# Physiological, but not fitness, effects of two interacting haemoparasitic infections in a wild rodent

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# Abstract

In contrast to the conditions in most laboratory studies, wild animals are routinely challenged by multiple infections at once, and these infections can interact in complex ways. This means that the impact of a parasite on its host's physiology and fitness cannot be fully assessed in isolation, and requires consideration of the interactions with other co-infections. Here we examine the impact of two common blood parasites in the field vole (*Microtus agrestis*): *Babesia microti* and *Bartonella* spp., both of which have zoonotic potential. We collected longitudinal and cross-sectional data from four populations of individually tagged wild field voles. This included data on biometrics, life history, ectoparasite counts, presence/absence of microparasites, immune markers and, for a subset of voles, more detailed physiological and immunological measurements. This allowed us to monitor infections over time and to estimate components of survival and fecundity. We confirm, as reported previously, that *B. microti* has a preventative effect on infection with *Bartonella* spp., but that the reverse is not true. We observed gross splenomegaly following *B. microti* infection, and an increase in IL-10 production together with some weight loss following *Bartonella* spp. infection. However, these animals appeared otherwise healthy and we detected no impact of infection on survival or fecundity due to the two haemoparasite taxa. This is particularly remarkable in the case of *B. microti* which induces apparently drastic long-term changes to spleen sizes, but without major adverse effects. Our work sheds light on the ecologies of these important zoonotic agents, and more generally on the influence that interactions among multiple parasites have on their hosts in the wild.



*Keywords*: Disease ecology; Co-infection; Immunology; *Babesia microti*; *Bartonella*; *Microtus agrestis*

# 1. Introduction

An individual animal in its natural environment is likely to harbour multiple parasitic infections (Petney and Andrews, 1998; Cox, 2001). These parasites can interact with one another in complex ways, meaning that even low virulence infections can indirectly influence the host's physiology and fitness by changing the course of more virulent infections (Randall et al., 2013). Understanding how parasites interact in a natural setting provides a vital complement to more controlled, laboratory-based studies (Pedersen and Babayan, 2011), and in particular it allows us to study parasites in the context of the natural co-infections which may occur.

Two parasites may interact with one another in several distinct ways. Commonly, one parasite may adversely affect the host's condition, or cause a shift in immunity, that then makes it easier for another to invade, or to increase in abundance (Cox, 2001). Alternatively, one infection can make it harder for a second to become established (van Duivenvoorde et al., 2010; Randall et al., 2013), for example through direct competition for resources (Johnson and Buller, 2011), cross-reactivity of antibodies (Naus et al., 2003), or a shift in host cytokine production (Graham et al., 2007). These interactions can give counter-intuitive outcomes for the host: one type of infection (particularly if it has low virulence) may have a net positive effect on host fitness if it reduces the abundance or prevalence of a second pathogen (Randall et al., 2013; Wuerthner et al., 2017). These indirect effects are important when considering treatment or removal of a parasite during disease management, and species or strains of pathogen should not be considered in isolation. It is vital to understand any changes in susceptibility to other parasites due to the intervention that might lead to unintended negative consequences (Graham, 2008; Fenton, 2013; Johnson et al., 2015).

In wild field voles, *Microtus agrestis*, a negative interaction has been described between two common taxa of blood parasites, *Babesia microti* and *Bartonella* spp. (Telfer et al., 2010; Sherlock et al., 2013). The protozoan *B. microti* infects a number of rodent species, often without obvious symptoms. It is transmitted by ixodid ticks, which can also spread the infection to humans, causing

63 babesiosis (Telford et al., 1993; Homer et al., 2000). The course of an infection varies, but a typical
64 sequence in rodents consists of acute and chronic phases. The acute phase lasts several weeks,
65 during which time *B. microti* infects the red blood cells (RBCs) of the host and may cause severe
66 anaemia. Between 20 and 50% of the RBCs can be infected, the packed cell volume (PCV) may be
67 reduced to as little as 20%, and the spleen becomes greatly enlarged (Van Peenen and Healy, 1970;
68 Cullen and Levine, 1987; Watkins et al., 1991). Mortality in otherwise healthy animals is low,
69 however, and after 3-4 weeks most have little or no evidence of infection in the blood. Nonetheless,
70 previously infected animals retain a chronic carrier status (Lykins et al., 1975; Homer et al., 2000)
71 and *B. microti* remains detectable by PCR indefinitely (Bown et al., 2008).

72 *Bartonella* is a genus of gram-negative bacteria that infects a wide range of mammalian
73 hosts (Breitschwerdt and Kordick, 2000). It is transmitted by blood-sucking arthropods, primarily
74 fleas (Bown et al., 2004; Gutiérrez et al., 2015), and invades the RBCs of the host (Breitschwerdt and
75 Kordick, 2000). Similar to *B. microti,* some *Bartonella* spp. can be transmitted to humans via
76 arthropod vectors, where they cause several different diseases including bartonellosis and cat
77 scratch disease (Anderson and Neuman, 1997; Oksi et al., 2013; Vayssier-Taussat et al., 2016).

78 In field voles, *Bartonella* spp. (henceforth "*Bartonella*") and *B. microti* show a negative
79 interaction, with few animals showing coinfection despite relatively high prevalence of the individual
80 infections (Telfer et al., 2010). This interaction appears to be unidirectional; that is, *B. microti*
81 reduces the chance of infection with *Bartonella*, but the reverse is not the case (Sherlock et al.,
82 2013). Particularly in light of the zoonotic potential of the two infections, it is valuable to study them
83 in combination to understand the fitness effects on the host, and to learn more about the dynamics
84 of their interaction.

85 Here, we aim to explore the consequences of *Bartonella* and *B. microti* infections for wild-caught
86 field voles. We make use of longitudinal data from multiple captures of the same individuals to
87 examine infection sequences and changes in immunology over time to help separate cause from

effect. We also use more detailed physiological and immunological data taken from destructive cross-sectional sampling. We confirm the findings of Sherlock et al. (2013) that *B. microti* reduces susceptibility to *Bartonella*, but not the reverse. We find major physiological changes in the case of *B. microti* infection (characterised by splenomegaly) but, surprisingly, no evidence of adverse fitness consequences. Finally, we are unable to find any support for the hypothesis that *B. microti* infection has any indirect, positive effect on the host via its negative interaction with *Bartonella*.

# 2. Materials and methods

This paper uses data that have been previously analysed in Jackson et al. (2011) and Jackson et al. (2014). Here we give a short summary of the data collection methods; for more details see the two references above. We carried out all procedures under UK Home Office licence regulations.

## *2.1. Fieldwork*

Wild field voles were trapped at four different sites in Kielder Forest, Northumbria, UK: two sites in 2008-2009 and a further two in 2009-2010. At each site, 150 Ugglan small mammal traps (Grahnab, Sweden) were laid out in a grid spaced approximately 3-5 m apart. During monthly trapping sessions, traps were checked five times over the course of 3 days, and 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 across multiple sessions. On capture, we recorded sex and body mass of the voles, as well as reproductive status (males were considered to be reproductively active if they had descended testes; females if they were pregnant or had perforate vaginas). We also conducted a thorough visual inspection of the fur to count ectoparasites including ticks and fleas, and took a drop of blood from the tail into 500 µl of RNAlater (Fisher Scientific, UK), for use in pathogen detection and immune assays (see 2.3 and 2.4 below).

## 2.2. Cross-sectional data

At each trapping session we also retained a small number of individuals (up to 20) that were transported to the laboratory. These individuals were killed and dissected in order to collect more detailed, invasive measurements. For this component of the study, we focused on male voles only: we did not wish to remove pregnant females from the wild population, and data collected from non-pregnant females would therefore represent a biased sample.

Voles were killed by an overdose of chloroform, following which they were immediately weighed and then exsanguinated. The resulting blood samples were divided into two aliquots: one was used in pathogen detection (see Section 2.3), and the other for estimation of PCV. The latter blood sample was spun for 3 min in a microhaematocrit centrifuge (Hawksley, UK) to separate cells from plasma and the ratio of the two (PCV) was calculated. We measured body length from snout to vent (SVL), removed the spleen and measured its wet mass before setting up splenocyte cultures (see Section 2.5). We conducted a thorough search of the fur for ectoparasites.

## 2.3. Pathogen detection

We extracted DNA from blood samples and amplified pathogen-specific sequences using PCR. See Bown et al. (2008) for details of *B. microti* detection and Telfer et al. (2005) for *Bartonella*. For longitudinal samples, we used an aliquot of the tail blood samples and extracted DNA using isopropyl alcohol precipitation (see Jackson et al., 2014 for details). For cross-sectional samples in 2008, we used blood collected on filter paper and DNA extraction using Tris-EDTA buffer, as described in Bereczky et al. (2005). As this method appeared to have a lower sensitivity than expected, in 2009 we switched to extracting from pelleted RBCs after centrifugation, using alkaline digestion as described in Bown et al. (2003).

## 2.4. Blood immunology

We used two-step reverse transcription quantitative PCR to estimate the expression levels of the genes Interferon gamma (IFNγ), Gata3 and IL-10 in peripheral blood samples. Having only a

limited volume of blood from each individual, we selected these three genes as broadly representative of Th1 (IFNγ), Th2 (Gata3) and regulatory/anti-inflammatory (IL-10) immune responses. Samples were run in duplicate, and each 96-well plate included no-template controls. Expression was standardised against Ywhaz and Sdha as endogenous control genes, which were identified by geNorm analysis (Vandesompele et al., 2002) as the most stably expressed in the blood samples from a panel of seven candidate house-keeping genes. Expression values were normalised relative to a calibrator sample, using the ΔΔCT method (Livak and Schmittgen, 2001).

### *2.5. Splenocyte cultures*

After disaggregating spleen cells and lysing RBCs, we incubated the splenocytes (at a concentration of 2 x $10^6$ cells per ml, total volume 300 µl) for 96 h in two assays: one stimulated with the mitogen phytohaemagglutinin-M (40 µg per ml; Sigma, UK), and the other an unstimulated control. Assays for each individual and condition were replicated four times. After the incubation period, we estimated gene expression levels using two-step reverse transcription quantitative PCR, amplifying the sequences for IFNγ, IL-2 and T-box transcription factor TBX21 (T-bet) as markers for the Th1 immune response. Ywhaz was selected as the endogenous control gene in this case, being the most stably expressed in the splenocytes out of seven candidate house-keeping genes. Detailed methodology can be found in Jackson et al. (2011).

### *2.6. Statistical analysis*

Prior to statistical modelling, we transformed (natural log + 1) gene expression variables and quantitative parasite measures, and removed any rows containing missing values relevant to the model in question. Because different models used different sets of variables, this led to varying sample sizes (see Table 1).

All models included fixed effects of season and site (with “year” also implicitly accounted for, as site is nested within year), and for reproductive status and sex in cases where both factor levels were present. Further predictors are listed separately under individual models and in Table 1. We

modelled season as a sinusoid curve with a period of one year, using one sine and one cosine curve combined, following previous evidence that several infections show periodic variation in prevalence over the course of the year (Telfer et al., 2010). During model selection, these two curves were treated as a single unit.

We calculated an index of body condition as the residual values from a linear model of body mass against SVL, with the inclusion of second and third order polynomial terms (as allometry predicts a cubic relationship between length and mass). This index represents how much lighter or heavier an individual is compared with a prediction based on its length, with greater mass likely to indicate greater energy reserves and hence better condition (Schulte-Hostedde et al., 2005).

For model selection, a full sub-model set was generated and models were ranked according to their AICc (Akaike Information Criterion, with correction for small sample size) values. Models with $\Delta AIC < 2$ relative to the lowest value were considered to be equally supported as the best models to explain the data and were averaged. "Full" (as opposed to "conditional") coefficients are quoted in the final model, meaning that any terms not appearing in a given component model were assigned a coefficient of zero before averaging.

Since previous work indicates that *B. microti* infection reduces the chance of acquiring *Bartonella* (Sherlock et al., 2013; also see section 3.5 in this paper), we tested for indirect effects of *B. microti* on expression of selected genes, condition and fitness measures, via its effect on *Bartonella*. For models in which both *B. microti* and *Bartonella* were predictors, and *Bartonella* was found to be significant, we ran a further model in which we excluded *Bartonella* as a predictor. Our hypothesis was that, if the removal of *Bartonella* from the model caused a clear change in the size or direction of effects of *B. microti* infection, then this would be evidence of an indirect effect.

#### *2.6.1. Spleen mass, PCV and body mass*

Each of spleen mass, PCV and body mass were modelled using similar Generalised Linear Models (GLMs), with predictors including *Bartonella* and *B. microti* infection status, SVL (3$^{rd}$ order

polynomial) and, except in the body mass model, condition. Due to the way in which we defined condition (see Section 2.6 above), if it were included as a predictor in the body mass model it would create a circular argument as, together with SVL, it would explain 100% of the variation in body mass. Due to the slight change in extraction method described in Section 2.3, we also included interaction terms of *B. microti* and *Bartonella* with year.

### *2.6.2. Immunology from spleen cultures*

We created a summary measure for the expression levels of genes coding for our three Th1-associated cytokines (IFNγ, Tbet and IL-2) by Principal Components analysis. The first Principal Component represented 42.6% of the variation and correlated positively with all three gene expression variables, so we used this as our Th1 index. To test for an association between Th1 cytokines and haemoparasite infection, we ran a GLM with the Th1 index as the response variable. Predictors included presence/absence of *B. microti* and *Bartonella*, and their interactions with year (see Section 2.6.1 above),

### *2.6.3. Immunology from peripheral blood*

We used the longitudinal data to investigate the immunological changes that followed infection. We constructed three separate models of the changes in expression for Gata3, IL-10 and IFNγ, respectively, based on the tail blood samples. We selected all intervals for which an individual was captured in successive trapping sessions (i.e. less than 5 weeks apart) and was free from the infection in question at the start of the interval. For individuals with more than one such interval, we selected one interval at random in order to avoid pseudoreplication (we had previously attempted a mixed modelling approach, with individual as a random factor to allow the inclusion of all intervals, but the models did not converge because many individuals had only one valid interval). In order to verify that results were robust to the subsample obtained, we obtained models from 100 different random subsamples and recorded the proportion of these in which each predictor was significant (i.e. the 95% confidence interval for the given model coefficient did not overlap with 0).

We modelled the change in gene expression over the sampling interval (natural log(x2/x1) / interval length, where x1 is the expression at the start of the interval and x2 is the expression at the end) as our response variable. Predictors included the infection status for both *B. microti* and *Bartonella* (N = uninfected, B = infected) at both the start and end of the interval. This resulted in four possibilities: NN (uninfected), NB (acute infection, acquired during the interval), BB (chronic infection, acquired previously) and BN (cleared infection, for *Bartonella* only). As *B. microti* is known to persist indefinitely (Lykins et al., 1975; Telford et al., 1993; Bown et al., 2008), we excluded three intervals in which *B. microti* was apparently cleared (BN), on the assumption that these represented technical errors. Body mass was also included as a predictor, with a quadratic term to allow for a non-linear relationship.

### *2.6.4. Susceptibility to infection*

Using the longitudinal data, the effect of immunology on the acquisition of infection was evaluated. In two separate models, we examined the probability of an individual acquiring either *Bartonella* or *B. microti* during a given time interval. We selected time intervals as described in Section 2.6.3 above. The outcome of whether or not the individual became infected during the focal interval was modelled using a binomial GLM. Predictors included the level of tick and flea infestation, body mass (with a quadratic term) and expression of Gata3, IL10 and IFNγ, all measured at the start of the interval in question.

### *2.6.5. Mortality*

We analysed mortality rates within the longitudinal samples, excluding individuals that were only captured on a single occasion, on the assumption that many of these would have been non-resident. Recapture rates were very high: a simple Capture-Mark-Recapture (CMR) model (Cormack-Jolly-Seber) with constant values for recapture and survival gave a mean recapture rate of 0.89 (95% confidence limits 0.85 – 0.92). As a result, we judged that to calculate separate recapture and survival probabilities for each model term would greatly increase the number of model parameters

for little ultimate gain in accuracy. Therefore, we decided against fitting a full CMR model and made the simplifying assumption that an individual had died if it was released and never recaptured at subsequent sampling sessions. Although we used all time intervals in order to classify survival, we excluded intervals of more than one session (> 5 weeks) from the final model, on the basis that infection status is more likely to change (undetected) during longer intervals.

Survival was modelled using a Cox proportional hazards model, with predictors including *B. microti* and *Bartonella* infection status and body mass (with a quadratic term), all measured from the start of the sampling interval. Assessment of the proportional hazards assumption using Schoenfeld residuals (Schoenfeld, 1982) showed that hazards for the "site" term were not constant over time. We therefore stratified by site, after which all remaining terms showed proportional hazards.

### *2.6.6. Fecundity*

We used pregnancy rates to estimate fecundity among adult females. Again, our analysis was based on intervals between two consecutive sessions (excluding those from February and November, which are outside the breeding season), with predictors taken from the start of the interval and the outcome (pregnancy) measured at the end of the interval. Pregnancy was modelled using a Cox proportional hazards model, with the inclusion of a "cluster" term which adjusts variance estimates to allow for individuals with multiple pregnancy events. Given that the vole gestation period is approximately 3 weeks (Ranson, 1934), it is safe to assume that a female recorded as pregnant at two consecutive sampling sessions has had two different pregnancies. Predictors included *B. microti* and *Bartonella* infection status and body mass (with a quadratic term).

### *2.6.7. Software*

We carried out all analysis in R version 3.3.1 (R Core Team, 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria), making use of the following packages: "tidyverse" for data processing and visualisation (Wickham, 2016. tidyverse: Easily Install and Load "Tidyverse" Packages. R package version 1.0.0); "MuMIn" for

model averaging (Barton, 2016. MuMIn: Multi-Model Inference. R package version 1.15.6); and "survival" for Cox proportional hazards models (Therneau, 2015. A Package for Survival Analysis in S. R package version 2.38).

# 3. Results

## *3.1. Both Bartonella and B. microti were prevalent in the sample populations*

A total of 920 voles were tagged over the course of the longitudinal study, with a total of 1665 trapping instances, yielding between one and seven records per individual (mean = 1.8 captures). In addition, 345 male voles were destructively sampled, forming the cross-sectional dataset. *Bartonella* infections were highly prevalent within our study animals. Of the individuals captured three or more times, 91% were infected with *Bartonella* at some point during their capture history. In the same set of individuals, *B. microti* had a lower, but still considerable, prevalence of 37% during the course of the study (Table 2).

## *3.2. Babesia microti is associated with splenomegaly, and Bartonella with a reduction in body mass*

There was a strong, positive association between spleen mass and *B. microti* infection, with spleens of infected animals predicted to be 0.17 g heavier (95% Confidence Interval (CI) = 0.14-0.20) than those of uninfected individuals, which had a mean mass of 0.14 g (Table 3, Fig. 1, Supplementary Table S1).

PCV was not associated with either *B. microti* or *Bartonella* infections (Table 3, Supplementary Table S1).

We found an association between *Bartonella* and body mass, with infected voles 1.1 g lighter (95% CI = 0.1-2.1) than uninfected voles at the same time of year and at the same site (Table 3, Supplementary Table S1). *Babesia microti* did not feature as a predictor in the final model, even if the *Bartonella* term was excluded (Supplementary Table S2).

### 3.3. *Haemoparasitic infection was not associated with changes in the Th1 immune response*

Our index measuring expression of genes coding for Th1-associated proteins was not significantly associated with either *B. microti* or *Bartonella* infections (Table 4, Supplementary Table S3).

### 3.4. *An increase in IL10 expression follows Bartonella infection*

Expression of IL10 increased significantly upon initial infection with *Bartonella* (0.636 log units per 30 days, 95% CI = 0.87 – 3.36; significant in 81% of subsamples; a change of 0.636 log units corresponds to a 1.89-fold increase; Fig. 2). Expression then appeared to decline (relative to uninfected animals), both in individuals that remained infected (BB) and those that cleared the infection (BN), although the 95% CI for both of these changes included zero (Table 5, Supplementary Table S4). *Babesia microti* did not feature as a predictor in the final model, even if the *Bartonella* term was excluded (Supplementary Table S5). Neither Gata3 nor IFNγ expression were significantly influenced by infection status (Table 5).

### 3.5. *Babesia microti reduces infection rates by Bartonella, but not vice versa*

Susceptibility to *Bartonella* was significantly lower in individuals that had a pre-existing *B. microti* infection (odds ratio (OR) = 0.33, 95% CI = 0.14 – 0.76; significant in 94% of subsamples; Table 6, Supplementary Table S6). The reverse was not true; *Bartonella* infection did not influence an individual's susceptibility to *B. microti*. Males were more susceptible than females to *B. microti* infection (OR = 4.01, 95% CI = 1.65 – 9.78; significant in 100% of subsamples; Table 6, Supplementary Table S6).

### 3.6. *No loss of fitness was detected from infections*

Neither *B. microti* nor *Bartonella* infections significantly influenced the estimated vole mortality rates (*Bartonella* OR = 1.03, 95% CI = 0.85 – 1.26; *B. microti* OR = 0.97, 95% CI = 0.79 –

1.18; Table 7, Supplementary Table S7, Fig. 3), nor did they affect female fecundity, measured as the probability of pregnancy over time (*Bartonella* OR = 0.99, 95% CI = 0.84 – 1.16; *B. microti* OR = 0.96, 95% CI = 0.80 – 1.15; Table 8, Supplementary Table S8, Fig. 4).

# 4. Discussion

*Babesia microti* and *Bartonella* infections appear to have little impact on the fitness of field voles, despite provoking clear physiological changes. A substantial proportion of the vole population has greatly enlarged spleens due to *B. microti* infection, and yet has similar rates of mortality and pregnancy to uninfected animals. While *Bartonella* infection is associated with a reduction in body mass, this poorer condition does not translate to any detectable loss of fitness in the two measures that we examined.

It is notable that the voles can support these infections with apparently little deleterious impact, given the associated physiological changes. Most conspicuously, we find that *B. microti*-infected field voles have highly enlarged spleens compared with uninfected voles, as has been recorded in many other host species (Lykins et al., 1975; Cullen and Levine, 1987; Watkins et al., 1991; Telford et al., 1993; Homer et al., 2000). Given that splenomegaly affects *B. microti*-positive individuals almost universally (Fig. 1), we infer that it is not restricted to the initial, acute phase of infection, but rather it affects the animal throughout the full course of infection, including when the vole has carrier status. An enlarged spleen indicates proliferation of lymphocytes, and/or that large numbers of RBCs are being recycled from the blood (Eichner, 1979; Jiao et al., 2001), presumably to remove the pathogen-containing cells. Despite this, we find no evidence of anaemia associated with *B. microti*, meaning it is likely that new RBCs are also being produced at an elevated rate to make up for those being removed. A study in another vole species, *Microtus montanus*, found a similar pattern of splenomegaly without anaemia in *B. microti*-infected individuals, and showed that erythrocytes were on average younger in these individuals (Watkins et al., 1991). We would expect

334 such energetic expenditure to have a negative impact on the animal's condition or fitness, and yet,
335 despite substantial sample sizes, we have not found evidence for any such impact.

336 We did not find any increase in expression of genes coding for Th1-related cytokines such as
337 IFNγ associated with either of the focal infections. One might predict stimulation of the Th1 pathway
338 in response to infections such as these, in accordance with its function in targeting intracellular
339 parasites (Abbas et al., 1996). Indeed, in the laboratory, *B. microti* has been shown to stimulate
340 increased expression of IFNγ and IL-2 in mice during a period 1 - 3 weeks p.i. (Chen et al., 2000).
341 Similarly, mice experimentally infected with *Bartonella henselae* show an increase in secretion of
342 IFNγ indicative of a Th1 response (Arvand et al., 2001; Kabeya et al., 2007), and in cats, a stronger
343 Th1 response leads to reduction in *B. henselae* bacteraemia (Kabeya et al., 2009).

344 By contrast, we observed an increase in expression of IL-10 following initial infection with
345 *Bartonella*. IL-10 is a regulatory cytokine that reduces inflammatory responses, and in other host-
346 pathogen systems it has been shown to play a role in tissue damage associated with inflammation
347 (Hunter et al., 1997; Brown et al., 1999; Sanni et al., 2004). Kabeya et al. (2007) observed an increase
348 in IL-10 secretion following *B. henselae* infection, which acted to reduce the strength of the Th1
349 response. The induction of IL-10 production by *Bartonella* may therefore help the parasite to persist
350 in its host (Kabeya et al., 2007) but, given the lack of detectable fitness cost in our study, it is also
351 possible that IL-10 might minimise negative impacts on host health by reducing inflammation. The
352 addition of data on parasite loads would allow more detailed investigation of this possibility.

353 Our study provides independent corroboration of the finding of Sherlock et al. (2013), that
354 *B. microti* protects against *Bartonella* infection, but that the reverse is not true. To date we have
355 been unable to establish a mechanism for the inhibition. One possibility is that *B. microti* might
356 cause changes in immunology that then make the individual better able to resist initial infections
357 with *Bartonella*. Immunological changes are a common way for concurrent infections to interact
358 (Cox, 2001). For example, increased production of cytokines such as Tumour Necrosis Factor has

359 been credited for mediating protection against protozoan diseases such as malaria and *B. microti*
360 after infection with tuberculosis (Clark, 2001). However, on the basis of the genes examined in this
361 study, we found no evidence that *B. microti* induces changes in immune status, nor that immune
362 status influences *Bartonella* acquisition. We note, however, that levels of gene expression do not
363 necessarily correlate directly with concentrations of their protein products (Vogel and Marcotte,
364 2012), and therefore direct data on circulating cytokines would allow us to investigate these
365 immunological interactions more thoroughly.

366 Alternatively, the interaction between *B. microti* and *Bartonella* might be physiological. The
367 most conspicuous feature of *B. microti* infection is splenomegaly, and this is associated with an
368 increased number of reticulocytes in the blood compared with mature erythrocytes (Watkins et al.,
369 1991). There is some evidence that both *B. microti* (Borggraefe et al., 2006) and *Bartonella* (Scheff et
370 al., 1956) are less likely to infect reticulocytes than mature erythrocytes. Therefore, by maintaining
371 high reticulocyte populations, *B. microti*-infected individuals may limit the capacity of *Bartonella* and
372 *B. microti* to reproduce, hence lowering the probability of the infections establishing/re-establishing.

373 In cases of negative interactions between two different parasites, we might predict an
374 indirect, positive effect of one infection on the host via a reduction in abundance or prevalence of
375 the other. For example, Wuerthner et al. (2017) found that a trematode infection in amphibians can
376 have a positive fitness effect by reducing ranaviral loads. However we found no evidence of a similar
377 positive effect of *B. microti* in the current study. For example, although we found that *Bartonella*
378 causes a reduction in body mass, this did not translate into a positive effect of *B. microti* on mass on
379 exclusion of *Bartonella* from the model. This may simply be because the impacts of *Bartonella* on an
380 individual's health are relatively small, and therefore any indirect effects conferred are too small to
381 be detected.

382 While we found little evidence for negative health consequences of these infections in the
383 voles, it is plausible that our sampling method overlooked the most acutely infected individuals. In

384 theory, an individual that was recorded as free of infection could subsequently become infected and
385 die before recapture. In this case, the infection would not be observed, making it impossible to
386 ascribe that death to the parasite. Given the high prevalence of both parasites among the sampled
387 populations, we can be confident that our conclusions apply to a sizable number of infected
388 individuals, but we cannot rule out that some individuals are more acutely affected. It is also
389 possible that we missed some fitness consequences of infection (e.g. effects on number of offspring)
390 by focusing on mortality and pregnancy rates.

391 Overall, our study shows that *B. microti* and *Bartonella* have little negative impact on their
392 field vole hosts, despite provoking clear immunological and physiological changes that, at least in the
393 case of *B. microti*, appear to persist for long periods of time. This demonstrates the importance of
394 using direct measures of fitness components wherever possible to assess the impact of parasites in
395 natural populations, rather than relying on assumptions based on pathology or prevalence of
396 infection.

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402

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532

533

**Figures**

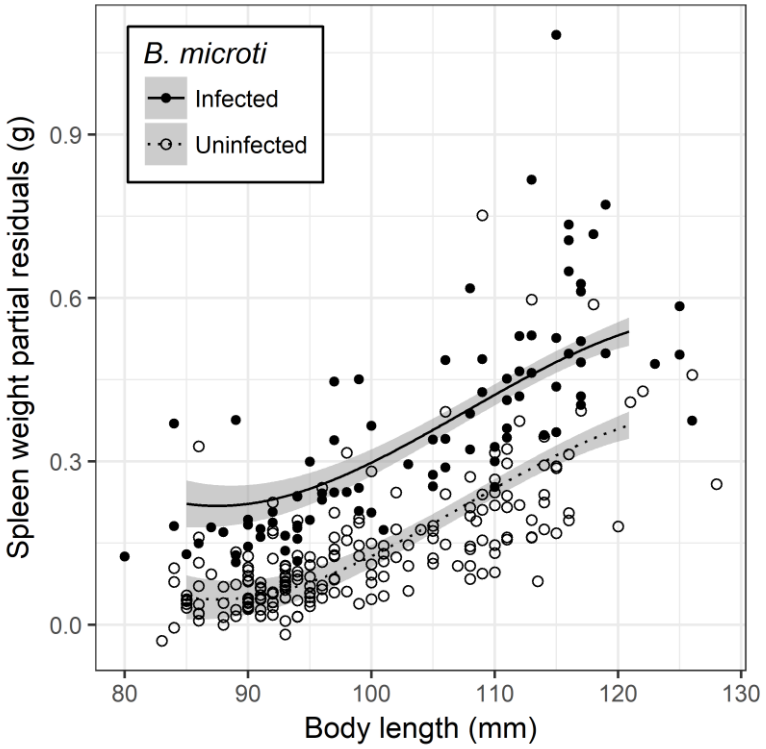
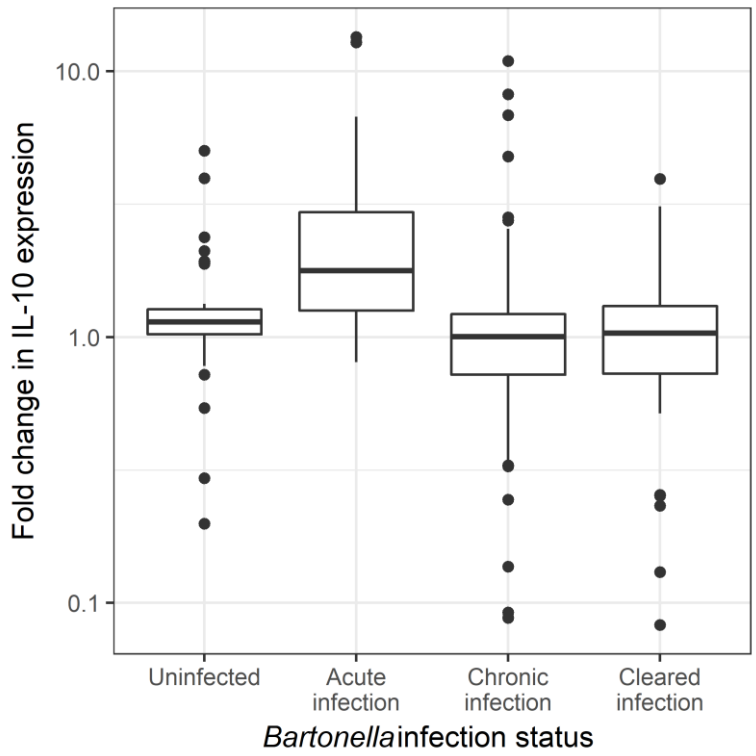
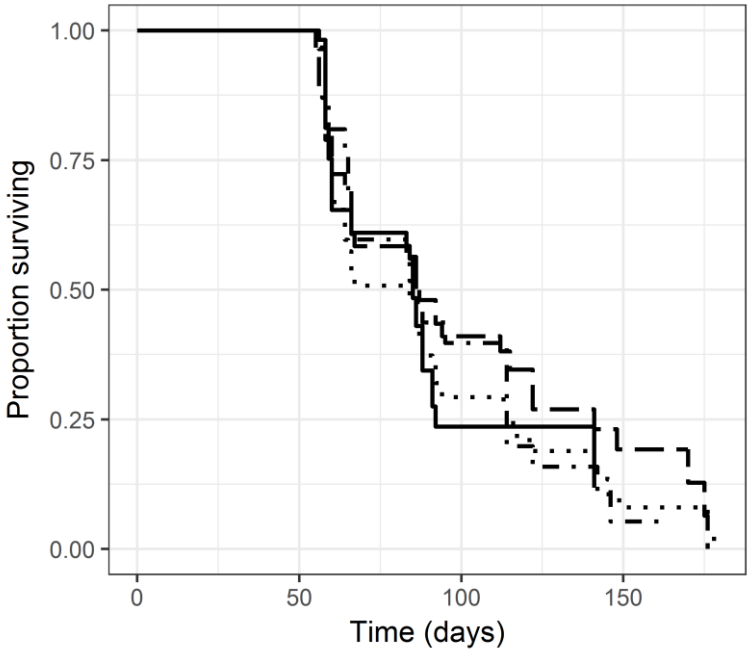



Fig. 1. Variation in *Microtus agrestis* spleen weight with body length and *Babesia microti* infection status. Spleen weight has been calculated as partial residuals from the model given in Table 4. The effects of *Bartonella*, breeding status and site have been standardised to their modal values, and condition and season to their median values. Points show partial residuals for each individual (solid = infected with *B. microti*, outline = uninfected), lines show model predictions (solid = infected, dotted = uninfected) ± standard error in grey.




542

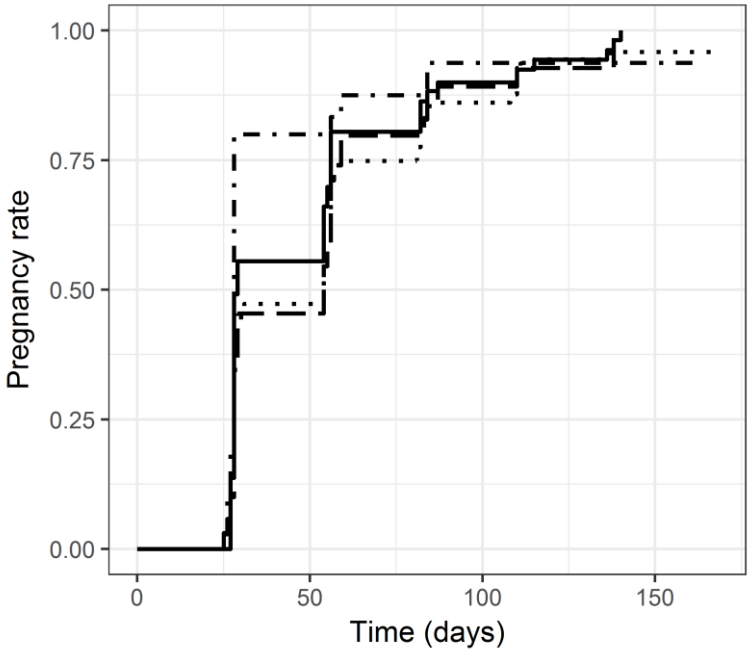
543 Fig. 2. Fold changes in IL-10 expression in vole splenocytes according to *Bartonella* infection status.
544 Changes are standardised for a typical sampling interval (30 days) and plotted on a log scale. Values
545 have been converted to partial residuals using the model given in Table 5. The effects of *Babesia*
546 *microti*, sex, breeding status and site have been standardised to their modal values, and weight
547 change and season to their median values. Boxes show the inter-quartile range (IQR) with a thick
548 horizontal line for the median. Whiskers extend to the smallest/largest values that are within 1.5 x
549 IQR of the box, and values beyond that are shown as individual points.




550

551 Fig. 3. Survival of voles according to infection status. Lines show the proportion of individuals
552 estimated from capture records to have survived at different time points. Solid line = neither *Babesia*
553 *microti* nor *Bartonella* infection (93 records, 34 events), dashed line = *B. microti*-infected (97 records,
554 36 events), dotted line = *Bartonella*-infected (278 records, 117 events), dot and dash line = both
555 infections (52 records, 23 events). Note that the reported 100% survival rate over the first 50 days is
556 an artefact due to our exclusion of individuals with only a single capture record.

557




558

559 Fig. 4. Female vole fecundity according to infection status. Lines show the proportion of individuals
560 with at least one recorded pregnancy at different time points. Solid line = neither *Babesia microti*
561 nor *Bartonella* infection (73 records, 44 events), dashed line = *B. microti*-infected (33 records, 20
562 events), dotted line = *Bartonella*-infected (156 records, 83 events), dot and dash line = both
563 infections (15 records, 7 events).

564

565 **Table 1.** Summary information of model specifications including predictors from each full model before

566 simplification.

| Predictors | Th1 index | Spleen mass | Packed Cell Volume | Body mass | Change in Gata3 | Change in IL10 | Change in IFNγ | Susceptibility to Bartonella | Susceptibility to B. microti | Mortality | Fecundity |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Site | Y | Y | Y | Y | Y | Y | Y | Y | Y | Y | Y |
| Season | Y | Y | Y | Y | Y | Y | Y | Y | Y | Y | Y |
| Sex | | | | | Y | Y | Y | Y | Y | Y | |
| Mating status | Y | Y | Y | Y | Y | Y | Y | Y | Y | Y | |
| Snout to vent length (3rd order polynomial) | | Y | Y | Y | | | | | | | |
| Body mass (2nd order polynomial) | | | | | | | | Y | Y | Y | Y |
| Body mass change | | | | | Y | Y | Y | | | | |
| Condition | | Y | Y | | | | | | | | |
| *Babesia microti* | Y | Y | Y | Y | Y | Y | Y | Y | | Y | Y |
| *Bartonella* | Y | Y | Y | Y | Y | Y | Y | | Y | Y | Y |
| Ticks | | | | | | | | Y | Y | | |
| Fleas | | | | | | | | Y | Y | | |
| IL10 | | | | | | | | Y | Y | | |
| Gata3 | | | | | | | | Y | Y | | |
| IFNγ | | | | | | | | Y | Y | | |
| Year:*B. microti* | Y | Y | Y | Y | | | | | | | |
| Year:*Bartonella* | Y | Y | Y | Y | | | | | | | |
| Dataset[a] | C | C | C | C | L | L | L | L | L | L | L |
| Sample size | 142 | 285 | 278 | 286 | 192 | 181 | 191 | 121 | 190 | 296 | 127 |
| Degrees of freedom | 11 | 15 | 15 | 14 | 10 | 10 | 10 | 14 | 14 | 11 | 9 |

567

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

569

570 **Table 2.** Prevalence of selected parasites within/on the sampled voles.

| Parasite | Point prevalence[a] | "Lifetime" prevalence[b] |
|---|---|---|
| *Bartonella* | 0.57 | 0.91 |
| *Babesia microti* | 0.18 | 0.37 |
| Flea | 0.54 | 0.92 |
| Tick | 0.21 | 0.57 |

571

572 [a] The probability that, at any given capture, an individual will be found to be infected with the parasite.

573 [b] The proportion of individuals with three or more captures in which the parasite has been detected on at least
574 one occasion.

575

**Table 3.** The effects of infection status and body size of voles on three physiological parameters: spleen mass, packed cell volume, and body mass, estimated from Generalised Linear Models.

| Predictor | Spleen mass (g) model | | | Packed Cell Volume (%) model | | | Body mass (g) model | | |
|---|---|---|---|---|---|---|---|---|---|
| | Coef[a] | LCI[b] | UCI[c] | Coef[a] | LCI[b] | UCI[c] | Coef[a] | LCI[b] | UCI[c] |
| Intercept | **0.186** | 0.134 | 0.238 | **51.2** | 49.7 | 52.7 | **24.8** | 23.5 | 26.2 |
| Snout to vent length (SVL) | **1.738** | 1.314 | 2.162 | -9.2 | -22.9 | 4.5 | **110.5** | 99.6 | 121.4 |
| $SVL^2$ | **0.242** | 0.027 | 0.456 | **-14.3** | -24.8 | -3.8 | **10.8** | 3.8 | 17.7 |
| $SVL^3$ | **-0.271** | -0.472 | -0.071 | | | | **-16.8** | -23.4 | -10.2 |
| Mass residuals | **0.012** | 0.009 | 0.016 | | | | | | |
| *Babesia microti* (infected) | **0.172** | 0.143 | 0.201 | | | | | | |
| *Bartonella* (infected) | | | | | | | **-1.1** | -2.1 | -0.1 |
| Status (breeding) | | | | | | | **2.3** | 0.9 | 3.7 |
| Season (cos) | | | | | | | **-3.2** | -4 | -2.3 |
| Season (sin) | | | | | | | **0.6** | 0 | 1.3 |
| Site 2 | | | | 0.1 | -1.4 | 1.6 | -0.8 | -1.8 | 0.1 |
| Site 3 | | | | **-2.8** | -4.9 | -0.7 | **2.2** | 0.8 | 3.6 |
| Site 4 | | | | **-2.8** | -5 | -0.5 | **1.6** | 0.2 | 3.1 |

[a] Estimated coefficient averaged across the set of candidate models. Coefficients in bold have a 95% confidence interval that does not include zero. Some terms with confidence intervals that overlap zero are not shown – see Supplementary Table S1 for full model details.

[b] Lower confidence interval (2.5%)

[c] Upper confidence interval (97.5%)

586 **Table 4.** The effects of vole infection status on an index of expression of Th1-associated genes.

| Predictor | Coef[a] | LCI[b] | UCI[c] |
|---|---|---|---|
| Intercept | 0.163 | -0.343 | 0.668 |
| *Babesia* | 0.614 | -0.0981 | 1.33 |
| *Bartonella* | 0.185 | -0.545 | 0.916 |
| Season (cos) | -0.217 | -0.677 | 0.243 |
| Season (sin) | **-0.718** | -1.13 | -0.305 |

587

588 [a] Estimated coefficient averaged across the set of candidate models. Coefficients in bold have a 95%
589 confidence interval that does not include zero. Some terms with confidence intervals that overlap
590 zero are not shown – see Supplementary Table S3 for full model details.

591 [b] Lower confidence interval (2.5%)

592 [c] Upper confidence interval (97.5%)

593

594

**Table 5.** Change in gene expression: log fold change per 30 days, estimated from GLMs.

| | IL10 model | | | Gata3 model | | | IFNγ model | | |
|---|---|---|---|---|---|---|---|---|---|
| | Coef[a] | LCI[b] | UCI[c] | Coef[a] | LCI[b] | UCI[c] | Coef[a] | LCI[b] | UCI[c] |
| Intercept | 0.36 | -0.123 | 0.846 | 0.063 | -0.081 | 0.21 | 0.072 | -0.015 | 0.159 |
| *Babesia microti* (new infection) | 0.261 | -0.174 | 0.699 | | | | | | |
| *B. microti* (chronic infection) | -0.153 | -0.468 | 0.165 | | | | | | |
| *Bartonella* (new infection) | **0.636** | 0.261 | 1.008 | | | | | | |
| *Bartonella* (chronic infection) | -0.204 | -0.537 | 0.129 | | | | | | |
| *Bartonella* (cleared infection) | -0.345 | -0.741 | 0.048 | | | | | | |
| Status (breeding) | | | | **-0.105** | -0.192 | -0.015 | | | |

[a] Estimated coefficient averaged across the set of candidate models. Coefficients in bold have a 95% confidence interval that does not include zero. Some terms with confidence intervals that overlap zero are not shown – see Supplementary Table S4 for full model details.

[b] Lower confidence interval (2.5%)

[c] Upper confidence interval (97.5%)

603 **Table 6.** Susceptibility of voles to *Babesia microti* and *Bartonella* infections: estimated log odds ratios from

604 binomial Generalised Linear Model.

| | **Bartonella model** | | | **B. microti model** | | |
|---|---|---|---|---|---|---|
| | Coef[a] | LCI[b] | UCI[c] | Coef | LCI | UCI |
| Intercept | 0.88 | -0.71 | 2.48 | -2.56 | -3.73 | -1.38 |
| *B. microti* (infected) | **-1.12** | -1.96 | -0.27 | | | |
| *Bartonella* (infected) | | | | -0.06 | -0.51 | 0.39 |
| Sex (M) | 0.1 | -0.43 | 0.62 | **1.39** | 0.5 | 2.28 |
| Body mass | | | | 3.24 | -1.69 | 8.16 |
| Body mass$^2$ | | | | **7.6** | 2.52 | 12.7 |

605

606 [a] Estimated coefficient averaged across the set of candidate models. Coefficients in bold have a 95%

607 confidence interval that does not include zero. The binomial model returns log odds ratios, so odds

608 ratio = $e^{coef}$. Some terms with confidence intervals that overlap zero are not shown – see

609 Supplementary Table S6 for full model details.

610 [b] Lower confidence interval (2.5%)

611 [c] Upper confidence interval (97.5%)

612

613

614

615

**Table 7.** Cox proportional hazards model of individual vole mortality.

| | *Coef*[a] | *LCI*[b] | *UCI*[c] |
|---|---|---|---|
| *Bartonella (infected)* | 0.037 | -0.160 | 0.233 |
| *Babesia microti (infected)* | -0.034 | -0.233 | 0.165 |
| *Sex (M)* | **0.526** | 0.206 | 0.846 |
| *Status (breeding)* | **0.445** | 0.044 | 0.845 |
| *Body mass* | **-23.100** | -28.800 | -17.500 |
| *Body mass*$^2$ | **4.680** | 0.690 | 8.68 |
| *Season (cos)* | -0.133 | -0.887 | 0.621 |
| *Season (sin)* | **1.370** | 0.990 | 1.760 |

[a] Estimated coefficient averaged across the set of candidate models. Coefficients in bold have a 95% confidence interval that does not include zero. Some terms with confidence intervals that overlap zero are not shown – see Supplementary Table S7 for full model details.

[b] Lower confidence interval (2.5%)

[c] Upper confidence interval (97.5%)

624 **Table 8.** Cox proportional hazards model of female vole fecundity.

| | *Coef*[a] | *LCI*[b] | *UCI*[c] |
|---|---|---|---|
| *Babesia microti (infected)* | -0.01 | -0.18 | 0.15 |
| *Bartonella (infected)* | -0.04 | -0.22 | 0.14 |
| *Body mass* | **-3.97** | -7.58 | -0.35 |
| *Body mass*$^2$ | **-3.84** | -6.76 | -0.91 |
| *Season (cos)* | **-0.64** | -1.09 | -0.19 |
| *Season (sin)* | 0.09 | -0.24 | 0.43 |

625 [a] Estimated coefficient averaged across the set of candidate models. Coefficients in bold have a 95%
626 confidence interval that does not include zero. Some terms with confidence intervals that overlap
627 zero are not shown – see Supplementary Table S8 for full model details.

628 [b] Lower confidence interval (2.5%)

629 [c] Upper confidence interval (97.5%)

630

631