10* significantly increasing with the number of T alleles (odds ratio = 1.74; 95% CI = 1.03–2.94; $p = .04$; Figure 3). However, the *Il17* genotype only explained a small proportion of variation in *Il10* expression ($r^2 = .026$).

4 | DISCUSSION

We have used a wild rodent system to provide evidence that, (1) the reproductive success (a measure of fitness), and (2) the response to infection of mature individuals (a measure of health) are both predicted by immune expression in the early stages of life. We have also found evidence to suggest that these patterns vary between different parts of the immune network.

We were able to identify three early life co-expression clusters among the immune-associated genes we studied. Given the tendency for cross-regulation among mammalian immune pathways (Atallah et al., 2020), it was unsurprising to see genes involved in different pathways, and with mixed pro- and anti-inflammatory functions, within the same clusters. This might happen, for example, because some responses increase at the same time as their negative feedbacks, or because responses within one pathway alter expression patterns within another pathway. Nonetheless, we saw very clear functional bias, with the largest cluster (cluster 1) containing predominantly pro-inflammatory markers such as *Il1* signalling cascade members (Dinarello, 2018), *Ifng* (Schroder et al., 2004), *Tgfb1* (Han et al., 2012) and *Retnlg* (Nagaev et al., 2006), but also genes with more nuanced functions in the promotion and suppression of inflammation, including *Tgfb1* itself (Li & Flavell, 2008) and *Orai1* (Shaw & Feske, 2012). This 'pro-inflammatory' cluster was distinct from a second cluster (cluster 2) containing predominantly immune down-regulatory markers including *Foxp3* (Shevach, 2009), *Tollip* (Zhang & Ghosh, 2002) and *Irf2* (Blanco et al., 2000), but also *Irf9* (Jefferies, 2019) which is a driver of interferon effector responses. A third cluster (cluster 3) containing *Gata3*, *Il10* and *Il17* may provide new insights into immune function, given that it contained a functionally mixed, counter-intuitive set of genes and was, at the same time, one of the clusters best linked to host health and fitness.

We have previously found *Gata3* expression in field voles to be associated with infection tolerance (Jackson et al., 2014). Thus, the

co-expression of *Gata3* and the anti-inflammatory cytokine gene *Il10* found here, in blood, could be consistent with some shared role for these genes in infection tolerance mechanisms, given the ability of IL10 to limit immunopathology (Coomes et al., 2017; Couper et al., 2008). In our blood samples, *Il10* expression was, in turn, positively associated with *Il17* expression. IL17, whose mRNA expression we found to be an early-life indicator of reproductive success, is typically associated with pro-inflammatory immunopathology but may also be protective against the numerous primary microbial insults that young voles, lacking acquired immunity, may encounter (Xu & Cao, 2010). The correlation between *Il10* and *Il17* expression could thus reflect IL10 production to safely regulate the inflammatory effects of IL17 and prevent immunopathology (Gazzinelli et al., 1996; Gu et al., 2008; Kulcsar et al., 2014; Li et al., 1999). We argue that observations, like these, on wild rodents can help us to define which of the possible immunological interactions known from laboratory studies predominate and shape the trajectory of immune phenotypes in individuals exposed to pathogens, nutritional challenge or other stressors within the natural environment.

Having identified early-life co-expression clusters we asked whether variation in these clusters affected later-life health and fitness. Our aim was not to comprehensively describe all possible associations, but to focus on those which we thought could generate the most important new insights. Associations, including with infection susceptibility, survival and fitness, were described in cluster 3 (Figure 3). However, there will be others (both within and beyond our network) which require further study. Within cluster 3, elevated early-life *Il10* expression was associated with a 10-fold increase in susceptibility to *Bartonella* infection later in life and a threefold reduction in survivorship. It is somewhat surprising that no associations were found with another intra-erythrocyte parasite, *B. microti*, which may have been expected to be associated with similar immune genes. We also found that early-life *Il10* expression was predicted by an *Il17* genetic polymorphism, although the effect size was small. This suggests that some components of early-life effects may be laid down at birth and confirms a genetic basis for immune expression. The observed genetic effect, as previously shown in the present system and others, acts through the network of interacting cytokines such that polymorphism at one cytokine gene can affect expression of other cytokines and immune phenotypes (Dinarello, 2007; Li et al., 2016; Salnikova et al., 2020; ter Horst et al., 2016; Turner et al., 2011; Viney & Riley, 2017; Wanelik et al., 2018).

A challenge for future work will be to establish how this polymorphism acts. For example, an adverse *Il17* genotype may make an individual more likely to become infected with *Bartonella*, with infection stimulating *Il10* expression. Alternatively, that genotype may predispose an individual to express *Il10* early in life (and perhaps lead to other immune-expression variations) with that pattern of immune expression increasing the likelihood of *Bartonella* infection. The positive association between *Il10* expression in the earliest samples and *Bartonella* infection at that time supports but cannot positively establish the former, since *Bartonella* infection before even the earliest samples is possible. Furthermore, it is unclear whether it is *Bartonella* infection or the pattern of immune expression that reduces survival.

We also found a significant association between *Il10* expression in breeding adults and the probability of being infected with *Bartonella* later in life. It is perhaps not surprising that we found this association, as both *Il10* expression and *Bartonella* infection in this case were measured in contemporaneous samples (taken in later life) and our previous work shows that *Il10* expression increases soon after acquisition of *Bartonella* (Taylor et al., 2018). Furthermore, we found no significant association between *Il10* expression early and later in life, suggesting that the later-life association was not driven by its early-life counterpart.

Elevated early-life expression of another cluster 3 gene, *Il17*, was associated with a fivefold increase in the likelihood of reproduction. By contrast, *Il17* expression in breeding adults was not associated with reproduction. *Il17* expression in early and later life was unrelated and, furthermore, no genetic effects on *Il17* expression were detected, including no effect of polymorphisms in *Il17* itself. Only a limited panel of polymorphisms was considered, making it impossible to rule out genetic effects. Nonetheless, it is likely that, even if some undetected genetic effects occurred, these would be of relatively small effect size, as for the genetic effect detected for *Il10* expression here, or for other genetic effects on immune expression reported in wild voles (Guivier et al., 2010; Turner et al., 2011; Wanelik et al., 2018). Thus, it is likely that much *Il10* and *Il17* expression is determined by environmental exposures, which drives early-life immune phenotypes influencing later development into reproductively mature adults. In the case of *Il17*, these exposures could include, for example, a bacterial infection or composition of the gut microbiome early in life.

In this study, we have demonstrated how the immunophenotypic die is sometimes cast early in naturally occurring vertebrates: certain immune expression profiles early in life, however determined, are a powerful predictor of later-life outcomes. Previous evidence for this has been scarce. A number of studies have shown an association between broad-brush measures of immune expression in nestling birds and recruitment from the nest (Cichoń & Dubiec, 2005; López-Rull et al., 2011; Moreno et al., 2005), an almost contemporaneous effect. Bowers et al. (2014) went a step further, and showed an association between a broad-brush measure of immune expression (PHA responsiveness), longevity and reproductive success. Here, we provide a more granular analysis, linked to a gene-level understanding of the molecular immunological network. We describe two examples in which the early-life expression of important, conserved cytokine genes, *Il10* and *Il17*—which are co-expressed in early life and thus perhaps mechanistically linked—profoundly influence health and fitness in later life.

AUTHOR CONTRIBUTIONS

M.B., J.E.B., J.A.J. and S.P. designed the study. C.H.T. and I.M.F. conducted the laboratory work, with assistance from others. K.M.W. analysed the data. All authors wrote the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

Data accessibility: Phenotypic data are available from the NERC EDS. Environmental Information Data Centre: https://doi.org/10.5285/e5854431-6fa4-4ff0-aa02-3de68763c952 Paterson et al. (2022) and from the University of Liverpool's

Research Data Catalogue: https://doi.org/10.17638/datacat.liverpool.ac.uk/1850 Paterson (2022a). Genotype data are also available from the University of Liverpool's

Research Data Catalogue: https://doi.org/10.17638/datacat.liverpool.ac.uk/1849 Paterson (2022b).

BENEFIT SHARING STATEMENT

Benefits from this research accrue from sharing of our data and results on public databases as described above.

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SUPPORTING INFORMATION

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