Differences in the ecology of *Bartonella* infections of *Apodemus flavicollis* and *Myodes glareolus* in a boreal forest

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SUMMARY

The epidemiology of *Bartonella* species infecting *Apodemus flavicollis* and *Myodes glareolus* in a forest in Eastern Poland was followed for 2 years using mark-recapture. Infections could be acquired in any month, but prevalence, and probability of infection, peaked in the summer. There were significant differences in the pattern of infections between the two species. Both hosts were primarily infected as juveniles, but the probability of infection was highest for *A. flavicollis*, which, evidence suggests, experienced longer-lasting infections with a wider range of *Bartonella* genotypes. There was no evidence of increased host mortality associated with *Bartonella,* although the infection did affect the probability of recapture. Animals could become re-infected, generally by different *Bartonella* genotypes. Several longer lasting, poorly resolved infections of *A. flavicollis* involved more than 1 genotype, and may have resulted from sequential infections. Of 22 *Bartonella gltA* genotypes collected, only 2 (both *B. grahamii*) were shared between mice and voles; all others were specific either to *A. flavicollis* or to *M. glareolus,* and had their nearest relatives infecting *Microtus* species in neighbouring fields. This heterogeneity in the patterns of *Bartonella* infections in wild rodents emphasizes the need to consider variation between both, host species and *Bartonella* genotypes in ecological and epidemiological studies.

Key words: *Bartonella*, *Apodemus*, *Myodes*, rodents, rodent epidemiology, parasite communities.

INTRODUCTION

Myodes glareolus and *Apodemus* spp. (*A. flavicollis* and *A. sylvaticus*), the dominant sylvatic rodents in Eurasia (e.g. Flowerdew, 1985; Stenseth *et al*. 2002), are central to woodland food webs as well as being implicated in the transmission of numerous zoonotic diseases including hanta virus, borreliosis, tick-borne encephalitis and babesiosis. Bacteria of the genus *Bartonella* are also well-known blood parasites of wild rodents (e.g. Healing, 1981; Birtles *et al*. 1994, 2001; Bajer *et al*. 2001; Holmberg *et al*. 2003). *Bartonella* may be present at high prevalence in rodent populations, and forms part of a network of pathogens that interact in a complex but predictable manner (Telfer *et al*. 2010). It has also come under scrutiny as a potential opportunistic zoonotic pathogen of humans (e.g. Iralu *et al*. 2006). The importance of this pathogen is reflected in numerous accounts describing molecular variation in rodent bartonellae (e.g. Inoue *et al*. 2008; Berglund *et al*. 2009, 2010; Paziewska *et al*. 2011), but nevertheless there are few studies of the epidemiology of natural populations of *Bartonella* in wild rodents. The most detailed (e.g. Birtles *et al*. 2001) suggests that infections are self-limiting in the same way as laboratory infections (Koesling *et al*. 2001), that many genetic variants of *Bartonella* circulate within the host population (Birtles *et al*. 2001; Inoue *et al*. 2008), and that re-infections with the same genotype are rare (Birtles *et al*. 2001); however, such studies have, at least in Europe, been focused on relatively small and discontinous populations of rodents (e.g. Birtles *et al*. 2001; Telfer *et al*. 2008). The present study was undertaken to investigate *Bartonella* epidemiology in *A. flavicollis* and *M. glareolus* within their core distributional area in the forests of Central Europe, based on a 2-year longitudinal study in an old managed forest in north-eastern Poland. It adopts a mark-recapture and modelling methodology to compare the distribution, duration and genetic composition of infections in the 2 species of rodent.

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MATERIALS AND METHODS

Bank voles (*M. glareolus*) and yellow-necked mice (*A. flavicollis*) were live-trapped in a longitudinal study in managed forest at Urwitałt, in the Mazury Lake District, NE Poland. Rodents were trapped

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monthly from June 2007 to May 2009 (with a 3-month break in winter) along 3 trapping lines, with a distance between them of not greater than 300 m. Detailed trapping protocols have been described previously (Paziewska *et al*. 2010, 2011), and conformed to permission granted by the Polish Ethical Committee (permit number 737/2007). For all captured rodents the trap line was noted, along with species identity, sex, reproductive status (scrotal/non-scrotal for males; pregnant/lactating/vagina perforate for females), body weight, body length and fur condition. Animals were released on the line of capture. All ectoparasites (ticks, fleas and mites) were collected into 90% ethanol. Rodents were marked with numbered ear tags (World Precision Instruments Inc., Sarasota FL, USA) when first captured. For re-captures, tag number, body weight, body length, and reproductive status were assessed and ectoparasites collected. For each rodent at each capture, blood (50 μl) was taken from the tail vein directly into 200 μl of 0·001 M EDTA and stored at −20 °C for molecular analysis.

Each of the collected blood samples was subsequently analysed in the laboratory. DNA was isolated using a commercial kit (Genomic Mini AX Blood, AxyGen, USA) and *Bartonella* spp. were detected and identified by PCR amplification of a citrate synthase (*gltA*) gene fragment, using primers Bh.CS778p and Bh.CS1134n with PCR conditions as described (Norman *et al*. 1995). Amplicons were viewed on a 1% agarose gel, and for 91 amplicons, including all for which rodents were apparently infected twice, the *gltA* fragment was sequenced on both strands (Genomed S.A., Poland).

Analysis

Two datasets were compiled for the collected rodents. The first included all animals sampled, including those collected only once, and was used to estimate rodent abundance and survival, overall *Bartonella* prevalence, and probability of lifetime infection or re-infection. The second data set included only those animals for which complete or almost complete (captured at least 3 times, no more than 1 trapping session missing between first capture and final disappearance) trapping histories were available and were used to estimate duration of infection. A subset of this dataset included all animals first captured as juveniles for which complete or almost complete trapping histories were available.

Apodemus flavicollis and *M. glareolus* were sorted into 3 age categories according to body weight, body length, fur condition and reproductive condition and trapping history. Animals were classified as juveniles (physiologically immature), young adults (large enough to reproduce in the year of their birth, although not necessarily sexually active) and overwinterers (adult animals born in the previous calendar year). Overwinterers were defined by recapture history, date of capture (all animals caught in March and April, and adults in May, were by definition overwinterers) and by physical condition. For *M. glareolus*, juveniles were defined as being less than 15 g, body length less than 85 mm, sexually inactive, and with juvenile pelage. For *A. flavicollis* juveniles were defined on the basis of body length (less than 100 mm). Such animals could weigh up to 30 g, although the majority were less than 25 g. Juvenile pelage was lost in *A. flavicollis* at a body mass of ca. 20 g, but animals between 20 g and 30 g were never observed with mature gonads and were therefore also considered as juveniles (Flowerdew, 1984; Perkins *et al*. 2009). The juvenile phase for *M. glareolus* lasts approximately 8 weeks (Kozakiewicz, 1976; Bajer *et al*. 2001). There is less certainty over the duration of this phase for *A. flavicollis*; females reach adult size in ca. 8 weeks, but males may require up to 4–5 months to reach maturity (Amori and Luiselli, 2011). Animals were allocated to spring or autumn birth cohorts based on estimated birth date, determined from the date of capture and estimated age at this time, and from their future trapping history (i.e. it was considered unlikely that an animal would overwinter twice).

Rodent abundance was estimated using catch per unit effort and mark-recapture methods. The former was calculated as the mean number of individuals of each rodent species captured during the first 3 nights on each line in each month ($D = N/T \times H \times 10^{-4}$, where D = rodent density, N = number of captured rodents, T = number of traps, H = number of trapping hours; see Bajer *et al*. 2002). Population sizes based on mark-recapture data were calculated using the POPAN model (MARK 6.0 software, White and Burnham, 1999) with an open-population parameterization of the Jolly-Seber model (Schwarz and Arnason, 1996, 2006).

The probability of recapture (p) and of survival (φ) for each month (for rodents infected and not infected with *Bartonella* spp.) was calculated in MARK 6.0 software (White and Burnham, 1999). For each rodent species, tests (RELEASE) for conformity to the assumptions of the Cormack-Jolly-Seber model were applied. The best base model was then estimated (using RECAPTURES ONLY model in MARK) from a full model including full time-dependence, and age and sex of rodents for both survival and recapture probabilities ($p_{time*age*sex}$, $\Phi_{time*age*sex}$). Time dependency was established using survey number, rather than season, month or year, because the study lacked sufficient replication of years, seasons or months and because the peak in rodent abundance during the second autumn prevented direct comparison of the 2 years. By stepwise elimination of factors and comparison of the Aikake Information Criterion (AIC; the models with $\Delta AIC < 2$ were treated as

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comparable) and remaining deviance, the most parsimonious base model was established. This base model was then used to establish 2 multistate RECAPTURES ONLY models taking account of infection status with *Bartonella* (Model A: 2-state, *infected*, PCR-positive or *uninfected*, PCR-negative; Model B: 3-state, *infected*, PCR positive, *uninfected*, PCR negative with no previous history of infection and *recovered*, PCR negative with a known previous history of infection), which allowed estimation of survival and recapture probability for infected and uninfected rodents by partitioning Φ into S, the true survival function, and ψ, the probability that the animal moves between states (uninfected to infected, infected to uninfected in the 2-state model, uninfected to first infection, first infection to recovered, recovered to subsequent infection, subsequent infection to recovered in the 3-state model) in a particular month. This model assumes that the probability of survival from time t to $t+1$ is independent of the state of the rodent (infected/uninfected) in the next time-interval, $t+1$ (Cooch and White, 2010). Cohort identity was not included in the final model because it was not independent of survey number; however, separate models incorporating cohort as a factor while controlling for time-interval were developed to investigate differences in survivorship and recapture probability between cohorts.

Factors potentially explaining variance in prevalence data (rodent species, trapping session, trapping line, sex of rodents) were analysed using χ^2 statistics, and only the significant results are presented. The probability of being infected once or more was analysed using variants of the multistate models.

RESULTS

Rodent populations

Altogether 663 bank voles (1335 total captures) were marked; 316 (48%) were recaptured at least once, and 162 (24%) more than once. Of these 162 animals, complete capture histories (recaptured in every month from first capture to final disappearance) were available for 113; recapture histories were missing only a single month for a further 42 animals, giving a total of 155 animals with complete or nearly complete capture histories. Within this set of detailed histories, 64 animals were first captured as juveniles (<8 weeks old). Of 414 *A. flavicollis* marked (744 captures in total), 168 (41%) were subsequently recaptured at least once and 81 (20%) more than once. Of the 81 animals, complete capture histories were available for 33, and histories missing 1 month for a further 29 giving a complete/nearly complete data set for 62 animals, including 29 first collected as juveniles (<8 weeks old). Both species were more abundant in 2008, and catch per unit effort data suggested that abundance of bank voles in July 2008 was more than twice as high, and in the peak months of August and September 2008 four times as high, as in the corresponding months in 2007. Abundance subsequently declined again, but in November and December 2008 remained twice as high as in the same months of 2007. For mice, abundance in 2008 was 1·4 times (July) to 3·4 times (August) higher than in corresponding months in 2007. In spring 2009 a significant decrease in rodent abundance was observed, with the lowest point in April, when no individuals of *A. flavicollis* were caught (Supplementary File 1, online version only). Population estimates based on mark-recapture analysis confirmed these patterns, although the differences between the two years were not as marked as suggested by catch per unit effort data; mark-recapture estimates suggested that the *M. glareolus* population was 14%–71% larger in July–September 2008 compared to 2007, while there was little difference in the abundance of *A. flavicollis* between years (Fig. 1A).

In 2007 the abundance of both rodent species was estimated to be similar, with slightly greater abundance of *A. flavicollis* in the beginning of the trapping season (June, July) and for *M. glareolus* in the rest of the year (August-November). In 2008 the abundance of *M. glareolus* was higher than that of *A. flavicollis* throughout the year, and by spring 2009 vole abundance was 25–30 times higher than that of mice. Rodent species composition differed between the lines; bank voles were most numerous on line 3, and least abundant on line 2, where the highest densities of yellow-necked mice were observed (Supplementary File 2, online version only). Some rodents moved between the lines; most were *A. flavicollis* (30 animals, 18% of those recaptured at least once), but 29 *M. glareolus* (9% of recaptures) also moved.

Age and reproductive status of rodents

Both rodent species reproduced between early March and mid-September. The latest lactating *M. glareolus* were captured in September 2007 and October 2008, while the latest lactating *A. flavicollis* was captured a month earlier in both years. There were thus 2 main cohorts in each year, with some overlap during mid-summer. Animals born in the summer months (July and August) matured but did not breed in the autumn of their birth, and the survivors then overwintered to become reproductively active in the spring of the following year (overwinterers). All animals collected in March and April had mature pelage, and the weight distribution of previously unmarked animals (new captures) matched that of marked, known overwintered, recaptures. The earliest pregnant females of both species were found in April. There is therefore no evidence

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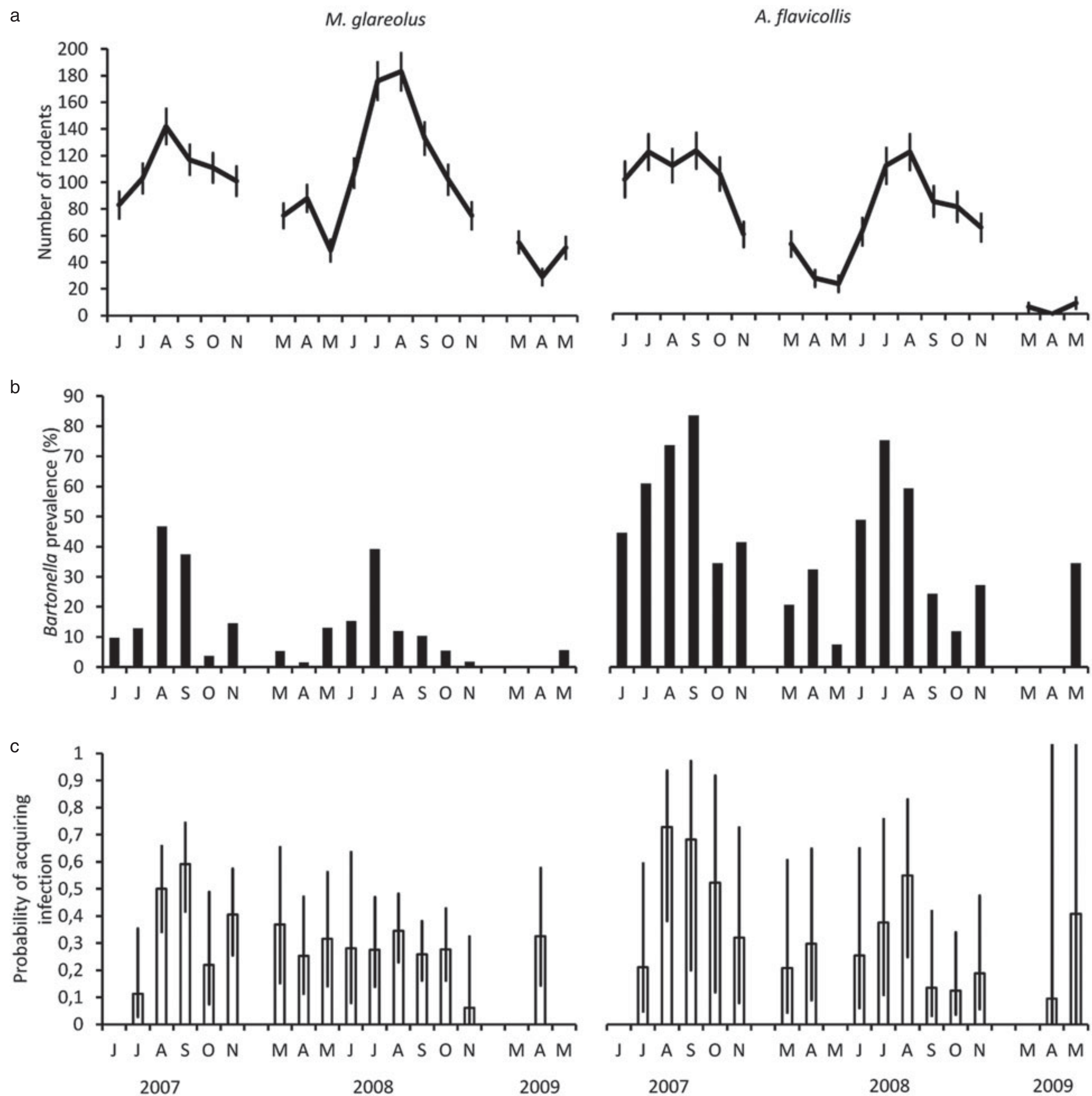

Fig. 1. Rodent abundance ± s.e., calculated in Mark 6.0 (White and Burnham, 1999) using mark-recapture estimates (Popan, Schwarz and Arnason. 1996, 2006). (A) *Bartonella* prevalence in examined rodents in following months of the study (survey *infection: $\chi^2 = 186{\cdot}79$, d.f. = 17, $P < 0{\cdot}001$ for *Myodes glareolus*, $\chi^2 = 174{\cdot}55$, d.f. = 16, $P < 0{\cdot}001$ for *Apodemus flavicollis*). (B) Probability of acquiring a new infection in successive trapping sessions with 95% CL, calculated in Mark 6.0 (White and Burnham, 1999) using multistate Recaptures Only model (C).

for reproduction before April, and the earliest young-of-the-year animals appeared in May, when all were clearly immature. Most of the overwintered cohort died during the course of the spring and summer at an age of 6–9 months, although the longest-lived over-wintering *M. glareolus* was first marked in August 2007, and was last captured in October 2008 (14 months). Spring-born animals (May, June) reproduced in the summer of their birth. A single reproducing spring-born female *M. glareolus* was collected in June 2007, but the main reproductive season for these animals was July-September. A small proportion (4 *A. flavicollis* and 22 *M. glareolus*, including the individual with a 14-month mark history) then overwintered to become sexually active again in the following spring. Because there were therefore approximately 2 cohorts of rodents present in each year, each animal was assigned to its birth cohort, numbered sequentially; cohort 0 consisted of animals born in autumn 2006 and present in summer 2007, cohort 1 represented animals born in spring 2007 and reproducing in late summer/autumn 2007, giving birth to overwintering rodents of cohort 2, which bred in spring 2008. Animals born in spring 2008 represented cohort 3 and their offspring born in late summer/autumn 2008 were assigned to

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cohort 4. Rodents born in spring 2009 were treated as cohort 5.

Models of rodent survival and recapture probabilities

Tests (Release) for non-violation of the assumptions of the Cormack-Jolly-Seber model were applied; for both species, conformity to a CJS model was supported (*A. flavicollis,* $\chi^2=44{\cdot}78$, d.f. = 85, P=, 0·999; *M. glareolus,* $\chi^2=92{\cdot}27$, d.f. = 115, $P=0{\cdot}941$). The global model ($p_{time*age*sex}$, $\Phi_{time*age*sex}$) was simplified stepwise, using AIC and deviance, to assess the most parsimonious base model. The simplest base model for *A. flavicollis* was with recapture probability constant while survival varied with time (p_{const}, Φ_{time}, AICc = 1325·5, number of parameters, np = 17, deviance, dev = 524·5; see Supplementary File 3, online version only). For *M. glareolus*, the most parsimonious model included recapture rate and survival as time (p_{time}, Φ_{time}, AICc = 2160·5, np = 33, dev = 722·3). For *M. glareolus*, survivorship of the 2007 cohorts and the autumn-born 2008 cohort was not significantly different, but survivorship of the spring-born 2008 cohort was much poorer (best model: $p_{cohort1*time, cohort2*time, cohort3*time, cohort4*time}$, $\varphi_{cohort1=cohort2=cohort4=const, cohort3*time}$; AICc = 1995·2, np = 39, dev = 387·0) (Fig. 2A), especially during the first part of the summer of 2008. For *A. flavicollis* (Fig. 2B), survivorship of the two 2007 cohorts and the spring-born 2008 animals was indistinguishable, but the survivorship of the autumn 2008 cohort was especially poor, corresponding with the almost complete disappearance of this species from the study area in the spring of 2009 (best model: p_{time}, $\varphi_{cohort1=cohort2=cohort3=const, cohort4=const}$; AICc =, 1158·9 np = 18, dev = 403·2).

Bartonella *spp.: prevalence, probability and duration of infection*

Based on PCR, the overall *Bartonella* spp. prevalence was 16·0% in bank voles and 48·2% in yellow-necked mice ($\chi^2=245{\cdot}0$, d.f. = 1, $P<0{\cdot}001$), with substantial differences between months for each of the rodent species (Fig. 1B). Prevalence peaked in the summer months. In 2007 this occurred in August for *M. glareolus* (37·4%) and September for *A. flavicollis* (67·9%), before declining in autumn. In 2008 the peak for both rodent species occurred in July (38·0% for *M. glareolus* and 71·2% for *A. flavicollis*), and there was then a rapid decline (e.g. to 11·1% for *M. glareolus* in August). This decline corresponded to the increase in rodent abundance, and poorer survivorship, in late summer and early autumn 2008. In spring 2009, when the abundance of hosts was low (no *A. flavicollis* captured in April), only 4 rodents were infected with *Bartonella*.

When using models with full time dependence the probability of acquiring new infection mirrored seasonal prevalence (Fig. 1C). The cumulative life-time probability of infection differed significantly between the 2 rodents ($\chi^2=39$, d.f. = 1, $P<0{\cdot}001$); for *A. flavicollis*, 94% animals for which complete or near-complete recapture histories were available experienced infection during their lifetimes. For *M. glareolus*, only 48% of equivalent animals experienced infection. Amongst animals which had been followed since juvenile (64 *M. glareolus* and 28 *A. flavicollis*), the majority of animals had experienced their first infection by the time they were adult (Fig. 3). Thirty-eight% of *M. glareolus* and 82% of *A. flavicollis* were first infected when juveniles, while only 18% of bank voles and 11% of mice become infected for the first time as adults. These proportions of rodents first infected at different ages differed between rodent species ($\chi^2=3{\cdot}9$, d.f. = 1, $P=0{\cdot}048$), and again, substantially more *A. flavicollis* (93%) became infected compared to *M. glareolus* (56%; $\chi^2=11{\cdot}9$, d.f. = 1, $P=0{\cdot}001$). A small number of both *A. flavicollis* and *M. glareolus* became infected more than once during their lifetimes. Using a 3-state (uninfected/infected/recovered) multistate model, it was possible to demonstrate that the probability of subsequent infection did not differ significantly from the probability of being infected for the first time: i.e. probability of first infection $0{\cdot}09\pm0{\cdot}01$ and $0{\cdot}31\pm0{\cdot}05$, probability of subsequent infection $0{\cdot}08\pm0{\cdot}03$ and $0{\cdot}25\pm0{\cdot}06$ for *M. glareolus* and *A. flavicollis*, respectively.

The duration of *Bartonella* spp. infection could be calculated only for rodents captured at least 3 times, with at least 1 month uninfected both before and after the infection. Only 29 *M. glareolus* and 17 *A. flavicollis* satisfied these criteria. The mean duration of infection for this group of *M. glareolus* was 1·17 months, while for 17 *A. flavicollis* mean duration was slightly longer, 1·59 months. No errors could be obtained for these estimates, and so they were supplemented using data from the recorded length of all observed infections (including those without a clear month uninfected before or after infection). A regression line fitted to these data (Fig. 4) suggested a 50% clearance time for *M. glareolus* of $43{\cdot}4\pm4{\cdot}3$ days, and for *A. flavicollis* of $67{\cdot}9\pm3{\cdot}4$ days. The shorter-lived *M. glareolus* infections were more likely to include both beginning and end, and therefore to be estimated accurately, and examples of infections persisting for 2, 3 and 4 months in *A. flavicollis* were observed, while in 1 mouse *Bartonella* was detected in 6 successive months (Fig. 5). Infections persisting for 2 months were more prevalent in young *M. glareolus*, and only 1 example of a 2-month infection in an overwintering vole has been observed (Fig. 5). This pattern was also seen in the smaller subset of voles ($n=19$) that were collected as juvenile animals and followed until they

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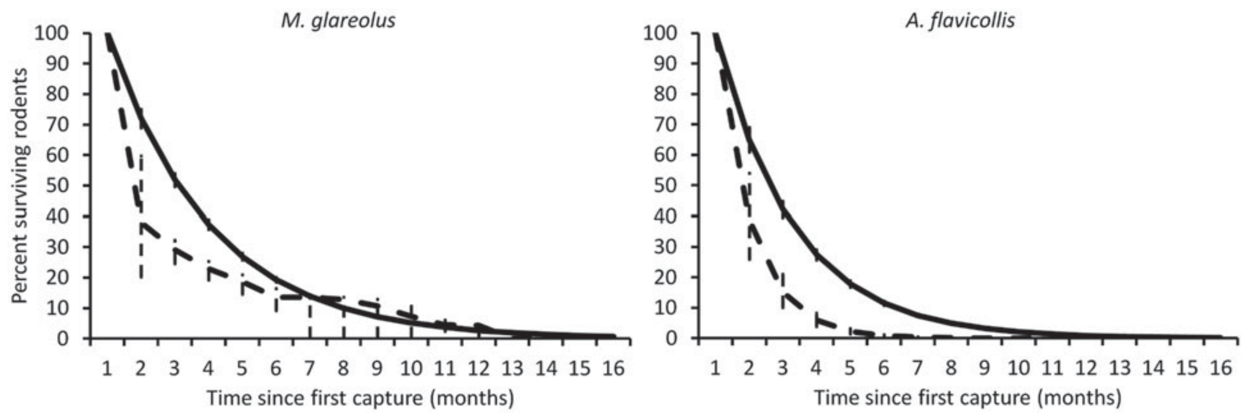

Fig. 2. Survivorship curves (with 95% CL) for different cohorts of rodent populations calculated in MARK 6.0 (White and Burnham, 1999) using RECAPTURES ONLY; solid lines represent cohorts 1, 2 and 4 for *Myodes glareolus* and cohorts 1, 2 and 3 for *Apodemus flavicollis*; dashed lines represent cohort 3 for *M. glareolus* and cohort 4 for *A. flavicollis*.

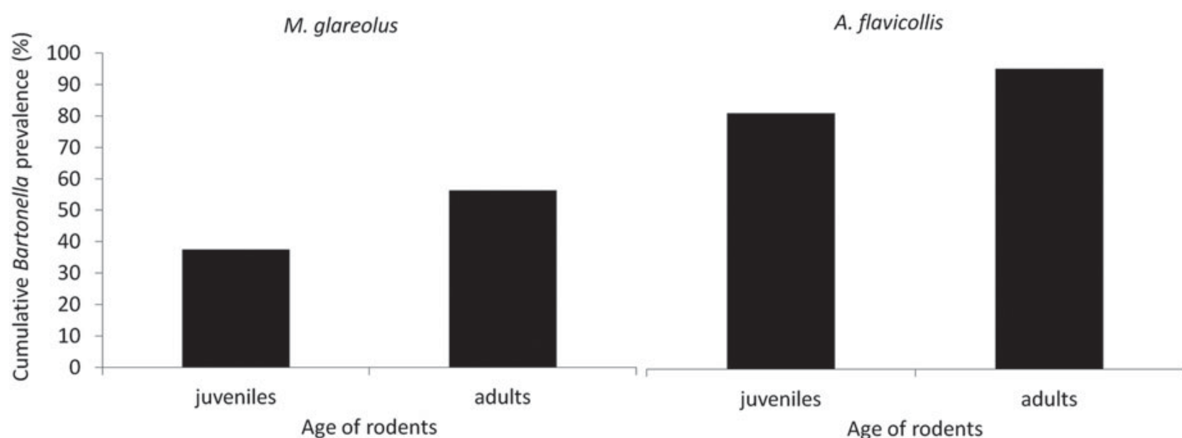

Fig. 3. Cumulative prevalence of *Bartonella* spp. in *Myodes glareolus* and *Apodemus flavicollis*, depending on the age of the rodents, analysed using a subset of animals first captured as juveniles, with complete, or nearly complete recapture histories.

disappeared from the trap lines. Unlike voles, infections lasting more than 1 month in *A. flavicollis* were more prevalent in the oldest mice (Fig. 5). These differences were not, however, significant, and are based on small sample sizes.

Bartonella *spp. infection and the probability of rodent recapture*

Since the difference in apparent course of infection of *Bartonella* between *M. glareolus* and *A. flavicollis* could have been related to differences in the effect of the pathogen on rodent survival and/or recapture, the multistate analysis was superimposed upon the base model to estimate survivorship and recapture probability. This demonstrated no significant difference between survivorship of infected and uninfected *M. glareolus* (S_{time}, $p_{inf/uninf*time}$, $\psi_{inf/uninf}$; AICc = 2628·9, np = 52, dev = 757·9; see Supplementary File 3, online version only), although the probability of recapture depended upon the

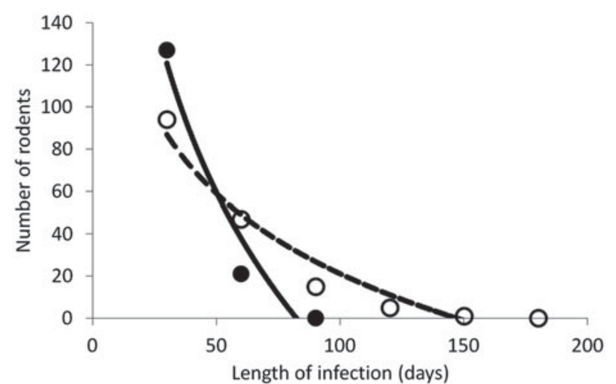

Fig. 4. Length of *Bartonella* spp. infection in *Myodes glareolus* (solid circles and solid line; $y = -119{\cdot}6\ln(x) + 527{\cdot}63$, $R^2 = 0{\cdot}9529$) and *Apodemus flavicollis* (empty circles and dashed line; $y = -54{\cdot}68\ln(x) + 272{\cdot}92$, $R^2 = 0{\cdot}9482$).

animal being infected; for *Bartonella*-positive rodents the probability of recapture was significantly lower than for uninfected ones (Fig. 6). On the other

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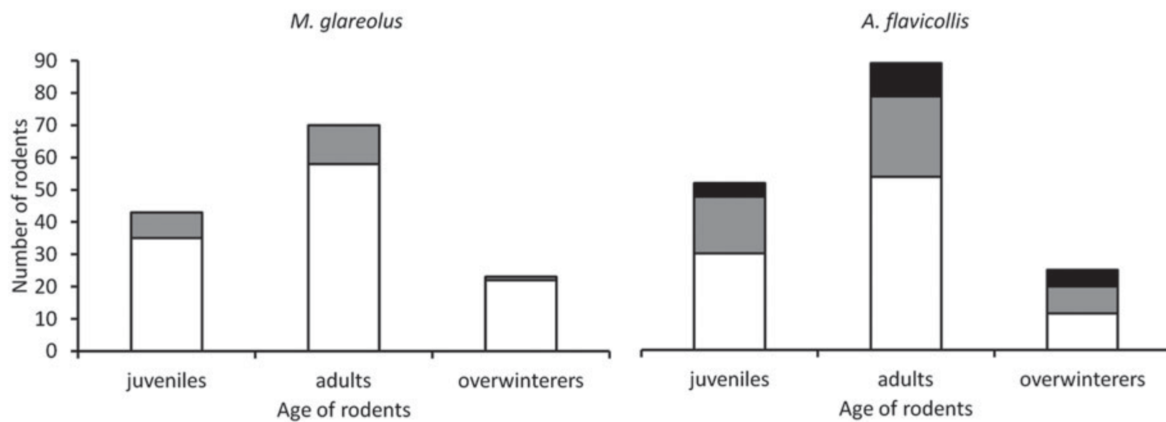

Fig. 5. Length of *Bartonella* spp. infection in *Myodes glareolus* ($\chi^2 = 2{\cdot}65$, D.F. = 2, $P = 0{\cdot}265$) and *Apodemus flavicollis* ($\chi^2 = 5{\cdot}08$, D.F. = 4, $P = 0{\cdot}280$) from rodents of different age groups; white- infections lasting 1 month, grey- 2 months, black- 3 months and more.

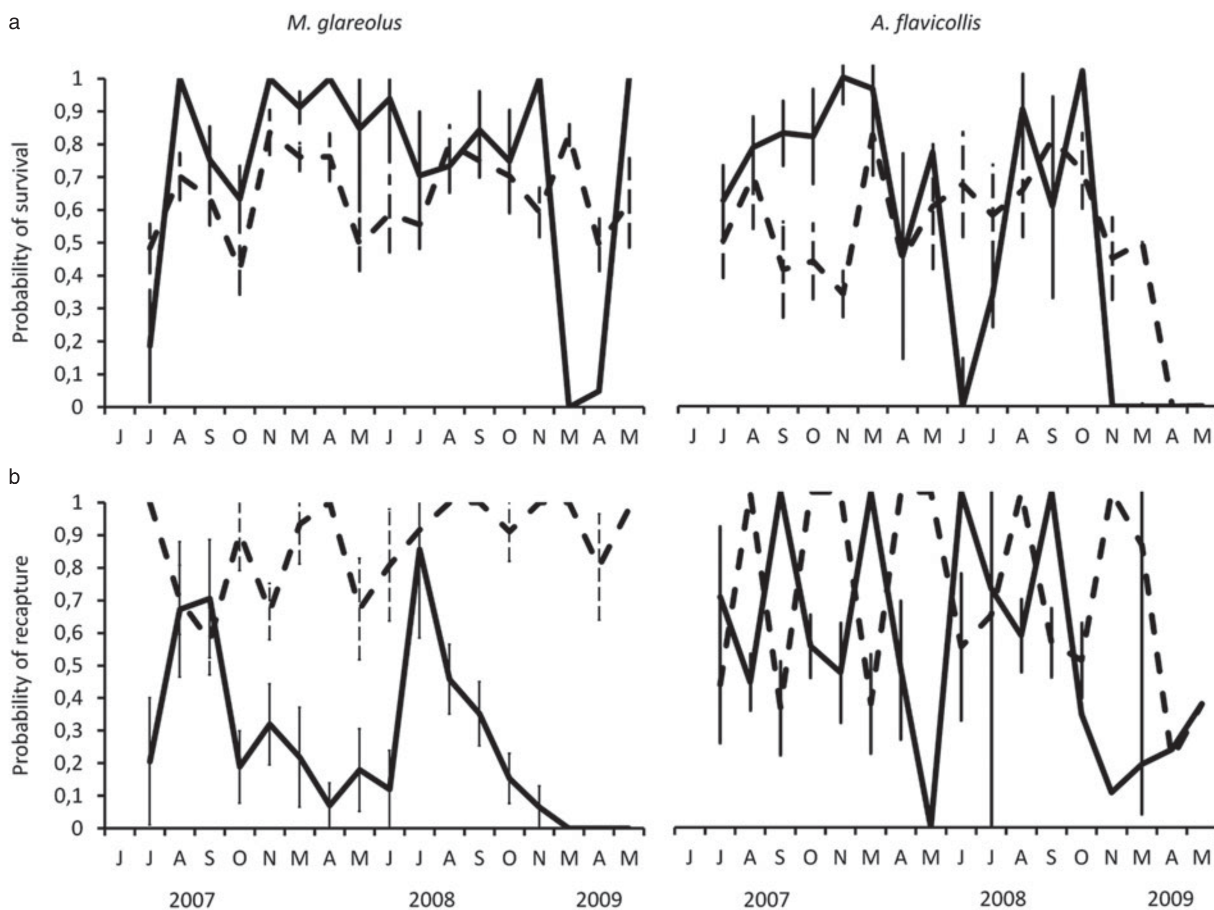

Fig. 6. Probability of survival (A) and of recapture (B) of infected (solid lines) and uninfected (dashed lines) *Myodes glareolus* and *Apodemus flavicollis* with 95% CL calculated in in MARK 6.0 (White and Burnham, 1999) using multistate RECAPTURES ONLY model.

hand, infected *A. flavicollis* appeared to survive somewhat better during the summer period than their uninfected counterparts ($S_{inf/uninf*time}$, $p_{inf/uninf}$, $\psi_{inf/uninf*time}$; AICc = 1699·0, np = 50, dev = 615·7, Fig. 6). However, dAICc between this model, and one which does not distinguish between the survivorship of infected and uninfected animals (S_{time}, $p_{inf/uninf}$, $\psi_{inf/uninf*time}$; AICc = 1701·8, np = 38, dev = 645·9), was only 2·8. Both of these models included full time dependence of acquiring/losing infection and both assumed that the probability of recapture depended on the infection; unlike *M. glareolus*, the probability of recapture was higher for infected *A. flavicollis* (0·67) than for uninfected (0·54).

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Molecular diversity of Bartonella *based on gltA gene fragment*

Sequencing of 34 *Bartonella* amplicons from *M. glareolus* and 57 from *A. flavicollis* revealed 22 *gltA* variants of *B. grahamii, B. taylorii* and *B. birtlesii* (for full details of molecular characterization see Paziewska *et al.* 2011). Only 3 *gltA* variants (Ur29, 30 and 31 according to the nomenclature of Paziewska *et al.* 2011) could be referred to *B. grahamii*. These variants differed from each other by a single base change, and only 1 (Ur31) was common, accounting for 9 of the 34 (26%) *M. glareolus* infections and 10 (17%) of those from *A. flavicollis* (see Table 1). *B. birtlesii* was only amplified once, from *A. flavicollis*. The greatest diversity of infections was caused by *B. taylorii* (70% of infections in *M. glareolus*, 77% in *A. flavicollis*), which was divided into 3, only distantly related (see Paziewska *et al.* 2011) clades. The most diverse, *B. taylorii* clade A, consisted of 11 variants, differing from each other by a maximum of 6 base changes within *gltA*. These variants showed a clear differentiation between the hosts infected. Variants Ur02–Ur11 were collected only from *A. flavicollis*, accounting for 35 of the 57 (63%) of the *Bartonella* positives sequenced from this host. The 2 variants Ur14 and Ur15 (differing from each other by a single base change) were, however, found only in *M. glareolus* (19 amplicons, 55% of all *Bartonella* positives sequenced from this host). *B. taylorii* clade B was represented by only 2 variants (Ur21 and Ur25), differing from each other by 3 bases at the *gltA* locus; the 6 sequenced examples of this clade were all recorded from *A. flavicollis*. Three variants (Ur17, 18, 19) of *B. taylorii* clade C were also collected, all 5 sequenced examples coming from *M. glareolus*. These variants also differed from each other by up to 3 base changes. A further '*B. taylorii*-like clade' (see Paziewska *et al.* 2011) was collected once from *A. flavicollis*. A final clade, Ur27, collected once from *A. flavicollis*, was a hybrid between *B. grahamii* and *B. taylorii* clade C (see Paziewska *et al.* 2011). Overall then, only the 2 *B. grahamii* variants infected both species of rodents. These data are summarized in Table 1.

In bank voles, a significant change in distribution of *Bartonella* species between the 2 years of trapping was observed ($\chi^2 = 4{\cdot}08$, D.F. = 1, $P = 0{\cdot}043$), as *B. taylorii,* responsible for 50% of infections in 2007, became more dominant, and accounted for 80% of all infections in 2008. Genotypes were equally distributed between the trapping lines, although the greatest number of different variants (14) was observed on line 2 where *A. flavicollis* was most abundant (line 2, see Supplementary File 2, online version only), compared to line 1 (9 variants) and line 3 (8 variants) (Table 1).

Molecular analysis also gave further insight into the duration of infections and re-infections. Not all amplicons were sequenced, and priority was given to obvious re-infections (i.e. 2 episodes of *Bartonella* infection separated by 1 or more months) for characterization of the *Bartonella* genotypes involved. A small number ($n = 5$) of continuous 2-month infections from *M. glareolus* were characterized by sequencing in both months; 4 consisted of the same *Bartonella gltA* clade sequenced in both months, but 1 individual (*M. glareolus* no. 3 in Table 2) was infected by *B. taylorii* Ur14 in the first month and *B. grahamii* Ur31 in the second month, suggesting that a minority of 2-month infections in *M. glareolus* may have been due to mixed infections or re-infection. A more general difference between *Bartonella* genotypes was also noted between 1- and 2-month infections in *M. glareolus*. All 1-month infections in which *gltA* genotype was confirmed by sequencing ($n = 4$) were referable to *B. taylorii*, whereas of ten 2-month infections, only 5 were referrable to *B. taylorii*, while 4 were *B. grahamii* and 1 was mixed. Within *A. flavicollis*, experiencing longer contiguous infections, the situation was much more heterogenous. Even where infections were sequenced in successive months, 4 out of 5 examples demonstrated a change of genotype (Table 2); the exception retained the same *gltA* genotype but was recombinant at other housekeeping genes (Paziewska *et al.* 2011). Sequencing of *Bartonella* amplicons from 2 *M. glareolus* and 4 *A. flavicollis* which were considered to have been re-infected (i.e. at least 1 month uninfected between infections, Table 2) revealed 5 cases of different *Bartonella* strains involved in the successive infections (Table 2), and only 1 *M. glareolus* was infected with the same genotype after a clear 2-month break in infection. One animal, an *A. flavicollis* (no. 4 in Table 2), was infected with the same genotype 1 month before, and 2 months after, a 2-month infection. Another *A. flavicollis* (no. 8), *Bartonella*-positive for 4 consecutive months (July-October), was infected successively during this period with 3 different genotypes of *Bartonella*, and in one month a mixed infection was detected. A mixed infection was also observed for another mouse in 2 successive months; the mixed infections were identified during proof-reading of chromatograms, and were not confirmed by cloning.

DISCUSSION

The present work has confirmed previous observations (Birtles *et al.* 2001; Kosoy *et al.* 2004) that *Bartonella* infections of wild rodents are transient and follow a course of infection similar to laboratory mice, which cleared *B. grahamii* infections in 45–60 days (Koesling *et al.* 2001). However, significant differences in the epidemiology of infections between the 2 host species were apparent. Prevalence was much higher in *A. flavicollis* than in *M. glareolus*, because infections of *M. glareolus* were significantly shorter,

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Table 1. Distribution of different genotypes of *Bartonella* (based on *gltA* variants) in rodents captured on different lines

Bartonella species and clade (as in Paziewska *et al*. 2011)	Variant number (as in Paziewska *et al*. 2011)	*M. glareolus* Number of isolates from rodents captured on			*A. flavicollis* Number of isolates from rodents captured on		
		Line 1	Line 2	Line 3	Line 1	Line 2	Line 3
B. taylorii-like	Ur01				1		
B. taylorii clade A	Ur02					2	
	Ur 04					1	
	Ur 05				1		2
	Ur 06				6	6	2
	Ur 07					1	
	Ur 08					1	
	Ur 09				3	6	2
	Ur 10					1	
	Ur 11					1	
	Ur 14	6	3	8			
	Ur 15			2			
B. taylorii clade C	Ur 17					1	
	Ur 18						1
	Ur 19				2	1	1
B. taylorii clade B	Ur 21	1	1	2			
	Ur 25		1				
recombinant	Ur 27				1		
B. grahamii	Ur 29						1
	Ur 30	1			1	1	
	Ur 31	3	2	4	2	4	4
B. birtlesii	Ur 33					1	

typically clearing within 1 or at most 2 months, similar to the results obtained from laboratory mice by Koesling *et al*. (2001). *A. flavicollis* on the other hand experienced substantially longer infections, lasting up to 6 months in 1 case, and many animals were infected from their first capture until their final disappearance. The duration of infection was such that for many *A. flavicollis*, despite several recaptures while infected, neither the start date nor the end date of the infection could be known. Re-infections were observed in both species, but were harder to detect in the more continuously infected *A. flavicollis*.

It could be argued that differences in the ecology of the 2 rodent species, unrelated to *Bartonella* infection, could lead to this difference in *Bartonella* epidemiology, prompting our multistate analysis of rodent survival using the mark-recapture software Mark. Increased mortality of *M. glareolus* relative to *A. flavicollis*, or a difference in vagility, could for example cut short observed infections, giving an apparently shorter patent period for *Bartonella* in this host. Nevertheless, the evidence suggests that the opposite may be true. For 3 of the 4 cohorts of each species, the probability of survival for *M. glareolus* was marginally better than that of *A. flavicollis*, possibly because these animals are less vagile and a smaller proportion migrated permanently away from the trap lines than did *A. flavicollis*. The multistate recapture model incorporating *Bartonella* infection status also suggested that, if *Bartonella* infection has any effect, it is to improve survival of *A. flavicollis* over the summer months when *Bartonella* prevalence was highest. A similar trend was also observed in *M. glareolus*, but was not significant. Interpretation of such apparent improvements in survival following infection with a pathogen are fraught with difficulty (e.g. Telfer *et al*. 2002), but we would argue that, whatever the reason for the apparent improvement, it is unlikely that infections of *M. glareolus* are differentially curtailed by impaired survival. Curiously, the recapture rate of infected *M. glareolus* was depressed, although there was no significant difference in survival rate; it is possible that *Bartonella* impacts upon recapture probability by depressing activity during the patent period. This reduction in trappability may have led to slight underestimation of infection length in this species, because animals were not always captured during the patent period. However, there still seems little doubt that infections in *M. glareolus* were substantially shorter than those in *A. flavicollis*. Further evidence that infections were not curtailed by mortality in *M. glareolus* comes from an inspection of animals for which the start or end date of the infection was

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Table 2. Genotypes of *Bartonella* (*gltA* variants, as in Paziewska *et al*. 2011) in rodents with confirmed re-infections and in those with confirmed 2-month infections with the same genotype

(N, blood sample negative; P, blood sample positive, not sequenced; M, mixed infection; *the isolates turned out to be different while using a different genetic marker.)

	Rodents in which re-infections were detected										Rodents with confirmed 2-month infections				
	M. glareolus			*A. flavicollis*							*M. glareolus*				*A. flavicollis*
Month	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
III									Ur06*			N			
IV									Ur06*			N			
V															
VI	N				P	Ur31					Ur31		Ur21		
VII	N	Ur31		Ur06	Ur09	Ur17	M	Ur31		Ur06	Ur31		Ur21	Ur14	
VIII	P	P		Ur09			M	Ur06				Ur31	N	Ur14	
IX	Ur31	N	Ur31	Ur09		Ur31	Ur02	M		Ur09	N	Ur31	N	N	
X	N	N	Ur14	N				Ur09		Ur07		N	N		Ur02
XI	Ur21	Ur31	N	Ur06	Ur02								N		Ur02

unknown. Approximately twice as many *M. glareolus* in which the end date of the infection (but not the start date) was known compared to those for which the start date alone was known are represented in the database, further suggesting that failure to catch animals which were young enough to be uninfected was a greater problem than differential loss from the system of infected animals. Loss of infected animals could have been an issue with *A. flavicollis*, making the longer infections in this species even more striking. It seems likely therefore that the difference in *Bartonella* persistence in the 2 rodent species is a property of the bacterium-rodent interaction, and not an artifact of rodent ecology. Kosoy *et al*. (2004) similarly recorded infections of up to 4 months in *Sigmodon hispidus*, suggesting that rodent species naturally vary in their response to *Bartonella*.

However, there is another explanation of the longer infections in *A. flavicollis*. When *Bartonella* genotype was considered, it was clear that infections of *M. glareolus*, composed of only 6 *gltA* genotypes, were far more homogenous than those of *A. flavicollis*, within which 18 genotypes could be found. Furthermore, there was greater heterogeneity within contiguous infections (sampled in successive months) in *A. flavicollis*, and between successive infections. The multiple genotypes present in the blood of *A. flavicollis* suggest that either multiple infections with different infections are frequent in this species, or sequential infections are so common that there is no observable break between them, giving the appearance of a long primary infection. A pattern of successive invasion or sequential detection of different *Bartonella* genotypes has also been noted in long-term, poorly resolving infections of the North American deer mouse (*Peromyscus maniculatus*) by Bai *et al*. (2011). *Bartonella* has a 3-phase life cycle (Dehio, 2008), with an initial short phase infecting endothelial cells, followed by invasion of red blood cells, within which the bacteria persist until the red blood cell is either ingested by a haematophagous insect or recycled by the host. The third phase is in the gut of the insect vector. It is assumed that immunity to *Bartonella* develops, and once developed, is long-lasting (Karem *et al*. 1999; Koesling *et al*. 2001). Koesling *et al*. (2001) further showed that passive transfer of immune serum could clear bacteria from the bloodstream, and hypothesized that this was due to blocking of bacterial release from endothelial cells. In the present study secondary infections predominantly involved a different *gltA* variant to the primary genotype, further suggesting that immunity to particular strains may play a part in *Bartonella* epidemiology. One protein which does evoke a strong antibody response is the species-specific VirB5 protein (known as the 17 kDa antigen), suggesting that antibody responses to this protein may play a significant part in immunity to infection (Anderson *et al*. 1995;

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Sweger *et al.* 2000). We have shown this virulence gene to be highly variable in some of the *B. taylorii, B. grahamii* and *B. birtlesii* clades included within the present study (Paziewska *et al.* manuscript submitted), and to be poorly correlated with *gltA* genotype. It may therefore represent an important source of molecular heterogeneity allowing co-infection of numerous *Bartonella* strains within the natural rodent population. *Bartonella* spp. are highly recombinant (Paziewska *et al.* 2011) and the frequency of recombination is likely to generate novel strains able to break immunity to previous variants. The collection of different isolates from concurrent or sequential infections of *A. flavicollis*, and the collection of 1 isolate which appeared to have undergone recombination, suggests that *Bartonella* isolates may undergo genetic exchange and recombination within *A. flavicollis*, but probably not as frequently within *M. glareolus*. This may in turn contribute to the longer span of primary infections in *A. flavicollis*.

Infection with *Bartonella* was most frequent during the summer months. When considering only those animals collected for the first time as juveniles, it is clear that the infections begin early in life, and that most *A. flavicollis* (but not *M. glareolus*) have been infected by the time they reach adulthood. An important question concerning transmission of *Bartonella*, especially in *M. glareolus*, is the extent to which infections are acquired within the nest. Four voles weighing less than 10 g with juvenile pelage were found to be PCR positive for *Bartonella*. Such animals must have only recently begun independent foraging, and since rodent *Bartonella* infections undergo an initial endothelial phase (Dehio, 2008), it is likely that they had become infected within the nest. Infections of weanling *M. glareolus* in this way, especially with the observed reduction in trappability of infected voles, could lead to a reduction in apparent infection duration in this species, and more data are needed, particularly on infections in such very young animals. Transmission is thought to occur via blood-feeding arthropods, either lice (as in *B. quintana*, see Maurin and Raoult, 1996) or more likely in the case of woodland rodents, by fleas (Bown *et al.* 2004; Morick *et al.* 2011). Transmission of pathogens from mother to offspring via fleas within the nest has been entirely ignored in the case of small mammals, although it is known to be a factor of considerable significance for bird evolutionary biology (e.g. Richner *et al.* 1993). Although the prevalence of re-infections is relatively low (only 10 re-infections in *M. glareolus* were observed), the probabilities of first infection and re-infection as calculated by the MARK software using a 3-state model are similar. The apparent deficit of re-infections may therefore be due to mortality of rodents, which experience monthly survivorship of no better than approximately 70%. Thus, although infections of overwintered animals can be identified at a population level, the probability of these occurring in a particular individual are small, and most rodents become infected as juveniles or young adults. This illustrates the relative lack of power associated with the detection of re-infection or immunity in species, such as rodents, which experience high monthly mortality.

The present work has shown that the apparently uniform infection of small mammals with *Bartonella* is in fact heterogenous, dependent upon host species, with *M. glareolus* experiencing short, resolving infections and rarely becoming re-infected, while *A. flavicollis* experience longer, poorly resolved infections which may be made up of several sequential (or multiple) *Bartonella* genotypes. A further important difference is the almost complete segregation of *Bartonella* clades between one host species or the other. Of the 22 *Bartonella gltA* variants isolated, only 2 were shared (both *B. grahamii*, differing by a single base substitution, see Paziewska *et al.* 2011) and the majority of *Bartonella* diversity (16 unique clades) was recorded from *A. flavicollis*. Apart from the 2 shared clades, all other genotypes from *A. flavicollis* and *M. glareolus* within the forest had their nearest relatives infecting *Microtus* in neighbouring fields (Paziewska *et al.* 2011). This may relate to migratory behaviour of both voles and mice, moving into neighbouring disused fields during the summer (Paziewska *et al.* 2010, 2011), and providing opportunity for transfer of *Bartonella* belonging to different clades between these disparate ecosystems. The distribution of *Bartonella* clades relative to host species does imply that only the *B. grahamii* clades transmit freely between *A. flavicollis* and *M. glareolus*. The reduced overall prevalence and decline of *B. grahamii* relative to *B. taylorii* in voles in 2008, which may have been related to the decline in abundance of *A. flavicollis* during that year, and the longer duration of infections in *A. flavicollis* all suggest that the mouse is a more competent host for *Bartonella* transmission. An implication of these findings is that either most different *Bartonella* genotypes are strictly specific to 1 rodent species, which seems unlikely given the ability of *B. grahamii* to infect laboratory mice (e.g. Koesling *et al.* 2001), or there is some barrier to transmission, possibly related to vector biology. This latter possibility remains to be investigated, although there is close overlap in the flea communities infecting both *A. flavicollis* and *M. glareolus* (Harris *et al.* 2009).

The impact of *Bartonella* on the rodent populations included in this study appears to be minimal, although the possibility that *Bartonella* may act as a modulator of, and be modulated by, other, possibly more pathogenic, parasites is clear (Telfer *et al.* 2010). Of the other possible blood parasites with which it may interact, *Babesia microti* is present in

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M. glareolus and *A. flavicollis* in Urwitałt forest (see Bajer *et al.* 2001; Siński *et al.* 2006; Welc-Falęciak *et al.* 2008) but *Anaplasma phagocytophilum* has never been found, and its competent vector, *Ixodes trianguliceps* (Bown *et al.* 2006) is absent. On the other hand, a haemotropic *Mycoplasma* species (listed as *Haemobartonella* by Bajer *et al.* 2001) is common at Urwitałt (up to 30% prevalence in *Myodes*), the haemoflagellate *Trypanosoma evotomys* occurs in *M. glareolus* and the apicomplexan *Hepatozoon* has a prevalence of ca. 15% (see Bajer *et al.* 2001). Based on statistical inference, interactions occur between *Bartonella* (all described as *B. grahamii* by Bajer *et al.* 2001) and both *Haemobartonella* and *T. evotomys* at Urwitałt (Bajer *et al.* 2001). The principal significance of *Bartonella* infections of woodland rodents remains the suspicion that these bacteria can become opportunistic zoonotic pathogens of humans living in or visiting the forest (Breitschwerdt *et al.* 2009) and that, because of their propensity for recombination (Inoue *et al.* 2008; Berglund *et al.* 2010; Paziewska *et al.* 2011), novel disease-causing strains may emerge from forest settings. The clearest message to emerge from the present work is that heterogeneity in epidemiology of *Bartonella* circulating in different hosts within a single site may play a crucial role in creating the opportunity for these pathogens to undergo genetic change.

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