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Investigating the persistence of tick-borne pathogens via the R_0 model

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SUMMARY

In the epidemiology of infectious diseases, the basic reproduction number, R_0 , has a number of important applications, most notably it can be used to predict whether a pathogen is likely to become established, or persist, in a given area. We used the R_0 model to investigate the persistence of 3 tick-borne pathogens; *Babesia microti*, *Anaplasma phagocytophilum* and *Borrelia burgdorferi* sensu lato in an *Apodemus sylvaticus*-*Ixodes ricinus* system. The persistence of these pathogens was also determined empirically by screening questing ticks and wood mice by PCR. All 3 pathogens behaved differently in response to changes in the proportion of transmission hosts on which *I. ricinus* fed, the efficiency of transmission between the host and ticks and the abundance of larval and nymphal ticks found on small mammals. Empirical data supported theoretical predictions of the R_0 model. The transmission pathway employed and the duration of systemic infection were also identified as important factors responsible for establishment or persistence of tick-borne pathogens in a given tick-host system. The current study demonstrates how the R_0 model can be put to practical use to investigate factors affecting tick-borne pathogen persistence, which has important implications for animal and human health worldwide.

Key words: tick-borne disease, zoonosis, basic reproduction number, *Ixodes ricinus*.

INTRODUCTION

In infectious disease epidemiology, the basic reproduction number, R_0 , is defined as the average number of secondary cases caused by one infected individual entering a population consisting solely of susceptible individuals (Anderson and May, 1990; Diekmann *et al.* 1990; Hartemink *et al.* 2008). R_0 has a number of important applications. It has a threshold value such that if $R_0 > 1$, a pathogen will persist should it be introduced, whilst $R_0 < 1$ suggests it will die out. R_0 is also a measure of the risk that an outbreak may occur and, when an outbreak does occur, it gives a measure of the initial rate of exponential increase of infected individuals. The proportion of a population that requires vaccination in order to prevent an outbreak is also determined using R_0 (Anderson and May, 1990; Diekmann *et al.* 1990; Hartemink *et al.* 2008). R_0 , however, is difficult to define in natural systems due to indeterminate variability in susceptibility, infectivity and contact rates among individuals. This problem is often compounded by the presence of multiple host species and transmission routes (Hartemink *et al.* 2008). Given the importance of R_0 in the epidemiology of infectious diseases there have been many attempts to define R_0 for tick-borne infections (Randolph, 1998; Norman *et al.* 1999;

Randolph *et al.* 1999; Caraco *et al.* 2002; Rosa *et al.* 2003; Ghosh and Pugliese, 2004; Rosa and Pugliese, 2007). More recently, next generation matrix methods have been employed to address the complexities of infections in natural systems (Hartemink *et al.* 2008) which has resulted in the most comprehensive and biologically correct estimation of R_0 for tick-borne infections.

Tick species of the genus *Ixodes* are important vectors of numerous pathogens worldwide (Parola and Raoult, 2001). Throughout Europe, *I. ricinus* is the vector of *Babesia microti*, *Anaplasma phagocytophilum* and *Borrelia burgdorferi* sensu lato, the agents of human babesiosis, human granulocytic anaplasmosis and Lyme borreliosis respectively (Duh *et al.* 2001; Parola, 2004; Stanzak *et al.* 2004). To be a competent vector, more than 1 developmental stage of *I. ricinus* must acquire a bloodmeal from a given host species. For trans-stadial transmission, larvae and nymphs that feed on an infected host, develop to the next instar, and infect a new host during their subsequent feed as nymphs or adults, thereby maintaining a cycle of infection. (Randolph and Storey, 1999). In some cases, ticks can also acquire an infection by feeding alongside infected ticks, without the need for systemic infection of the host (Jones *et al.* 1987; Randolph *et al.* 1996). In Europe, rodents host both larvae and nymphs of *I. ricinus* (Milne, 1949; Gern *et al.* 1998; Liz *et al.* 2000; Karbowski, 2004) and are competent transmission hosts of *B. microti*, *A. phagocytophilum* and *B. burgdorferi* s.l. *B. microti*

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is a small mammal specific pathogen whilst *A. phagocytophilum* infects both small mammals and large mammals such as deer, although it is thought that separate *A. phagocytophilum* strains exist in discrete small mammal and large mammal cycles (Bown *et al.* 2009). Members of the *B. burgdorferi* s.l. complex utilize a range of vertebrate transmission hosts, for example, the *B. valaisiana* genospecies is associated with birds and *B. afzelii* with rodents (Kurtenbach *et al.* 2002). Deer are not considered competent transmission hosts of *B. burgdorferi* s.l. (Telford *et al.* 2006). In some locations, as in Ireland, nymphs of *I. ricinus* may be found in extremely low numbers or be completely absent from small mammals (Gray *et al.* 1999, 2000; Harrison *et al.* 2010). This has led to the suggestion that small mammals may not always be important transmission hosts of tick-borne infections (Gray *et al.* 1999, 2000).

We used empirical data from Ireland, where the incidence of nymphs of *I. ricinus* on small mammals is low, and previously published tick, and pathogen-specific, data to parameterize the R_0 model of Hartemink *et al.* (2008). This model was then used to predict whether infections of *B. microti*, *A. phagocytophilum*, and *B. burgdorferi* s.l. were likely to persist in small mammal populations. The model was also used to investigate how changes in the proportion of transmission-competent hosts on which *I. ricinus* had fed, the transmission efficiency of pathogens to and from ticks and hosts, and the abundance of larvae and nymphs on hosts, affects pathogen persistence in small mammals. Predictions of the model were validated by screening small mammals and ticks for pathogens by PCR.

MATERIALS AND METHODS

Calculation of R_0

In the current study, R_0 was calculated as a function of h_c , the proportion of competent hosts on which *I. ricinus* is feeding, for *B. microti*, *A. phagocytophilum* and *B. burgdorferi* s.l. using the next-generation matrix method of Hartemink *et al.* (2008). Each element in the matrix was calculated using previously published tick-related and pathogen-specific parameters and tick-related parameters describing the distribution of life stages of *I. ricinus* on *A. sylvaticus* specific to the current study (Tables 1 and 2). As there is a paucity of literature regarding the transmission efficiency of *B. microti* and *A. phagocytophilum* from *I. ricinus* to small mammal hosts (β_{T-V}) and from small mammal hosts to *I. ricinus* (β_{V-T}), R_0 was calculated using low, medium and high transmission efficiency scenarios for these pathogens using transmission coefficients of 0.1, 0.5, and 0.9 respectively. In the case of *B. burgdorferi* s.l. previously published transmission coefficients were used.

Table 1. Ecological parameters for *Ixodes ricinus* derived from the literature and the current study (adapted from Hartemink *et al.* (2008).)

(Numbers in superscript refer to the following sources: ¹Randolph and Craine (1995), ²Randolph (2004), ³Current study, ⁴Gray (2002), ⁵Randolph, unpublished. All parameters not taken from the current study were cited by Hartemink *et al.* (2008). Please refer to the Appendix for equations used to calculate each element within the next generation matrix and for the structure of the matrix.)

Parameter	Description	Estimate
E	Eggs per adult	2000 ^{1,2}
s_L	Survival probability from egg to feeding larvae	0.05 ¹
s_N	Survival probability from feeding larvae to feeding nymph	0.1 ¹
s_A	Survival probability from feeding nymph to feeding adult	0.1 ¹
C_{LL}	Mean number of larvae co-feeding with a larva	11.99 ³
C_{NL}	Mean number of nymphs co-feeding with a larva	1 ³
C_{AL}	Mean number of adults co-feeding with a larva	0 ³
C_{LN}	Mean number of larvae co-feeding with a nymph	47.67 ³
C_{NN}	Mean number of nymphs co-feeding with a nymph	0 ³
C_{AN}	Mean number of adults co-feeding with a nymph	0 ³
C_{LA}	Mean number of larvae co-feeding with an adult	0 ³
C_{NA}	Mean number of nymphs co-feeding with an adult	0 ³
C_{AA}	Mean number of adults co-feeding with an adult	0 ³
N_{LH}	Average number of larvae on competent host	7.87 ³
N_{NH}	Average number of nymphs on competent host	0.02 ³
N_{AH}	Average number of adults on competent host	0 ³
D_L	Days of attachment of larva	2.5 ⁴
D_N	Days of attachment of nymph	3.5 ⁵
D_A	Days of attachment of adult	12 ⁵

To investigate what impact the abundance of larval and nymphal stages on hosts may have on the ability of these tick-borne pathogens to become established, or persist, in the current study, R_0 was calculated using a fixed value of h_c (corresponding to the value obtained for *A. sylvaticus* from bloodmeal analysis) across a range of mean loads of larval and nymphal ticks using a medium level transmission coefficient of 0.5 for *B. microti* and *A. phagocytophilum* and previously published transmission coefficients for *B. burgdorferi* s.l.

R_0 was calculated via the spectral decomposition of the parameterized next-generation matrix that yields a set of eigenvalues, the largest of which is R_0 . The matrix was decomposed using the eigen (matrix) function in package base of the R software

Table 2. Ecological parameters for *B. microti*, *A. phagocytophilum* and *B. burgdorferi* s.l. (adapted from Hartemink *et al.* (2008).)

(Numbers in superscript refer to the following sources: ¹Randolph (1995), ²Gray *et al.* (2002), ³Telford *et al.* (1986), ⁴Hodzic *et al.* (2001), ⁵Ogden *et al.* (1998), ⁶Bown *et al.* (2003), ⁷Randolph *et al.* (1996), ⁸Gern and Rais (1996), ⁹Randolph and Craine (1995), ¹⁰Randolph, unpublished, ¹¹Kurtenbach *et al.* (1994), ¹²Hubálek and Halouzka (1998). ^aAbsent or inefficient co-feeding transmission, ^bsuggested low, medium and high transmission efficiency scenarios, ^cabsent or inefficient transovarial transmission. References 7–12 cited by Hartemink *et al.* (2008). Please refer to the Appendix for equations used to calculate each element within the next generation matrix and for the structure of the matrix.)

Description	<i>B. microti</i>	<i>A. phagocytophilum</i>	<i>B. burgdorferi</i> s.l.	
<i>i</i>	Systemic infection duration	2.5 days ¹	40 days ³	120 days ⁷
θ	Efficiency from tick to tick	0 ^a	0 ^{4,a}	0.56 ⁸
p_L	Efficiency from competent host to larva	0.1/0.5/0.9 ^b	0.1/0.5/0.9 ^b	0.5 ⁹
p_N	Efficiency from competent host to nymph	0.1/0.5/0.9 ^b	0.1/0.5/0.9 ^b	0.5 ¹⁰
p_A	Efficiency from competent host to adult	0.1/0.5/0.9 ^b	0.1/0.5/0.9 ^b	0.4 ¹¹
q_L	Efficiency from larva to competent host	0 ^{1,2}	0 ^{5,6,c}	0.8 ¹⁰
q_N	Efficiency from nymph to competent host	0.1/0.5/0.9 ^b	0.1/0.5/0.9 ^b	0.8 ¹⁰
q_A	Efficiency from adult to competent host	0.1/0.5/0.9 ^b	0.1/0.5/0.9 ^b	0.8 ¹⁰
r_A	Efficiency from adult to egg	0 ^{1,2,c}	0 ^{5,6,c}	0.1 ¹²

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Study sites

Five sites supporting mixed broadleaf and coniferous woodland sites in Northern Ireland were sampled over 8 weeks from May until July 2007. Sites were selected on the basis that they had resident populations of red deer, *Cervus elaphus*, (2 sites) or fallow deer, *Dama dama*, (3 sites) and were therefore likely to have ticks present.

Small mammal samples

In total, 180 Self-set snap traps were deployed in pairs at 15 m intervals in vegetation adjacent to forest tracks. Traps were set after 6 pm in the evening and collected before 8 am the following morning. Each mouse was stored separately in a sealed sample bag that was also searched for unattached ticks. Ticks were removed from each mouse using fine forceps and a stiff bristle brush paying particular attention to the margins of the pinna. The total number of ticks was recorded per mouse and identified to species using standard keys (Snow, 1978; Arthur, 1963). Their developmental stage was recorded as larvae, nymph or adult. Blood of mice was sampled by cardiac puncture using a sterile 5 ml, 21-gauge syringe and needle, blood was stored in individual 1.5 ml microcentrifuge tubes at -20°C prior to DNA extraction.

Sampling of questing ticks

The abundance of questing ticks was assessed using a standardized drag sampling technique. A 1 m \times 1 m square piece of towelled material, weighted and spread out with bars at the leading and rear edge was dragged along a 15 m transect of trackside grass

at 1 m s^{-1} with a total of 20 transects per forest site. Ticks were removed from the drag after each transect using fine forceps and stored in 70% ethanol. Ticks were identified to species level using standard keys, counted, and the developmental stage recorded. In addition to ticks collected from standardized drag sample transects, additional drag samples were conducted to increase the sample size of ticks available for screening for tick-borne pathogens. All sites were sampled for questing ticks at the same time as small mammal trapping (May, June and July, 2007).

DNA extraction

DNA was extracted from blood by alkaline digestion (Bown *et al.* 2003). First, 0.5 ml of 1.25% ammonia solution was added to 50 μl of blood in a Sure-Lock microcentrifuge tube (Fisher Scientific, Loughborough, UK) and heated to 100°C for 20 min. Tubes were centrifuged, opened and heated until half the initial volume remained. The solution was diluted 1 in 10 with sterile, deionized distilled water. The same method was used to extract DNA from ticks that had first been macerated using a pipette tip. DNA extracts of ticks were not diluted. Only nymphal and adult ticks were tested for the presence of pathogens.

Detection of pathogens via polymerase chain reaction (PCR)

An Apicomplexa-specific PCR targeting the 18S rRNA gene was used to test for the presence of *Babesia microti* (Simpson *et al.* 2005). *A. phagocytophilum* and *B. burgdorferi* s.l. infections were detected using a real-time PCR assay as previously described by Courtney *et al.* (2004). Samples positive for *A. phagocytophilum* were subjected to a second,

nested PCR assay targeting the *mSP4* gene for sequence determination (De La Fuente *et al.* 2005; Bown *et al.* 2007). Samples positive for *B. burgdorferi* s.l. were subjected to a second, nested PCR targeting the 5S-23S intergenic spacer region (Rijpkema *et al.* 1995). All PCRs included negative controls in a ratio of 1:5 and positive controls. Amplification products were purified using a Qiaquick PCR purification kit (Qiagen) and sequences determined using a commercial sequencing service (Macrogen, Korea). Sequence data from successfully sequenced amplification products were used to search for other closely related sequences using the NCBI nucleotide BLAST database. Sequences were aligned and compared using BioEdit v7.0.9© (Ibis Biosciences, California, USA).

Bloodmeal analysis

Bloodmeal analysis, to identify hosts that questing *I. ricinus* nymphs had fed on as larvae, was conducted using a published reverse line blot (RLB) protocol (Humair *et al.* 2007). Five probes were used ('*Apodemus*', 'bird', '*Capreolus*', '*Sciurus*' and '*Sorex*') as they represent the most likely vertebrate hosts present at study sites, targeting *Apodemus sylvaticus*, birds, deer, squirrel spp. and *Sorex minutus* respectively.

RESULTS

The basic reproduction number, R_0

Values of R_0 plotted as a function of h_c (the proportion of competent hosts on which *I. ricinus* is feeding), for *B. microti*, *A. phagocytophilum* and *B. burgdorferi*, s.l. are presented in Fig. 1.

In the case of *B. microti*, the threshold value for R_0 was never reached regardless of the proportion of competent hosts on which *I. ricinus* had fed or the transmission efficiency scenario employed. This was also the case for *A. phagocytophilum* under low transmission efficiency. However, for medium and high transmission scenarios the proportion of competent hosts on which *I. ricinus* was required to feed upon in order for the threshold value to be reached were 30% and 9% respectively. When h_c was fixed at 11.45% (representing 11 out of 96 positive reactions obtained for *A. sylvaticus* during bloodmeal analysis) the transmission coefficient required to produce a value of $R_0 > 1$ for *A. phagocytophilum* was 0.795. In contrast, the threshold value of R_0 was rapidly achieved for *B. burgdorferi* s.l. with only 2.55% of competent hosts required to be feeding *I. ricinus* for the threshold to be reached.

A plot of the interaction between mean number of larvae and nymphs on a competent host and R_0 for each pathogen is presented in Fig. 2. In the case of *B. microti*, increasing the mean number of larvae and

nymphs on the host slowly increased the value of R_0 , but even at unrealistically high tick burdens (80 larvae and 80 nymphs) the threshold value of R_0 was not reached. In the case of *A. phagocytophilum*, however, the threshold value was achieved much more rapidly, requiring, only a single larvae and 30 nymphs or 20 larvae and a single nymph for the threshold value to be achieved. Similarly, in the case of *B. burgdorferi* s.l. the value of R_0 increased rapidly with increasing tick load, requiring only a single larvae and a single nymph for the threshold value of R_0 to be reached.

Tick distribution

A total of 233 questing ticks consisting of 100 larvae, 129 nymphs and 4 adults were collected from standardized drag samples. The only tick species identified was *I. ricinus*. Densities were generally low with a mean abundances per $m^2 \pm$ s.e. for larvae, nymphs and adults of 0.086 ± 0.019 , 0.067 ± 0.014 , and 0.003 ± 0.001 respectively. A total of 1168 ticks consisting of 1165 larvae, 3 nymphs and 0 adults were collected from wood mice, giving an overall nymph:larvae ratio of 1:388. Again, the only tick species recovered was *I. ricinus*. Mean tick burdens per mouse \pm s.e. for larvae, nymphs and adults were 7.871 ± 1.087 , 0.020 ± 0.011 and 0 respectively. The distribution of ticks on wood mice was over-dispersed, with a small proportion of the host population (20%) feeding the majority of larvae (72%) and all nymphs.

Pathogen detection

In addition to the 100 nymphs and 4 adult ticks collected by standardized drag samples, a further 167 nymphs and 6 adults were collected by non-standardized drags. In total, 137 wood mice and 277 ticks (267 nymphs and 10 adults) were tested for the presence of *B. microti* and *A. phagocytophilum* whilst the 277 ticks were also tested for *B. burgdorferi* s.l. Three *I. ricinus* nymphs tested positive for the presence of *A. phagocytophilum* but no wood mice or adult ticks were positive. Of the 277 ticks screened for the presence of *B. burgdorferi* s.l. 20 nymphs were positive. No samples were positive for the presence *Babesia microti*.

Sequence analyses

(a) *A. phagocytophilum*. Of the 3 tick samples that tested positive for *A. phagocytophilum*, 2 (R14 and R49) were sequenced successfully. R14 and R49 were not identical but shared 96.3% similarity. R14 was identical to a strain found in a dog in Slovenia (GenBank Accession no. EF442004), whilst R49 was most closely related to strains recovered from red

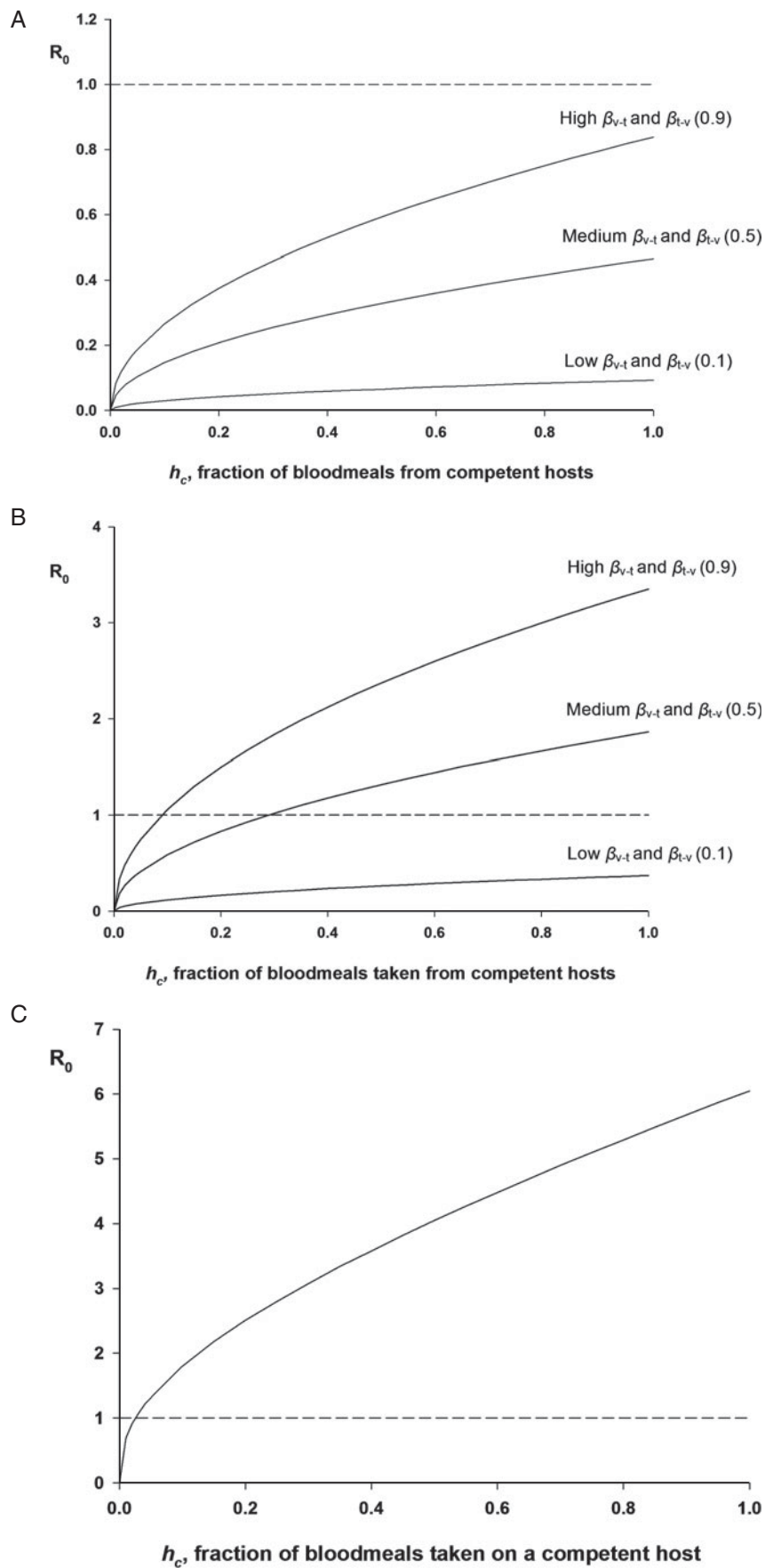


Fig. 1. R_0 plotted as a function of h_c , the fraction of bloodmeals taken on a competent host, for (a) *Babesia microti*, (b) *Anaplasma phagocytophilum* (both under low, medium and high transmission efficiency scenarios) and (c) *Borrelia burgdorferi* s.l. using previously published transmission coefficients (cited by Hartemink *et al.* 2008).

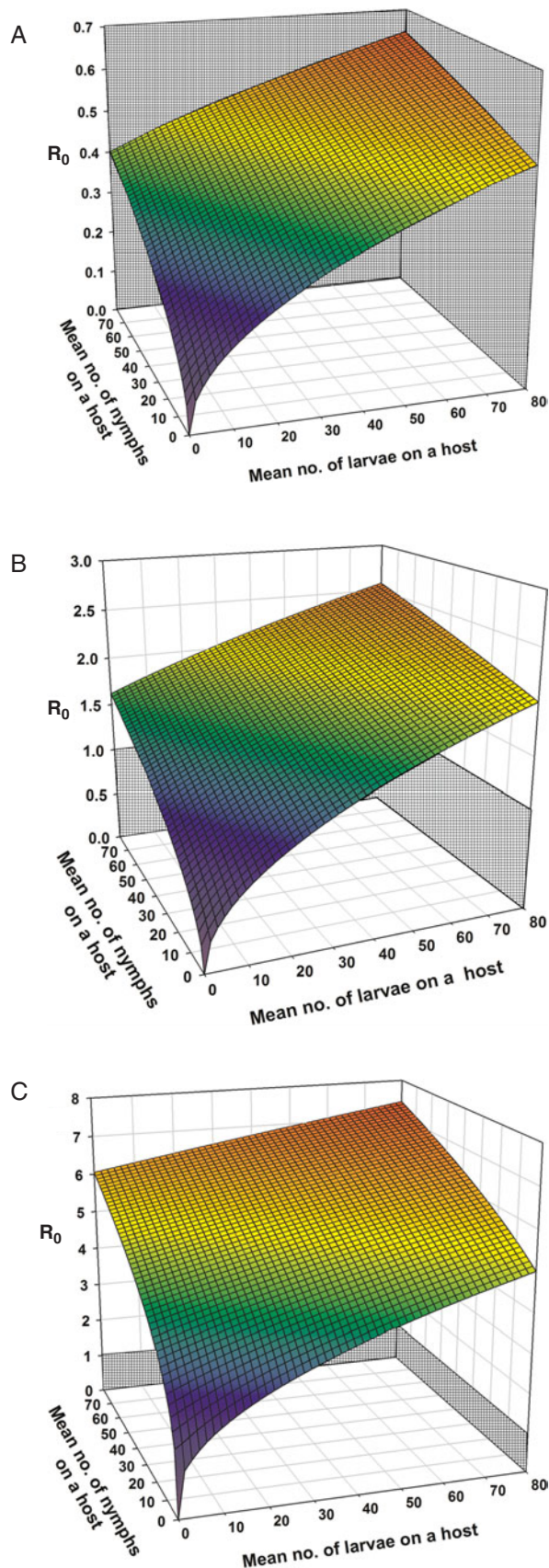


Fig. 2. Interaction of mean larval and nymphal abundance of *Ixodes ricinus* on *Apodemus sylvaticus* and R_0 for (a) *Babesia microti*, (b) *Anaplasma phagocytophilum* and (c) *Borrelia burgdorferi* s.l. assuming a medium level transmission efficiency of 0.5 for (a, b), previously

and roe deer in Slovakia and a lamb from Norway sharing 98.0% sequence similarity (EU180065, EF442003 and EU240474, respectively). All percentage similarities are across 301 base pairs.

(b) *B. burgdorferi* s.l. Of the 20 ticks that tested positive for *B. burgdorferi* s.l. 13 were sequenced successfully. R6, R17, R21, R57, T5, T61 and TC64 were most closely related to the *B. garinii* genospecies (AB178361) sharing 96.4%–98.2% sequence similarity. TC33 and TC19 were most closely related to the *B. afzelii*-type strain (GQ369937) with 93.2% and 98.6% sequence similarity and L1, TC16, R64 and T1 were most closely related to the *B. valaisiana* genospecies (L30134) with 93.5%–97.3% similarity. Therefore, 85% of *B. burgdorferi*-positive samples successfully sequenced were bird-associated genospecies whilst 15% were associated with rodents. All percentage similarities are across 225 base pairs.

Bloodmeal analysis

A total of 170 questing *I. ricinus* nymphs collected from 4 sites were included in bloodmeal analysis, 83 of which yielded positive reactions. DNA from more than 1 host was found in 13/83 positive reactions resulting in 96 host identifications made from 83 positive reactions. Birds were the most important hosts for *I. ricinus* nymphs feeding as larvae and were present in 51 out of 96 host identifications. Deer were the second most important hosts (18/96) followed by wood mice and pygmy shrews (both 11/96). Squirrels were the least important hosts for larval ticks, present in only 5 out of 96 host identifications. None of the ticks that tested positive for *A. phagocytophilum* yielded reactions in the bloodmeal analysis. Sixteen of the 20 nymphs that tested positive for *B. burgdorferi* s.l. were included in bloodmeal analysis. Of the 8 *B. burgdorferi* s.l. positive samples identified as the *B. valaisiana* genotype by sequence analysis, 4 gave positive reactions all of which indicated that the ticks had previously fed on birds. Of the 3 *B. burgdorferi* s.l. positive samples identified as *B. garinii*, 1 gave a positive host identification indicating that this tick had also fed on a bird. Neither of the samples identified as *B. afzelii* by sequence analysis gave positive host identifications. Two samples which gave positive host identifications were of mixed origin, both of which included a deer and shrew signal.

published transmission coefficients (cited by Hartemink *et al.* 1998) for (c) and $h_c = 11.45\%$ for all 3 pathogens. Hatching indicates areas of the plot where $R_0 < 1$.

DISCUSSION

The basic reproduction number, R_0 , responded differently for each pathogen in response to the proportion of competent hosts on which *I. ricinus* fed and the mean abundance of larval and nymphal ticks on hosts. Values of R_0 suggested that *B. microti* could not persist given the distribution of life-history stages of ticks on wood mice, even if the transmission coefficients were high, if ticks fed solely on competent reservoir hosts, or if tick larval and nymphal tick burdens were unrealistically high. This was supported by the absence of *B. microti* in wood mice and questing ticks when screened by PCR. The inability of *B. microti* to become established or persist in this system is likely to be a product of the short period of infectivity that this pathogen has for ticks of 1–4 days (Randolph, 1995).

In the case of *A. phagocytophilum*, the threshold value of R_0 was achieved, but only when the proportion of competent hosts on which *I. ricinus* had fed was greater than that of the current study or when the transmission coefficient was unrealistically high. *A. phagocytophilum* was not detected in small mammals but *A. phagocytophilum* was found in questing ticks. However, sequence analysis revealed that the strains were most closely related to those recovered from large mammals across Europe suggesting that other, large mammal, hosts of *I. ricinus* present at the study site were responsible for these infections. Moreover, Bown *et al.* (2009) observed that different *A. phagocytophilum* strains exist in discrete enzootic small mammal and large mammal cycles. The prevalence of infection of *I. ricinus* nymphs was low (1.12%) and the probability of a mouse feeding a nymph was also low (2.02%). Even if different strains of *A. phagocytophilum* were capable of utilizing both large and small mammals the probability of a nymph infected with *A. phagocytophilum* feeding on a mouse was extremely low (0.02% or 1 in 5000) making the spillover of *A. phagocytophilum* from larger to small mammals highly unlikely. Therefore, it is highly probable that the *A. phagocytophilum* strains present in the current study were involved in an ungulate-tick cycle and that no *A. phagocytophilum* cycles were present in wood mice.

In contrast to *B. microti* and *A. phagocytophilum*, the threshold value of R_0 for *B. burgdorferi* s.l. was achieved rapidly, requiring *I. ricinus* to feed on a much smaller proportion of competent hosts than encountered in the current study (2.55%). This threshold value was reached using realistic transmission coefficients and required fewer larval and nymphal tick abundances to feed on mice than that recorded in the current study. Values of R_0 indicated that small mammals alone could maintain cycles of infection of *B. burgdorferi* s.l. without the need for alternative transmission hosts. s.l. This suggestion

was at least partially supported by the identification of *B. afzelii*, a rodent-associated *Borrelia* genospecies (Kurtenbach *et al.* 2002), in questing ticks. However, the origin of the *B. afzelii* infections could not be determined by bloodmeal analysis. Squirrels are also competent reservoirs of this *Borrelia* genospecies (Craine *et al.* 1997) and it is possible that they were the origin of the infection.

Differences in the response of R_0 between *B. microti* and *A. phagocytophilum* most likely lie in differences in the systemic infection duration. Clinical infections of *B. microti* have been detected for up to 31 days post-infection by PCR in the USA (Vannier *et al.* 2004). As previously mentioned, Randolph (1995) observed that in the actual period of infectivity for ticks feeding on an infected host is 1–4 days using British strains. *A. phagocytophilum* infections have been detected by PCR for up to 40 days post-infection (Telford *et al.* 1996) but the actual period of infectivity is unknown. If, like *B. microti*, the period of infectivity is much less than the period where the infection can be detected by PCR then the threshold value of R_0 would be more difficult to achieve and infection cycles of *A. phagocytophilum* less likely to develop.

The ability of *B. burgdorferi* s.l. to become established more readily in the wood mouse-tick system than other pathogens is a product of its relatively long systemic infection duration and the secondary route of infection available via efficient co-feeding transmission (Randolph *et al.* 1996).

As expected, wood mice were infected almost exclusively with larvae and only 3 nymphs were recovered. The resultant small nymph to larvae ratio (1:388) is comparable to those found elsewhere in Ireland (1:∞ and 1:650 (Gray *et al.* 1992) and 1:105 (Gray *et al.* 1999) but is generally much smaller than those recorded across the rest of Europe (min=1:7, max=1:185, mean=1:44, $n=19$) (Matuschka *et al.* 1991; Humair *et al.* 1993; Talleklint and Jaenson, 1994; Kurtenbach *et al.* 1995; Humair *et al.* 1999; Randolph and Storey, 1999; Randolph *et al.* 1999)). It has been suggested that climatic conditions, such as humidity and temperature, can determine the distribution of tick life stages on hosts (Randolph and Storey, 1999). Ticks are prone to desiccation and immature stages are more susceptible than adults due to their smaller surface area to volume ratio, higher metabolic rate and limited fat reserves (Randolph and Storey, 1999). As a result, different life stages quest at different heights in vegetation, with larvae questing close to the moist litter layer and nymphs and adults questing progressively higher (Gigon, 1985). Experimental data have shown that nymphs, when confronted by increasingly dry conditions, quest lower in vegetation and feed more frequently on small mammals (Randolph and Storey, 1999). Ireland has a temperate maritime climate and generally has higher levels of precipitation and lower temperatures

than other locations across Europe (BIOCLIM variables; BIO12-annual precipitation and BIO1-annual mean temperature, www.worldclim.org/bioclim). Therefore, it is likely that nymphs in Ireland quest higher in vegetation than individuals in drier locations and, as a result, do not encounter small mammals as frequently. Low nymph to larvae ratios may limit the development of enzootic tick-borne pathogen cycles in small mammals. However, the distribution of *I. ricinus* on small mammals is often over-dispersed and this must be taken into account when assessing if tick-borne pathogen cycles are likely to be present, or develop, in a given area (Nilsson and Lundqvist, 1978; Craine *et al.* 1995; Randolph *et al.* 1999). For example, Randolph *et al.* (1999) found that the same 20% of small mammal hosts fed 61% of larvae and 72% of nymphs whilst a similar observation was made in the current study (20% of hosts fed 72% of larvae and all nymphs). This coincident aggregated distribution has important implications for the transmission of tick-borne pathogens as it allows small numbers of nymphs to feed alongside, and potentially infect, large numbers of larvae (Randolph *et al.* 1999). Therefore, even small nymph to larvae ratios, such as those found in Ireland may be epidemiologically significant.

Sequence analysis indicated that 2 bird-associated genospecies of *B. burgdorferi* s.l. were also present in *I. ricinus* nymphs, *B. valaisiana* and *B. garinii* (Kurtenbach *et al.* 2002). Bloodmeal analysis revealed that birds were the most important hosts of larval *I. ricinus* and that ticks infected with *B. valaisiana* and *B. garinii* had previously fed on birds. Therefore, it is not surprising that bird-associated *Borrelia* genospecies were the most common infections present. Present data suggest that birds are important hosts of larval *I. ricinus* and have a more important role in the epidemiology of *B. burgdorferi* s.l. in Ireland than small mammals. This suggestion is supported by previous studies in Ireland that also found bird-associated *Borrelia* genospecies to be the most common *Borrelia* infections present in questing ticks and that wood mice were rarely infected with *B. burgdorferi* s.l. (Kirstein *et al.* 1997; Gray *et al.* 1999, 2000).

The current study highlights how individual variation in the ecological parameters of tick-borne pathogens and their vectors can greatly affect the probability of establishment and persistence of pathogens within a system. We believe the R_0 model of Hartemink *et al.* (2008) and the methods currently presented provide a potentially valuable tool in the control of tick-borne pathogens, allowing the identification of factors responsible for tick-borne pathogen persistence which could be utilized in management decisions. The view that small mammals have a more limited role in the epidemiology of tick-borne infections where nymphs of *I. ricinus* are rare on small mammals is supported.

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REFERENCES

- Anderson, R. M. and May, R. M. (1990). Modern vaccines: Immunisation and herd immunity. *Lancet* **335**, 641–645.
- Arthur, D. R. (1963). *British Ticks*. 1st Edn. Butterworths, London.
- Bown, K. J., Begon, M., Bennett, M., Woldehiwet, Z. and Ogden, N. H. (2003). Seasonal dynamics of *Anaplasma phagocytophilum* in a rodent-tick (*Ixodes trianguliceps*) system, United Kingdom. *Emerging Infectious Diseases* **9**, 63–70.
- Bown, K. J., Lambin, X., Ogden, N. H., Begon, M., Telford, G., Woldehiwet, Z. and Birtles, R. J. (2009). Delineating *Anaplasma phagocytophilum* ecotypes in coexisting, discrete enzootic cycles. *Emerging Infectious Diseases* **15**, 1948–1954.
- Bown, K. J., Lambin, X., Ogden, N. H., Petrovec, M., Shaw, S. E., Woldehiwet, Z. and Birtles, R. J. (2007). High-resolution genetic fingerprinting of European strains of *Anaplasma phagocytophilum* by use of multilocus variable-number tandem-repeat analysis. *Journal of Clinical Microbiology* **45**, 1771–1776.
- Caraco, T., Glavanakov, S., Chen, G., Flaherty, J. E., Ohsumi, T. K. and Szymanski, B. K. (2002). Stage-structured infection transmission and a spatial epidemic: a model for Lyme disease. *The American Naturalist* **160**, 348–359.
- Courtney, J. W., Kostelnik, L. M., Zeidner, N. S. and Massung, R. F. (2004). Multiplex real-time PCR for detection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi*. *Journal of Clinical Microbiology* **42**, 3164–3168.
- Craine, N. G., Nuttall, P. A., Marriott, A. C. and Randolph, S. E. (1997). Role of grey squirrels and pheasants in the transmission of *Borrelia burgdorferi* sensu lato, the Lyme disease spirochaete, in the U.K. *Folia Parasitologica* **44**, 155–160.
- Craine, N. G., Randolph, S. E. and Nuttall, P. A. (1995). Seasonal variation in the role of grey squirrels as hosts of *Ixodes ricinus*, the tick vector of the Lyme disease spirochaete, in a British woodland. *Folia Parasitologica* **42**, 73–80.
- De La Fuente, J., Massung, R. F., Wong, S. J., Chu, F. K., Lutz, H., Meli, M., Von Loewenich, F. D., Grzeszczuk, A., Torina, A. and Caracappa, S. (2005). Sequence analysis of the *msp4* gene of *Anaplasma phagocytophilum* strains. *Journal of Clinical Microbiology* **43**, 1309–1317.
- Diekmann, O., Heesterbeek, J. A. P. and Metz, J. A. J. (1990). On the definition and the computation of the basic reproduction ratio R_0 in models for infectious diseases in heterogeneous populations. *Journal of Mathematical Biology* **28**, 365–382.
- Duh, D., Petrovec, M. and Avsic-Zupanc, T. (2001). Diversity of *Babesia* infecting European sheep ticks (*Ixodes ricinus*). *Journal of Clinical Microbiology* **39**, 3395–3397.
- Gern, L., Estrada-Pena, A., Frandsen, F., Gray, J. S., Jaenson, T. G., Jongejans, F., Kahl, O., Korenberg, E., Mehl, R. and Nuttall, P. A. (1998). European reservoir hosts of *Borrelia burgdorferi* sensu lato. *Zentralblatt für Bakteriologie: International Journal of Medical Microbiology* **287**, 196–204.
- Gern, L. and Rais, O. (1996). Efficient transmission of *Borrelia burgdorferi* between co-feeding *Ixodes ricinus* ticks (Acari: Ixodidae). *Journal of Medical Entomology* **33**, 189–192.
- Ghosh, M. and Pugliese, A. (2004). Seasonal population dynamics of ticks, and its influence on infection transmission: A semi-discrete approach. *Bulletin of Mathematical Biology* **66**, 1659–1684.
- Gigon, F. (1985). *Biologie d'Ixodes ricinus L. sur le Plateau Suisse—Une contribution à l'écologie de ce vecteur*. Unpublished Ph.D. thesis, University of Neuchâtel, France.
- Gray, J., von Stedingk, L. V., Gurtelschmid, M. and Granstrom, M. (2002). Transmission studies of *Babesia microti* in *Ixodes ricinus* ticks and gerbils. *Journal of Clinical Microbiology* **40**, 1259–1263.
- Gray, J. S. (2002). Biology of *Ixodes* species ticks in relation to tick-borne zoonoses. *Wiener klinische Wochenschrift* **114**, 473–478.

- Gray, J. S., Kahl, O., Janetzki, C. and Stein, J. (1992). Studies on the ecology of Lyme disease in a deer forest in County Galway, Ireland. *Journal of Medical Entomology* **29**, 915–920.
- Gray, J. S., Kirstein, F., Robertson, J. N., Stein, J. and Kahl, O. (1999). *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks and rodents in a recreational park in south-western Ireland. *Experimental and Applied Acarology* **23**, 717–729.
- Gray, J. S., Robertson, J. N. and Key, S. (2000). Limited role of rodents as reservoirs of *Borrelia burgdorferi* sensu lato in Ireland. *European Journal of Epidemiology* **16**, 101–103.
- Harrison, A., Scantlebury, M. and Montgomery, W. I. (2010). Body mass and sex-biased parasitism in wood mice *Apodemus sylvaticus*. *Oikos* **119**, 1099–1104.
- Hartemink, N. A., Randolph, S. E., Davis, S. A. and Heesterbeek, J. A. P. (2008). The basic reproduction number for complex disease systems: Defining R_0 for tick-borne infections. *The American Naturalist* **171**, 743–754.
- Hodzic, E., Borjesson, D. L., Feng, S. and Barthold, S. W. (2001). Acquisition dynamics of *Borrelia burgdorferi* and the agent of human granulocytic ehrlichiosis at the host-vector interface. *Vector-Borne and Zoonotic Diseases* **1**, 149–158.
- Hubálek, Z. and Halouzka, J. (1998). Prevalence rates of *Borrelia burgdorferi* sensu lato in host-seeking *Ixodes ricinus* ticks in Europe. *Parasitology Research* **84**, 167–172.
- Humair, P. F., Douet, V., Cadenas, F. M., Schouls, L. M., Van De Pol, I. and Gern, L. (2007). Molecular identification of bloodmeal source in *Ixodes ricinus* ticks using 12S rDNA as a genetic marker. *Journal of Medical Entomology* **44**, 869–880.
- Humair, P. F., Rais, O. and Gern, L. (1999). Transmission of *Borrelia afzelii* from *Apodemus* mice and *Clethrionomys* voles to *Ixodes ricinus* ticks: differential transmission pattern and overwintering maintenance. *Parasitology* **118**, 33–42.
- Humair, P. F., Turrian, N., Aeschlimann, A. and Gern, L. (1993). *Borrelia burgdorferi* in a focus of Lyme borreliosis: epizootiologic contribution of small mammals. *Folia Parasitologica* **40**, 65–70.
- Jones, L. D., Davies, C. R., Steele, G. M. and Nuttall, P. A. (1987). A novel mode of arbovirus transmission involving a nonviraemic host. *Science* **237**, 775–777.
- Karbowiak, G. (2004). Zoonotic reservoir of *Babesia microti* in Poland. *Polish Journal of Microbiology* **53**, 61–65.
- Kirstein, F., Rijpkema, S., Molkenboer, M. and Gray, J. S. (1997). Local variations in the distribution and prevalence of *Borrelia burgdorferi* sensu lato genospecies in *Ixodes ricinus* ticks. *Applied and Environmental Microbiology* **63**, 1102–1106.
- Kurtenbach, K., De Michelis, S., Etti, S., Schäfer, S. M., Sewell, H. S., Brade, V. and Kraiczy, P. (2002). Host association of *Borrelia burgdorferi* sensu lato—the key role of host complement. *Trends in Microbiology* **10**, 74–79.
- Kurtenbach, K., Dizij, A., Seitz, H. M., Margos, G., Moter, S. E., Kramer, M. D., Wallich, R., Schaible, U. E. and Simon, M. M. (1994). Differential immune responses to *Borrelia burgdorferi* in European wild rodent species influence spirochete transmission to *Ixodes ricinus* L. (Acari: Ixodidae). *Infection and Immunity* **62**, 5344–5352.
- Kurtenbach, K., Kampen, H., Dizij, A., Arndt, S., Seitz, H., Schaible, U. E. and Simon, M. M. (1995). Infestation of rodents with larval *Ixodes ricinus* (Acari: Ixodidae) is an important factor in the transmission cycle of *Borrelia burgdorferi* sl in German woodlands. *Journal of Medical Entomology* **32**, 807–817.
- Liz, J. S., Anderes, L., Sumner, J. W., Massung, R. F., Gern, L., Rutti, B. and Brossard, M. (2000). PCR detection of granulocytic ehrlichiae in *Ixodes ricinus* ticks and wild small mammals in western Switzerland. *Journal of Clinical Microbiology* **38**, 1002–1007.
- Matuschka, F. R., Fischer, P., Musgrave, K., Richter, D. and Spielman, A. (1991). Hosts on which nymphal *Ixodes ricinus* most abundantly feed. *The American Journal of Tropical Medicine and Hygiene* **44**, 100–107.
- Milne, A. (1949). The ecology of the sheep tick, *Ixodes ricinus* L. Host relationships of the tick, Part 2 Observations on hill and moorland grazings in northern England. *Parasitology* **39**, 173–197.
- Nilsson, A. and Lundqvist, L. (1978). Host selection and movements of *Ixodes ricinus* (Acari) larvae on small mammals. *Oikos* **31**, 313–322.
- Norman, R., Bowers, R. G., Begon, M. and Hudson, P. J. (1999). Persistence of tick-borne virus in the presence of multiple host species: tick reservoirs and parasite mediated competition. *Journal of Theoretical Biology* **200**, 111–118.
- Ogden, N. H., Bown, K., Horrocks, B. K., Woldehiwet, Z. and Bennett, M. (1998). Granulocytic Ehrlichia infection in ixodid ticks and mammals in woodlands and uplands of the UK. *Medical and Veterinary Entomology* **12**, 423–429.
- Parola, P. (2004). Tick-borne rickettsial diseases: emerging risks in Europe. *Comparative Immunology, Microbiology and Infectious Diseases* **27**, 297–304.
- Parola, P. and Raoult, D. (2001). Ticks and tick-borne bacterial diseases in humans: an emerging infectious threat. *Clinical Infectious Diseases* **32**, 897–928. doi: 10.1086/319347.
- Randolph, S. E. (1995). Quantifying parameters in the transmission of *Babesia microti* by the tick *Ixodes trianguliceps* amongst voles (*Clethrionomys glareolus*). *Parasitology* **110**, 287–295.
- Randolph, S. E. (1998). Ticks are not insects: consequences of contrasting vector biology for transmission potential. *Parasitology Today* **14**, 186–192.
- Randolph, S. E. (2004). Tick ecology: processes and patterns behind the epidemiological risk posed by ixodid ticks as vectors. *Parasitology* **129**, 37–65.
- Randolph, S. E. and Craine, N. G. (1995). General framework for comparative quantitative studies on transmission of tick-borne diseases using Lyme borreliosis in Europe as an example. *Journal of Medical Entomology* **32**, 765–777.
- Randolph, S. E., Gern, L. and Nuttall, P. A. (1996). Co-feeding ticks: epidemiological significance for tick-borne pathogen transmission. *Parasitology Today* **12**, 472–479.
- Randolph, S. E., Miklisova, D., Lysy, J., Rogers, D. J. and Labuda, M. (1999). Incidence from coincidence: patterns of tick infestations on rodents facilitate transmission of tick-borne encephalitis virus. *Parasitology* **118**, 177–186.
- Randolph, S. E. and Storey, K. (1999). Impact of microclimate on immature tick-rodent host interactions (Acari: Ixodidae): implications for parasite transmission. *Journal of Medical Entomology* **36**, 741–748.
- Rijpkema, S. G., Molkenboer, M. J., Schouls, L. M., Jongejans, F. and Schellekens, J. F. (1995). Simultaneous detection and genotyping of three genomic groups of *Borrelia burgdorferi* sensu lato in Dutch *Ixodes ricinus* ticks by characterization of the amplified intergenic spacer region between 5S and 23S rRNA genes. *Journal of Clinical Microbiology* **33**, 3091–3095.
- Rosa, R. and Pugliese, A. (2007). Effects of tick population dynamics and host densities on the persistence of tick-borne infections. *Mathematical Biosciences* **208**, 216–240.
- Rosa, R., Pugliese, A., Norman, R. and Hudson, P. J. (2003). Thresholds for disease persistence in models for tick-borne infections including non-viraemic transmission, extended feeding and tick aggregation. *Journal of Theoretical Biology* **224**, 359–376.
- Simpson, V. R., Panciera, R. J., Hargreaves, J., McGarry, J. W., Scholes, S. F. E., Bown, K. J. and Birtles, R. J. (2005). Myocarditis and myositis due to infection with *Hepatozoon* species in pine martens (*Martes martes*) in Scotland. *The Veterinary Record* **156**, 442–446.
- Snow, K. R. (1978). *Identification of Larval Ticks Found on Small Mammals in Britain*. 1st Edn. The Mammal Society, Reading, Berks, UK.
- Stanzak, J., Gabre, R. M., Kruminis-Lozowska, W., Raciewicz, M. and Kubica-Biernat, B. (2004). *Ixodes ricinus* as a vector of *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum* and *Babesia microti* in urban and suburban forests. *Annals of Agriculture and Environmental Medicine* **11**, 109–114.
- Talleklint, L. and Jaenson, T. G. T. (1994). Transmission of *Borrelia burgdorferi* sl from mammal reservoirs to the primary vector of Lyme borreliosis, *Ixodes ricinus* (Acari: Ixodidae), in Sweden. *Journal of Medical Entomology* **31**, 880–886.
- Telford, S. R., Dawson, J. E., Katavolos, P., Warner, C. K., Kolbert, C. P. and Persing, D. H. (1996). Perpetuation of the agent of human granulocytic ehrlichiosis in a deer tick-rodent cycle. *Proceedings of the National Academy of Sciences, USA*, **93**, 6209–6214.
- Telford, S. R., Mather, T. N., Moore, S. I., Wilson, M. L. and Spielman, A. (2006). Incompetence of Deer as reservoirs of *Borrelia burgdorferi*. *Annals of the New York Academy of Sciences* **539**, 429–430.
- Vannier, E., Borggraefe, I., Telford, S. R., Menon, S., Brauns, T., Spielman, A., Gelfand, J. A. and Wortis, H. H. (2004). Age-associated decline in resistance to *Babesia microti* is genetically determined. *The Journal of Infectious Diseases* **189**, 1721–1728. doi: 10.1086/382965.

APPENDIX

Structure of the next generation matrix (a), a schematic version of the matrix indicating the location of the various transmission routes used by pathogens (b) and a list of equations used to calculate each element within the matrix (c) (taken from Hartemink *et al.* 2008). Equations utilize tick- and pathogen-specific parameters derived from the literature and the current study (Tables 1 and 2).

$$(a) \quad K = \begin{pmatrix} k_{11} & k_{12} & k_{13} & k_{14} & 0 \\ k_{21} & k_{22} & k_{23} & 0 & k_{25} \\ k_{31} & k_{32} & k_{33} & 0 & k_{35} \\ k_{41} & k_{42} & k_{43} & 0 & k_{45} \\ k_{51} & k_{52} & k_{53} & 0 & 0 \end{pmatrix}$$

$$(b) \quad \begin{pmatrix} \text{transovarial} & \text{transovarial} & \text{transovarial} & \text{transovarial} & 0 \\ \text{cofeeding} & \text{cofeeding} & \text{cofeeding} & 0 & \text{host} \rightarrow \text{L} \\ \text{cofeeding} & \text{cofeeding} & \text{cofeeding} & 0 & \text{host} \rightarrow \text{N} \\ \text{cofeeding} & \text{cofeeding} & \text{cofeeding} & 0 & \text{host} \rightarrow \text{A} \\ \text{tick} \rightarrow \text{host} & \text{tick} \rightarrow \text{host} & \text{tick} \rightarrow \text{host} & 0 & 0 \end{pmatrix}$$

$$(c) \quad \begin{aligned} k_{11} &= s_L s_N s_A E r_A, \\ k_{12} &= s_N s_A E r_A, \\ k_{13} &= s_A E r_A, \\ k_{14} &= E r_A, \\ k_{15} &= 0, \\ k_{21} &= (s_L \theta_{LL} C_{LL} + s_L s_N \theta_{NL} C_{LN} + s_L s_N s_A \theta_{AL} C_{LA}) h_c, \\ k_{22} &= (s_N \theta_{NL} C_{LN} + s_N s_A \theta_{AL} C_{LA}) h_c, \\ k_{23} &= (s_A \theta_{AL} C_{LA}) h_c, \\ k_{24} &= 0, \\ k_{25} &= \frac{p_L i N_{LH}}{D_L} \\ k_{31} &= (s_L \theta_{LN} C_{NL} + s_L s_N \theta_{NN} C_{NN} + s_L s_N s_A \theta_{AN} C_{NA}) h_c, \\ k_{32} &= (s_N \theta_{NN} C_{NN} + s_N s_A \theta_{AN} C_{NA}) h_c, \\ k_{33} &= (s_A \theta_{AN} C_{NA}) h_c, \\ k_{34} &= 0, \\ k_{35} &= \frac{p_N i N_{NH}}{D_N} \\ k_{41} &= (s_L \theta_{LA} C_{AL} + s_L s_N \theta_{NA} C_{AN} + s_L s_N s_A \theta_{AA} C_{AA}) h_c, \\ k_{42} &= (s_N \theta_{NA} C_{AN} + s_N s_A \theta_{AA} C_{AA}) h_c, \\ k_{43} &= (s_A \theta_{AA} C_{AA}) h_c, \\ k_{44} &= 0, \\ k_{45} &= \frac{p_A i N_{AH}}{D_A} \\ k_{51} &= (s_L q_L + s_L s_N q_N + s_L s_N s_A q_A) h_c, \\ k_{52} &= (s_N q_N + s_N s_A q_A) h_c, \\ k_{53} &= s_A q_A h_c, \\ k_{54} &= 0, \\ k_{55} &= 0. \end{aligned}$$