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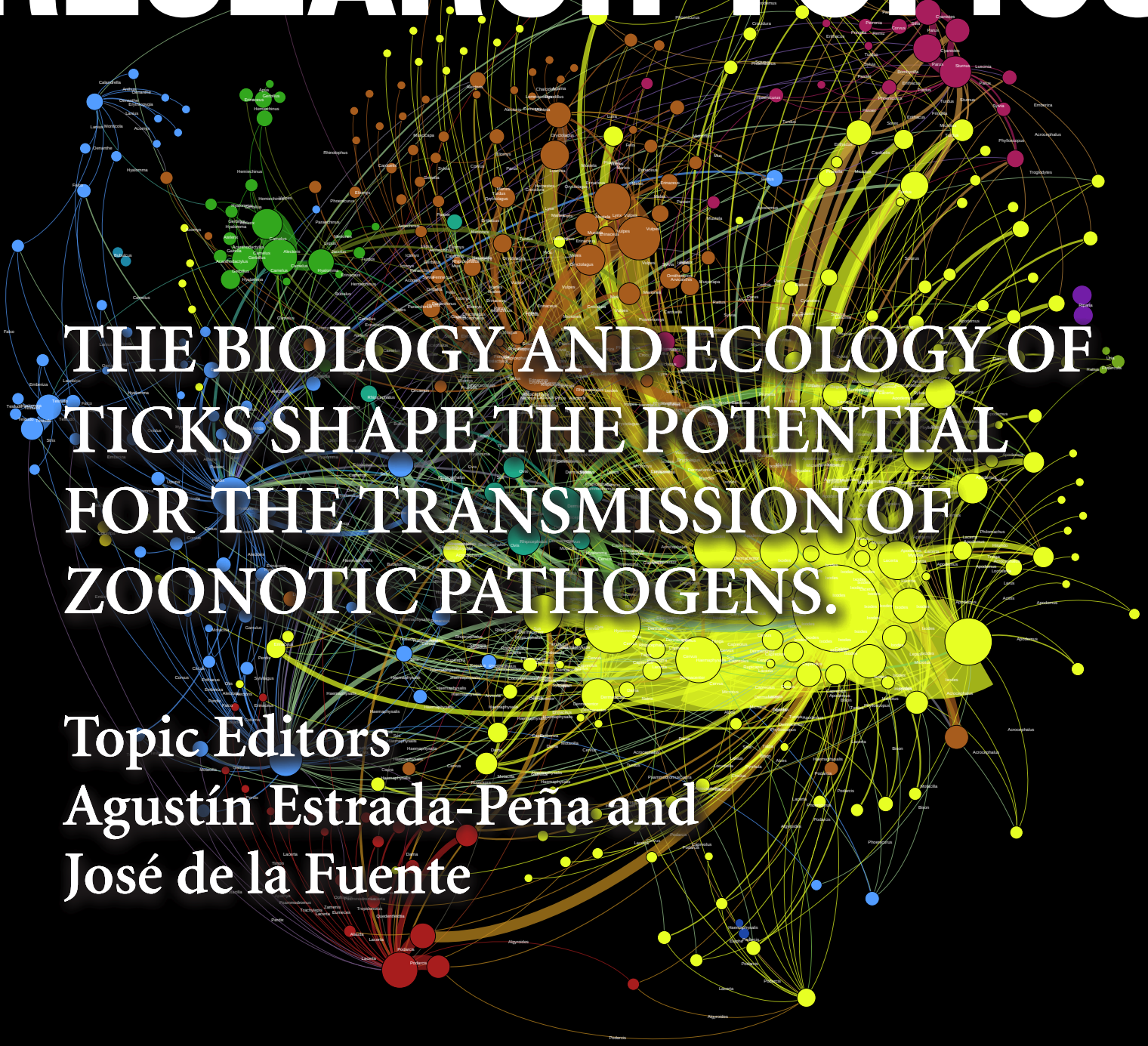
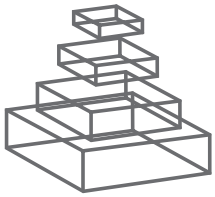


THE BIOLOGY AND ECOLOGY OF TICKS SHAPE THE POTENTIAL FOR THE TRANSMISSION OF ZOONOTIC PATHOGENS.

Topic Editors
Agustín Estrada-Peña and
José de la Fuente

frontiers in
CELLULAR AND INFECTION MICROBIOLOGY



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THE BIOLOGY AND ECOLOGY OF TICKS SHAPE THE POTENTIAL FOR THE TRANSMISSION OF ZOONOTIC PATHOGENS

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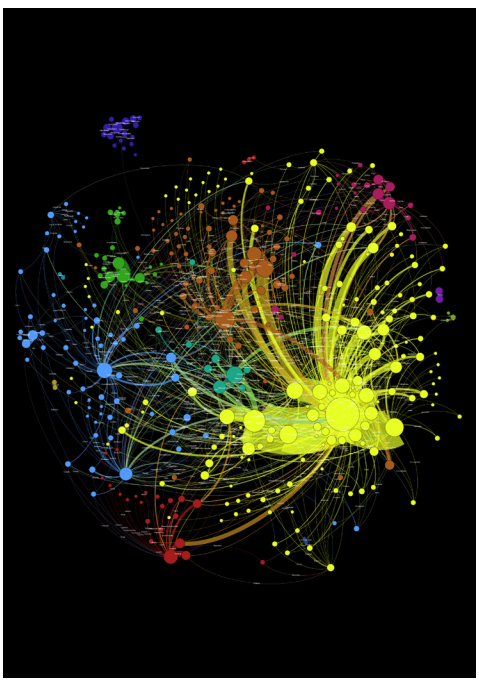

Ticks are noticeable by the high diversity of pathogens they can transmit, most of them with implications in human and animal health. Ticks are arachnids, meaning that they do not share the biological and ecological features of the mosquitoes and other parasitic Diptera. The natural foci of tick-borne pathogens may be as large as a continent, or be restricted to small portions of a country, without apparently too many similar features. The life cycle of the ticks involved three developing instars. The precise relationships of ticks and their hosts, the specific seasonal pattern of activity of ticks, and the still poorly known molecular relationships between ticks and the pathogens they can transmit, make these vectors a specially fecund field of research. Importantly, extensive studies on the biological and ecological relationships of ticks and abiotic (climate and vegetation) conditions have revealed the fine-tuning of the ticks and the pathogens they transmit, together with the biological effects of host and the driving features by the climate.

The studies on tick-transmitted pathogens have been on the rise in the last years. There is a growing interest in understand the somewhat complex relationships between the landscape,

the climate, the vectors and the pathogens, because the concerns of spread, probably driven by subtle changes in climate and man made alterations of the landscape. Studies on Lyme borreliosis are addressing the interesting issue of the relationships between the climate, the tick activity patterns, and the selection of strains according to the reservoir availability. Furthermore, the expanding field of habitat suitability modeling has been applied with different degrees of success to evaluate and quantify the risk of disease transmission. In such exponentially growing field, revisionary books are clearly welcome additions to the bibliographical tools of researchers. It is however necessary the compilation of works devoted to explore the tip of the iceberg in the field of research.

In this Research Topic, we wish to summarize and review the studies on ecology, molecular biology, and tick-host-pathogens interactions, provided to resolve the important issues of ticks and pathogens. We want not only the results obtained by newly developed molecular tools, but rigorous reviews of the most recent advances in these issues. This Topic will cover aspects of both human and animal health, with special interest on zoonoses. Aspects of the biology of the ticks, as affecting the transmission of pathogens, are of special interest in this Topic. Studies on ticks of the poorly known family Argasidae, as related to their involvement on pathogen transmission, are especially welcome. We also wish to describe the perspective of the field in the future. Finally, the presentation of ongoing original works is greatly encouraged.

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CELLULAR AND INFECTION MICROBIOLOGY

EDITORIAL
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Toward a multidisciplinary approach to the study of tick-borne diseases

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"The biology and ecology of ticks shape the potential for the transmission of zoonotic pathogens" is a collection of research and review articles related to the study of tick-borne diseases, with a focus on the methodology to explore the basic relationships between the tick-transmitted pathogens and the environment. It is well-known that a multidisciplinary point of view is necessary in order to develop a global vision of this growing problem. Therefore, in order to promote this holistic approach to the knowledge of tick-borne diseases, this research topic, which will be assembled as an e-book, contains collaborations of entomologists, epidemiologists, virologists, parasitologists, bacteriologists, zoologists, molecular biologists, and veterinarians. As stated in the title, the book addresses some of the factors that are behind the emergence and/or reemergence of tick-borne diseases. Contributions include, among others, interesting reviews on the immune system of ticks, the dissemination of pathogens by bird-carried ticks, an assessment of the main methodological gaps in the research of tick ecology and the epidemiology of tick-transmitted pathogens, the control of tick-borne pathogens by anti-tick vaccines, or reviews about prominent tick-transmitted diseases.

It is now well-known that several climatic, environmental, and sociodemographic changes that have occurred over the past years are behind the resurgence of some tick transmitted pathogens worldwide. Other than the impact on the animal production systems and the derived problems in the husbandry, there are serious concerns about how tick-transmitted pathogens would affect human and animal health. Global change, defined as the impact of human activity on the fundamental mechanisms of biosphere functioning, includes not only climate change, but also habitat transformation, water cycle modification, biodiversity loss, and synanthropic incursion of alien species into new territories (Gortázar et al., 2014). All these factors may affect the ecological relationships of ticks with their hosts and the microorganism community and pathogens they carry (Estrada-Peña et al., 2014). We thus opted to present here compilations and reviews regarding some of the most important tick-transmitted pathogens, together with some facts regarding tick as a system such as tick stress response that have been overlooked in other reviews.

As editors of "The biology and ecology of ticks shape the potential for the transmission of zoonotic pathogens," we would like to acknowledge all coauthors for their valuable and interesting contributions and we wish the readers of this e-book a productive and enjoyable reading of some of the most innovative work related to tick-borne diseases.

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Changing geographic ranges of ticks and tick-borne pathogens: drivers, mechanisms and consequences for pathogen diversity

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The geographic ranges of ticks and tick-borne pathogens are changing due to global and local environmental (including climatic) changes. In this review we explore current knowledge of the drivers for changes in the ranges of ticks and tick-borne pathogen species and strains via effects on their basic reproduction number (R_0), and the mechanisms of dispersal that allow ticks and tick-borne pathogens to invade suitable environments. Using the expanding geographic distribution of the vectors and agent of Lyme disease as an example we then investigate what could be expected of the diversity of tick-borne pathogens during the process of range expansion, and compare this with what is currently being observed. Lastly we explore how historic population and range expansions and contractions could be reflected in the phylogeography of ticks and tick-borne pathogens seen in recent years, and conclude that combined study of currently changing tick and tick-borne pathogen ranges and diversity, with phylogeographic analysis, may help us better predict future patterns of invasion and diversity.

Keywords: range change, invasion, Ixodes, Lyme, genetic diversity

INTRODUCTION

Change in geographic range is one process whereby infectious diseases emerge or re-emerge (Kilpatrick and Randolph, 2012). Many ticks and tick-borne diseases are of significance for human or animal health, so professionals in these fields have a keen interest in being able to identify, understand, and predict changes in their geographic ranges. Ticks and tick-borne pathogens that are increasing their geographic ranges are effectively invasive species and the processes of invasion, consequences for their genetic diversity, and their potential impact on the natural ecosystems that they invade remain mostly unstudied. Examples of ticks and tick-borne pathogens that have changed their range in recent decades include northward expansion of *Ixodes scapularis* and *I. ricinus* and the pathogens they transmit (Lindgren et al., 2000; Ogden et al., 2009; Léger et al., 2013; Medlock et al., 2013), as well as *Rhipicephalus* and *Amblyomma* spp. ticks (reviewed in Léger et al., 2013). Here we review the processes involved in range changes of ticks and tick-borne pathogens, and their consequences for genetic diversity of ticks and tick-borne pathogens, which in turn can have importance for understanding the ecology, pathogenicity and diagnostic detectability of tick-borne pathogens (e.g., Ogden et al., 2011). We investigate two epidemiological/ecological processes involved in range change: (i) the factors involved in the maintenance of tick-borne pathogen transmission cycles that may or may not permit expansions of ticks and tick-borne pathogen populations, and may or may not permit them to become endemic once they arrive in new locations; and (ii) the mechanisms whereby ticks and tick-borne pathogens are dispersed from locations where they are endemic allowing invasion of new, ecologically-suitable locations. Then we review possible expected consequences of these processes for the diversity of tick-borne pathogens (using the Lyme borreliosis group of spirochaetes as an example), and how we would expect them to be reflected in the phylogeography of ticks and *Borrelia burgdorferi* sensu lato (s.l.: the agent of Lyme disease, also called Lyme borreliosis). We show that many current hypotheses are supported by observations, which gives confidence that in the future we may, by combined study of the ecology of tick and tick-borne pathogen invasion, their genetic diversity and their phylogeography, be better able to predict future patterns of invasion and diversity.

PROCESSES INVOLVED IN TICK-BORNE PATHOGEN MAINTENANCE

The basic reproduction number (R_0) is an index of the rate of reproduction of a species or strain under the conditions occurring at a particular point in space and time. Therefore, R_0 provides an index of the fitness of species and strains at locations where they occur, or in new locations into which ticks and tick-borne pathogens are invading. In this section we review the key variables affecting the basic reproduction number (R_0) of tick-borne pathogens using the simplified equation of Randolph (1998):

$$R_0 = \frac{Nf\beta_{V-T}\beta_{T-T}\beta_{T-V}p^nF}{H(r+h)}$$

Where N = number of tick vectors, H =number of susceptible reservoir hosts, β_{t-v}, β_{t-t}, β_{t-v} =pathogen transmission coefficients [respectively tick-to-vertebrate host, tick-to-tick (transtadially or transovarially) and vertebrate host-to-tick], p = vector daily survival probability, n =development duration for ticks (and p^n =interstadial survival rate), r =daily rate of loss of infectivity of host, h =host daily mortality rate, f =probability tick feeds on an individual of a host species, F = tick birth rate.

N, THE DENSITY OF TICKS

Tick density must be above a threshold to support tick-borne pathogen transmission cycles, and the higher the density of ticks, the more efficient transmission cycles are likely to be (Norman et al., 1999; Ogden et al., 2007). Tick density has two principal determinants: density of hosts (be they reservoir competent hosts or not) and tick mortality rates. In any one location, host density is in turn determined by the biotic and abiotic features of the community at that location (Begon et al., 2005). These features will also partly determine the range of host species, which may have consequences for pathogen transmission (see subsequent sections) and for rates of on-host tick mortality. While many tick species of importance for human and animal health are exophilic host generalists to a greater or lesser degree (e.g., *I. ricinus*, *I. persulcatus* and *I. scapularis*), some ticks are host specialists that are often nest-living (nidicolous, e.g., *I. trianguliceps, I. muris*). The density of hosts for the latter, the specialists, will in most cases be lower than for the former in most locations. Both feeding ticks and free-living ticks (i.e., those undergoing development from one instar to the next, those in diapause or those questing for a host) suffer mortality. Apart from host densities, rates of mortality of ticks that are not feeding on hosts are likely the main determinant as to whether a particular habitat is suitable for tick population invasion, establishment and maintenance. The capacity of a habitat to provide non-feeding ticks with refugia from extreme temperatures, desiccation, drowning or perhaps predation, within the limits provided by climate, will determine the mortality rates of ticks in that habitat (Lindsay et al., 1995, 1998; Ogden et al., 2006a). Ambient temperature also acts as a determinant of mortality rates of ticks that interplays with habitat qualities. The colder the climate the longer is the tick's lifecycle and, given constant daily, per-capita mortality rates, the less likely a larval tick is to survive to be a mated adult (Ogden et al., 2005). Below a threshold of temperature conditions tick populations will die out, or fail to invade. However, what that threshold is precisely, for a particular habitat, will depend on the mortality rates of the ticks in that habitat (Ogden et al., 2005, 2006a). It should be noted that temperature-independent (frequently daylength-dependent) behavioral or developmental diapause (Gray, 1991) occurs in the lifecycle of some tick species, in which case the duration of the lifecycle may be less influenced by temperature. In habitats that support off-host survival of ticks, host grooming (Levin and Fish, 1998) and effects of the innate and acquired immune responses of the host (which may of course be linked) are important causes of tick mortality (Randolph, 1979; Wikel, 1999; Ogden et al., 2002a,b). Mortality due to host grooming and acquired resistance are density-dependent in many species (Randolph, 1979; Levin and Fish, 1998; Goodwin et al., 2001; Shaw et al., 2003), the latter due to density-dependent activation of cell-mediated acquired resistance (Dizij and Kurtenbach, 1995; Ogden et al., 2002b), and may be a key mechanism for density-dependent regulation of tick populations (Randolph, 1998). Different host species may have a greater or lesser capacity to groom or express innate or acquired resistance so the host species range and relative abundance will determine the overall proportion of ticks that survive feeding (Dizij and Kurtenbach, 1995; Keesing et al., 2009). Thus, climatic conditions and habitat (acting together on tick mortality) as well as host range and abundance are all key determinants of range spread for ticks and tick borne diseases by acting on tick density.

f, THE PROBABILITY THAT A TICK FEEDS ON AN INDIVIDUAL OF A RESERVOIR COMPETENT HOST SPECIES

The probability that a tick finds an individual host of a species that is a competent reservoir depends on the range and relative densities of these two types of host species (Tsao, 2009). This is effectively the theory behind the "dilution effect" of biodiversity (Ostfeld and Keesing, 2000; Dobson et al., 2006; Keesing et al., 2006), and although the effect of biodiversity *per se* as an inhibitor of tick-borne disease transmission in all circumstances is under question (Wood and Lafferty, 2013), high abundance of reservoir incompetent hosts in some circumstances likely inhibit transmission cycles (Ogden and Tsao, 2009; Tsao, 2009), for example when pathogens display host associations as seen for *Borrelia* species in Europe (Kurtenbach et al., 2002, 2006). Thus, rates at which ticks find a reservoir-competent host are largely determined by the composition of the host community, and the relative densities of reservoir host species.

TRANSMISSION COEFFICIENTS $\beta_{T-V}, \beta_{T-T}, \beta_{V-T}$

Transmission coefficients (i.e., the efficiency) of transstadial transmission (β_{t-v} and then β_{v-t}) and (if it occurs) transovarial transmission (β_{t-v} and then β_{t-t}) are functions of the pathogen-tick interaction and key determinants of the basic capacity of the tick-borne pathogen to invade: if a tick-borne pathogen is poorly adapted to ticks in regions in which it is invading, these transmission coefficients will be lower, reaching zero if the vector is completely incompetent. Therefore, the geographic occurrence of competent tick vector species absolutely impacts the geographic range of tick-transmitted pathogens, although genetic change by random mutations can mean that tick-borne pathogens become adapted to transmission by new tick vector species (Margos et al., 2013). This is, of course, not a consideration where ticks and the pathogens they transmit are invading together or sequentially.

Whether or not tick-to-host transmission occurs (i.e., whether or not a host can acquire infection), depends on the innate susceptibility of individuals of the host community to infection, i.e., the capacity of the innate immune response of the host to kill the pathogen at the point of infection (Kurtenbach et al., 2006). While tick saliva contains many components that may inhibit the innate immune response, some functions are not affected and different host species may be differentially susceptible to different tick-borne pathogen species or strains (Kurtenbach et al., 2002). Host-to-tick transmission coefficients depend on the capacity of the pathogen to multiply in the host and disperse widely within

the skin or blood of the host from which they can be transmitted to uninfected feeding ticks. This is a function of the capacity of the pathogen to evade the host innate and acquired immune responses. In general greater multiplication corresponds with greater transmission coefficients, but higher rates of pathogen multiplication in the host may result in a "cost" in terms of host mortality rates (Ogden et al., 2007 and see below).

Host-to-tick transmission coefficients are usually highest in early acute stages of infection, and then decline to low levels (assuming the pathogen is capable of more long-term immune evasion and persistence in the host) or to zero if host immunity is complete (Ogden et al., 2007). Some strains of *B. burgdorferi* s.s. are, however, capable of causing infections of wild rodents that result in persistently high host-to-tick transmission coefficients (Hanincová et al., 2008). The degree to which infecting and infection-acquiring instars (usually nymphs and larvae respectively) feed sequentially on hosts within a short enough time period determines whether infection-acquiring instars feed on hosts during the acute rather than the post-acute/chronic phases of infection, or on hosts that have recovered from infection (Ogden et al., 2007). Thus, the degree to which these tick instars are seasonally active at the same time (Randolph et al., 1999; Gatewood et al., 2009) is a determinant of the "average" host-to-tick transmission coefficient from the reservoir host population. The more persistent acute infections are though (i.e., the lower is r), the less impact tick seasonality will have.

Co-feeding transmission, where it occurs, may augment coefficients of host-to-tick transmission associated with systemic host infections (Randolph et al., 1996). The occurrence of co-feeding transmission depends on the capacity of pathogens to be transmissible by this route, and for individual host species to support co-feeding transmission. It also depends on patterns of coincident feeding of infecting instars and infection-acquiring instars on the same host at the same time, and therefore on seasonal activity patterns of the ticks (Randolph et al., 1999; Gatewood et al., 2009). Thus, overall the potential transmission coefficients for a particular pathogen at a particular location will depend on (i) the degree of competence of the vectors present, (ii) the composition of the host community and the relative densities of different reservoir host species, as well as (iii) the effects of seasonal tick activity on β_{v-t} and co-feeding transmission. Genetic change of the tick-borne pathogens can result in changes to vector and host competence, as well as the dynamics of host infections that determine β_{v-t}.

P^N THE INTERSTADIAL TICK SURVIVAL RATE

The main determinants of interstadial survival are the daily per-capita mortality rates of engorged ticks in the environment, the duration of the development period, and the time it takes for a moulted tick to find a host. Daily per-capita mortality rates are determined by the qualities of habitat that determine mortality rates of ticks when off-host as discussed above. Development duration is determined largely by ambient temperature; the warmer the temperature, the faster is the development and the lower is the total proportion of ticks that will die (reviewed in Ogden et al., 2005). Microclimate (i.e., habitat-modified climate) affects tick activity which, combined with host densities, affects the rate at which ticks find a host (Ogden et al., 2005). In addition, interstadial survival rate will depend on the quality and quantity of the tick's last meal, which determines the fat reserves on which the moulted tick relies for host seeking (Randolph et al., 2002). In turn this depends on the host innate and acquired resistance to ticks: ticks that feed on hosts expressing resistance may not die but feed less successfully (Dizij and Kurtenbach, 1995; Ogden et al., 2002a). There is a roughly quadratic relationship between temperature and host seeking activity with activity inhibited at low and high temperatures (Vail and Smith, 1998, 2002). Low relative humidity at one extreme, and intense rainfall at the other are inhibitory of host-seeking activity (Randolph, 1997; Vail and Smith, 1998, 2002) so rainfall frequency and intensity can also have a quadratic relationship with host-seeking activity. Interstadial tick survival rate therefore depends on a combination of climate, habitat and host density.

F, THE TICK BIRTH RATE

Tick birth rate depends on the proportions of adult female ticks that successfully mate, which is determined by the densities of suitable hosts (as female ticks must feed to reproduce), and the fecundity of individual female ticks, which depends on the quality and quantity of the female tick's meal. The latter will be determined by the community of adult tick hosts and their innate and acquired immune responses to the ticks (reviewed in Ogden et al., 2005). Thus, the tick birth rate will depend on the densities and species range of hosts for adult ticks.

H, THE NUMBER OF SUSCEPTIBLE RESERVOIR HOSTS

This depends on the host species range and densities of each competent reservoir host species (Tsao, 2009).

h, THE HOST MORTALITY RATE

Host mortality rates are intrinsic to each species within the habitats and communities in which they live. However, mortality of hosts does occur that is directly attributable to infection with tick-borne pathogens. Although this is most commonly recognized in domesticated animals, infections of wild animals with tick-borne pathogens can results mortality, and morbidity that may indirectly increase host mortality by increasing vulnerability to other causes of mortality such as predation (Reid et al., 1978). *B. burgdorferi* s.l. is frequently considered non-pathogenic for wild animal hosts such as *P. leucopus* (Wright and Nielsen, 1990). However, there is evidence of pathology associated with infection in juvenile *P. leucopus* (Moody et al., 1994) and in dusky-footed woodrats, *Neotoma fuscipes* (Brown and Lane, 1994). Potential effects of tick-borne pathogens on mortality rates of hosts may be particularly high in newly-invaded regions. Co-evolution of hosts and tick-borne pathogens, resulting in some reductions in pathogen-induced mortality rates, could be expected in endemic regions (Woolhouse et al., 2002). However, immediately after invasion, tick-borne pathogens could have greater impact on mortality rates in pathogen- and tick-naïve host populations. Thus, host mortality rates are intrinsic to the location, and may be increased by invading parasites and pathogens, although to date there is no evidence that this has a significant inhibitory effect on invasion of tick-borne pathogens.

r, THE RATE AT WHICH INFECTED HOSTS LOSE THEIR INFECTIVITY

This is determined by the individual relationship between the pathogen and host and, for the most part, the rate at which the latter acquires immunity to the former (Ogden et al., 2007; Tsao, 2009). The more persistent host infections are, the more ticks an infected host can infect and most vector-borne pathogens have evolved strategies for persistence in the host by evading the host acquired immune response (Kurtenbach et al., 2006). Some studies suggest that the evolution of investment in acquired immune responses by wild hosts depends on the 'pace of life' which is inversely related to their normal life span (Previtali et al., 2012). While this may be a general pattern, there are clearly exceptions. The white-footed mouse *Peromyscus leucopus* can all but eliminate infection with some, but not all, strains of *B. burgdorferi* s.s. suggesting that these mice do acquire immunity to some strains (Hanincová et al., 2008), while *B. burgdorferi* s.s. outer surface genes show evidence of strong balancing selection thought to be driven by host immune responses of the predominanting rodent reservoirs (Qiu et al., 2002). For a particular pathogen at a particular location *r* will, therefore, be determined by the composition of the host community.

As ticks and pathogens become established in new locations and the effective reproduction number (rather than the basic reproduction number) becomes a more meaningful index of the abundance of ticks and pathogens, host acquired immunity (and the capacity of the community overall to acquire immunity/resistance to ticks and pathogens) becomes a more crucial determinant of N, p^n, H, and h. The capacity of the host community to express such resistance/immunity will again be a function of the range of species in that community.

In summary, the main factors that determine the capacity for tick and tick-borne pathogen populations to expand and drive invasion, and the suitability of any one location for invasive ticks and tick-borne pathogens to become established are (i) climate (temperature and rainfall); (ii) habitat; and (iii) host species range and density, and (iv) for tick-borne pathogens the presence of, and adaptation to competent vector species. Generalist exophilic tick vectors and generalist pathogens will have greater capacity to invade new regions than specialist ticks and tick-borne pathogens that have a narrow, specialized niche breadth. Climate, habitat and host species range and abundance are highly linked, and together affect suitability for tick invasion; changes in one factor alone is unlikely to drive population or range expansion while one of the other factors is limiting. The rate of range expansion would, however, be expected to vary according to how many limiting factors must change. For example, if habitat and host abundance are suitable, responses to a warming climate may be more rapid than if the warming climate must also drive changes in habitat and host densities for these to become suitable for tick and pathogen invasion. Changes in just one of these factors that are detrimental to tick and tick-borne pathogen persistence would, however, drive range contraction even if the other two factors remain within parameter ranges suitable for tick and tick-borne pathogen persistence. As described above, superimposed on these three main drivers of tick and tick-borne pathogen range change, is the possibility of tick-borne pathogens expanding their populations subsequent to genetic changes that produce novel strains that are capable of exploiting new, or enhancing transmission amongst existing, tick vectors and reservoir host species.

CAPACITY FOR TICKS AND TICK-BORNE PATHOGENS TO BE DISPERSED

ABUNDANCE IN SOURCE LOCATIONS

For a location to be a source, the ticks and pathogens must be sufficiently abundant, and the effective reproduction number sufficiently high, that export of ticks and pathogens (i) is precisely balanced by immigration from elsewhere, (ii) is precisely balanced by the rate of reproduction, or (iii) merely releases the ticks and pathogens from density-dependent regulation. If these criteria are not met then ticks and pathogens will die out in the source location. In most circumstances this means that source tick and pathogen populations are undergoing expansion or that tick and pathogen densities are constrained at equilibrium by density-dependent regulation mechanisms.

CAPACITY FOR SPREAD BY HOSTS

Ticks do not fly and are not capable of wind-borne spread so the only way ticks and their pathogens can be dispersed is on or in their hosts. Thus, dispersal is intimately dependent on host movement behavior in dispersal over local scales, and migratory behavior over long distances. In both cases, these may be highly idiosyncratic to the individual locations, tick and pathogen species. Some general patterns can be established regarding long distance dispersal on migratory birds and animals, which may have broadly predictable north-south directions and specific routes through which ticks are dispersed. An example is the dispersion of *I. scapularis* into Canada which has been predicted on the basis of dispersion by migratory passerines (Ogden et al., 2008), and empirical observations support these predictions (Leighton et al., 2012). Northward range expansion of several small mammal species due to climate change (e.g., Myers et al., 2009) may also facilitate vector and pathogen spread. It is possible, however, that climate change may unlink seasonal coincidence of host migration and tick activity as the timing of both could be impacted by changing temperatures (Marra et al., 2005; Ogden et al., 2008) and this may have idiosyncratic impacts of tick and pathogen dispersion (Ogden et al., 2013).

For tick-borne pathogens it has been predicted that dispersal patterns will match those of reservoir hosts. As Lyme borreliosis group spirochetes vary in their host specificity, this hypothesis has been tested utilizing species with narrow reservoir host ranges (see the following and Kurtenbach et al., 2006).

EXPECTED PATTERNS OF TICK AND TICK-BORNE PATHOGEN GENETIC DIVERSITY IN EXPANDING AND CONTRACTING POPULATIONS

In the following, our discussion focuses on patterns of genetic diversity of pathogens exemplified by the group of bacteria containing the causative agents of Lyme disease, *B. burgdorferi* s.l. Past and present pattern of genetic diversity are likely to be evidenced in genes or sequences showing neutral evolution. Increasingly for *B. burgdorferi* s.l. Multi-Locus Sequence Typing (MLST) of housekeeping genes that show neutral variation is being recognized as a highly useful tool for phylogeographic studies (Margos et al., 2008).

It would be expected that tick and pathogen populations in source locations for range expansion would show genomic evidence of current or past expansion. Evidence of population expansion may be found in mismatch distributions of pairwise nucleotide differences amongst different sequence types/haplotypes in the populations (Schneider and Excoffier, 1999). Adaptive radiation may also be expected when populations expand, although adaptive radiation may lead to host specialization (multiple niche polymorphism), and a narrowing niche for tick and pathogen that may potentially reduce the capacity for dispersion and establishment to new locations or environments (Kempf et al., 2009). Observed patterns of diversity will vary depending on the processes of population expansion, and the time point at which the observation is made. In general, it would be expected that pioneer populations of ticks and tick-borne pathogens at the leading edges of expansion would have constrained genetic diversity, particularly if the new environments are in some way suboptimal, e.g., due to limited or fragmented suitable habitat (Hill et al., 2006). Theoretical studies have suggested that expected patterns of diversity change will depend on whether a range expansion is "pushed," i.e., the range expansion occurs due to population expansion at the edge of the source location, or "pulled" by pioneers seeding new populations well ahead of the source population range edge (Roques et al., 2012). In the former, expansion is a slower process whereby ecological factors such as the Allee effect constrain population growth allowing diversity in the source population to keep pace with geographic spread. In the latter, genetic diversity in colonizers at the leading edge of range expansion is lower than that in the main body of the population due to stochastic effects of survival of individuals at the colonization front. Long-range dispersal of ticks and tick-borne pathogens by migratory birds may enhance the likelihood that isolated founder populations form the colonization front within which founder effects, i.e., limited genetic diversity, are observed (Mayr, 1963). Skewed diversity may occur at the front of population expansion due to the process of "surfing"; high rates of reproduction at the expanding edge increase mutation rates and new alleles can "surf" the wave of population growth and expand and survive at the leading edge when they may not survive at such abundance, or at all, in a stationary population (Klopfstein et al., 2006). How long and where any of these effects may be seen will depend on the speed at which invasions occur and the degree of connectivity of new populations and the body of the source population (e.g., Banks et al., 2010).

Alongside the northward expansion of the northern limit of the geographic ranges of ticks and tick-borne pathogens with climate change, the southern limit of those ranges may also migrate northwards, i.e., populations at the southern limits may become extinct as climate becomes unsuitable for tick survival (Brownstein et al., 2005; Estrada-Peña and Venzal, 2006). Alternatively, changing environmental conditions may result in changes in tick questing behavior as observed in the Mediterranean for *I. ricinus* (Baptista et al., 2004; Pérez-Eid, 2007) and the southern range of *I. scapularis* (Stromdahl and Hickling, 2012), and adaptation of spirochetes to nidiculous ticks (as perhaps seen in southeastern parts of the US, Lin et al., 2004). Indeed, the consequences of decreasing environmental suitability for ticks and tick-borne pathogens would be expected to be declining diversity because fewer novel genotypes survive, while genotypes that are least resistant to the new environmental stress become extinct (e.g., Wu et al., 2013). The likelihood of extinction will depend on the capacity for 'evolutionary rescue' of stressed populations, which in turn depends on the speed of environmental change, immigration rates and the population size, and thus the likelihood that the existing pool of different geno- and phenotypes of ticks and pathogens will contain one or more that is resistant to the changing conditions (Bell, 2013). The severity of the environmental change will also likely determine whether or not refugia may exist within which islands of tick and tick-borne pathogens remain following range contraction. It may, however, be surprising how small such refugia might be. Researchers in Indiana found a 50 ha patch of woodland surrounded by cropland that was sufficient to sustain rodents, *I. scapularis* and *B. burgdorferi* s.s. transmission cycles (Piesman, 2002). Refugial populations could be expected to show divergence from the main population due to genetic drift and narrowed genetic diversity compared to the main population because persistence in likely sub-optimal conditions would be expected to provide a population bottleneck (Provan and Bennett, 2008).

The long term consequences of expansions and contractions of tick and tick-borne pathogen populations would be expected to be reflected, to a greater or lesser degree, in the phylogeography of these organisms. Phylogeography may reveal the genetic relatedness of different genotypes or alleles, directions of ancestry, occurrence and time points at which population events such as expansions and contractions occurred, and how current geographic patterns relate to these observations (Margos et al., 2012). Phylogeography of ticks and tick-borne pathogens is particularly interesting at present because phylogeographic patterns may hold information on effects of past climate changes in the form of glacial and interglacial period cycles, which may predict effects of current anthropogenic climate change on tick and tick-borne pathogen occurrence and diversity (Hofreiter and Stewart, 2009).

OBSERVED PATTERNS OF GENETIC DIVERSITY IN TICKS AND THE TICK-BORNE BACTERIAL SPECIES COMPLEX *BORRELIA BURGDORFERI* SENSU LATO

The origin of *B. burgdorferi* s.l. is thought to be Europe (Margos et al., 2008), and consistent with this, the species complex is differentiated in Europe into well-defined species that are frequently host-specialists providing evidence of past adaptive radiation (see below). In contrast, the predominant species in North America is the host generalist *B. burgdorferi* s.s. (Hanincová et al., 2006). Recent expansion of *I. scapularis* and *B. burgdorferi* s.s. in northern USA and into and within Canada provides an opportunity to study what occurs to diversity in tick and pathogen populations undergoing population and range expansions. Pairwise mismatch analysis of mitochondrial gene sequences of *I. scapularis* show the expected evidence of the expansion of northeastern *I. scapularis* populations (Qiu et al., 2002; **Figure 1**) due to recent environmental changes. In particular, reversal of post-Columbian deforestation by land use changes that permitted re-forestation and expansion of populations of tick hosts (particularly deer) are thought to have driven the expansion of *I. scapularis* and *B.*

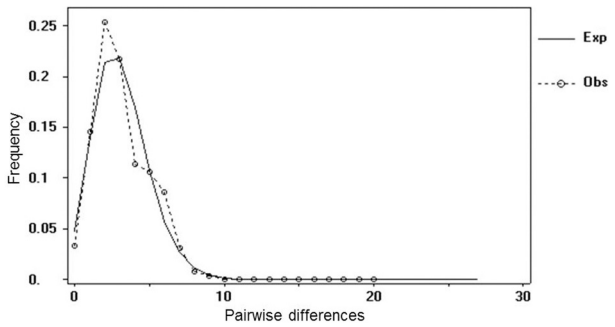

FIGURE 1 | Pairwise mismatch distribution of 55 mtDNA Cox1 haplotypes of *I. scapularis* in Canada (Obs) compared against the expected distribution for a population under expansion (Exp). This graph represents a re-analysis of the data in Mechai et al. (2013) using only one example of each haplotype, to clarify the similarity of observed and expected frequency distributions.

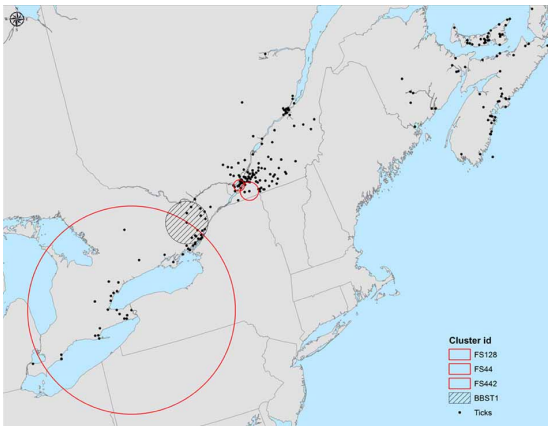

FIGURE 2 | Spatial cluster analysis of 55 haplotypes of mtDNA Cox1 sequences from *Ixodes scapularis* (see Mechai et al., 2013 for details) and MLST sequence types of *B. burgdorferi* within these ticks collected in passive surveillance in southern Canada. Three significant clusters of Cox1 haplotypes were identified respectively in south and south-western Quebec and Southern Ontario (red circles). One small-scale cluster of one *B. burgdorferi* ST (ST-01) was also found in south eastern Ontario (hatched circle). Because this ST has been found previously in the northeastern US, and because of the small radius of the cluster, this cluster likely represents a founder event.

burgdorferi s.s. populations. This resulted in the Lyme disease epidemic in northern USA that began in the late 1970s, even though some Lyme disease cases had been seen before then (Wood and Lafferty, 2013). Therefore, expansion of *I. scapularis* and *B. burgdorferi* s.s. populations was driven by change in habitat and linked changes in host densities, and occurred within climatically-suitable locations. Increasing deer densities are also likely driving the expansion of *I. ricinus* populations in northern Europe (Medlock et al., 2013). Expansion of populations of *I. scapularis* and *B. burgdorferi* s.s. in north eastern and north central USA, with invasion of new locations, continues today and as ecological conditions (particularly habitat and host abundance) have become more stable, adaptive radiation of *B. burgdorferi* s.s., leading to multiple niche polymorphism, has been suggested (Brisson and Dykhuizen, 2004; Kurtenbach et al., 2006; Brinkerhoff et al., 2010). It is thought that the strain structure of *B. burgdorferi* s.s. may be partly determined by different geographic patterns of tick seasonality. Activity of larval and nymphal *I. scapularis* has greater seasonal coincidence in the north Midwest US compared to the northeastern States (Gatewood et al., 2009) meaning that strains with greater host adaptation may be favored in the northeastern States (Ogden et al., 2007). Studies in Michigan suggest that range expansion of *I. scapularis* in the northern Midwest USA is "pushed" by expanding source populations, although there is evidence of possible refugial *B. burgdorferi* s.s. populations transmitted by nidicolous ticks in locations ahead of expanding *I. scapularis* populations (Hamer et al., 2010).

The *I. scapularis* and *B. burgdorferi* s.s. expansion process now extends to invasion of southern Canada. Analysis of field and surveillance data suggest that invasion of these species is taking place along trajectories determined by all three environmental factors: climate, habitat and host range and abundance (Ogden et al., 2010; Bouchard et al., 2011, 2013; Leighton et al., 2012). In this region the occurrence of small-scale (<30 km radius) spatial clusters of ticks carrying the same mitochondrial gene haplotypes support the hypothesis that *I. scapularis* populations in south eastern Canada have (or have until recently) arisen from isolated founder events (**Figure 2**). Empirical observations (Ogden et al., 2010; Leighton et al., 2012) are consistent with a hypothesis of climate change-driven range expansion of *I. scapularis* in this region (Ogden et al., 2006b). The occurrence of small-scale clusters of ticks collected in surveillance that carried specific MLST sequence types of *B. burgdorferi* s.s., also support the occurrence of founder populations of *B. burgdorferi s.s.* within founder populations of *I. scapularis* (**Figure 2**). Evidence for founder events of *B. burgdorferi* s.s. at the geographic scale of a single woodland have been identified by MLST analysis in south eastern Canada (Ogden et al., 2010). Evidence of founder events in the form of clusters of ticks Infected with *B. burgdorferi* s.s. that carried the same allele of the outer surface protein C (*ospC*) gene of *B. burgdorferi* s.s. have also been found in Canada (**Figure 3**). Although *ospC* is maintained under balancing selection, a high degree of linkage disequilibrium in the northeastern part of the US amongst *B. burgdorferi* s.s. genes suggests that phylogeographic patterns may be partially congruent between *ospC* and MLST, although this may not be the case for all *Borrelia* species and geographic ranges (Barbour and Travinsky, 2010; Hellgren et al., 2011). Together these observations suggest that expansion into Canada is being 'pulled' by establishment of founder populations. This supports the hypothesis that recent invasion in this region has occurred via introduction by migratory birds (Ogden et al., 2008) rather than by terrestrial hosts. The Great Lakes and Appalachians have likely posed significant geographic barriers to introduction by terrestrial hosts from the USA to regions of Canada from the Maritimes to Western Ontario. However, the occurrence of one large-scale cluster of ticks carrying the same mitochondrial gene haplotype (radius > 300 km; **Figure 2**) could provide evidence of a novel genotype 'surfing' the rapid growth of *I. scapularis* populations in southern Canada (Leighton et al., 2012), and in the near future range expansion of *I. scapularis* and *B. burgdorferi* s.s. may follow

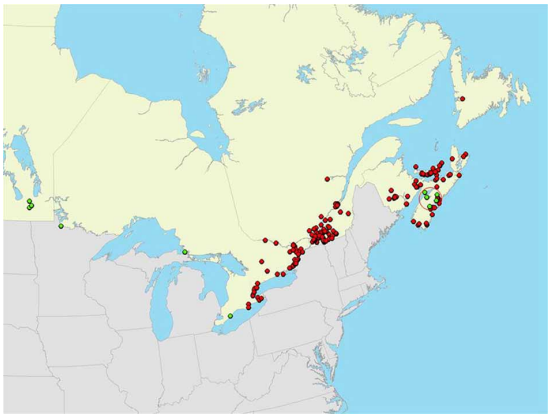

FIGURE 3 | Geographic distribution of *B. burgdorferi*-infected *Ixodes scapularis* ticks collected in passive surveillance in Canada in which the *ospC* major groups were identified in Ogden et al. (2011). A significant spatial cluster of ticks infected with *B. burgdorferi* carrying *ospC* major group I is indicated by the red circle. Reproduced with permission from Ogden et al. (2011).

the "pushed" pattern seen in the US with greater importance of terrestrial hosts in tick and pathogen dispersion.

At a continental scale, analysis of MLST data of North American *B. burgdorferi* s.s. populations identified barriers to gene flow amongst northeastern, Midwestern and western populations of *B. burgdorferi* s.s. that are at least partly consistent with our known history of land use changes (Hoen et al., 2009; Margos et al., 2012). MLST data also identify the potential for multiple expansions of *B. burgdorferi* s.s. in North America over a long millennial time scale (Hoen et al., 2009; Margos et al., 2012) that could imply past expansions of *B. burgdorferi* s.s. associated with the onset of climate warming in interglacial periods.

In contrast to the generalist *Borrelia burgdorferi* s.s., which may utilize a diverse range of vertebrates as reservoir hosts including birds and rodents (Hanincová et al., 2006), several European species of the Lyme borreliosis group of spirochetes show distinct pattern of host associations, i.e., the range of reservoir hosts that is able to support completion of the transmission cycle is narrower and consists of either rodent or avian species (reviewed by Kurtenbach et al., 2006). This feature of the ecology of *B. burgdorferi* s.l. in Europe has allowed the hypothesis that host associations determine dispersal rates and geographic patterns of tick-borne pathogens (Vollmer et al., 2011, 2013). These studies showed that populations of the rodent-associated *B. afzelii*, were highly structured and Western and Eastern European populations were identified (Vollmer et al., 2013). The observed pattern of population structure follows that of potential rodent reservoir hosts of *B. afzelii* following the last glacial maximum (Hewitt, 1999; Tougard et al., 2008). Similarly, for *B. lusitaniae*, a species that is transmitted by lizards, distinct populations were found in Portugal south (Grandola) and north (Mafra) of Lisbon using MLST (Vitorino et al., 2008). Different ecological conditions prevail in those habitats and the lizard populations of the Iberian Peninsula are highly geographically structured accordingly (Paulo et al., 2002, 2008). As expected, populations of *Borrelia* species that utilize avian reservoir hosts, such as *B. garinii* and *B. valaisiana*, were spatially mixed in Europe and identical MLST sequence types were found in different geographic regions (e.g., England, France, Latvia and Germany) (Vollmer et al., 2011). At a wider continental scale, however, population structure was found even for the bird-associated *Borrelia* species, *B. garinii*, some Asian *B. garinii* sequence types formed a distinct clade in phylogenetic analysis (Vollmer et al., 2013). Additional diversity may be added to *B. garinii* populations via an overlap of marine and terrestrial transmission cycles (Gómez-Díaz et al., 2011).While expansion of *I. scapularis* and *B. burgdorferi* s.s. populations in northeastern US may both show evidence of recent expansion (Qiu et al., 2002), studies have shown no or weak concordance of the phylogeography of these species across the northeastern and Midwestern regions of North America (Humphrey et al., 2010). We have, however, raised the hypothesis that patterns of *Peromyscus* spp. mouse phylogeography may reflect that of *B. burgdorferi* s.s. in North America (Margos et al., 2012). Together these data support the hypothesis that dispersal of tick-borne pathogens is strongly dependent on dispersal behavioral traits of their reservoir hosts.

A further determinant of the phylogeography of *B. burgdorferi* s.l. in Europe is vector competence. In Eastern Europe (from Estonia/Latvia to the Moscow region) populations of *I. ricinis* and *I. persulcatus* (the main Western European vector and the main Asian vector of tick-borne pathogens, respectively) occur sympatrically (Lindquist and Vapalahti, 2008). Certain *B. burgdorferi* s.l. strains are absent in *I. ricinus* (for example NT29 and related strains) while some *Borrelia* species are not or rarely detectable in *I. persulcatus* suggesting vector incompetence for those (Korenberg et al., 2002; Bormane et al., 2004; Masuzawa et al., 2005). The observation that *I. persulcatus* appears to be vector incompetent for *B. burgdorferi* sensu stricto (s.s.) has important implications for historic dispersal and the colonization of the American continent by this species. It suggests that *B. burgdorferi* s.s. and related species either originated in North America (Marti Ras et al., 1997) or invaded the continent from the east (Margos et al., 2008). Also, *B. bavariensis* comprises European and Asian rodent-transmitted strains were previously incorporated into the species *B. garinii* (e.g., Korenberg et al., 2002; Margos et al., 2013; Scholz et al., 2013). However, NT29 and related strains (the Asian variant of *B. bavariensis*) have been associated exclusively with *I. persulcatus* while ospA type 4 (the European variant of *B. bavariensis*) are transmitted by *I. ricinus* (Fingerle et al., 2008). These data suggested that a single clone of *B. bavariensis* adapted to a different vector and was subsequently able to spread into a new geographic region (Margos et al., 2013).

Courtesy of current expansion of *I. scapularis* and *B. burgdorferi* s.s. populations in the USA and Canada we now we have the opportunity to study these processes in real time. Amongst the three key drivers of range change, climate and to some extent habitat are quantifiable, categorisable and measurable allowing some prediction of invasion probabilities (see for example Ogden et al., 2006a,b and Ogden et al., 2008). However, our capacity to predict host species ranges and densities in different locations, and predict host dispersal rates and trajectories, is more limited. Improved knowledge of these factors and their

interactions would improve our capacity to develop and calibrate models that predict future patterns of invasion, evolution and diversity of *B. burgdorferi* s.l. associated with current environmental changes. Confidence in such predictions would depend on validation, which is difficult to do prospectively. However, the phylogeographic record may allow us to validate models by hindcasting past population expansions and contractions associated with glacial and interglacial periods, and comparing expected results against currently observed phylogeography. More precise estimates of the molecular clock of selectively neutral loci would be particularly useful for this purpose.

CONCLUSION

Here we have outlined processes involved in the expansion and invasion of tick and tick-borne pathogen populations and identified changes in climate, habitat and hosts as key factors in range changes for ticks and tick-borne diseases. Host movements are the main mechanism of dispersal of ticks and tick-borne pathogens that permits invasions. We have also reviewed expected patterns of diversity of ticks and tick-borne pathogens in zones of emergence and geographic spread and we identify that for the Lyme borreliosis spirochaetes and vectors many observed patterns are consistent with the patterns expected from theoretical studies or studies of other species. Studies of the agent of Lyme disease *B. burgdorferi* s.l. suggest that the phylogeographic and ancestral patterns held within the genome of tick-borne pathogens may depend closely on host behavior and dispersion, and provide clues as to how their populations expanded and contracted associated with past climatic changes. Study of host dispersion and current range expansions may help us to develop better predictive models of effects of current climate change on patterns of invasion and diversity of ticks and tick-borne pathogens, while the phylogeographic record of past population expansions and contractions may allow us to validate these models.

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Sex-biased differences in the effects of host individual, host population and environmental traits driving tick parasitism in red deer

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The interactions between host individual, host population, and environmental factors modulate parasite abundance in a given host population. Since adult exophilic ticks are highly aggregated in red deer (*Cervus elaphus*) and this ungulate exhibits significant sexual size dimorphism, life history traits and segregation, we hypothesized that tick parasitism on males and hinds would be differentially influenced by each of these factors. To test the hypothesis, ticks from 306 red deer—182 males and 124 females—were collected during 7 years in a red deer population in south-central Spain. By using generalized linear models, with a negative binomial error distribution and a logarithmic link function, we modeled tick abundance on deer with 20 potential predictors. Three models were developed: one for red deer males, another for hinds, and one combining data for males and females and including "sex" as factor. Our rationale was that if tick burdens on males and hinds relate to the explanatory factors in a differential way, it is not possible to precisely and accurately predict the tick burden on one sex using the model fitted on the other sex, or with the model that combines data from both sexes. Our results showed that deer males were the primary target for ticks, the weight of each factor differed between sexes, and each sex specific model was not able to accurately predict burdens on the animals of the other sex. That is, results support for sex-biased differences. The higher weight of host individual and population factors in the model for males show that intrinsic deer factors more strongly explain tick burden than environmental host-seeking tick abundance. In contrast, environmental variables predominated in the models explaining tick burdens in hinds.

Keywords: host-parasite, polygynous, cervidae, tick, sexual segregation

INTRODUCTION

Tick distribution in their hosts is frequently found to be highly aggregated in a few individuals within the host population, which determines that a few hosts are responsible for feeding large amounts of ticks (Shaw and Dobson, 1995; Shaw et al., 1998). This ecological feature of tick-host interactions greatly conditions the transmission of pathogens between ticks and their hosts (Perkins et al., 2003). The probability of tick-borne pathogen transmission at the tick-host interface largely depends on the burden of ticks feeding in a single infected individual, especially when co-feeding transmission is of great relevance for the epidemiology of the pathogen (Perkins et al., 2003). Thus, identifying factors driving tick-host relationships in each tick-host system is crucial to both prevent undesired effects on target and accidental hosts that may be highly susceptible to certain tick-borne pathogens and to reduce risks of transmission to humans of zoonotic pathogens.

Higher individual macroparasite burdens would be expected to be associated with lower immune capacity to fight against parasites (Vicente et al., 2007a), though recent studies link higher macroparasite burdens to host activity traits (Boyer et al., 2010), body mass (Kiffner et al., 2013) or to other effects linked to tick distribution in the environment (Calabrese et al., 2011). Since exophilic ticks are highly aggregated in the environment (Ruiz-Fons and Gilbert, 2010), the rate of host-tick effective contacts at a local spatial scale would consequently be expected to be higher for hosts displaying higher activity and higher body surface. However, many studies have dealt with the immunocompetence handicap hypothesis (Folstad and Karter, 1992) driving the burden of macroparasites in their hosts (Hughes and Randolph, 2001; Malo et al., 2009). The immunocompetence handicap hypothesis basically proposes that testosterone has a dual effect on males, enhancing expression of secondary sexual traits and depressing the immune system. Thus, better males could allow overexpression of sexual traits while overcoming negative effects related to immunocompetence reduction. Therefore, physical (morphology), ecological (behavior), and physiological

(testosterone levels) factors have been considered as main drivers of parasitism in mammals (Moore and Wilson, 2002; Alzaga et al., 2009; Kiffner et al., 2013).

Sex-biased parasitism has been reported in many different host-parasite systems, often displaying a male-biased parasitism in highly dimorphic species (Moore and Wilson, 2002; Kiffner et al., 2013), especially those subjected to greater intraspecific competition for resources (Bacelar et al., 2011). Resource partitioning in self-maintenance, reproduction and defense against parasites is the result of a basic trade-off experienced by animals (Clutton-Brock et al., 1982). Mating system in polygynous mammals may carry over drastic resource allocation changes in individuals, especially in males, whose priorities in mating are more important than those related to immunocompetence (Rolff, 2002). Red deer (*Cervus elaphus*) males display a "live hard, die young" strategy (Carranza et al., 2004) in contrast to females that tend to allocate resources to self-maintenance, offspring rearing, and immunity, which has been deemed as one of the main sexual behavioral traits enhancing higher parasite loads in male red deer (Vicente et al., 2007a,b). Sex-related effects on tick burdens in mammals are controversial. Several authors described a clear sex-related effect on tick burden in white-tailed deer (*Odocoileus virginianus*; Schulze et al., 1984; Kitron et al., 1992; Schmidtmann et al., 1998), while recent studies in German roe deer (*Capreolus capreolus*) found no sex-related effect on tick burdens (Vor et al., 2010; Kiffner et al., 2011). The latter authors assume these host-species related differences in wild ruminants could be linked to sexual dimorphism, since dimorphism is higher in white-tailed than in roe deer, but it also could be linked to other factors related to sexual segregation.

Since adult exophilic ticks are highly aggregated in red deer and this wild ruminant exhibits a significant sexual size, resource allocation in immunity and behavioral dimorphism (e.g., Clutton-Brock et al., 1982), we hypothesized that parasitism by ticks—i.e., tick abundance on hosts—on each sex would be differentially influenced by host individual, host population and environmental factors. Predicting tick burdens in hinds with factors identified to drive tick burdens in males, and vice-versa, could be accurate only in the case of factors having the same weight on parasite burdens in both sexes. Otherwise, prediction of tick burdens, and hence identification of key hosts for tick-borne pathogens, would need to differentially consider host sex.

MATERIALS AND METHODS

STUDY AREA AND HOST INDIVIDUAL TRAITS

The study area comprised a 900 ha hunting estate located in Ciudad Real province (south-central Spain: 38°55′N, 0°36′E; 600–850 m a. s. l.) of which 700 ha are dedicated to game rearing for hunting purposes, mostly Iberian red deer (*C. elaphus hispanicus*) and Eurasian wild boar (*Sus scrofa*) but also small numbers of mouflon (*Ovis aries musimon*) and aoudad (*Ammotragus lervia*). Orography in the estate is formed by hill chains (up to 100 m high) bordering three main valleys. Hills are covered by Mediterranean shrub ecosystem composed by scattered *Quercus ilex* trees and shrub dominated by *Cystus* spp. *Pistacia* spp., *Rosmarinus* spp., *Erica* spp., *Arbutus unedo*, and *Phyllirea* spp. Valleys are dedicated to grow seasonal cereal crops for game feeding. Climate is continental Mediterranean with cold winters and very hot and dry summers and rainfall—ranging 300–700 mm annually—is highly seasonal. Supplementary food (mixed cereal-leguminous pellets) is available *ad-libitum* along the year for deer on selective feeders located at the bottom of valleys (8 feeding points). Additionally, water supply is maintained all-over the year in eight water ponds distributed along riverbeds in valleys.

Over 7 years, from 2004 to 2010, hunter harvested red deer were surveyed for ticks immediately after being shot. The whole deer body was surveyed for ticks, which were counted and collected. Every tick from lowly parasitized animals (<30 ticks) was collected while a representative subsample of ticks were collected in highly parasitized individuals (>30 ticks). Every immature stage located was collected from deer carcasses. Collected ticks were identified to species level (Manilla, 1998; Estrada-Peña et al., 2004; Apanaskevich and Horak, 2008; Apanaskevich et al., 2008).

Every deer surveyed was subjected to a detailed necropsy to detect any lesion caused by macro or microparasites (e.g., tuberculosis-like lesions; see Vicente et al., 2006) in different organs, weighed, sexed, and biometrically characterized—total length, hind foot length, and thoracic perimeter (measured to the nearest 0.1 cm). During necropsies, spleen was weighed to the nearest 0.1 g and kidney fat index (KFI) was calculated as an estimation of body fat (Santos et al., 2013). Age was determined for young individuals (<2 years old) based on tooth eruption patterns (Sáenz de Buruaga et al., 1991) and by incisor 1 sectioning in >2 year old animals (Klevezal and Kleinenberg, 1967). Deer age was categorized into 5 classes: (1) fawns (0–1 year old); (2) yearlings (1–2 years old); (3) subadults (2–3 years old); (4) adults (4–10 years old); and (5) old (>10 years old). Maximum recorded age was 23 years. Individual host data throughout sex and age class is shown in **Table 1**.

HOST POPULATION TRAITS

Host abundance is a key factor influencing host-seeking tick burdens in the environment at local geographic scales that could greatly condition tick burden in individual hosts (Ruiz-Fons et al., 2012). At the short time-scale (i.e., a year) the influence of host abundance on tick environmental abundance is difficult to measure since individual ticks may take up to several years to complete their life cycle. However, at the long-time scale changes in key host availability between years may be reflected in changes in host-seeking tick abundance. Wild ungulates are key hosts for adults of the predominant tick species in the estate (*Hyalomma lusitanicum* and *Rhipicephalus bursa*; see Ruiz-Fons et al., 2006) so annual censuses for the most abundant ungulate species in the estate, that is, red deer and wild boar, were used as predictors for tick burden models. The effect of host abundance in previous years on current tick burdens was tested by considering deer and wild boar abundance in years t-1 and t-2 (see Ostfeld et al., 2006). Censuses were performed by experienced observers (gamekeepers) who counted individuals approaching feeders at the bottom of the valleys (total counts) during the red deer rut season. For further details on the census procedure see Rodríguez-Hidalgo et al. (2010).

ENVIRONMENTAL VARIABLES

Climatic conditions (e.g., temperature and hydric stress) greatly condition tick phenology, activity, and survival (Estrada-Peña et al., 2011). Adult tick burden in an individual host at a given time is a function of the ticks encountered by the individual within a two week period since this is the average time adult *Hyalomma* ticks remain feeding in their host (Estrada-Peña et al., 2011). Thus, meteorological data at the short time scale, i.e., in 30 days before each animal was surveyed, were considered as a proxy of climatic constraints of tick activity. Considering a 30 days period aimed to buffer the occurrence of any stochastic meteorological event that could have momentarily affected tick questing behavior and consequently tick burdens on hosts. Meteorological data—temperature and precipitation—on a daily basis were obtained from a meteorological station (Spanish Meteorological Agency reference station 4210E; http://www.aemet.es) located in the study hunting estate (**Table 2**). The actual evapotranspiration (AET)—a measure of hydric stress experienced by ticks in its off-host period—was calculated on the basis of temperature and precipitation data using the formula proposed by Turc (1954), as follows:

$$\mathrm{AET} = \frac{P}{\sqrt{0.9 + \left(\frac{P^2}{L^2}\right)}}$$

where "P" is accumulated precipitation in mm and "L" is defined by:

$$L = 300 \times 25t + 0.05t^3$$

being "t" the mean temperature in °C.

Table 1 | Average values, associated standard error and range (within brackets) of host individual variables [total length (TL; cms), thoracic perimeter (TP; cms), hind foot length (HF; cms), and kidney fat index (KFI; %)] throughout sex and age class of studied deer.

Sex	Age class	TL	TP	HF	KFI
Male	Fawn	133.8 ± 2.7 (104–149)	93.4 ± 2.6 (68–115)	45.3 ± 0.9 (34–51)	88.0 ± 21.3 (9.5–385.5)
	Yearling	165.8 ± 1.3 (149–178)	115.0 ± 1.9 (101–132)	51.8 ± 0.4 (48–56)	4.3 ± 6.4 (4.6–107.7)
	Subadult	174.9 ± 2.4 (161–187)	115.9 ± 1.7 (108–127)	53.1 ± 1.7 (49–62)	41.6 ± 7.8 (15.2–85.6)
	Adult	187.7 ± 1.1 (158–213)	125.8 ± 0.7 (111–155)	53.3 ± 0.2 (48–61)	65.7 ± 8.5 (4.9–455.3)
	Old	188.8 ± 2.6 (174–203)	125.0 ± 1.9 (116–134)	52.8 ± 0.7 (49–56)	48.9 ± 14.0 (7.5–106.4)
	Subtotal male	178.1 ± 1.5 (104–213)	120.2 ± 1.0 (68–155)	52.2 ± 0.3 (34–62)	63.0 ± 6.5 (4.6–455.3)
Female	Fawn	128.5 ± 3.5 (95–152)	86.1 ± 2.6 (64–106)	44.3 ± 0.8 (35–49)	78.0 ± 19.3 (7.4–247.0)
	Yearling	148.4 ± 2.9 (130–161)	108.0 ± 6.5 (88–162)	49.1 ± 0.7 (45–53)	122.2 ± 23.2 (61.0–241.0)
	Subadult	160.6 ± 2.3 (147–174)	105.2 ± 2.0 (91–114)	49.3 ± 0.3 (47–51)	74.7 ± 17.6 (20.0–232.0)
	Adult	162.4 ± 1.1 (133–184)	110.3 ± 0.9 (89–130)	48.7 ± 0.2 (43–53)	93.8 ± 8.4 (2.4–263.5)
	Old	166.6 ± 1.7 (160–178)	110.2 ± 1.7 (104–120)	48.7 ± 0.4 (47–51)	119.7 ± 25.0 (30.9–283.9)
	Subtotal female	157.2 ± 1.3 (95–184)	106.0 ± 1.0 (64–130)	48.2 ± 0.2 (35–53)	94.4 ± 6.6 (2.4–283.9)

STATISTICAL MODELLING AND ANALYTICAL DESIGN

For descriptive analyses of parasitization rates the statistical uncertainty was assessed by calculating the 95% confidence interval for each of the proportions according to the expression $95\%\mathrm{C.I.} = 1.96[p(1-p)/n]^{1/2}$ (where "p" is the proportion in its unitary value and "n" is the sample size) and expressed in percentage.

Using an inductive approach we quantified the effect of the main factors able to explain tick burdens on red deer, at individual level. Predictors were considered in generalized linear models

Table 2 | Deer, wild boar, total ungulate (deer + boar + mouflon + aoudad) counts, and average values of climatic variables (and associated standard error within brackets) associated to deer sampling date in the hunting estate throughout year.

Year	Deer_C	Wild boar_C	Tot_Ung_C	AvT_M[a]	AP_M[b]	AET_M[c]
2002	363	160	600	NA	NA	NA
2003	365	60	504	NA	NA	NA
2004	286	40	395	20.1 (1.2)	8.0 (2.1)	0.97 (6.4×10^{-3})
2005	400	140	626	22.0 (1.2)	8.3 (3.2)	0.25 (7.3×10^{-2})
2006	392	100	559	15.3 (1.2)	47.8 (5.2)	0.99 (4.8×10^{-4})
2007	425	200	693	16.5 (1.0)	47.3 (3.5)	0.99 (1.0×10^{-4})
2008	418	150	636	13.0 (0.8)	68.2 (4.7)	0.93 (4.2×10^{-2})
2009	434	16	514	20.4 (0.5)	31.7 (1.8)	0.89 (3.2×10^{-2})
2010	332	48	458	8.9 (1.1)	151.6 (9.4)	0.99 (8.2×10^{-6})
Average	379.4	101.5	553.8	—	—	—

[a]AvT_M, average mean daily temperature (°C) values of 30 days before sampling (bs); [b]AP_M, accumulated precipitation (mm) of 30 days bs; [c]AET_M, actual evapotranspiration (mm) of 30 days bs. NA, Not applicable.

with a negative binomial distribution and a logarithmic link function (Cameron and Trivedi, 1998), and the final models (three, see below) were obtained using a forwards-backwards stepwise procedure based on Akaike Information Criteria (AIC; Akaike, 1974). We opted for the negative binomial distribution due to high levels of overdispersion in the data when models were fitted with Poisson distributions. The multicolineality among predictors included in the final models was assessed using predictor's variance inflation factor (VIF). VIFs were calculated—for each predictor and model—as the inverse of the coefficient of non-determination for a regression of a given predictor on all others (see Zuur et al., 2010).

Because we were interested if tick-burdens were affected differentially in male and female deer, we developed three models: a model for red deer males, a model for hinds, and, finally, a model combining data for males and hinds and including "sex" as factor. If parasitization by ticks on red deer males and hinds responded to the explanatory factors in a differential way, it would be not possible to precisely and accurately predict the tick burden on hinds using the model fitted on males (and/or vice-versa), or with the model that combines data for males and hinds. However, if parasite loads on males and hinds similarly responded to the explanatory factors, then any of the independent models could precisely determine the rates of either sex, and in this occasion better adjust terms and more accurate predictions could be attained with the model carried out by combining data from males and hinds than with the independent model for each sex. Two analytical procedures were used in order to compare the model in the previous terms: variation partitioning and cross-validation.

Variation partitioning procedures (see Borcard et al., 1992) were used to estimate the variation of the final models explained independently by each factor (pure effects) and the variation explained simultaneously by two or more factors (overlaid effects; see **Figure A1**). Note that a factor is a group of related-predictors; in this study three factors: individual host, host population and environment. For this purpose, we determined the total amount of deviance explained by the final model. Subsequently, we developed the partial models, i.e., models adjusted independently with the predictors related to each factor (individual host: Ind, host population: Pop, and environment: Env), as well as with those of each pair of factors (Ind + Pop, Ind + Env, and Pop + Env), and estimated the amounts of deviance explained by each of these six partial models. Values of the deviance explained by the final model (Ind + Pop + Env) and those explained by the partial models were subjected to subtraction rules in order to split up the different sections of the explained variation (see Alzaga et al., 2009). A complete scheme of each part of deviance and the subtraction rules used for their determination, is reported in Appendix. Briefly, the proportion of variation explained exclusively—independently of the other factors—by the individual host, for instance, was obtained with the following subtraction rule: I = (Ind + Pop + Env) − (Pop + Env); the proportions explained exclusively by the other factors were obtained in a similar way. The amount of variation attributable to the intersection of two factors (e.g., individual host and host population) was obtained with the subtraction rule: IP = (Ind + Pop + Env) − Ind − P − E; where P is the explained variation by the pure effect of host population and E is the pure effect of environment. The amount of variation attributable to the intersections between individual and environmental factors (IE) and between population and environmental factors (PE) were calculated in a similar way, and the amount attributable to the intersections between the three factors together (IPE) was obtained with the subtraction rule: IPE = (Ind + Pop + Env) − E − P − I − EP − IP − EI. Therefore, we determined a value for each part of deviance explained and knew how much corresponded to its pure effect and how much to intersections between two or three factors. This procedure was carried out on each of the three final models. The proportions of explained deviance for each factor were standardized to make them comparable among models; for this purpose they were expressed in relation to the proportion of deviance explained for the final model (e.g., Alzaga et al., 2009; Pérez-Ramírez et al., 2012).

Cross-validation is a procedure for assessing how the results of a statistical model can be generalized to an independent data set (Picard and Cook, 1984). Under our analytical design, we are interested in how the results of the model developed on the dataset for a given sex can be used to explain variation in the response variable on the dataset for another sex (validation dataset). Similarly, we assessed the performance of the model developed by combining data for males and hinds, which was calibrated using an 70% random sample (training dataset) and was validated against the remaining 30% of the data (validation dataset). On each dataset and under this crossed framework, we binned predictions from the model into 10 evenly sized intervals of increasing predicted burdens. Assessment was carried out by plotting the mean observed against predicted abundance, in each interval on the validation datasets (see Pearce and Ferrier, 2000). The basic premise is that as the burdens predicted by the model increase (e.g., model for males), there should be a similar increase in the observed burdens in the validation dataset (in this case, on hinds dataset).

Statistical analyses were carried out in R 2.15.2 (R Core Team, 2012). The "MASS" library was used for model development (Venables and Ripley, 2002), the "HH" package for the VIF analyses (Heiberger, 2012), and the "ggplot2" package for the calibration plots (Wickham, 2009).

RESULTS

Tick data from 306 red deer—182 males and 124 females—were gathered for the 7 years of study (average deer no./year: 25.5; range: 12–64; **Table 3**). The 59.5% (95%CI: 54.0–65.0) of deer were parasitized by ticks, the major part only by adults (59.2%; 95%CI: 53.7–64.7). Out of the 4009 ticks counted on deer, 1772 were collected (1761 adults and 11 nymphs). Adults belonged mainly to *Hy. lusitanicum* ($n = 1750$; 98.8%), *Rh. bursa* ($n = 9$; 0.5%), *Rh. sanguineus* ($n = 1$; 0.05%), and *Dermacentor marginatus* ($n = 1$; 0.05%) and nymphs belonged to *Hy. lusitanicum* ($n = 9$; 0.5%) and *Rh. bursa* ($n = 2$; 0.1%). Annual average adult tick abundance per deer experienced a decrease along study years (**Table 4**).

Predictors included in the three final models are summarized in **Table 5**. VIFs obtained for the predictors included in final

Table 3 | Data on the number of tick parasitized deer (PosT) with respect the total number (N) of analyzed deer throughout sex and age class.

Sex	Age class	PosT/N	PrevT	Col-AvT	Cou-AvT	Col-AvA	Cou-AvA
Male	Fawn	2/20	10.0	0.2 (1–2)	0.2 (0–2)	0.1 (0–2)	0.1 (0–2)
	Yearling	21/24	87.5	7.7 (0–49)	14.5 (0–50)	7.5 (0–49)	14.4 (0–50)
	Subadult	10/11	90.9	10.7 (0–36)	15.6 (0–60)	10.1 (0–36)	14.8 (0–60)
	Adult	104/118	88.1	9.5 (0–67)	24.0 (0–125)	9.2 (0–67)	23.6 (0–125)
	Old	9/9	100.0	17 (5–47)	39.3 (0–140)	16.9 (5–47)	39.0 (0–140)
	Subtotal male	146/182	80.2	8.6 (0–67)	20.4 (0–140)	8.4 (0–67)	20.0 (0–140)
Female	Fawn	2/16	12.5	0.3 (0–2)	0.3 (0–2)	0.2 (0–2)	0.2 (0–2)
	Yearling	2/10	20.0	1.2 (0–11)	1.2 (0–11)	1.2 (0–11)	1.2 (0–11)
	Subadult	4/13	30.8	1.1 (0–6)	1.7 (0–12)	1.1 (0–6)	1.7 (0–12)
	Adult	22/72	30.6	1.4 (0–21)	2.2 (0–36)	1.4 (0–21)	2.2 (0–36)
	Old	6/12	50.0	5.5 (0–25)	8.5 (0–49)	5.5 (0–25)	8.5 (0–49)
	Unknown	0/1	0.0	0.0 (0–0)	0.0 (0–0)	0.0 (0–0)	0.0 (0–0)
	Subtotal female	36/124	29.0	1.6 (0–25)	2.4 (0–49)	1.6 (0–25)	2.4 (0–49)
TOTAL		182/306	59.5	5.8 (0–67)	13.1 (0–140)	5.6 (0–67)	12.9 (0–140)

Average number of ticks/deer collected (Col_AvT) and counted (Cou_AvT) as well as collected (Col_AvA) and counted (Cou_AvA) adult ticks are displayed. Values within brackets represent minimum and maximum collected and counted ticks and adult ticks per deer. The female with unknown age was not considered for modeling purposes.

models showed that no biased predictions are expected due to collinearity-derived problems (VIFs < 2.21, <2.32, and <1.99, for the model for males, for hinds, and for males and hinds, respectively). A higher amount of deviance was explained for the model of males (53.97%) than for the other models (46.26% and 50.76%, for the model of hinds and the model of males and hinds, respectively). When data for males and females were considered in a model, "sex" was a relevant predictor and a significantly higher number of ticks was detected on males than on hinds (see also **Table 3**). The observed increasing tick burden with deer age was evidenced in both males and females (**Table 5**). Predictors related to the three considered factors (i.e., individual host, host population and environment) were selected for the three final models; but according to test-values, the relevance of the predictors varied among the models (**Table 5**).

Variation partitioning procedures showed that the amount of variation explained by the pure factors and the overlaid effects were quite different among the three models, mainly between the model for males and that for hinds (**Figure 1**). In the model for males, individual host and host population factors explained a higher amount of variation than in the model for hinds. For the hind model most of variation could be explained by the environmental factor. Finally, the model combining data from males and hinds showed an intermediate situation between the independent models for each sex, with a similar amount of variation explained by the host population factor than in the model of hinds, and a similar amount of variation explained by the environmental factor than in the model of males.

Finally, cross-validation showed that the independent models for each sex were not able to accurately explain the parasitization rate on the other sex (**Figure 2**). To this respect, the worst performance was obtained when the model for hinds was applied to the dataset of males (**Figure 2A**). The model for males precisely, but not accurately, explained tick parasitization on hinds, mainly for individuals with higher parasitization rates (**Figure 2B**); the model was precise because the observed abundance monotonically increased with predicted abundance (mainly for the higher intervals of predicted abundance), and it was not accurate because predictions overestimated the observed abundances. Finally, a model was adjusted by pooling data for males and females, and this model again overestimated the higher intervals of predicted abundance (**Figure 2C**). This combined model was closer to the response of males than to that of hinds.

DISCUSSION

Identification of factors driving tick parasitism on hosts has been a relevant issue in ecology (Moore and Wilson, 2002; Boyer et al., 2010; Calabrese et al., 2011), and is currently a relevant topic in tick-borne disease epidemiology (Perkins et al., 2003; Ruiz-Fons et al., 2012). Most studies have centered attention to small mammals (Brunner and Ostfeld, 2008; Alzaga et al., 2009; Boyer et al., 2010) and few attention has been paid to large mammals. Large mammals—such as red deer—are key hosts for many epidemiologically relevant tick species (Ruiz-Fons and Gilbert, 2010; Ruiz-Fons et al., 2012), especially in Mediterranean environments (Ruiz-Fons et al., 2006). In south-central Spain, red deer are abundant (Acevedo et al., 2008) and have experienced a notable increase in the last three decades (Apollonio et al., 2010), which would have consequences for tick ecology and for zoonotic tick-borne pathogen epidemiology (e.g., *Anaplasma phagocytophilum*—(de la Fuente et al., 2005)—or Crimean-Congo haemorrhagic fever virus—Estrada-Peña et al., 2013). Mating system (Miller et al., 2007),

Table 4 | Average number of ticks/deer collected (Col_AvT) and counted (Cou_AvT) and average number of adult ticks/deer collected (Col_AvA) and counted (Cou_AvA) throughout year and season.

Year	Season	N	Col_AvT	Cou_AvT	Col_AvA	Cou_AvA
2004	Winter	0	NS[a]	NS	NS	NS
	Spring	0	NS	NS	NS	NS
	Summer	3	14.3 (6–19)	29.3 (9–60)	14.0 (6–19)	28.2 (9–57)
	Autumn	9	17.2 (4–47)	39.4 (4–140)	17.0 (2–47)	39.2 (2–140)
Subtotal 2004		12	16.5 (4–47)	36.9 (4–140)	16.3 (2–47)	36.5 (2–140)
2005	Winter	2	0.0 (0–0)	0.0 (0–0)	0.0 (0–0)	0.0 (0–0)
	Spring	1	25.0 (25–25)	49.0 (49–49)	25.0 (25–25)	49.0 (49–49)
	Summer	24	6.5 (0–49)	14.0 (0–50)	6.5 (0–49)	13.9 (0–50)
	Autumn	9	8.6 (0–31)	18.2 (0–48)	8.6 (0–31)	18.2 (0–48)
Subtotal 2005		36	7.2 (0–49)	15.2 (0–50)	7.1 (0–49)	15.2 (0–50)
2006	Winter	20	4.7 (0–22)	5.7 (0–28)	4.4 (0–22)	5.3 (0–28)
	Spring	0	NS	NS	NS	NS
	Summer	17	12.9 (0–67)	23.4 (0–125)	11.1 (0–67)	20.7 (0–125)
	Autumn	19	8.2 (0–27)	28.5 (0–120)	8.2 (0–27)	28.5 (0–120)
Subtotal 2006		56	8.4 (0–67)	18.8 (0–125)	7.7 (0–67)	17.8 (0–125)
2007	Winter	25	4.6 (0–14)	18.3 (0–80)	4.6 (0–14)	18.2 (0–80)
	Spring	1	0.0 (0–0)	0.0 (0–0)	0.0 (0–0)	0.0 (0–0)
	Summer	10	11.4 (0–24)	21.7 (0–60)	10.9 (0–21)	21.1 (0–60)
	Autumn	28	4.7 (0–28)	13.4 (0–80)	4.7 (0–28)	13.4 (0–80)
Subtotal 2007		64	5.7 (0–28)	16.4 (0–80)	5.6 (0–28)	16.3 (0–80)
2008	Winter	9	2.6 (0–15)	2.8 (0–16)	2.6 (0–15)	2.8 (0–16)
	Spring	0	NS	NS	NS	NS
	Summer	1	5.0 (5–5)	12.0 (12–12)	5.0 (5–5)	12.0 (12–12)
	Autumn	30	2.5 (0–25)	3.7 (0–28)	2.5 (0–25)	3.7 (0–28)
Subtotal 2008		40	2.6 (0–25)	3.7 (0–28)	2.6 (0–25)	3.7 (0–28)
2009	Winter	1	0.0 (0–0)	0.0 (0–0)	0.0 (0–0)	0.0 (0–0)
	Spring	0	NS	NS	NS	NS
	Summer	6	9.7 (1–23)	13.2 (1–40)	9.5 (1–23)	13.0 (1–40)
	Autumn	53	4.7 (0–19)	11.3 (0–80)	4.7 (0–19)	11.4 (0–80)
Subtotal 2009		60	5.1 (0–23)	11.3 (0–80)	5.1 (0–23)	11.4 (0–80)
2010	Winter	30	0.0 (0–0)	0.0 (0–0)	0.0 (0–0)	0.0 (0–0)
	Spring	0	NS	NS	NS	NS
	Summer	2	12.0 (11–13)	12.0 (11–13)	12.0 (11–13)	12.0 (11–13)
	Autumn	6	8.5 (3–21)	11.2 (4–30)	8.5 (3–21)	11.2 (4–30)
Subtotal 2010		38	2.0 (0–21)	2.4 (0–30)	2.0 (0–21)	2.4 (0–30)
TOTAL	Winter	87	2.7 (0–22)	6.9 (0–80)	2.3 (0–22)	6.7 (0–80)
	Spring	2	12.5 (0–25)	24.5 (0–49)	12.5 (0–25)	24.5 (0–49)
	Summer	63	9.8 (0–67)	18.3 (0–125)	9.2 (0–67)	17.4 (0–125)
	Autumn	154	5.8 (0–47)	14.4 (0–140)	5.8 (0–47)	14.4 (0–140)

[a] *NS, No samples.*

Table 5 | Statistical parameters (coefficient/test-value and significance: ns: non-significant, #0.1, *0.05, **0.01, and ***0.001) of the generalized lineal models (negative binomial error distribution and logarithmic link function) carried out to predict tick burden on red deer.

Predictor (factor)	Model for males	Model for hinds	Model for males and hinds
TL (Ind)	0.0213/3.58***	0.0316/1.20 ns	0.0400/4.45**
AvT_M (Env)	0.0873/6.74***	0.1376/3.63***	0.0962/6.54***
Age class (Ind)	0.6245/5.18***	0.5950/1.70#	0.5337/3.54***
Year (Env)	−0.4869/−7.16***		
Deer_C (Pop)	0.0158/5.94***		0.0083/3.22**
Deer_C t-2 (Pop)		0.0097/1.84#	
ETA_M (Env)	1.1880/4.40***		1.7497/4.63***
KFI (Ind)	−0.0033/−3.19**		−0.0032/−2.50*
AP_M (Env)	−0.0067/−2.49*	−0.0313/−3.64***	−0.0189/−6.47***
Wild boar_C t-2 (Pop)			−0.0101/5.42***
Sex(females) (Ind)[a]			−1.5922[a]/−5.19***
Intercept	965.5163/7.11***	−11.5223/−2.77**	−10.6220/−6.35***

*Individual host (Ind), host population (Pop), and environmental (Env) factors; predictors coded as in **Tables 1–2**.*

[a] *Coefficient for females in relation to males.*

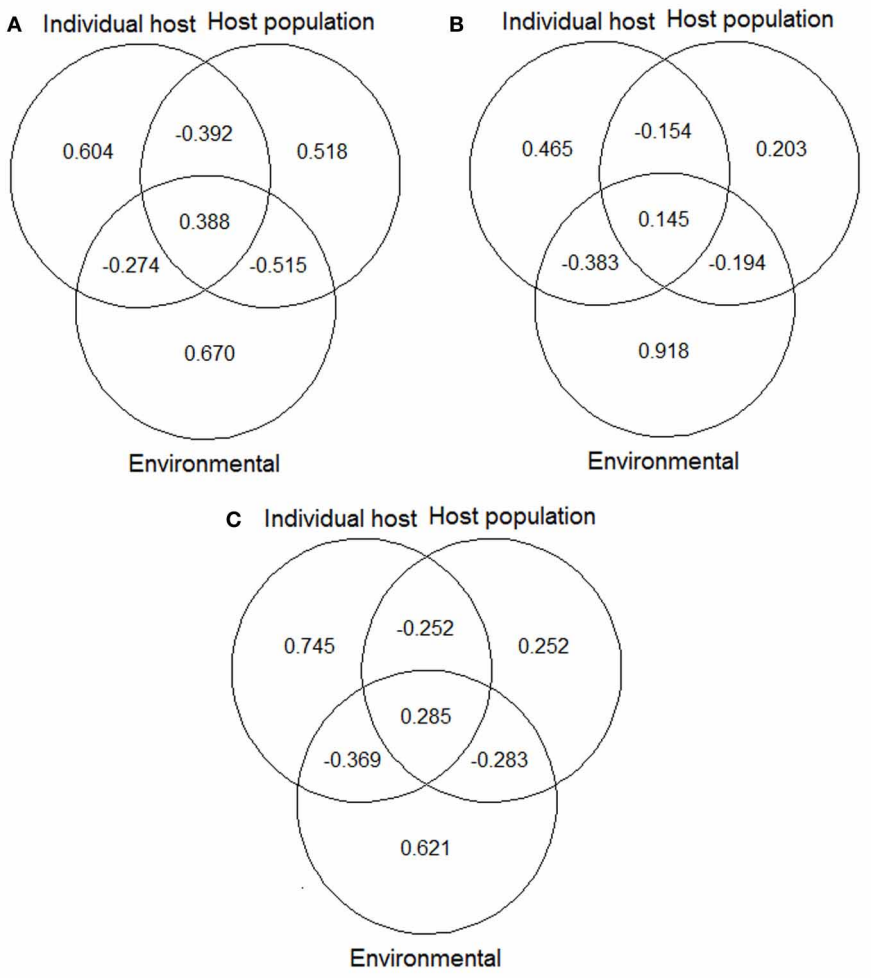

FIGURE 1 | Variation partitioning of the deviance explained by final models: (A) model for red deer males; (B) model for hinds; and (C) model for males and hinds. Values shown in the diagrams are the proportions of variation of each final model that can be explained exclusively by individual host, host population and environmental factors, and by the combined effect of these factors. See **Table 5** for details about predictors included in each of the abovementioned models/factors. The "VarPart" function was used for producing the plots (Barbosa et al., 2013).

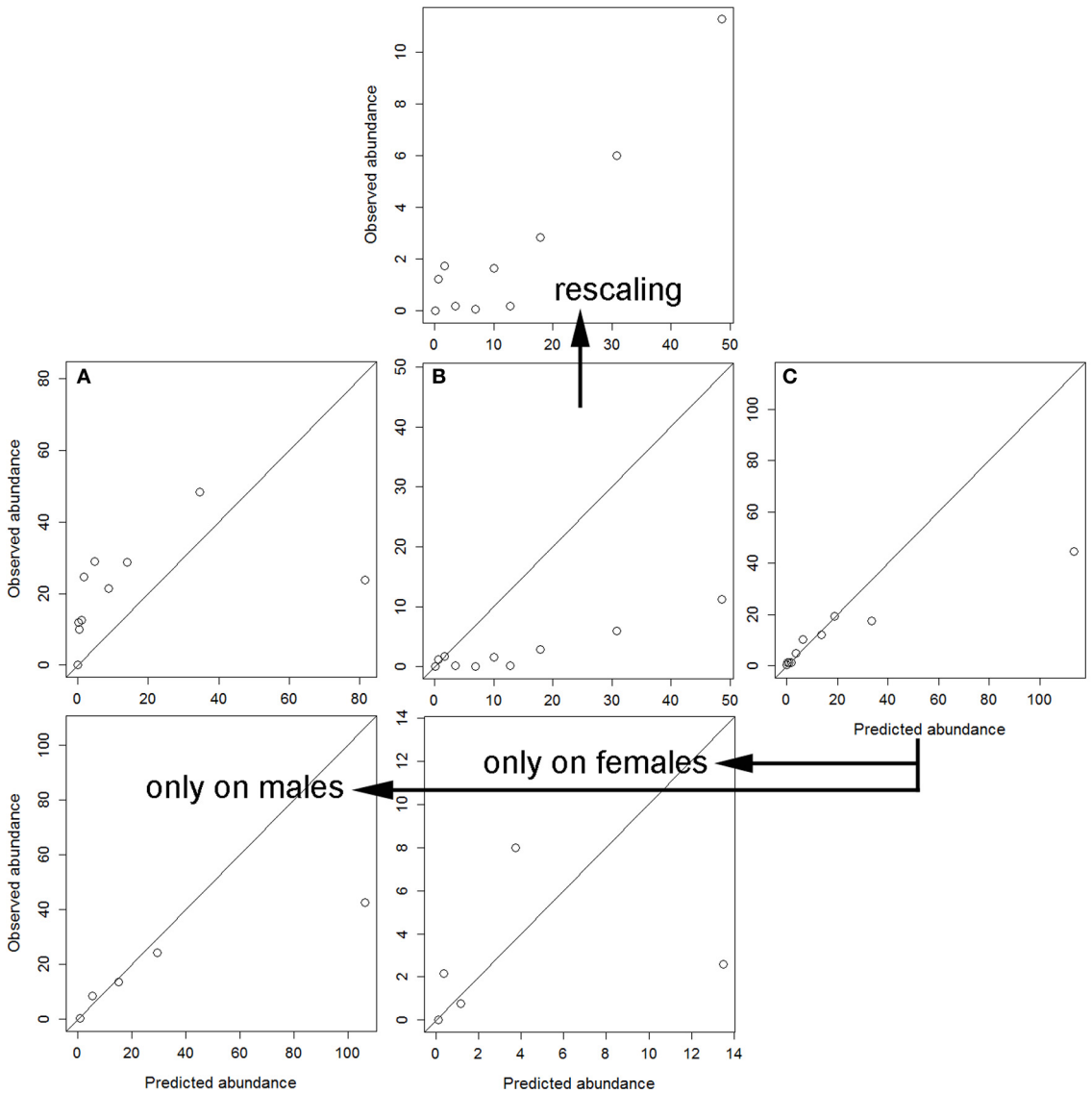

FIGURE 2 | Calibration's assessment of the three models (see Table 5) under a cross-validation procedure: (A) predictions from the model for hinds on the dataset for males; (B) predictions from the model for males on the dataset for hinds (also rescaling the observed abundance axis); and (C) predictions from the model for males and hinds on the validation dataset, also independently for males and females (only five intervals were used in these last cases due to sample size).

sexual dimorphism (Moore and Wilson, 2002), intraspecific competition (Bacelar et al., 2011), space use (Boyer et al., 2010), testosterone levels (Hughes and Randolph, 2001), and even effects of environmental host-tick interactions (Calabrese et al., 2011), have been proposed as relevant factors driving sex-biased parasitism in mammals. Identifying factors driving tick-deer interactions at the individual level is thus a crucial issue for efficiently preventing and controlling tick-borne disease risks. In this study, deer males were evidenced as primary targets of exophilic ticks, mainly *Hyalomma* spp., and we showed that parasitism on each sex was differentially determined by host individual, host population and environmental factors.

DIFFERENTIAL DRIVERS OF TICK PARASITISM IN MALES AND HINDS

Even when tick burdens on males and hinds were modeled with the same set of predictors, each sex specific model was not able to accurately predict tick burdens on animals of the other sex (**Figure 2**). This cross-validation procedure allows us to suggest that tick burden in red deer are driven by different traits on males and hinds (see also Vicente et al., 2007a). In addition, the model for males was able to predict—with moderate precision, better for higher tick abundance—burdens on hinds. This was likely due to the over-dominance of the environmental factor in the model for hinds. Parasites benefit from situations in which hosts are not in good conditions (Murray et al., 1998). Variations in condition along the year in food supplemented populations are lower

in hinds than in males (Santos et al., 2013; see also Rodríguez-Hidalgo et al., 2010), since the latter are strongly affected by the rut period. Likely, this can be one of the reasons by which tick parasitism on males is more dependent on intrinsic factors that parasitism on hinds. Finally, the model combining data from males and females was better adjusted to variation in males than in hinds, evidencing again that parasitism in hinds is likely a simplification of the process in males.

Differential effects of host individual, host population and environmental factors in relation to the life cycle of parasites were evidenced in other mammal species (Alzaga et al., 2009). In European hare (*Lepus europaeus*), Alzaga et al. (2009) showed that the individual factor was the more explicative factor of tick burdens, followed by environmental and host population factors. In our study, we found relevant differences in the effects of each factor in relation to host sex, but in male and global models the effect of the individual factor was not clearly inferior to the others. Differences between the study on hares and the present study are likely related to the ecology of the host-tick system, i.e., ecological traits of host species but also of ticks, since different tick species were found parasitizing European hare and deer. In our study model, individual host and host population factors were more relevant for males than for hinds. In contrast, the environmental factor was more important explaining burdens on hinds. Potential reasons mediating these sex-biased differences are discussed below.

HOST INDIVIDUAL FACTOR DRIVING TICK PARASITISM IN RED DEER

Individual predictors such as size and age were positively related to tick abundance in red deer, while KFI was negatively related in the model for males. Size—measured by total length—and body mass were highly correlated in our data set (Spearman's rho = 0.904, $p < 0.001$), and consequently both influence tick burden in a positive proportional direction. Body size was selected as the most appropriate measure of animal's body surface exposed to questing ticks because body mass could be modulated by *ad-libitum* availability of supplementary food. Similar results relating KFI and parasitism in males were obtained for red deer parasitized by *Elaphostrongylus cervi* in south-central Spain (Vicente et al., 2007b), suggesting a close relationship between KFI and macroparasite burden in red deer males in Mediterranean ecosystems. One trait responsible for this male-biased pattern in tick burdens could be related to resource allocation due to mating system, that can be also responsible for the higher relevance of the individual factor explaining tick burden on males than on hinds. This is coherent with results obtained for roe deer, in which sex-biased tick parasitism was only caused by a bias in male and female hunting seasons (Vor et al., 2010; Kiffner et al., 2011), a particular trait that did not account in our study where both sexes were surveyed in every season of the year. In contrast to roe deer males, red deer males defend big harems of several tens of females (Clutton-Brock et al., 1982). Keeping a higher number of hinds away from other males would make red deer males invest more resources in mating than those needed by roe deer males for the same purpose and this may be reflected in the immune system and finally on sex-biased parasitism in red deer males (Vicente et al., 2007b; Corbin et al., 2008). Likely, the apparent absence of any effect of KFI in the model for hinds could be related to the fact that KFI is significantly higher in hinds than in males and it remains constant throughout the year (Santos et al., 2013). Finally, the inverse relationship between KFI and tick burden in combination to the effect of body size, can explain the increasing trend on tick burden with host age.

Sex-related behavioral differences in the use of feeding and water points that could have led to differences in questing tick encounter rates by males and hinds, were discarded on the basis of a study on habitat selection of sympatric wild ungulates in the study estate (Sicilia, 2011). In this study, no sex-biased selection of feeders and water points were observed during summer—when natural food and water are scarce in our territory. It was also observed that both sexes actively selected shrub nearby feeding and water points during daytime and accessed feeders from dusk to dawn, for which no sex-related differences in daily time spent in different habitats were evidenced. If deer spent most of their daily time in feeding and water points this would have been reflected by higher questing tick abundances in these points. However, data from a monthly year-round survey on questing ticks performed in the study hunting estate (F. Ruiz-Fons, unpublished data) showed that higher questing tick abundances are present in the ecotone between shrub and pasture and not in pasture surrounding feeding and water points, being these results coherent with those from habitat use studies (Sicilia, 2011).

Another individual trait that could rely behind male-biased tick parasitism is innate genetic resistance. Fernández-de-Mera et al. (2009a)—in the same study red deer population—found that red deer presenting major histocompatibility complex class II (*MHC-II*) *DRB-2* haplotype 2 displayed significantly higher probability of being lowly parasitized by ticks with respect individuals displaying the other three most abundant *MHC-II-DRB-2* haplotypes in the estate. Data from a second study was re-analyzed for this study and showed that haplotype 2 was more frequent in hinds than in males (Fernández-de-Mera et al., 2009b), which could relate to the male-biased parasitism observed in our study. This hypothesis should be targeted in future experimental and field studies to properly identify its influence on male-biased tick parasitism.

HOST POPULATION DENSITY DRIVING TICK PARASITISM IN RED DEER

Density of hosts was selected in the independent models for each sex as related to tick burden, probably due to the fact that host densities regulate the percentage of adult ticks in the population that find a host and reproduce, thus contributing to densities of host-seeking ticks (Ruiz-Fons et al., 2012). Host population factor was able to explain a much higher amount of variation in the model for males than in the model for hinds. Likely these findings are again related to behavioral differences between sexes. Hinds live in groups and group size depends in a higher extent on antipredatory behavior—hunting resembles predation in our study population—than of the population density (Jedrzejewski et al., 2006), also in Mediterranean environments (Soriguer et al., 1994). Thus, population density may not be a key factor in determining tick transmission rates in hinds. In contrast, males are more solitary than females (Clutton-Brock et al., 1982), and the contacts in males should be closely dependent of the population density, mainly in the rut season (Carranza et al., 1996).

Wild boar are efficient hosts for *Hyalomma* spp. ticks (Ruiz-Fons et al., 2006), which was evidenced by the positive residual

effect of wild boar counts two years prior to survey on tick burdens in both sexes. This residual effect could be perhaps related to the lower abundance of wild boar with respect red deer in the study hunting estate that would make wild boar not to be very relevant in maintaining questing tick abundance.

ENVIRONMENTAL FACTOR DRIVING TICK PARASITISM IN RED DEER

Environmental factor captured most of the variation explained in tick burdens in individual models, especially in hinds (**Table 5**; **Figure 1**). Climate modulates both tick activity and survival during their off-host period (Estrada-Peña et al., 2011; Ruiz-Fons et al., 2012) and modulates host-seeking tick abundance. Environmental tick abundance seems to be related to tick burdens in hinds and contribute together with host population and host individual traits to tick burdens in males (**Table 5**). The effect of climatic variables, with positive influences of average temperature and AET and negative influence of precipitation, may be related to the preponderance of the xerophilic *Hy. lusitanicum* in the study estate which peaks in late spring and early autumn when mean temperatures are high.

FINAL STATEMENT

The higher weight of host individual and host population factors in the model for males show that intrinsic deer factors are more efficient predictors of tick burden than environmental host-seeking tick abundance, at least when food availability is not a constraint. According to these results, controlling ticks in males such as acaricide spread on males through selective feeders or application of anti-tick vaccines to males only, would hypothetically result in a reduction of tick burdens in hinds since host-seeking tick abundance would be reduced significantly. Whether such an specific tick control measure on males would result in an immediate increase of tick burdens on hinds or in a substantial reduction should be specifically tested in the future.

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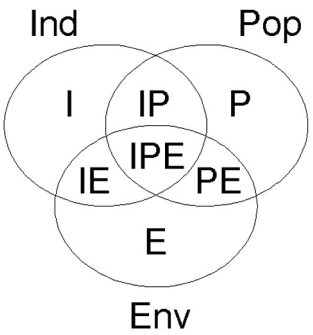
APPENDIX



$$I = (Ind+Pop+Env) - (Pop+Env)$$
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$$E = (Ind+Pop+Env) - (Ind+Pop)$$
$$IP = (Ind+Pop+Env) - Env - I - P$$
$$PE = (Ind+Pop+Env) - Ind - P - E$$
$$IE = (Ind+Pop+Env) - Pop - I - E$$
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FIGURE A1 | Scheme of the parts in which the deviance explained by a final model can be split by variation partitioning procedures, and the subtraction rules used for this purpose. For the variation partitioning we first determined the total amount of deviance explained by the final model, and secondly we developed partial models, i.e., the models adjusted independently with the predictors related to each factor (individual host: Ind; host population: Pop; and environment: Env), as well as with those of each pair of factors (Ind + Pop, Ind + Env, and Pop + Env), and estimated the amounts of deviance explained by each of these six partial models. The values of the deviance explained for the final model (Ind + Pop + Env) and for the partial models were used in the following subtraction rules.

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Ecology, biology and distribution of spotted-fever tick vectors in Brazil

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Spotted-fever-caused *Rickettsia rickettsii* infection is in Brazil the major tick-borne zoonotic disease. Recently, a second and milder human rickettsiosis caused by an agent genetically related to *R. parkeri* was discovered in the country (Atlantic rainforest strain). Both diseases clearly have an ecological background linked to a few tick species and their environment. Capybaras (*Hydrochoerus hydrochaeris*) and *Amblyomma cajennense* ticks in urban and rural areas close to water sources are the main and long-known epidemiological feature behind *R. rickettsii*-caused spotted-fever. Unfortunately, this ecological background seems to be increasing in the country and disease spreading may be foreseen. Metropolitan area of São Paulo, the most populous of the country, is embedded in Atlantic rainforest that harbors another important *R. rickettsii* vector, the tick *Amblyomma aureolatum*. Thus, at the city–forest interface, dogs carry infected ticks to human dwellings and human infection occurs. A role for *R. rickettsii* vectoring to humans of a third tick species, *Rhipicephalus sanguineus* in Brazil, has not been proven; however, there is circumstantial evidence for that. A *R. parkeri*-like strain was found in *A. ovale* ticks from Atlantic rainforest and was shown to be responsible for a milder febrile human disease. *Rickettsia*-infected *A. ovale* ticks are known to be spread over large areas along the Atlantic coast of the country, and diagnosis of human infection is increasing with awareness and proper diagnostic tools. In this review, ecological features of the tick species mentioned, and that are important for *Rickettsia* transmission to humans, are updated and discussed. Specific knowledge gaps in the epidemiology of such diseases are highlighted to guide forthcoming research.

Keywords: ecology, Brazil, tick species, *Rickettsia*, human spotted-fever

INTRODUCTION

Spotted-fever group (SFG) rickettsiae are chiefly transmitted by ticks and may cause mild to severe human infectious disease. These agents are found worldwide and are transmitted by an array of tick species, each one with specific ecological requirements. Thus, epidemiology of the various rickettsioses is determined by specific vector tick geographic and micro environmental distribution.

Brazil is a country with continental dimensions and encompasses diverse biomes such as rainforests (Amazonic and Atlantic), savannah (Cerrado), open fields (Pampas), semi-arid areas (Caatinga), and floodplains (Pantanal). A both numerous and rich fauna is superimposed to these ecological assembly, including 64 tick species (Dantas-Torres et al., 2009, 2012; Labruna and Venzal, 2009; Nava et al., 2010a). Furthermore, it also upholds a diverse microbiota together with *Rickettsia* spp. Curiously, until 2000 only one SFG, *Rickettsia rickettsii*, was known in the country but during the last 12 years this number jumped to five with the inclusion of *R. parkeri, R. rhipicephali, R. amblyommii*, and *R. felis* (Labruna et al., 2011a).

Whereas spotted-fever-caused *R. rickettsii* infection is in Brazil the major and long-known human tick-borne disease (Magalhães, 1952; Lemos et al., 1994; Angerami et al., 2012), only recently was a second and milder tick-borne SFG human rickettsiosis discovered (Spolidorio et al., 2010). Such late discovery is linked to recent use of more appropriate diagnostic tools for *Rickettsia* and an increased research of tick ecology in the country. These more contemporary requirements for diagnosis are understandable if one considers that human rickettsiosis in Brazil is most of the time overshadowed by several other febrile illnesses. Thus, diseases such as dengue fever, common flu, leptospirosis, meningococical meningitis, and others are blamed for or delay diagnosis of human rickettsiosis. In fact, ecological background linked to vector tick species and their environment is of utter importance to provide the first and many times the sole information for timely diagnosis and effective treatment of human patients.

We herein update ecology, biology, and distribution of spotted-fever tick vectors in Brazil and that are important for

Rickettsia transmission to humans. Specific knowledge gaps in the epidemiology of such diseases are highlighted to guide forthcoming research.

Rickettsia rickettsii—BRAZILIAN SPOTTED-FEVER (BSF)

Spotted-fever by *R. rickettsii* infection has been reported in Brazil since the 1920s and is caused by the same agent of the North American Rocky Mountain spotted-fever (reviewed by Labruna, 2009). However, vector tick species in Brazil are different and thus epidemiology of *R. rickettsii*-caused spotted-fever is quite different from that of North America. Hence, the frequent name in publications "BSF." BSF is a severe, acute disease and fatality rates are between 30 and 40% (Angerami et al., 2006). The disease is more frequently reported in the southeastern region of the country, encompassing the states of Minas Gerais, Rio de Janeiro, São Paulo, Espírito Santo, and Paraná (**Figure 1**). The highest incidence has occurred in São Paulo, the most populous Brazilian state, where during 2012 there were 68 confirmed cases that resulted in 37 fatalities (54% fatality rate), highlighting BSF absolutely as the vector-borne disease with highest fatality rate in southeastern Brazil (São Paulo Public Health Department).

There has been a clear reemergence of BSF since of the end of 1980s and ecological factors seem to play a major role in that (Labruna, 2009). Undoubtedly, tick vectors are a central link between the *Rickettsia* source and humans and thus tick ecology is basis for BSF epidemiology. In Brazil two tick species, *Amblyomma cajennense,* and *A. aureolatum*, are considered main vectors of BSF whereas *Rhipicephalus sanguineus* is a suspected vector, and may play a role in transmission in particular situations.

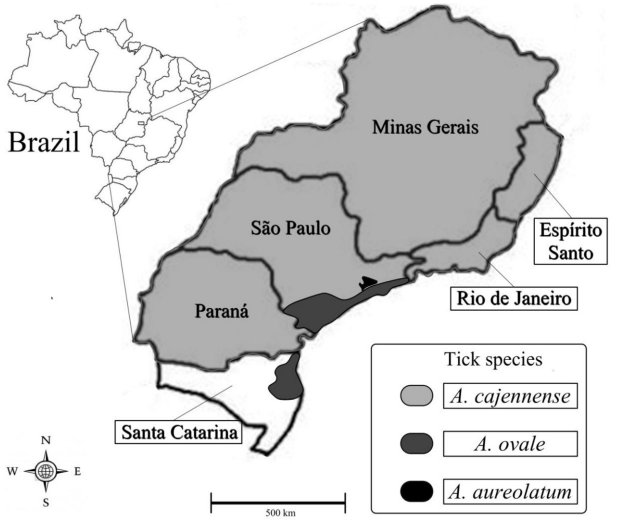

FIGURE 1 | Locations of spotted-fever vector ticks in Brazil. Light gray: States from Brazil where *A. cajennense* is proven or suspected vector of *R. rickettsii* to humans. Dark gray: areas where Atlantic rainforest *Rickettsia*-infected *A. ovale* were already found. Black: area with *A. aureolatum* transmission of *R. rickettsii* to humans.

Amblyomma cajennense

In southeast Brazil, location of most BSF cases, the disease occurs chiefly within a well-known ecological background: high *A. cajennense* environmental infestations maintained by one of its major hosts, the capybaras (*Hydrochoerus hydrochaeris*). At the same time, this background is far from being understood and several features are unknown.

A. cajennense is a tick species with wide distribution within the Neotropical region with tick populations from Southern Texas, USA, to South of South America as far as latitude 29°S (Estrada-Peña et al., 2004). However, care must be taken with this alleged geographical range. It has been recently proposed that this tick species is in fact a complex of species (Labruna et al., 2011b; Mastropaolo et al., 2011) and BSF vectoring by what is known today as *A. cajennense* probably differs according to tick populations. It is thus important to clearly define the tick species from the *A. cajennense* complex of southeast Brazil. From here on, information provided will refer solely to this specific tick population from southeast Brazil.

A. cajennense is a three-host tick species (Guglielmone et al., 2006a) and high environmental infestations in southeast Brazil are associated to hosts such horses and capybaras which feed the more host specific adult stages as well (Labruna et al., 2001; Oliveira et al., 2003; Heijden et al., 2005; Pacheco et al., 2007). This tick species has a 1-year life cycle (Serra Freire, 1982; Labruna et al., 2002; Oliveira et al., 2003) driven by a behavioral diapause of larvae (Labruna et al., 2003). Although egg hatching may occur in summer, larvae seek for hosts only in autumn, a behavior triggered by decrease of day length and temperature (Cabrera and Labruna, 2009). Thus, adults predominate in spring and summer, larvae in autumn and winter, and nymphs in winter and spring. Most BSF cases occur during nymph season (Pinter et al., 2011). Although other factors may be involved, high aggressiveness of nymphs to humans, smaller size that many times precludes host awareness, and a wider spread over the infested area are related to such seasonality of *A. cajennense*-vectored BSF. In addition, laboratory experiments have shown low vector competence of *A. cajennense*-infected larvae, contrasting to high vector competence of infected nymphs (Soares et al., 2012).

Many ecological features of *A. cajennense* from southeast Brazil are unknown, but a few observations indicate that it is being favored by anthropogenic factors. Should this tick species have adequate host supply, it thrives in green areas with at least small amount of shadow provided by herbaceous vegetation or small trees. Labruna et al. (2001), for example, observed at stud farms that presence of *A. cajennense* was statistically associated with the presence of at least one mixed overgrowth pasture (presence of undesired plants such as bushes and shrubs in pasture). This tick species is frequently found associated to riparian forests close to human settlements (Souza et al., 2006) as well. In fact such riparian forests are preferred habitats of capybaras, and a frequent place for contact with humans seeking for leisure.

Under natural conditions, *A. cajennense* is a tick species associated to the Cerrado biome, the Brazilian savannah (Knight, 1992; Szabó et al., 2007a; Veronez et al., 2010). At the same time it is generally absent from the Atlantic rainforest, but it will soon appear at degraded areas of such Biome (Szabó et al., 2009).

Within the Cerrado, *A. cajennense* is more linked to forestall phytophisiognomies (Veronez et al., 2010) probably to avoid desiccation. On the other way round, high humidity of rainforests seems to be deleterious to this tick species (Labruna et al., 2005a; Szabó et al., 2009).

Capybara (*H. hydrochaeris*), the largest living rodent, is a semi-aquatic and gregarious species and is widely distributed in South America (review by Pachaly et al., 2001). This rodent is a suitable host for *A. cajennense* ticks and capybara populations are associated to high environmental infestations (Heijden et al., 2005; Souza et al., 2006; Perez et al., 2008; Queirogas et al., 2012). Moreover, this host was experimentally proven to be an as amplifier host of *R. rickettsii* for *A. cajennense* ticks (Souza et al., 2009). Populations of this rodent have increased in southeastern Brazil, notably in human-altered landscape. According to Ferraz et al. (2007), capybaras populations expanded in such areas favored by various factors such as hunting prohibition by Brazilian federal law, high reproductive capacity, decline in natural predators, and rising agricultural production and which provides food. Unfortunate coincidence provided adequate habitat for both capybaras and *A. cajennense* ticks at human-altered landscape. Thus, both are abundant close to human settlements in riparian forests and at habitats with water bodies such as urban and peri-urban parks, garden of condominiums, companies, and similar.

R. rickettsii can be found in *A. cajennense* ticks in endemic areas (Guedes et al., 2005), but it is a rare event (Sangioni et al., 2005; Pacheco et al., 2009). Experimental data shows *that A. cajennense* is inadequate host for *R. rickettsii*. This tick species has a low efficiency to maintain the bacterium through successive generations, and *R. rickettsii* infection rates of ticks decline drastically throughout the successive tick generations (Soares et al., 2012). At the same time, Souza et al. (2009) demonstrated that *R. rickettsii* could infect capybaras without causing clinical illness and that rickettsemia for, approximately 10 days, was capable to infect ticks. Thus, capybaras can act as amplifier host of *R. rickettsii* in *A. cajennense* ticks populations in Brazil but a continuous supply of *Rickettsia*-naive capybaras (usually juvenile animals) is needed for a regular creation of new lineages of infected ticks. In this regard, it is intriguing that BSF is endemic in a few locations but absent in others with high populations of both capybara and *A. cajennense*. Therefore, other and unknown factors also play a role to establish or restrain endemicity.

Amblyomma dubitatum

Capybaras are considered the main host for all stages of another three-hosted tick, *A. dubitatum* Neumann, 1899 (=*A. cooperi*) as well (Nava et al., 2010b) and in southeast Brazil infestation of this rodent with both tick species is a common feature (Heijden et al., 2005; Perez et al., 2008).

All findings of *A. dubitatum* in Brazil were concentrated in the biogeographical provinces of Cerrado, Atlantic Forest, Parana Forest, and *Araucaria angustifolia* Forest (Nava et al., 2010b). At the same time, its distribution area is lesser than that of its principal host, the capybara, suggesting that environmental variables rather than hosts determine the distributional ranges of this tick species (Nava et al., 2010b).

Within its range, it is tick species related to areas prone to flooding (Szabó et al., 2007b; Queirogas et al., 2012). In fact, Queirogas et al. (2012) observed that *A. dubitatum* was privileged over *A. cajennense* by river margins exposed to flooding at least once a year. Thus, river margins in the urban areas without additional drier vegetation for *A. cajennense* maintained overwhelmingly *A. dubitatum* tick populations. At the same time, the role of *A. dubitatum* as vector of human diseases is undetermined but it is commonly associated to *R. bellii* (Pacheco et al., 2009), as far as known, a non-pathogenic *Rickettsia* species that does not belong to the SFG. Although not as aggressive as *A. cajennense, A. dubitatum* was already shown to bite humans (Labruna et al., 2007). Thus, the relationship of *A. cajennense, A. dubitatum*, and *R. rickettsii* deserves further investigation.

In summary, the ongoing major scenario for BSF is the occurrence of human cases associated to tick bites at anthropicized areas with capybaras (**Figure 2**). These areas close to water bodies, either riparian forest fragments or arborized small dams, are many times used for leisure activities and are landscape types in

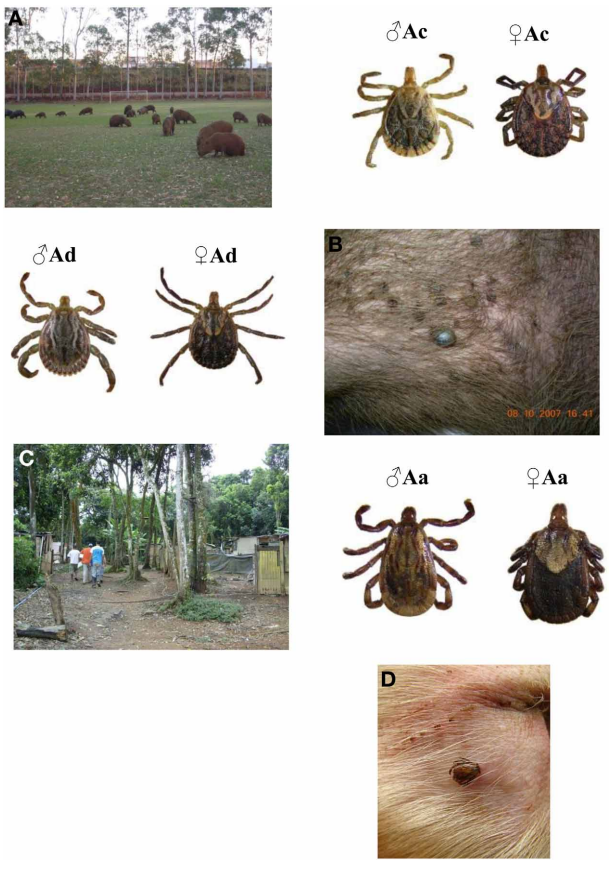

FIGURE 2 | Environmental backgrounds and ticks associated with Brazilian spotted-fever caused by human infection with *R. rickettsii* in Brazil. Capybaras in anthropized area **(A)**. Tick-infested capybara **(B)**. Male and female adults of *Amblyomma cajennense* (Ac) and *Amblyomma dubitatum* (Ad). Endemic human settlement at the border of the Atlantic rainforest and metropolitan area of São Paulo **(C)**. Dog ear with an attached *Amblyomma aureolatum* **(D)**. Adult couple of *Amblyomma aureolatum* (Aa).

expansion in the country. In these areas both capybaras and their ticks flourish and human tick bites are a frequent event particularly by *A. cajennense*, a tick species very aggressive to humans. Such scenario is widespread in southeast Brazil encompassing many municipalities. In a few of these, BSF is endemic but still a rare event, probably because of the low infection rate of the vector tick *A. cajennense*. Since *R. rickettsii*-infected tick populations in nature depends on parasite feeding on a rickettsemic host such as a non-immune capybara, *A. cajennense* infection rate at a particular area seems to be a dynamic process; it decreases over time but can have focal uprising when such a host (amplifier host) is bitten by an infected tick. An unknown feature is the very initial source of *Rickettsia* in previously non-endemic areas and more detailed studies comparing ecological features of capybara–*A. cajennense* relationships in endemic and non-endemic area is mandatory.

Finally, experimental studies have shown that both opossums (*Didelphis aurita*) and domestic dogs may be competent amplifier hosts of *R. rickettsii* to ticks (*A. cajennnense* and *R. sanguineus*, respectively) (Horta et al., 2009; Piranda et al., 2011). Nevertheless, capacity of infection of solely 5% of *A. cajennense* places the opossum as secondary source of amplification to *R. rickettsii* among tick population. Furthermore, neither dogs nor opossums feed an important number of *A. cajennense* ticks when compared to capybaras. However, since both animal species are usually frequent in BSF-endemic areas, where they frequently become infested by immature stages of *A. cajennense*, their role in the ecology of the disease should be investigated deeper.

Amblyomma aureolatum

A second but nonetheless important scenario associated to BSF in Brazil involves *A. aureolatum*. This scenario is much more constrained (**Figure 1**) because the environmental requirements and behavior of the vector tick have particular epidemiological features. Human BSF cases associated to *A. aureolatum* seem to occur when dogs are bitten by adult ticks during incursions into the rainforest and bring them back to human dwellings (**Figure 2**). From then on, two possible ways of human infection with *R. rickettsii* are supposed. In the main one, infected *A. aureolatum*, particularly males because they can remain on the dogs for several weeks, drop off from the dog accidentally (scratching, picked by humans) and bite humans (Pinter et al., 2004). The second possibility refers to infection of *R. sanguineus* ticks feeding on a dog parasitized by an infected *A. aureolatum* tick and will be discussed below. Although BSF cases caused in this scenario may have a wider distribution, most knowledge derives from studies at the metropolitan area of São Paulo (São Paulo State capital and other 38 municipalities) that is embedded in the Atlantic rainforest biome.

A. aureolatum is a Neotropical three-host tick, found in the eastern area of South America (Guglielmone et al., 2003). This tick species is associated with very humid habitat and cooler subtropical temperatures (Pinter et al., 2004). Thus, it is typically a tick from the Atlantic rainforest at higher altitudes in the Southeastern region (Sabatini et al., 2010) but can be found close to sea level in southern Brazil (Medeiros et al., 2011). Under laboratory conditions *A. aureolatum* is more susceptible to *R. rickettsii* infection than *A. cajennense* and is more efficient in maintaining the infection through 100% transstadial perpetuation, 100% transovarial transmission, as well as higher filial infection rates (Labruna et al., 2008, 2011c). At the same time, within BSF-endemic areas, infection rates of *A. aureolatum* by *R. rickettsii* have been reported to be around 1–10% (Pinter and Labruna, 2006; Ogrzewalska et al., 2012). Such relatively low infection rate might be explained, at least partially, by a deleterious effects caused by *R. rickettsii* in ticks (Niebylski et al., 1999; Labruna et al., 2011c).

In the case of *A. aureolatum*, no *R. rickettsii* amplifier host has been determined so far. In natural settings adults of *A. aureolatum* feed mainly on wild carnivore species (Guglielmone et al., 2003; Labruna et al., 2005b). The few host records for tick immature stages refer majorly to passerine birds, mainly the genus *Turdus* (Arzua et al., 2003; Ogrzewalska et al., 2012) and a few rodent species (Guglielmone et al., 2003). Additionally, a recent study (Ogrzewalska et al., 2012) observed that the BSF-endemic areas in São Paulo metropolitan area differed from the non-endemic areas by the presence of significantly smaller and more degraded forest patches in the former. Still the original amplifier source of the bacterium is undetermined and ecological features of endemic and non-endemic areas where *A. aureolatum* thrives should be compared further. For yet unknown reasons fatality rates of BSF at *A. aureolatum* transmission areas are higher (above 60%) and no obvious seasonal pattern for the disease can be detected (Angerami et al., 2012).

Rhipicephalus sanguineus

Transmission of BSF by a third tick species, *R. sanguineus*, in Brazil is by now speculative but demands awareness for its potential. In fact this tick species is the main vector of *R. conorii agent* of the Boutonneuse fever in the Mediterranean basin and human *R. rickettsii* infection caused by *R. sanguineus* tick bites were already shown in the USA (Demma et al., 2005) and Mexico (Eremeeva et al., 2011). However, and again, care must be taken with tick populations involved with this rickettsiosis. It is by now well determined that South America has at least two genetically, morphologically, biologically, and geographically distinct species that have been treated under the taxon *R. sanguineus*: one is found in tropical and subtropical areas and the other in the south of South America (Southern Brazil, Uruguay, Chile, and Argentina) (Oliveira et al., 2005; Szabó et al., 2005; Moraes-Filho et al., 2011; Nava et al., 2012). Observations from here on refer solely to the tropical *R. sanguineus* populations of Brazil.

R. sanguineus is a tick, introduced in the country with colonization and is always associated to dogs (Szabó et al., 2005) and biting of other animals, including man, should be considered accidental. Up to our knowledge, this tick species has never been reported from natural or anthropized vegetation in Brazil. At the same time, it might attain high infestations levels at dog dwellings and thus close or at human dwellings (Labruna and Pereira, 2001; Guglielmone et al., 2006a), and it is found on dogs all over Brazil (Szabó et al., 2001, 2010; Dantas-Torres et al., 2004; Labruna et al., 2005a; Castro and Rafael, 2006; Soares et al., 2006). Although human *R. sanguineus* tick-biting in Brazil was reported before (Dantas-Torres et al., 2006), it is a rare event if one considers

the frequent and close association of *R. sanguineus* and people. Importantly, it was shown in the laboratory that *R. sanguineus* is a competent *R. rickettsii* vector and, in opposition with that observed with *A. cajennense* and *A. aureolatum, R. rickettsii* did not elicit lethal effect on *R. sanguineus* (Piranda et al., 2011). Furthermore, naturally occurring infection of *R. sanguineus* with *R. rickettsii* in Brazil was already observed in BSF endemic areas by molecular tools and isolation in cell culture (Cunha et al., 2009; Gehrke et al., 2009; Moraes-Filho et al., 2009; Pacheco et al., 2011; Ogrzewalska et al., 2012).

Although human BSF infection transmitted by *R. sanguineus* in Brazil is still unproven, there is a very likely scenario for that. *R. sanguineus* ticks are mostly urban in the country (Szabó et al., 2001; Labruna, 2004; Labruna et al., 2005a) and thus held apart from *R. rickettsii* sources. However, there are many free-ranging dogs either ownerless or kept unrestrained by owners. In many instances, these animals wander between urban and natural areas and are infested with tick species from both environments (**Figure 3**) (Moraes-Filho et al., 2009; Queirogas et al., 2010). Under such conditions, *R sanguineus* ticks may feed on dogs previously or concomitantly harboring *R. rickettsii*-infected *A. cajennense* or *A. aureolatum* ticks and be infected during rickettsemia. In fact, dog infestation with *R. rickettsii*-infected *R. sanguineus* alongside infected *A. aureolatum* ticks in endemic areas has been recently reported (Moraes-Filho et al., 2009; Ogrzewalska et al., 2012). This very likely bridge of *R. rickettsii* to *R. sanguineus* infestation sites opens the gate for dissemination of the bacterium in ticks that apparently do not suffer its lethal effect and may propagate unrestrained. In this regard it is interesting to observe that in endemic areas *R. rickettsii* infection prevalence tend to be higher in *R. sanguineus* than *A. aureolatum* (Moraes-Filho et al., 2009; Ogrzewalska et al., 2012). Thus, we can suppose that at sites with high *R. sanguineus* (and dog) densities, *R. rickettsii* infection introduced by *Amblyomma* species may be overshadowed by infection of *R. sanguineus* populations. Fortunately, *R. sanguineus* is not as aggressive to humans in Brazil, and transmission might occur only in the case of occasional tick bites or by crushing of ticks picked from animals, more likely to people who handle dogs frequently.

Amblyomma and *Rhipicephalus* mixing may be particularly relevant at places with a constant influx of wandering dogs of unknown origin such as Zoonosis Control Centers and dog shelters. In a Zoonosis Control Center in a BSF-endemic area of Minas Gerais State, high prevalence of *R. rickettsii*-infected *R. sanguineus* was observed (Pacheco et al., 2011). Furthermore, recent death of four employees working at a dog shelter was attributed to BSF in Rio de Janeiro. In this case, the only tick species found at that location was *R. sanguineus*, and 97% of 117 tested dogs were seropositive to *R. rickettsii* antigen (Costa et al., 2012). Thus, such scenarios for *R. sanguineus* transmission of BSF should be deeply investigated.

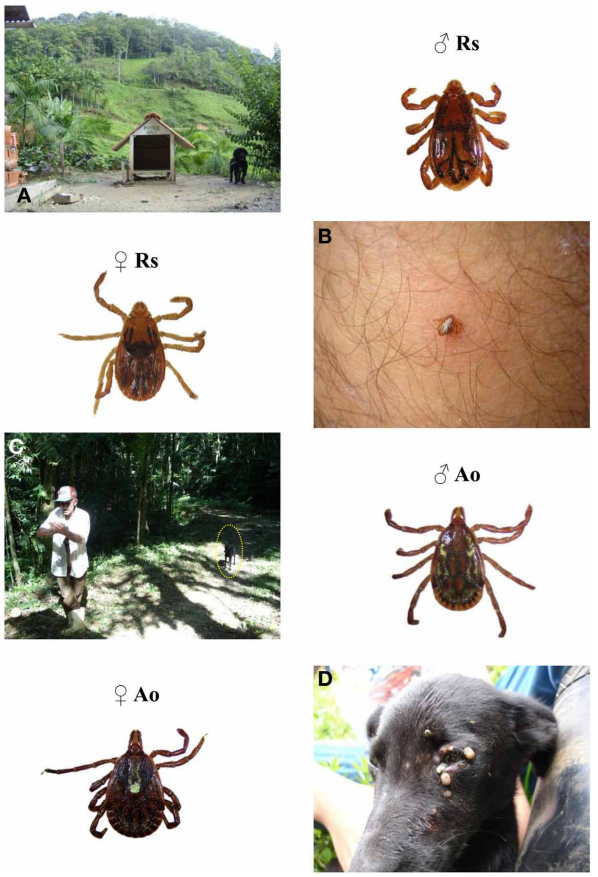

FIGURE 3 | Environments with potential for dog and human infestation with ticks associated to spotted-fever in Brazil. Dog restrained to its house providing conditions for the maintenance of *Rhipicephalus sanguineus* infestation but with potential for infestation with *Amblyomma ovale* or *Amblyomma aureolatum* in the nearby forest **(A)**. *Rhipicephalus sanguineus* bite of a human leg **(B)**. Adult couple of *Rhipicephalus sanguineus* (Rs). Atlantic rainforest with high *Amblyomma ovale* prevalence **(C)**. Dog infestation with *Amblyomma ovale* ticks at the same area **(D)**. Adult couple of *Amblyomma ovale* (Ao).

Amblyomma ovale AND ATLANTIC RAINFOREST *Rickettsia*

Only at the end of the last decade did the diagnoses of a second human tick-borne spotted-fever rickettsiosis occurred in the country (Spolidorio et al., 2010). The agent was a novel SFG strain closely related to *R. africae, R. parkeri*, and *R. sibirica* and caused a febrile illness but milder than BSF. The causative *Rickettsia* strain was named Atlantic rainforest due to the environment it was found (Sabatini et al., 2010; Spolidorio et al., 2010), but species definition was at the time controversial. A second clinical case due to this novel agent was subsequently reported in another region of the country (Silva et al., 2011), highlighting the possibility that Atlantic rainforest rickettsiosis could be much more frequent than currently known.

Atlantic rainforest *Rickettsia* strain was shown to be strongly associated with *A. ovale* ticks from the Atlantic rainforest and seems to have a wide range, at least in the south–southeastern Atlantic coast of Brazil (**Figure 1**) (Sabatini et al., 2010; Medeiros et al., 2011; Szabó et al., 2013). Within the studied areas, *A. ovale* tick populations attain infection levels of around 10% (Sabatini et al., 2010; Szabó et al., 2013). Importantly, adult of this tick species attaches to and feeds readily on dogs and is thus

frequently reported from dogs kept unrestrained in rural areas close to natural environments (Szabó et al., 2001, 2010, 2013; Labruna et al., 2005a; Sabatini et al., 2010). Moreover, *A. ovale* adult tick human bite is frequent (Labruna et al., 2005a; Guglielmone et al., 2006b; Szabó et al., 2006).

Under natural conditions, *A. ovale* is a three-host tick species with adults parasitizing carnivores whereas rodents are main hosts for immature feeding stages (Guglielmone et al., 2003; Labruna et al., 2005b; Szabó et al., 2013). *A. ovale* has wide distribution (Neotropical–Neartic) (Guglielmone et al., 2003) and has a surprising ecological plasticity being found in several Brazilian biomes, including Pantanal (Pereira et al., 2000), Amazon (Labruna et al., 2005a), Atlantic rainforest (Szabó et al., 2009), and Cerrado (Szabó et al., 2007a). Comparison of these populations from diverse environments is warranted to better define their relationships as well as vectoring capabilities.

Up to now, SFG *Rickettsia* infection of *A. ovale* was associated only with Atlantic rainforest populations where adult ticks were shown to quest on vegetation in high numbers (Szabó et al., 2013). *Rickettsia* amplifier host or reservoir in the forest has not been determined so far, but a small rodent, *Euryoryzomys russatus*, was shown to be an important host for *A. ovale* immatures as well as attained high seroconversion prevalence and high specific titers in one study (Szabó et al., 2013). Thus, humans can be *Rickettsia* infected if bitten by ticks during incursions into the forest or by ticks detached from the dogs. In the latter case, human infection is supposed to occur faster as *Rickettsia* reactivation (Hayes and Burgdorfer, 1982) should have occurred during feeding on the first host.

Importance of dogs in the epidemiology of the Atlantic rainforest rickettsiosis goes beyond possible *Rickettsia* reactivation in *A. ovale* ticks. Such hosts, that frequent the forest in endemic areas, are chiefly infested with *A. ovale* ticks (**Figure 3**), many *Rickettsia* infected, and all these dogs seroconvert, attaining very high titers against *R. parkeri* antigens (Sabatini et al., 2010; Medeiros et al., 2011; Szabó et al., 2013). Furthermore, Atlantic rainforest *Rickettsia* was detected by molecular tools in *A. aureolatum* and *R. sanguineus* on dogs co-infested with *A. ovale* ticks (Sabatini et al., 2010; Medeiros et al., 2011; Szabó et al., 2013). Thus, a role for *A. aureolatum* and *R. sanguineus* on dogs in the epidemiology of the disease also deserves investigation.

By this time, it can be supposed that many human cases formerly considered as mild BSF (Angerami et al., 2009) or other febrile illness were in fact Atlantic rainforest rickettsiosis. Undoubtedly a careful analysis of tick-borne human febrile illness along the Brazilian coast is mandatory to evaluate the range of the disease.

CONCLUDING REMARKS

As depicted from information above, agents of human tick-borne rickettsiosis in Brazil originate from wildlife. Unfortunately, the very initial source of such pathogenic *Rickettsia* is not determined and thus epidemiology of human infections has knowledge gaps. Whatever the origin of pathogenic *Rickettsia*, human activities can be blamed for amplification of both wildlife host (capybara) and tick infections as well as bridging from natural environment to human dwellings (unrestrained dogs). In all such cases, likelihood of human infection increases several fold. Nonetheless, recognition of the ecological background of each rickettsiosis is a major step to provide diagnosis, treatment and preventive measures. In this regard, increased capybara and *A. cajennense* populations at locations with human activities and dogs with access to wildlife environment are key features in the infection of human beings. Unfortunately, capybara populations are increasing in urban and peri-urban areas and control possibilities face a complex situation involving technical, ethical, and political aspects, as well as society issues. On the other hand, limiting dog access to wildlife areas seems to be a more feasible measure but which should rely on educational ground of animal owners as well as control of free-roaming ownerless dog populations.

We herein put together available information on tick ecology and *Rickettsia* to build the most probable epidemiology of known Rickettsiosis in Brazil. However, existing knowledge is still overwhelmingly restricted to southeast Brazil, and even in this region, it is not enough to provide a final picture. In this context, proper determination of tick species and their distribution in every geographic region is a prerequisite to unfold epidemiology of Rickettsiosis. Furthermore, there is an ongoing discovery of other *Rickettsia* species in the country and novel human Rickettsiosis with particular epidemiology might be revealed. A complicating factor is the fast man-induced landscape changes that alter existing host–tick relationships creating new scenarios for tick-borne diseases. Nonetheless, information gathered here is a good starting point to evaluate Rickettsial disease epidemiology in other geographical regions of the country as well.

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Anaplasma phagocytophilum—a widespread multi-host pathogen with highly adaptive strategies

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The bacterium *Anaplasma phagocytophilum* has for decades been known to cause the disease tick-borne fever (TBF) in domestic ruminants in *Ixodes ricinus*-infested areas in northern Europe. In recent years, the bacterium has been found associated with *Ixodes*-tick species more or less worldwide on the northern hemisphere. *A. phagocytophilum* has a broad host range and may cause severe disease in several mammalian species, including humans. However, the clinical symptoms vary from subclinical to fatal conditions, and considerable underreporting of clinical incidents is suspected in both human and veterinary medicine. Several variants of *A. phagocytophilum* have been genetically characterized. Identification and stratification into phylogenetic subfamilies has been based on cell culturing, experimental infections, PCR, and sequencing techniques. However, few genome sequences have been completed so far, thus observations on biological, ecological, and pathological differences between genotypes of the bacterium, have yet to be elucidated by molecular and experimental infection studies. The natural transmission cycles of various *A. phagocytophilum* variants, the involvement of their respective hosts and vectors involved, in particular the zoonotic potential, have to be unraveled. *A. phagocytophilum* is able to persist between seasons of tick activity in several mammalian species and movement of hosts and infected ticks on migrating animals or birds may spread the bacterium. In the present review, we focus on the ecology and epidemiology of *A. phagocytophilum*, especially the role of wildlife in contribution to the spread and sustainability of the infection in domestic livestock and humans.

Keywords: *Anaplasma phagocytophilum*, ecology, epidemiology, distribution, hosts, vectors

INTRODUCTION

The bacterium *Anaplasma phagocytophilum* has been known to cause disease in domestic ruminants (Europe) (Foggie, 1951) and horses (USA) (Gribble, 1969) for decades. More recently, the infection has been detected in several mammalian species, including humans, in areas on the northern hemisphere with endemic occurrence of *Ixodes* ticks. *A. phagocytophilum* as a bacterial species appears to be a generalist, infecting a wide range of animals. Multiple genetic variants of the bacterium have been characterized (Scharf et al., 2011) and subpopulations within the species are now being discussed. In this review, we present updated information especially concerning the ecology and epidemiology of *A. phagocytophilum*.

HISTORY

During an experimental study on louping-ill (LI) in Scotland last century, some sheep contracted an unknown fever reaction on tick-infested pastures. The fever reaction was transmitted to other sheep by blood inoculation, but gave no protection against a later LI-virus infection. The disease was given the provisional name "tick-borne fever" (TBF), and the responsible pathogen was assumed to belong to the class *Rickettsia* (Gordon et al., 1932, 1940). The name TBF is still used for the infection in domestic ruminants in Europe. Anecdotally it could be mentioned that the Norwegian synonym of TBF is "sjodogg," and this name was already used to describe a devastating illness in ruminants as early as year 1780 in a coastal area of western Norway (Stuen, 2003).

The causative agent of TBF was first classified as *Rickettsia phagocytophila* (Foggie, 1951). However, due to morphological resemblance with *Cytoecetes microti*, an organism found in the polymorphonuclear cells of the vole *Microtus pennsylvanicus* (Tyzzer, 1938), it was later suggested to include the TBF agent in the genus *Cytoecetes* in the tribe *Ehrlichia*, as *C. phagocytophila* (Foggie, 1962).

In 1974, the organism was named *Ehrlichia phagocytophila* in Bergey's manual of determinative bacteriology (Philip, 1974). The discovery of *E. chaffeensis* in 1986, causative agent of human monocytic ehrlichiosis (Maeda et al., 1987; Anderson et al., 1991), and the agent of human granulocytic ehrlichiosis (HGE) in 1994 (Bakken et al., 1994; Chen et al., 1994), initiated new studies on the host associations, epidemiology and taxonomy of the granulocytic *Ehrlichiae* (Ogden et al., 1998). Genus *Ehrlichia* was divided into three genogroups, of which the granulocytic group contained *E. phagocytophilum*, *E. equi* [described in horses (Gribble,

1969)] and the agent causing HGE. Later, a reclassification of the genus *Ehrlichia* was proposed, and based on phylogenetic studies, the granulocytic *Ehrlichia* group was renamed *Anaplasma phagocytophilum* (Dumler et al., 2001; Anonymous, 2002) (**Table 1**). However, it is still argued, whether the granulocytic *Anaplasma* should eventually be reclassified as distinct from the erythrocytic *Anaplasma* and returned to the previously published genus, *Cytoecetes* (Brouqui and Matsumoto, 2007).

CLINICAL CHARACTERISTICS

Natural infection with *A. phagocytophilum* has been reported, as already mentioned, in humans and a variety of domestic and wild animal species (Foley et al., 1999), whereas fatal cases have so far only been reported in sheep, cattle, horses, reindeer, roe deer, moose, dogs, and humans (Jenkins et al., 2001; Stuen, 2003; Franzén et al., 2007; Heine et al., 2007).

The main disease problems associated with TBF in ruminants are seen in young animals, and individuals purchased from tick-free areas and placed on tick-infested pastures for the first time. The most characteristic symptoms in domestic ruminants are high fever, anorexia, dullness, and sudden drop in milk yield (Tuomi, 1967a). However, the fever reaction may vary according to the age of the animals, the variant of *A. phagocytophilum* involved, the host species and immunological status of the host (Foggie, 1951; Tuomi, 1967b; Woldehiwet and Scott, 1993; Stuen et al., 1998). Abortion in ewes and reduced fertility in rams have also been reported. In addition, reduced weight gain in *A. phagocytophilum* infected bullocks and lambs have been observed (Taylor and Kenny, 1980; Stuen et al., 1992; Grøva et al., 2011).

A variable degree of clinical symptoms have also been detected in other mammals, such as fever, anorexia, depression, apathy, distal edema, reluctance to move, and petechial bleedings in horses, while the symptoms in dogs are characterized by fever, depression, lameness, and anorexia. In cats the predominant signs are anorexia, lethargy, hyperesthesia, conjunctivitis, myalgia, arthralgia, lameness, and incoordination (Egenvall et al., 1997; Björsdorff et al., 1999; Cohn, 2003; Franzén et al., 2005; Heikkilä et al., 2010).

In humans, clinical manifestations range from mild self-limiting febrile illness, to fatal infections. Commonly, patients express non-specific influenza-like symptoms with fever, headache, myalgias, and malaise (Bakken et al., 1994; Dumler, 1996). In addition, thrombocytopenia, leukopenia, anemia, and increased aspartate and alanine aminotransferase activity in sera are reported (Bakken and Dumler, 2008). However, most human infections probably result in minimal or no clinical manifestations. Reports from the US, indicate a hospitalization rate of 36%, of which 7% need intensive care, while the case fatality rate is less than 1% (Dumler, 2012). A recent cohort study from China however, describes a mortality of 26.5% (22/83) in hospitalized patients (Li et al., 2011).

Table 1 | Classification of genus *Anaplasma, Ehrlichia,* and *Neorickettsia* in the family *Anaplasmataceae* (modified after Dumler et al., 2001).

	Genus		
	Anaplasma	***Ehrlichia***	***Neorickettsia***
Species	*A. marginale*	*E. canis*	*N. risticii*
	A. bovis	*E. chaffeensis*	*N. sennetsu*
	A. ovis	*E. ewingii*	
	A. phagocytophilum	*E. muris*	
	A. platys	*E. ruminantium*	

DIAGNOSTIC AND LABORATORY METHODS

CLINICAL SIGNS

Clinical signs in ruminants may be sudden onset of high fever (>41°C) and drop in milk yield, while symptoms in horses, dogs, and cats may be more vague and unspecific. In humans, a flu-like symptom 2–3 weeks after tick exposure is an indicator of infection. However, laboratory confirmation is required to verify the diagnosis (Woldehiwet, 2010). To our knowledge, chronic infection has not yet been confirmed in any host, although persistent infections have been found to occur in several mammalian species.

DIRECT IDENTIFICATION

Light microscopy of blood smears taken in the initial fever period is normally sufficient to state the diagnosis. Stained with May-Grünwald Giemsa, the organisms appear as blue cytoplasmic inclusions in monocytes and granular leucocytes, especially neutrophils (Foggie, 1951). Electron microscopy may also confirm the diagnosis of acute *Anaplasma* infection in blood or organs. Single or multiple organisms are then identified in clearly defined cytoplasmic vacuoles (Tuomi and von Bonsdorff, 1966; Rikihisa, 1991). Immuno-histochemistry on tissue samples could also be performed to confirm the diagnosis (Lepidi et al., 2000).

POLYMERASE CHAIN REACTION (PCR) AND CULTIVATION

Several PCR techniques (conventional, nested, and real-time) for the identification of *A. phagocytophilum* infection in blood and tissue samples have been established primarily on basis of the *16S rRNA*, *groEL,* and *p44* genes (Chen et al., 1994; Courtney et al., 2004; Alberti et al., 2005a). Multiple variants of *A. phagocytophilum* have been genetically characterized. Identification and stratification into phylogenetic subfamilies have been based on cell culturing, experimental infections, PCR and sequencing techniques (Dumler et al., 2007). Cultivation of *A. phagocytophilum* in cell cultures has been described for variants isolated from human, dog, horse, roe deer, and sheep (Goodman et al., 1996; Munderloh et al., 1999; Björsdorff et al., 2002; Woldehiwet et al., 2002; Silaghi et al., 2011c).

SEROLOGY

The presence of specific antibodies may support the diagnosis. A complement fixation test, counter-current immunoelectrophoresis test and an indirect immunofluorescent antibody (IFA) test can be used (Webster and Mitchell, 1988; Paxton and Scott, 1989). Several ELISA tests have also been developed (Ravyn et al., 1998; Magnarelli et al., 2001; Alleman et al., 2006; Woldehiwet and Yavari, 2012). A SNAP®4Dx® ELISA test is commercially available

for rapid in-house identification of *A. phagocytophilum* antibodies in dog serum, but the kit has also been used successfully on horse and sheep sera (Granquist et al., 2010a; Hansen et al., 2010).

PATHOLOGY

An enlarged spleen, up to 4–5 times the normal size with subcapsular bleedings, has for decades been regarded as indicative of TBF in sheep (Gordon et al., 1932; Øverås et al., 1993). No other typical pathological changes have been described (Munro et al., 1982; Campbell et al., 1994; Lepidi et al., 2000). An enlarged spleen with subcapsular bleedings has also been observed in roe deer and reindeer (Stuen, 2003).

Relative sensitivity of the diagnostic tests used for laboratory diagnostic confirmation of *A. phagocytophilum* infection in humans is shown in **Table 2**.

TREATMENT, PREVENTION, AND CONTROL

The drug of choice is tetracycline (Woldehiwet and Scott, 1993; Dumler, 1996). Doxycyclin hyclate, given orally or intravenously, has been effective in treating clinical cases of human granulocytic anaplasmosis, and has led to clinical improvement in 24–48 h. In human patients, treated with doxycycline for 7–10 days, infections have resolved completely and relapses have never been reported. In patients at risk of adverse drug reactions, rifampin therapy should be considered (Bakken and Dumler, 2006).

Current disease prevention strategies in domestic animals are based on the reduction of tick infestation by chemical acaricides, for instance at turn out on tick pasture. This is mostly done be dipping or with a variety of pour-on applications (Woldehiwet and Scott, 1993; Stuen, 2003). This treatment has to be repeated during the tick season. In the UK, long-acting tetracycline has also been used as a prophylactic measure given before animals are moved from tick-free environment into tick-infested pasture (Brodie et al., 1986; Woldehiwet, 2007). However, there is a growing concern about the environmental safety and human health, increasing costs of chemical control and the increasing resistance of ticks to pesticides (Samish et al., 2004).

Biological tick control is becoming an attractive approach to tick management. Biological control of tick infestations has been difficult because ticks have few natural enemies. Studies so far have concentrated of bacteria, entomopathogenic fungi, and nematodes (Samish et al., 2004). However, the main challenge is to create a sustainable biological control of ticks in the natural habitat.

Table 2 | Relative sensitivity of diagnostic tests for *A. phagocytophilum* infection in humans (modified after Bakken and Dumler, 2006).

Duration of illness (days)	Blood smear microscopy	HL-60 cell culture	PCR	IFAT
0–7	Medium	Medium	High	Low
8–14	Low	Low	Low	Medium
15–30			Low	High
31–60				High
>60				High

Vaccines against *A. phagocytophilum* are not yet available. Several vaccine candidates have been suggested, but the development of an effective vaccine has so far been difficult (Ijdo et al., 1998; Herron et al., 2000; Ge and Rikihisa, 2006). In order to develop a vaccine, one challenge is to choose antigens that are conserved among all variants of *A. phagocytophilum*.

Vaccines against ticks are also an alternative option. The development of vaccines that target both ticks and pathogen transmission may provide a mean of controlling tick-borne infections through immunization of the human and animal population at risk or by immunization of the mammalian reservoir to minimize pathogen transmission (de la Fuente and Kocan, 2006). Gut-, salivary-, or cement antigen vaccines (recombinant Bm/Ba 86, Bm91, and 64TRP) have been tested, and TickGUARDPLUS and Gavac (both recombinant Bm86) are examples of commercially available vaccines from the early 1990's (Willardsen, 2004; Labuda et al., 2006; de la Fuente et al., 2007; Canales et al., 2009). Other vaccines that inhibit subolesin expression are now being tested. These vaccines cause degeneration of gut, salivary gland, reproductive -and embryonic tissues and causes sterility in male ticks (de la Fuente et al., 2006a,b,c). Tick vaccines are feasible control methods, cost-effective and environmentally friendly compared to chemical control (de la Fuente and Kocan, 2006).

TRANSMISSION AND COLONIZATION

A. phagocytophilum has, as its name implies, a partiality to phagocytic cells and is one of very few bacteria known to survive and replicate within neutrophil granulocytes (Choi et al., 2005). During tick feeding, neutrophil-associated-inflammatory-responses are modulated by various stimuli deployed by the tick sialome components (Beaufays et al., 2008; Guo et al., 2009; Heinze et al., 2012). Orchestration of vector—and bacterial interactions with the defensive mechanisms of the host animal seem to promote infection and transmission rather than controlling it, resulting in increased availability of infected cells in the circulating blood and at the site of tick bite (Choi et al., 2003, 2004; Granquist et al., 2010b; Chen et al., 2012). The low level of circulating organisms, detected between periods of bacteremia (Granquist et al., 2010c), may indicate temporary clearance of infected cells, possible margination of infected granulocytes to endothelial surface or immunologically modified intervals in generations of antigenically different organisms (Bakken et al., 1994; Beninati et al., 2006; Granquist et al., 2008). Because of the short-lived nature of circulating neutrophils, the role of these cells in establishing and maintaining infection has been questioned (Herron et al., 2005), however to date little is known about alternative cellular components involved in the invasion and colonization of *A. phagocytophilum* in the host organism (Granick et al., 2008).

A. phagocytophilum modulates the distribution of potential host cells and infected neutrophils, by inducing cytokine secretion and their receptors (Akkoyunlu et al., 2001; Scorpio et al., 2004) and promoting the loss of CD162 and CD62L (Choi et al., 2003). The bacterium further interacts with host cell ligands (Park et al., 2003; Granick et al., 2008), by surface exposed proteins known as adhesins (Yago et al., 2003; Ojogun et al., 2012) in order to facilitate internalization in the host cell (Wang et al., 2006).

The translocation of bacteria to the inside of host cells is receptor mediated and depending on transglutaminase activity (reviewed by Rikihisa, 2003). However, host cell specific differences to receptors and their components as well as their importance in the infection process seem to exist, which may explain why certain bacterial strains, e.g., ruminant *Ap* Variant 1 strain, are refractory to culture in commercially available cell lines (like the HL-60 cell line) (Carlyon et al., 2003; Herron et al., 2005; Reneer et al., 2006, 2008; Massung et al., 2007). Previous reports have shown that various tissues and cells are susceptible to infection by *A. phagocytophilum* (Klein et al., 1997; Munderloh et al., 2004). It has been shown that intravascular myeloid cells (mature) have a higher infection rate than cells located in the bone marrow which may indicate that precursor stages of myeloid cells express ligands different from mature neutrophils, thus being more refractory to binding and internalization of the organism (Bayard-Mc Neeley et al., 2004). The coincidence that *A. phagocytophilum* uses CD162 when infecting neutrophils, led to the hypothesis that endothelium may have a function in the pathogenesis of *A. phagocytophilum* infection *in vivo* (Herron et al., 2005). However, a field study of skin biopsies in sheep observed *A. phagocytophilum* in inflammatory cell infiltrates comprised of PMNs and macrophages in the dermis and subcutis, and occasionally restricted to the mid- and peripheral parts of the blood vessel walls during tick attachment, thus questioning the role of endothelium in the pathogenesis of *A. phagocytophilum* infection in in the earliest phases of tick bite inoculation (Granquist et al., 2010b). Interestingly A. *phagocytophilum* has the ability to delay host cell apoptosis by activation of an anti-apoptosis cascade (Sarkar et al., 2012). This is critical for intracellular survival and reproduction of *A. phagocytophilum* in the normally short lived neutrophil granulocytes (Yoshiie et al., 2000; Lee and Goodman, 2006). Unlike other Gram-negative bacteria, *A. phagocytophilum* lacks lipopolysaccharides and peptidoglycans, but compensates for the loss of membrane integrity by incorporation of cholesterol which allows the escape of Nod Like Receptor and Toll Like Receptor activation pathways to successfully infect vertebrate immune cells (Lin and Rikihisa, 2003a,b; Hotopp et al., 2006; Xiong et al., 2007). However, recent studies in mice have surprisingly shown that alternative pathways involving the Nod 1 and 2 associated receptor interacting protein 2 may be important in control and clearance of *A. phagocytophilum* infection (Sukumaran et al., 2012).

PERSISTENCE

A. phagocytophilum has been found to persist in several mammalian hosts, such as sheep, dog, cattle, horses, and red deer (Foggie, 1951; Egenvall et al., 2000; Stuen, 2003; Larson et al., 2006; Franzén et al., 2009). However, this may vary according to the variants of the bacterium involved.

The ability of *A. phagocytophilum* to persist in immunecompetent hosts between seasons of tick activity is a complex and coordinated interaction that through evolutionary steps, have left the genomes of *A. phagocytophilum* and related organisms, heavily reduced to comprise essential genes allowing for nearly infinite numbers of recombined antigens and macromolecular exchange with its host cell (Rikihisa, 2011; Rejmanek et al., 2012).

Cyclic bacteremias display as periodic peaks containing genetically distinct variants of major surface proteins (MSP) (Granquist et al., 2008, 2010a). The capacity to generate novel antigens when other organisms are already present (superinfection) results in persistence and maintenance of the organism in natural transmission cycles and possibly allows spatial spread in nature (Barbet et al., 2003; Rodriguez et al., 2005; Futse et al., 2008; Ladbury et al., 2008; Stuen et al., 2009). Variants of MSPs such as MSp2 (or P44) contain epitopes recognized by antibodies appearing subsequently, but not prior to the respective peaks of rickettsemia in which they are expressed (Barbet et al., 2003; Granquist et al., 2010c), indicating a true process of antigenic variation influenced by the host immune response. Sequence variation may be achieved by segmental gene conversion of a single polycistronic expression site by insertion of total or partial pseudogene sequences (Barbet et al., 2000; Granquist et al., 2008) with the possible formation of mosaics or chimeras (Rejmanek et al., 2012). The large repertoire of donor sequences in *A. phagocytophilum* suggests that this bacterium may however only require simple gene conversion to evade host immune surveillance (Lin et al., 2003). On the other hand, the close proximity of the partial recombinase gene, *recA*, which is commonly involved in homologous recombinations supports the theory that recombination of pseudogenes by insertion in the expression site occurs (Barbet et al., 2003; Lin et al., 2003).

VECTORS AND COMPETENT VECTORS OF *A. phagocytophilum*

A. phagocytophilum is transmitted by hard ticks of the *I. persulcatus*-complex. The main vector in Europe is *I. ricinus* (commonly known as sheep tick or castor been tick); in the Eastern US *I. scapularis* (deer tick or black-legged tick); in the Western US *I. pacificus* (Western black-legged tick), and in Asia *I. persulcatus* (taiga tick) (Woldehiwet, 2010). Vector competence has been proven for the American tick species *I. scapularis* (previously *I. dammini*), *I. pacificus,* and *I. spinipalpis* (Telford et al., 1996; Des Vignes et al., 1999; Zeidner et al., 2000; Teglas and Foley, 2006). Transovarial transmission has not been proven in *Ixodes* species, but in *Dermacentor albipictus*, which lifecycle involves a single host animal, representing a distinct ecological niche (Baldridge et al., 2009). As to current knowledge, a vertebrate reservoir host is necessary in nature for keeping the endemic cycle.

Prevalence data on molecular detection of *A. phagocytophilum* in questing ticks, show great variations within countries or continents where such studies have been performed. The infection rate in *I. scapularis* ranges from <1% up to 50% and in *I. pacificus* from <1% up to ∼10% in the US. Additionally, *A. phagocytophilum* has been detected in questing *I. dentatus*, *Amblyomma americanum*, *Dermacentor variabilis,* and *D. occidentalis* (**Table 4**; Goethert and Telford, 2003). In Asia, detection rates varied in *I. persulcatus* between <1% up to 21.6% and questing *I. ovatus*, *I. nipponensis*, *D. silvarum*, *Haemaphysalis megaspinosa*, *H. douglasii*, *H. longicornis,* and *H. japonica* also contained DNA of *A. phagocytophilum* (**Table 5**). The greatest number of studies has been performed on questing *I. ricinus* ticks in Europe, where the prevalence rates vary between and also within countries. On average, the *A. phagocytophilum*-prevalence in *I. ricinus* in Europe

ranges between <1% and ∼20%, in *I. persulcatus*-endemic areas in Eastern Europe between 1.7 and 16.7%, and additionally DNA of *A. phagocytophilum* has been detected in questing *D. reticulatus*, *H. concinna,* and *I. ventalloi* (**Table 3**). Detailed information on worldwide prevalence rates of *A. phagocytophilum* in unfed ticks from the vegetation can be found in **Tables 3–5**.

Based on molecular detection in questing ticks, *A. phagocytophilum* seems to appear in all countries across Europe. In the US, the majority of studies have been performed in Eastern and Western (California) parts. From Northern US such data are lacking for several geographical regions, however serological evidence indicate exposure to *A. phagocytophilum* in large parts of the continent (Dugan et al., 2006; Bowman et al., 2009; Villeneuve et al., 2011). Two recent studies revealed the presence of *A. phagocytophilum* in questing ticks also in the Southern US (Florida and Georgia) (Clark, 2012; Roellig and Fang, 2012). Only few studies have been carried out in Asia, namely in Russia, China, Japan, and Korea (**Table 5**). It seems likely that other parts of Asia also belong to the endemic area of this pathogen.

Additionally to the ticks mentioned above, molecular detections have been reported from the following tick species (collected engorged from animals): *I. hexagonus*, *I. trianguliceps*, *I. spinipalpis*, *I. ochotonae*, and *D. nutalli* (Zeidner et al., 2000; Bown et al., 2003; Foley et al., 2011; Yaxue et al., 2011; Silaghi et al., 2012a). However, the vector competence of a lot of the tick species in which *A. phagocytophilum* has been detected as well as their contribution to the endemic cycle of *A. phagocytophilum* remain to be investigated.

The tick species *I. ricinus, I. persulcatus, I. scapularis,* and *I. pacificus* are found ubiquitously in their distribution range, have an open questing behavior and a broad host range, including many mammalian species (Sonenshine, 1993). These tick species may consequently also transmit the bacterium from animal reservoir hosts to humans. Aside from these aforementioned antropophilic and exophilic ticks, the involvement of nidicolous, and more host-specific endophilic ticks have been discussed in the context of so-called niche cycles, which may additionally keep the infection in nature. Examples for such proposed niche cycles involve cottontail rabbits (*Sylvilagus* spp.), *I. dentatus* and *I. scapularis* in the US (Goethert and Telford, 2003); field voles (*Microtus agrestis*), *I. trianguliceps* and *I. ricinus* in the UK (Bown et al., 2003); and hedgehogs (*Erinaceus europaeus*), *I. hexagonus* and *I. ricinus* in Europe (Silaghi et al., 2012a). The mentioned animals harbor two to three developmental stages of both endophilic and exophilic tick species and can thus transmit the agent from the animal host to humans via the anthropophilic tick species. Considering the large number of host specific and/or nidicolous ticks all around the world, it is likely that more potential niche cycles will be uncovered in the future (Foley et al., 2011).

Due to the comparatively low prevalence of *A. phagocytophilum* in *I. pacificus* in the Western US, *I. spinipalpis* has been suggested as a bridging vector for HGA (Zeidner et al., 2000). This nidiculous tick species infests, among others, Mexican woodrats (*Neotoma mexicana*) (in which *A. phagocytophilum* DNA has also been detected) and also occasionally bites humans and may thus transmit the agent from zooendemic cycles to humans.

Infection rates reported in many studies are higher in adult ticks than in nymphs. Due to the transstadial transmission, but lack of transovarial transmission, larvae are considered free of *A. phagocytophilum*. Adult ticks have had an additional blood meal in comparison to nymphs, and thus twice the chance of acquiring the infection. Variations in prevalence in questing ticks have also been observed with regard to the year of collection and in-between study areas and different geographic locations (Levin et al., 1999; Wicki et al., 2000; Hildebrandt et al., 2002; Cao et al., 2003; Holman et al., 2004; Ohashi et al., 2005; Grzeszczuk and Stanczak, 2006; Wielinga et al., 2006; Silaghi et al., 2008, 2012b; Schorn et al., 2011; Overzier et al., 2013b).

When looking at these variations, it has to be taken into account, that variations can be due to local variations, such as habitat structure or host availability, variation in methodology and sampling approach. Most studies shown in **Tables 3–5** are single studies providing a spot prevalence, while studies including longitudinal data are scarce.

Variations in the prevalence of *A. phagocytophilum* in ticks may be attributed to several factors, such as the susceptibility of individual tick species, the susceptibility of certain tick populations, and the vector competence of tick species; the transmissibility of the *A. phagocytophilum* variant involved, the susceptibility of different host species, the susceptibility of individual hosts or host populations and the reservoir competence of the host. Especially the availability of different reservoir hosts and the adaptation strategy of *A. phagocytophilum* seem to be crucial factors in this variability. The availability of reservoir hosts depends on factors such as landscape structure and fragmentation (Medlock et al., 2013). In addition, effects exerted by changes in climate, demography, and agriculture may influence the tick distribution and density and their hosts.

HOSTS AND RESERVOIRS

Viable *A. phagocytophilum* organisms have been isolated from several hosts, such as cattle, sheep, goat, dog, horse, human, red deer (*Cervus elaphus*), roe deer (*Caperolus capreolus*), and white-tailed deer (WTD) (*Odocoileus virginianus*) (Foggie, 1951; Goodman et al., 1996; Munderloh et al., 1996; Woldehiwet et al., 2002; Massung et al., 2007; Stuen et al., 2010; Silaghi et al., 2011c). However, several prerequisites have to be fulfilled for a reservoir to be competent for a transstadially transmitted pathogen. A reservoir host must be fed on by an infected vector tick; it must take up a critical number of the infectious agent; it must allow the pathogen to multiply and survive for a period and it must allow the pathogen to find its way into other feeding ticks (Kahl et al., 2002). Several mammals may serve as hosts and reservoirs.

WILD RUMINANTS

In Europe, Asia, and America, *A. phagocytophilum* has been detected in local wild ruminant species (**Tables 6–8**). Wild ruminants such as WTD and roe deer are among the major feeding hosts for ticks in the Eastern US and Europe, respectively, and thus considered to contribute to a rapid increase in the population of ticks (Spielman et al., 1985; Vázquez et al., 2011; Medlock et al., 2013). WTD is considered one of the major reservoir hosts for an apathogenic variant (Ap-V1) of *A. phagocytophilum* in the Eastern

Table 3 | Molecular prevalence studies of *Anaplasma phagocytophilum* in questing ticks in Europe*.

Country	Tick species	Year of tick collection	No. of ticks	Prevalence in %	Method	References
Norway	*Ixodes spp.*	1998–1999	341	2.1[g]	PCR[a]	Jenkins et al., 2001
Norway	*Ixodes ricinus*		200	8.5		
			257	17.1		
		2006–2008[i]	145	3.4	qPCR[b]	Rosef et al., 2009
			235	0.4		
			348	14.9		
		2006	224	4.5	qPCR[b]	Radzijevskaja et al., 2008
		2011	87[adults]	4.6	qPCR[b]	Soleng and Kjelland, 2013
			133[nymphs]	0.8		
Sweden	*I. ricinus*	n.s.	151[nymphs]	6.6	PCR[a]	von Stedingk et al., 1997
		2007	1245[h]	11.5	qPCR[b]	Severinsson et al., 2010
Denmark	*I. ricinus*	1999–2000	106	23.6	PCR[a]	Skarphedinsson et al., 2007
Estonia	*I. ricinus*	2000	100	3	qPCR[a]	Mäkinen et al., 2003
		2006–2008	2474	1.7	qPCR[b]	Katargina et al., 2012
		2008–2010	112	2.7	nPCR[a]	Paulauskas et al., 2012
	I. persulcatus	2008–2010	31	6.5	nPCR[a]	Paulauskas et al., 2012
Latvia	*I. ricinus*	2008–2010	99	3.0	nPCR[a]	Paulauskas et al., 2012
	I. persulcatus	2008–2010	58	1.7	nPCR[a]	Paulauskas et al., 2012
Lithuania	*I. ricinus*	2006	140	3	qPCR[b]	Radzijevskaja et al., 2008
		2008–2010	277	2.9	nPCR[a]	Paulauskas et al., 2012
	D. reticulatus	2008–2010	87	8.0	nPCR[a]	Paulauskas et al., 2012
Russia	*I. ricinus*	1997–1998	295	13.6[g]	PCR[a], RLB	Alekseev et al., 2001a
		2002	80	8.8	nPCR[b]	Masuzawa et al., 2008
		2006–2008	82	13.4	qPCR[b]	Katargina et al., 2012
	I. persulcatus	2002	84	16.7	qPCR[b]	Eremeeva et al., 2006
		2002	119	2.5	nPCR[b]	Masuzawa et al., 2008
Poland	*I. ricinus*	2000	424	19.2	PCR[a]	Stanczak et al., 2002
		1999	533	4.5	PCR[a]	Skotarczak et al., 2003
		2001	701	14	PCR[a]	Stanczak et al., 2004
		n.s.	694	13.1	PCR[a]	Tomasiewicz et al., 2004
		2002	174	4.6	PCR[a]	Rymaszewska, 2005
		2002	73	4.1	PCR[b]	Skotarczak et al., 2006
		2000–2004	1474	14.1	PCR[a]	Grzeszczuk and Stanczak, 2006
		2005	684	10.2 2.8	PCR[a] PCR[c]	Chmielewska-Badora et al., 2007
		2004–2006	1620[h]	4.9	PCR[a]	Wójcik-Fatla et al., 2009
		2007–2008	1123[h]	8.5	PCR[a]	Sytykiewicz et al., 2012
		n.s.	40	2.5	PCR[b]	Richter and Matuschka, 2012
Slovakia	*I. ricinus*	2002	60	8.3	PCR[a]	Derdáková et al., 2003
		2003–2004	271	4.4	PCR[a]	Smetanová et al., 2006
		2006	68	4.4[g]	PCR[a]	Špitalská et al., 2008
		n.s.	180	1.1	PCR[e]	Derdáková et al., 2011
			102	7.8		
		n.s.	80	8	qPCR[d]	Subramanian et al., 2012

(Continued)

Table 3 | Continued

Country	Tick species	Year of tick collection	No. of ticks	Prevalence in %	Method	References
Belarus	*I. ricinus*	2006–2008	187	4.2	qPCR[b]	Katargina et al., 2012
		2009	453	2.6	nPCR[f]	Reye et al., 2013
Ukraine	*I. ricinus*	2006	84	3.6	PCR[a]	Movila et al., 2009
Moldova	*I. ricinus*	2005	198	9	PCR[a]	Koèi et al., 2007
		2006	156	5.1	PCR[a]	Movila et al., 2009
Bulgaria	*I. ricinus*	2000	112[adults]	33.9	PCR[c]	Christová et al., 2001
			90[nymphs,h]	2.2		
Hungary	*I. ricinus*	2006–2008	1800[h]	0.4	nPCR[a]	Egyed et al., 2012
Serbia	*I. ricinus*	2001–2004	287	13.9	nPCR[b]	Tomanovic et al., 2010
		2007–2009	27	3.7[g]	PCR[a]	Tomanovic et al., 2013
	D. reticulatus	2007–2009	53	1.9[g]	PCR[a]	Tomanovic et al., 2013
	Haemaphysalis concinna	2007–2009	35	2.9[g]	PCR[a]	Tomanovic et al., 2013
Slovenia	*I. ricinus*	1996	93	3.2	PCR[a]	Petrovec et al., 1999
	I. ricinus	2005–2006	442[h]	0.6	PCR, nPCR[a,f]	Smrdel et al., 2010
UK (Scotland)	*I. ricinus*	1996–1997	210[h]	0.27–2.0	PCR[a]	Alberdi et al., 1998
		1996–1999	1476	3.0	PCR[a]	Walker et al., 2001
UK (Wales)	*I. ricinus*	n.s.	60	7.0	nPCR[a]	Guy et al., 1998
UK (England)	*I. ricinus*	n.s.	44[adults]	9	nPCR[a]	Ogden et al., 1998
			65[nymphs]	6		
	I. ricinus	n.s.	70[adults]	1.4	nPCR[a]	Ogden et al., 1998
			70[nymphs]	1.4		
	I. ricinus	2004–2005	4256[nymphs]	0.7	qPCR[b]	Bown et al., 2009
			263[females]	3.4		
			321[males]	2.5		
The Netherlands	*I. ricinus*	2000–2004	704	0.6	PCR[a], RLB	Wielinga et al., 2006
Belgium	*I. ricinus*	2010	625	3.0	qPCR[a,j]	Lempereur et al., 2012
Luxembourg	*I. ricinus*	2007	1394	1.9	PCR[f]	Reye et al., 2010
France	*I. ricinus*	2003	4701[h]	15	PCR[a]	Halos et al., 2006
		2004	1065[nymphs]	0.4	PCR[a]	Ferquel et al., 2006
			171[adults]	1.2		
		2003	123[males]	4.3–9.4	nPCR[a]	Halos et al., 2010
			102[females]	2.2–10.7		
			3480[nymphs,h]	1.7–2.6		
		2006–2007	572	0.3	PCR[a]	Cotté et al., 2010
		2008	131	1.5	PCR[a]	Reis et al., 2011
Germany	*I. ricinus*	1999	492	1.6	PCR[a]	Fingerle et al., 1999
		2002	1963	2.6–3.1	nPCR[a]	Oehme et al., 2002
		2003	305	2.3	PCR[a]	Hildebrandt et al., 2002
		1999–2001	5424	1.0	nPCR[a]	Hartelt et al., 2004
		2003	127	3.9	PCR[a], RLB	Pichon et al., 2006
		2006	2862	2.9	qPCR[b]	Silaghi et al., 2008

(Continued)

Table 3 | Continued

Country	Tick species	Year of tick collection	No. of ticks	Prevalence in %	Method	References
		2006–2007	1000	5.4	PCR[a]	Hildebrandt et al., 2010b
		2005	1646	3.2	qPCR[b]	Schicht et al., 2011
		2009–2010	5569	9.0[g]	qPCR[b]	Schorn et al., 2011
		n.s.	542	4.1	PCR[b]	Richter and Matuschka, 2012
		2009[i]	539	8.7		
			128	9.4	qPCR[b]	Silaghi et al., 2012b
			115	17.4		
		2011–2012	4064	5.3[g]	qPCR[b]	Overzier et al., 2013b
Austria	*I. ricinus*	2000–2001	235	5.1	PCR[a]	Sixl et al., 2003
		n.s.	880	8.7	qPCR[f]	Polin et al., 2004
Switzerland	*I. ricinus*	n.s.	100	2	qPCR[a]	Leutenegger et al., 1999
		1998	1667	1.3	qPCR[a]	Pusterla et al., 1999
		1998	417	1.4	nPCR[a]	Liz et al., 2000
		1999	6071[h]	1.2	qPCR[a]	Wicki et al., 2000
		2008	100[nymphs]	2	qPCR[b]	Burri et al., 2011
		2009–2010	1476	1.5	qPCR[b]	Lommano et al., 2012
Italy	*I. ricinus*	n.s.	86	24.4	PCR[a]	Cinco et al., 1997
		2002	1014	9.9	nPCR[a]	Mantelli et al., 2006
		2000–2001	1931	4.4	PCR[a]	Piccolin et al., 2006
		1998	55[h]	9	PCR	Lillini et al., 2006
		2010	232	8.2	qPCR[b]	Aureli et al., 2012
		2006–2008	193	1.5	qPCR[b]	Capelli et al., 2012
Spain	*I. ricinus*	2004	104[nymphs]	8.6	PCR[a]	Portillo et al., 2005
			54[adults]	3.7		
		2005–2006	168	10.7	nPCR[a]	Portillo et al., 2011
		2004	n.s.	20.5	PCR[a]	Ruiz-Fons et al., 2012
Portugal	*n.s.*	Archival collection	300	0.3	nPCR[f]	de Carvalho et al., 2008
	I. ricinus	2003–2004	142[h]	4.0	PCR[a,b] PCR[b]	Santos et al., 2004
		n.s.	101	6.9		Richter and Matuschka, 2012
	I. ventalloi	2003–2004	93[h]	2.0	PCR[a,b]	Santos et al., 2004
Turkey European and Asian part)	*I. ricinus*	2008	241	2.7–17.5[i]	nPCR[a,b]	Sen et al., 2011

**This table does not claim completeness. It does not include studies with 0% prevalence and studies with mixed results for questing and engorged tick.*

nPCR, nested PCR; qPCR, real-time PCR; RLB, reverse line blot; n.s., not specified.

[a] *16S rRNA as gene target.*

[b] *Msp2 as gene target.*

[c] *AnkA as gene target.*

[d] *ApaG as gene target.*

[e] *Msp4 as gene target.*

[f] *GroEL as gene target.*

[g] *Total prevalence not specified in the paper, prevalence was calculated by the authors of the present manuscript.*

[h] *Study includes pools*

[i] *From different locations*

[j] *Commercial kit.*

Table 4 | Molecular prevalence studies of *Anaplasma phagocytophilum* in questing ticks in the USA*.

State	Tick species	Year of tick collection	No. of ticks	Prevalence in %	Method	References
New Hampshire	*Ixodes scapularis*	2007	509	0.2[e]	PCR	Walk et al., 2009
Rhode Island	*I. scapularis*	1996–1999	538	22.9	nPCR[a]	Massung et al., 2002
Connecticut	*I. scapularis*	1994	120	50.0	PCR[a]	Magnarelli et al., 1995
		1996–1997	1115	1.2–19.0[e]	PCR[a]	Levin et al., 1999
		1996–1999	375	13.3	nPCR[a]	Massung et al., 2002
New York	*I. scapularis*	2003–2004	25[females] 32[males] 62[nymphs]	40.0 50.0 27.0	nPCR[c]	Moreno et al., 2006
New Jersey	*I. scapularis*	2001	107	1.9	PCR[a]	Adelson et al., 2004
Pennsylvania	*I. scapularis*	2005	94	1.1	PCR[a]	Steiner et al., 2008
Wisconsin	*I. scapularis*	1998	636	3.8	PCR[a]	Shukla et al., 2003
		2006	100	14	nPCR[a]	Steiner et al., 2008
		2008	201	12.0	qPCR[b]	Lovrich et al., 2011
Indiana	*I. scapularis*	2003	68	11.8	nPCR[a]	Steiner et al., 2006
		2004	100	5	nPCR[a]	Steiner et al., 2008
Maine	*I. scapularis*	2003	100	16	nPCR[a]	Steiner et al., 2008
Maryland	*I. scapularis*	2003	348	0.3	PCR[a]	Swanson and Norris, 2007
Florida	*I. scapularis*	2004–2005	236	1.3	PCR[b]	Clark, 2012
	Amblyomma americanum	2004–2005	223	2.7	PCR[b]	Clark, 2012
Georgia	*I. scapularis*	2004–2005	808	20.0	nPCR[d]	Roellig and Fang, 2012
California	*Ixodes pacificus*	1995–1996	1112[adults,f] 47[nymphs,f]	0.8 2.1	nPCR[a]	Barlough et al., 1997a
		1997	84	1.2[e]	PCR[c]	Nicholson et al., 1999
		1996–1997	401[f]	2.0	nPCR[a]	Kramer et al., 1999
		1998	465[adults] 202[nymphs]	0 9.9	PCR[a]	Lane et al., 2001
		2000–2001	776	6.2	PCR[b]	Holden et al., 2003
		2002	234	3.4	nPCR[a]	Lane et al., 2004
		2000–2001	168	3.0	PCR[b]	Holden et al., 2006
		2005–2007	138	2.2[e]	qPCR[b]	Rejmanek et al., 2011
	Dermacentor variabilis	2000–2001	58	8.6	PCR[b]	Holden et al., 2003
	D. occidentalis	2000–2001	353	1.1	PCR[b]	Holden et al., 2003
		2003–2005; 2009–2010	513	0.2	nPCR[a]	Lane et al., 2010

*This table does not claim completeness. It does not include studies with 0% prevalence and studies with mixed results for questing and engorged ticks.
nPCR, nested PCR; qPCR, real-time PCR; n.s., not specified.
[a] 16S rRNA as gene target.
[b] Msp2 as gene target.
[c] GroESL as gene target.
[d] AnkA as gene target.
[e] Calculated by the authors of the present manuscript.
[f] Study includes pools.

US (Massung et al., 2005). Several genetic variants of *A. phagocytophilum* have been found in roe deer in Europe and there seem to be both potentially pathogenic and apathogenic variants occurring in roe deer (Silaghi et al., 2011b; Overzier et al., 2013a). A high roe deer density is associated with a high tick density (Jensen et al., 2000; Carpi et al., 2008; Rizzoli et al., 2009) and both presence and high density of roe deer seems to have a positive effect on the *A. phagocytophilum* prevalence (Rosef et al., 2009). Similarly, the density of WTD influences the density of *I. scapularis* ticks in the north-eastern US (Rand et al., 2003). For example, the elimination of WTD from certain areas lead to a drastic reduction of the occurrence of *I. scapularis* (Wilson et al., 1988). In a later study, however, there was no direct effect of a deer culling program on the occurrence of *I. scapularis* developmental stages (Jordan et al., 2007).

In the US, WTD has prevalence rates of *A. phagocytophilum* of up to 46.6% (**Table 6**), while detection of *A. phagocytophilum* in wild ruminants other than WTD are scarce so far. In Europe, roe deer show prevalence rates reaching up to 98.9% (Overzier et al., 2013a). Other deer species seem to contribute to the endemic

Table 5 | Molecular prevalence studies of *Anaplasma phagocytophilum* in questing ticks in Asia*.

Country	Tick species	Year of tick collection	No. of ticks	Prevalence in %	Method	References
Russia	*Ixodes persulcatus*	2003–2004	125	2.4	nPCR[a]	Rar et al., 2005
		2002	8	12.5	PCR[a]	Shpynov et al., 2006
		2003–2010	3751	3.0	nPCR[a]	Rar et al., 2011
China	*I. persulcatus*	1997	372[d]	0.8*	nPCR[a]	Cao et al., 2000
		1999–2001	1345	4.6	nPCR[a]	Cao et al., 2003
		2005	100	4.0	nPCR[a]	Cao et al., 2006
	Dermacentor silvarum	2005	286	0.7	nPCR[a]	Cao et al., 2006
Japan	*I. persulcatus*	n.s.	325	6.2	PCR[b]	Murase et al., 2011
		2010–2011	134	21.6[f]	nPCR[a]	Ybañez et al., 2012
	Haemaphysalis megaspinosa	2008	48	12.5	nPCR[a]	Yoshimoto et al., 2010
	H. douglasii	2011	35	6.3[f]	nPCR[c]	Ybañez et al., 2013
	I. persulcatus, I. ovatus	n.s.	130	4.6[e]	nPCR[b]	Wuritu et al., 2009
Korea	*H. longicornis*	2004	241[d]	1.1	nPCR[a]	Chae et al., 2008
	I. nipponensis	2004	5[male]	20	nPCR[a]	Chae et al., 2008

*This table does not claim completeness. It does not include studies with 0% prevalence and studies with mixed results for questing and engorged tick.
nPCR, nested PCR; n.s., not specified.
[a] 16S rRNA gene as target.
[b] Msp2 gene as target.
[c] GroEL gene as target.
[d] Study includes pools.
[e] I. persulcatus and I. ovatus.
[f] Total prevalence not specified in the paper, prevalence was calculated by the authors of the present manuscript.

cycles in Europe, and may also constitute efficient reservoir hosts, as the pathogen has been detected in red deer with up to 87% prevalence, in fallow deer (*Dama dama*) with up to 72%, and in sika deer (*Cervus nippon*) with up to 50% (**Table 7**). *A. phagocytophilum* has also been identified in deer species in Asia, namely sika deer and water deer (*Hydropotes inermis*) with prevalence rates of up to 46% and of 63.6%, respectively (Jilintai et al., 2009; Kang et al., 2011; **Table 8**). However, the studies that have been conducted in Asia on wild ruminants are too few as to draw any definite conclusion on the distribution of *A. phagocytophilum*.

SMALL MAMMALS

The second large group of animals that *A. phagocytophilum* is found in endemic countries are in small mammals such as rodents and insectivores. These animals also are major feeding hosts for ticks, especially for the developmental stages (Kiffner et al., 2011). DNA of *A. phagocytophilum* was found in different mouse, vole, other rodent and insectivore species in the US, Europe, and Asia (**Tables 6–8**).

Rodents

In Europe, yellow-necked mice (*Apodemus flavicollis*) were infected with ranges from <1 to 15%, wood mice (*Apodemus sylvaticus*) from <1 to 11% and bank voles (*Myodes glareolus*) from 5 to 19.2%. In mouse species, detection with higher prevalence rates represents only single studies, whereas detection in bank voles seemed higher and more consistent. This was also the case for other vole species in Europe (**Table 6**). In the UK, the field vole has been discussed as a potential small mammal reservoir (Bown et al., 2003). However, in several studies on rodents in Europe, no DNA of *A. phagocytophilum* has been detected or at such low prevalence rates, that a reservoir role of this group of animals in Europe remains unclear (Barandika et al., 2007; Silaghi et al., 2012b; **Table 6**).

On the contrary, in the Eastern US, the white-footed mouse (*Peromyscus leucopus*) is considered one of the major reservoir hosts for the human pathogenic variant (Ap-ha) (Massung et al., 2003). *P. leucopus* is found as the predominant small mammal in forested habitats throughout the Eastern and Central US and it is one of the major hosts for the larval stages of *I. scapularis* (Sonenshine, 1993). The white-footed mouse has reservoir competence for the AP-ha variant, but reservoir competence could not be shown for the apathogenic Ap-V1 variant (Massung et al., 2003), as opposed to the aforementioned WTD as a major reservoir hosts for Ap-V1 (Massung et al., 2005). Different lengths of infections with the two strains have also been shown in an experimental WTD study: Ap-V1 from tick cells resulted in lasting parasitemia, whereas infection with Ap-ha was short-lived (Reichard et al., 2009). By contrast, both Ap-V1 and Ap-ha were infectious for goats and goats are reservoir competent to Ap-V1 (Massung et al., 2006).

Ap-V1 was isolated from goats and *I. scapularis* and propagated in the ISE6 tick cell line, but it could not be cultivated in the human HL-60 cell line. This stands in contrast to *A. phagocytophilum* strains which have been isolated from human cases in the US, which readily grow in HL-60 cell lines (Horowitz et al.,

Table 6 | DNA-Detection of *Anaplasma phagocytophilum* in blood/spleen in vertebrate hosts in the Americas*.

Group of animals	Animal species	Country	No. of investigated	Prevalence in %	Method	References
Wild ruminants	White-tailed deer (*Odocoileus virginianus*)	USA	458	16.0	PCR[a,b]	Dugan et al., 2006
		USA (Wisconsin)	181	15	PCR[a]	Belongia et al., 1997
		USA (Minnesota)	266	46.6	PCR[b]	Johnson et al., 2011
		USA (Connecticut)	63	37.0	PCR[b]	Magnarelli et al., 1999
		USA (Pennsylvania)	38	28.9	nPCR[a]	Massung et al., 2005
		USA (Wisconsin)	18	5.6	PCR[b]	Michalski et al., 2006
			40	22.5		
		USA (Mississippi)	32	3.1	PCR[b]	Castellaw et al., 2011
	Black-tailed deer (*Odocoileus hemonius columbianus*)	USA (California)	15	26.7[d]	nPCR[a]	Foley et al., 1998
	Mule deer (*O. h. hemonius*)	USA (California)	6	83.3[d]	nPCR[a]	Foley et al., 1998
	Elk (*Cervus elaphus nannodes*)	USA (California)	29	31.0	nPCR[a]	Foley et al., 1998
Small mammals (rodents)	White-footed mouse (*Peromyscus leucopus*)	USA (Minnesota)	158	11.4	nPCR[a]	Walls et al., 1997
			98–150	20.0–46.8	PCR[b]	Johnson et al., 2011
		USA (Connecticut)	47	36.2	nPCR[a]	Stafford et al., 1999
			135	14.1	PCR[b]	Levin et al., 2002
	Meadow jumping mouse (*Zapus hudsonius*)	USA (Minnesota)	18	50.0	PCR[b]	Johnson et al., 2011
	Cotton mouse (*P. gossypinus*)	USA (Florida)	41	4.9	PCR[b]	Clark, 2012
	Deer mouse (*P. maniculatus*)	USA (Colorado)	63	20.6	PCR[a]	Zeidner et al., 2000
			55[d]	9.2[d]	PCR[b]	DeNatale et al., 2002
	Brush mouse (*P. boylii*)	USA (California)	n.s.	4.0	qPCR[b]	Foley et al., 2008b
	Pinyon mouse (*P. truei*)	USA (California)	5[e]	20.0	PCR[c]	Nicholson et al., 1999
	Western harvest mouse (*Rheithrodontomys megalotis*)	USA (California)	n.s.	6.3	qPCR[b]	Foley et al., 2008b
	Red-backed vole (*Clethrionomys gapperi*)	USA (Minnesota)	6	17.0	nPCR[a]	Walls et al., 1997
			73	15.1	PCR[b]	Johnson et al., 2011
	Meadow vole (*Microtus pennsylvanicus*)	USA (Minnesota)	14	14.3	PCR[b]	Johnson et al., 2011
	Prairie vole (*Microtus ochrogaster*)	USA (Colorado)	15	6.6	PCR[a]	Zeidner et al., 2000
	Eastern chipmunk (*Tamias striatus*)	USA (Minnesota)	23	4.3	nPCR[a]	Walls et al., 1997
		USA (Rhode Island)	19	57.9	nPCR[a]	Massung et al., 2002
	Chipmunk	USA (Minnesota)	43	88.4	PCR[b]	Johnson et al., 2011
	Least chipmunk (*T. minimus*)	USA (Colorado)	5	40.0	PCR[b]	DeNatale et al., 2002
	Redwood chipmunk (*T. ochrogenys*)	USA (California)	60	6.6	qPCR[b]	Nieto and Foley, 2008
			n.s.	6.9	qPCR[b]	Foley et al., 2008b
			141	10.6	qPCR[b]	Foley and Nieto, 2011
	Sonoma chipmunk (*T. sonomae*)	USA (California)	5	40	qPCR[b]	Nieto and Foley, 2008
			n.s.	50.0	qPCR[b]	Foley et al., 2008b
	Chipmunk	USA (California)	81	8.9	qPCR[b]	Foley et al., 2011
	Tamias sp.	USA (California)	50	16.7[d]	qPCR[b]	Rejmanek et al., 2011
	Golden-mantled ground squirrel (*Spermophilus lateralis*)	USA (Colorado)	8	13	PCR[b]	DeNatale et al., 2002
	Eastern gray squirrel (*Sciurus carolinensis*)	USA (California)	27	11.1	qPCR[b]	Nieto and Foley, 2008

(Continued)

Table 6 | Continued

Group of animals	Animal species	Country	No. of investigated	Prevalence in %	Method	References
			n.s.	18.8	qPCR[b]	Foley et al., 2008b
			9	11.1[d]	qPCR[b]	Nieto et al., 2010
	Western gray squirrel (*S. griseus*)	USA (California)	41	12.1	qPCR[b]	Nieto and Foley, 2008
			n.s.	15.8	qPCR[b]	Foley et al., 2008b
			37	10.8[d]	qPCR[b]	Nieto et al., 2010
			6[e]	n.a.	qPCR[b]	Foley et al., 2008a
	Douglas squirrel (*Tamiasciurus douglasii*)	USA (California)	2[e]	n.a.	qPCR[b]	Foley et al., 2008a
	Northern flying squirrel (*Glaucomys sabrinus*)	USA (California)	20	5	qPCR[b]	Nieto and Foley, 2008
			n.s.	16.7	qPCR[b]	Foley et al., 2008b
			24	4.2[d]	qPCR[b]	Foley et al., 2007
			4	25.0[d]	qPCR[b]	Rejmanek et al., 2011
	Cotton rat (*Sigmodon hispidus*)	USA (Florida)	31	45.2	PCR[b]	Clark, 2012
	Mexican wood rat (*Neatoma mexicana*)	USA (Colorado)	36	38.8	PCR[a]	Zeidner et al., 2000
			30[d]	15[d]	PCR[b]	DeNatale et al., 2002
	Dusky-footed woodrat (*Neatoma fuscipes*)	USA (California)	25[e]	68	PCR[c]	Nicholson et al., 1999
			35[e,f]	68.6	PCR[c]	Castro et al., 2001
			134	71	qPCR[b]	Drazenovich et al., 2006
			n.s.	4.3	qPCR[b]	Foley et al., 2008b
			42	11.8	qPCR[b]	Foley et al., 2011
			53	9.4[d]	qPCR[b]	Rejmanek et al., 2011
	Big free-tailed bat (*Nyctinomops macrotis*)	USA (California)	n.s.	1.8	qPCR[b]	Foley et al., 2008b
Small mammals (insectivores)	Short-tailed shrew (*Blarina* spp.)	USA (Minnesota)	29	17.2	PCR	Johnson et al., 2011
Reptiles and Snakes	Northern alligator lizard (*Elgaria coeruleus*)	USA (California)	3	33.3	qPCR[b]	Nieto et al., 2009
	Sage-brush lizard (*Sceloporus graciosus*)	USA (California)	4	25.0	qPCR[b]	Nieto et al., 2009
	Western fence lizard (*S. occidentalis*)	USA (California)	77	9.1	qPCR[b]	Nieto et al., 2009
	Pacific gopher snake (*Pituophis catenifer*)	USA (California)	5	20.0	qPCR[b]	Nieto et al., 2009
	Common garter snake (*Thamnophis sirtalis*)	USA (California)	1	100	qPCR[b]	Nieto et al., 2009
Other	Cottontail rabbit (*S. floridanus*)	USA (Massachusetts)	203	27	nPCR[a]	Goethert and Telford, 2003
	American black bear	USA (California)	80	4	qPCR[b]	Drazenovich et al., 2006
	Gray Fox (*Urocyon cinereoargenteus*)	USA (California)	70[f]	9	qPCR[b]	Gabriel et al., 2009
	Raccoon (*Procyon lotor*)	USA (Connecticut)	57	24.6	PCR[b]	Levin et al., 2002
Domestic animals	Cat (stray)	USA (Connecticut)	6	33.3	PCR[b]	Levin et al., 2002
	Dog	USA (Minnesota)	222	3	PCR[a]	Beall et al., 2008
			51[g]	37		

(Continued)

Table 6 | Continued

Group of animals	Animal species	Country	No. of investigated	Prevalence in %	Method	References
		USA (California)	97	7	qPCR[b]	Drazenovich et al., 2006
			184	7.6	qPCR[b]	Henn et al., 2007
		Brazil	253	7.1	qPCR[b]	Santos et al., 2011
	Horse	Guatemala	74	13	nPCR[a]	Teglas et al., 2005
	Cattle	Guatemala	48	51	nPCR[a]	Teglas et al., 2005

*This table does not claim completeness. It does not include studies with 0% prevalence and case reports.

nPCR, nested PCR; qPCR, real-time PCR; n.s., not specified.

[a] 16S rRNA as gene target.

[b] Msp2 as gene target.

[c] GroEL as gene target.

[d] Total prevalence/number not specified in the paper, prevalence/number was calculated by the authors of the present manuscript.

[e] Seropositive for Anaplasma phagocytophilum antibodies.

[f] Includes recaptures.

[g] Partially with symptoms.

1998; Massung et al., 2007), suggesting differing host specificity for these two strain types.

Apart from the white-footed mouse, *A. phagocytophilum* DNA has been detected in several rodent species such as voles and chipmunks in the Eastern US, cotton mice and cotton rats in Florida and several mouse-, chipmunk-, and squirrel-species as well as the dusky-footed woodrat (*Neotoma fuscipes*) in the Western US (**Table 7**). Prevalence ranges from 1.8 to 88.4%. The gray squirrel (*Sciurus carolinensis*) has also been found to be reservoir competent (Levin et al., 2002) and the redwood chipmunk (*Tamias ochrogenys*) and sciurid rodents are discussed as important reservoir hosts for *A. phagocytophilum* in the Western US (Nieto et al., 2010; Foley and Nieto, 2011). Similarly to other small mammals that have been suggested to maintain niche cycles, the redwood chipmunk hosts both antropophilic (*I. pacificus*) and nidicolous (*I. angustus*) ticks (Foley and Nieto, 2011).

In Asia, comparatively high prevalence rates in small mammals also seem to indicate a reservoir function of this group of mammals (**Table 8**). For example, in China, wood mice showed prevalence rates up to 10.0% (Zhan et al., 2008), Korean field mice (*A. peninsulae*) up to 25% (Zhan et al., 2010) and black-striped field mice (*A. agrarius)* up to 20.8% (Cao et al., 2006). In Korea, prevalence rates in the black-striped field mouse was also up to 23.6% (Kim et al., 2006) and therefore, *A. agrarius* has been discussed as one of the major reservoir host in Asian countries. In the Asian part of Turkey, however, all captured rodents were serologically negative for *A. phagocytophilum* (Güner et al., 2005).

Additionally to mice, voles, chipmunks, and squirrels, DNA of *A. phagocytophilum* has also been detected in rats on all three continents, in hamsters (China) and in a porcupine (Italy) (**Tables 6–8**).

Insectivores

There are very few published studies on the role of insectivores in the life cycle of *A. phagocytophilum*. The common shrew (*Sorex araneus*) has been discussed as a reservoir host for *A. phagocytophilum* in the UK (Bown et al., 2011). In that study, prevalence reached 18.7%. Other insectivores which have been investigated in Europe were the greater white-toothed shrew (*Crocidura russula*) and the European hedgehog (**Table 6**). DNA of *A. phagocytophilum* has also been detected in short-tailed shrews (*Blarina brevicauda*) with 17.2% prevalence in the US and in Asia in white-toothed shrews with 63.6% prevalence (**Tables 6**, **8**). Detection rates of *A. phagocytophilum* in insectivores were generally high, with average prevalence rates around 20%, reaching over 80%. However, the role of insectivores in the life cycle of *A. phagocytophilum* needs further investigation.

OTHER ANIMAL SPECIES

Apart from wild ruminants, rodents and insectivores, there are several other vertebrate species in which DNA from *A. phagocytophilum* has been isolated. Whether these contribute to the endemic cycle of *A. phagocytophilum* is currently not clear. Amongst these animals are mammals such as wild boars, foxes, and bears, but also birds and reptiles (**Tables 6–8**). The prevalence rates in these animal species seem similar to the potential reservoir hosts discussed above, but studies have been very few so a final conclusion is not yet possible. In the US, raccoons (*Procyon lotor*) have been found to be reservoir competent for *A. phagocytophilum* (Levin et al., 2002; Yabsley et al., 2008), while wild boar (*Sus scrofa*) has recently been discussed as a host for human pathogenic variants of *A. phagocytophilum* in Europe (Michalik et al., 2012).

The questions which remain open are whether many different animal species get infected only temporarily with potentially non-species specific strains of *A. phaogcytophilum* and constitute dead-end hosts such as human beings, whether they develop clinical signs of disease or if they contribute in any way to the endemic cycle.

DOMESTIC ANIMALS

Dogs in Europe were positive for DNA of *A. phagocytophilum* at about 1–6% prevalence, regardless whether they show symptoms of canine granulocytic anaplasmosis or not. By comparison, the

Table 7 | Detection of DNA of *Anaplasma phagocytophilum* in blood or tissue (majority spleen) of vertebrate hosts in Europe*.

Group of animals	Animal species	Country	No. of investigated	Prevalence in %	Method	References
Wild ruminants	Roe deer (*Capreolus capreolus*)	Denmark	237	42.6	qPCR[b]	Skarphedinsson et al., 2005
		UK	112	38.0	PCR[d], SB	Alberdi et al., 2000
			279	47.3	qPCR[b]	Bown et al., 2009
			5	20.0	qPCR[b]	Robinson et al., 2009
		Poland	166	9.6	PCR[a,c]	Michalik et al., 2009
			31	38.7	nPCR[a]	Hapunik et al., 2011
		Slovakia	2	50.0	PCR[a]	Smetanová et al., 2006
			30	50.0	PCR[a]	Stefanidesová et al., 2008
		Czech Republic	40	12.5	qPCR[a]	Hulínská et al., 2004
			10	30.0	nPCR[a]	Zeman and Pecha, 2008
		Germany	31	94.0	nPCR[a]	Scharf et al., 2011
			95	98.9	qPCR[b]	Overzier et al., 2013a
		Austria	121	43.0	qPCR[d]	Polin et al., 2004
			19	52.6	qPCR[b]	Silaghi et al., 2011b
		Switzerland	103	18.4	nPCR[a]	Liz et al., 2002
		Italy	96	19.8	PCR[a]	Beninati et al., 2006
			8	50.0	PCR[a,e]	Torina et al., 2008b
		Spain	29	38.0	nPCR[a]	Oporto et al., 2003
			17	18.0	PCR[e]	de la Fuente et al., 2008
	Red deer (*Cervus elaphus*)	Norway	8	87.5[g]	nPCR[a]	Stuen et al., 2013
		UK	5	80.0	qPCR[b]	Robinson et al., 2009
		Poland	88	10.2	PCR[a,c]	Michalik et al., 2009
			106	50.9	nPCR[a]	Hapunik et al., 2011
		Czech Republic	15	13.3	qPCR[a]	Hulínská et al., 2004
			21	86.0	nPCR[a]	Zeman and Pecha, 2008
		Slovakia	3	33.3[g]	PCR[a]	Smetanová et al., 2006
			49	53.1	PCR[a]	Stefanidesová et al., 2008
		Austria	7	28.6	qPCR[d]	Polin et al., 2004
			12	66.7	qPCR[b]	Silaghi et al., 2011b
		Spain	21	23.8[g]	nPCR[a]	Portillo et al., 2011
	Iberian red deer (*C. e. hispanicus*)	Spain	6	100	PCR[e]	Naranjo et al., 2006
	Fallow deer (*Dama dama*)	UK	58	21.0	qPCR[b]	Robinson et al., 2009
		Poland	44	20.5	PCR[a,c]	Michalik et al., 2009
			130	1.5	nPCR[a]	Hapunik et al., 2011
			50	14.0[g]	PCR[a]	Adaszek et al., 2012
		Czech Republic	15	13.3	PCR[a]	Hulínská et al., 2004
			2	50.0	nPCR[a]	Zeman and Pecha, 2008
		Italy	72	15.3	PCR[a]	Veronesi et al., 2011
			29	72.4	nPCR[a]	Ebani et al., 2011
	Sika deer (*Cervus nippon*)	UK	12	50.0	qPCR[b]	Robinson et al., 2009
		Poland	32	34.4	nPCR[a]	Hapunik et al., 2011
		Czech Republic	5	40.0	nPCR[a]	Zeman and Pecha, 2008
	Chamois (*Rupicapra rupicapra*)	Austria	23	26.1	qPCR[b]	Silaghi et al., 2011b
	Alpine ibex (*Capra ibex*)	Austria	18	16.7	qPCR[b]	Silaghi et al., 2011b
	Mouflon (*Ovis musimon*)	Czech Republic	28	4.0	nPCR[a]	Zeman and Pecha, 2008
			15	13.3	PCR[a]	Hulínská et al., 2004
		Slovakia	2	50.0	PCR[a]	Stefanidesová et al., 2008
		Austria	6	50.0	qPCR[b]	Silaghi et al., 2011b

(Continued)

Table 7 | Continued

Group of animals	Animal species	Country	No. of investigated	Prevalence in %	Method	References
	European bison (*Bison bonasus*)	Poland	26	58.0	nPCR[a]	Scharf et al., 2011
			5	57.7[g]	nPCR[a]	Matsumoto et al., 2009
Small mammals (rodents)	Yellow necked-mouse (*Apodemus flavicollis*)	Czech Republic	40	15.0	qPCR[a]	Hulínská et al., 2004
		Slovakia	38	5.3[g]	PCR[a]	Smetanová et al., 2006
		Germany	218	0.5	nPCR[a]	Hartelt et al., 2008
		Switzerland	69	2.9	nPCR[a]	Liz et al., 2000
	Wood mouse (*A. sylvaticus*)	UK	902[j]	0.8	nPCR[a]	Bown et al., 2003
		Switzerland	48	4.2	nPCR[a]	Liz et al., 2000
		France	18	11.1[g]	PCR[a]	Matsumoto et al., 2007
		Spain	162	0.6	PCR[b], RLB	Barandika et al., 2007
	Black-striped field mouse (*A. agrarius*)	Bulgaria	9	33.3	PCR[c]	Christová and Gladnishka, 2005
	Bank vole (*Myodes glareolus*)	UK	527	5.0	nPCR[a]	Bown et al., 2003
		Czech Republic	15	13.3	qPCR[a]	Hulínská et al., 2004
		Switzerland	78	19.2	nPCR[a]	Liz et al., 2000
		Germany	149	13.4	nPCR[a]	Hartelt et al., 2008
			36	5.5	qPCR[b]	Silaghi et al., 2012b
	Common vole (*Microtus arvalis*)	Germany	97	6.2	nPCR[a]	Hartelt et al., 2008
	Field vole (*Mi. agrestis*)	UK	163	6.7	nPCR[a]	Bown et al., 2006
			2402[j]	6.7	qPCR[b]	Bown et al., 2008
			1503[j]	6.3	qPCR[b]	Bown et al., 2009
	Root vole (*Mi. oeconomus*)	Poland	30	6.7[g]	nPCR[a]	Grzeszczuk et al., 2006
	Black rat (*Rattus rattus*)	Bulgaria	136	4.4	PCR[c]	Christová and Gladnishka, 2005
	Porcupine (Hystricidae)	Italy	1	100	PCR[a]	Torina et al., 2008a
Small mammals (insectivores)	Common shrew (*Sorex araneus*)	UK	76	1.3	PCR[a]	Bray et al., 2007
			647[j]	18.7	qPCR[b]	Bown et al., 2011
		Switzerland	5	20.0[g]	nPCR[a]	Liz et al., 2000
	European hedgehog (*Erinaceus europaeus*)	Germany	31	25.8	nPCR[a]	Skuballa et al., 2010
			48	85.4[g]	qPCR[b]	Silaghi et al., 2012a
	Greater white-toothed shrew (*Crocidura russula*)	Spain	6	16.7	PCR[b], RLB	Barandika et al., 2007
Birds	Blackbird (*Turdus merula*)	Spain	3	100	PCR[e]	de la Fuente et al., 2005b
	Chaffinch (*Fringilla coelobs*)	Spain	1	100	PCR[e]	de la Fuente et al., 2005b
	House sparrow (*Passer domesticus*)	Spain	18	6.0	PCR[e]	de la Fuente et al., 2005b
	Spanish Sparrow (*Passer hispaniolensis*)	Spain	3	33.0	PCR[e]	de la Fuente et al., 2005b
	Rock bunting (*Emberiza cia*)	Spain	1	100	PCR[e]	de la Fuente et al., 2005b
	Woodchat shrike (*Lanius senator*)	Spain	1	100	PCR[e]	de la Fuente et al., 2005b
	Magpie (*Pica pica*)	Spain	1	100	PCR[e]	de la Fuente et al., 2005b
	Long-tailed tit (*Aegithalos caudatus*)	Spain	1	100	PCR[e]	de la Fuente et al., 2005b

(Continued)

Table 7 | Continued

Group of animals	Animal species	Country	No. of investigated	Prevalence in %	Method	References
Other	European Brown bear (*Ursus arctos*)	Slovakia	74	24.3	PCR[a]	Vichová et al., 2010
	Red fox (*Vulpes vulpes*)	Poland	111	2.7	nPCR[a]	Karbowiak et al., 2009
		Czech Republic	25	4.0	PCR[a]	Hulínská et al., 2004
		Italy	150	16.6	nPCR[a]	Ebani et al., 2011
	Wild boar (*Sus scrofa*)	Poland	325	12	nPCR[a]	Michalik et al., 2012
		Slovakia	18	5.5[g]	PCR[a]	Smetanová et al., 2006
		Czech Republic	69	4.4	PCR[a]	Hulínská et al., 2004
		Slovenia	113	2.7[g]	PCR[a]	Galindo et al., 2012
			160	6.3	qPCR[f]	Zele et al., 2012
	Hare (*Leparus europaeus*)	Czech Republic	8	12.5	PCR[a]	Hulínská et al., 2004
Domestic animals	Cat	Germany	306	0.3[g]	qPCR[b]	Hamel et al., 2012a
		Germany	265	0.4	qPCR[b]	Morgenthal et al., 2012
	Dog	UK	120[k]	0.8[g]	PCR[a]	Shaw et al., 2005
		Poland	408	0.5	PCR[c]	Zygner et al., 2009
			242[k]	5.4	PCR[b]	Rymaszewska and Adamska, 2011
		Czech Republic	296[k]	3.4	nPCR[a]	Kybicová et al., 2009
		Germany	111	6.3	nPCR[a]	Jensen et al., 2007
			522[k]	5.7	qPCR[b]	Kohn et al., 2011
		Italy	46	2.8–21.7[i]	PCR[a,e]	Torina et al., 2008a
		Italy (Sardinia)	50[k]	7.5[g]	nPCR[d]	Alberti et al., 2005a
		Hungary/Romania	216	1.9	qPCR[b]	Hamel et al., 2012b
	Horse	Czech Republic	40	5	PCR[a]	Hulínská et al., 2004
		Netherlands	61[k]	9.8[g]	PCR[a], RLB	Butler et al., 2008)
		Italy	135[k]	8.1[g]	nPCR[a]	Passamonti et al., 2010
			5[k]	80.0[g]	PCR	Lillini et al., 2006
			134	0–4.7[i]	PCR[a,e]	Torina et al., 2008a
			300	6.7[g]	PCR[a]	Laus et al., 2013
			42	4.7	PCR[a,e]	Giudice et al., 2012
		Italy (Sardinia)	20[k]	15.0[g]	nPCR[d]	Alberti et al., 2005a
	Donkey	Italy	76	4	PCR[a,e]	Torina et al., 2008b
		Spain	3	100	PCR[e]	Naranjo et al., 2006
	Cattle	Czech Republic	55	5.5	PCR[a]	Hulínská et al., 2004
		France	20[j]	20.0[g]	PCR[a,d,e]	Laloy et al., 2009
		Switzerland	27[k]	4.0	qPCR[a]	Hofmann-Lehmann et al., 2004
			16[k]	13.0		
		Italy	78	17	PCR[a,e]	Torina et al., 2008b
			374	0–2.9[i]	PCR[a,e]	Torina et al., 2008a
		Spain	107	19	PCR[e]	de la Fuente et al., 2005b
			157	13	PCR[e]	Naranjo et al., 2006
	Sheep	Norway	32	37.5[g]	nPCR[a,e]	Stuen et al., 2013
		Denmark	43	11.6[g]	PCR[a]	Kiilerich et al., 2009
		Germany	255	4	nPCR[a]	Scharf et al., 2011
		Italy	200	11.5	PCR[a]	Torina et al., 2010
			286	0–3.8[i]	PCR[a,e]	Torina et al., 2008a
			90	3	PCR[a]	Torina et al., 2008b

(Continued)

Table 7 | Continued

Group of animals	Animal species	Country	No. of investigated	Prevalence in %	Method	References
	Sheep, goats	Slovakia, Czech Republic	323	2.8[h]	PCR[e]	Derdáková et al., 2011
	Goats	Switzerland	72	5.6[g]	qPCR[b]	Silaghi et al., 2011e
		Italy	134	0–3.5[i]	PCR[a,e]	Torina et al., 2008a

**This table does not claim completeness. It does not include studies with 0% prevalence and case reports.*

nPCR, nested PCR; qPCR, real-time PCR; RLB, reverse line blot, SB, Southern Blot.

[a] 16S rRNA as gene target.

[b] Msp2 as gene target.

[c] AnkA as gene target.

[d] GroEL as gene target.

[e] Msp4 as gene target.

[f] Commercial kit.

[g] Total prevalence not specified in the paper, prevalence was calculated by the authors of the present manuscript.

[h] Sheep only.

[i] Range represents confidence interval.

[j] Individuals sampled several times.

[k] Partially with symptoms.

prevalence rates in cats were much lower, with <0.5%. In horses, prevalence was higher ranging up to 80%, however, several of the studies investigated horses with symptoms of equine granulocytic anaplasmosis. Without any clinical signs, the prevalence in horses was less than 6.7% (**Tables 6–8**). Furthermore, several case reports and case series have been published on domestic animals in North America (e.g., Cockwill et al., 2009; Granick et al., 2009; Uehlinger et al., 2011), and serological studies have shown a wide evidence of dogs, cats, and horses being in contact with *A. phagocytophilum* in USA, Canada, and Asia (e.g., Magnarelli et al., 2001; Billeter et al., 2007; Bowman et al., 2009; Villeneuve et al., 2011; Bell et al., 2012; Ybañez et al., 2012). Additionally, serological and molecular evidence have been provided from North Africa (which also is an endemic area for *I. ricinus*) that horses and dogs become infected with *A. phagocytophilum* (M'Ghirbi et al., 2009, 2012). This important finding broadens the known geographic range of *A. phagocytophilum* to Africa as another continent.

The role of dogs as reservoir hosts has been discussed (Schorn et al., 2011). Furthermore, a report of granulocytic anaplasmosis has been described in another member of the canine family, a captive timber wolf (*Canis lupus*) (Leschnik et al., 2012). The question remains open whether dogs can contribute to the natural cycle of *A. phagocytophilum*: Is the infection persistent enough for subsequent ticks to become infected, and do dogs host enough nymphal stages of ticks to contribute to the spread? Animals which host mainly adult ticks cannot effectively contribute to the life cycle of *A. phagocytophilum*, as transovarial infection does not seem to occur.

DOMESTIC RUMINANTS

Infection with *A. phagocytophilum* has also been detected in several domestic ruminant species such as sheep, goats, cattle, and yaks (**Tables 6–8**). In Europe, domestic ruminants have been found infected with DNA with rates of up to 20% (cattle), 37% (sheep), and 5.6% (goats) (**Table 6**). However, larger scale molecular studies on domestic ruminants in Northern America are lacking, but cases of granulocytic anaplasmosis have been described in llama (*Lama glama*) and alpaca (*Vicugna pacos*) in California and Massachusetts, respectively (Barlough et al., 1997a,b; Lascola et al., 2009). Furthermore, serological evidence has been provided for *A. phagocytophilum* antibodies in cattle in Connecticut (Magnarelli et al., 2002).

SPREAD OF INFECTION

A. phagocytophilum may be spread between different geographic regions by both infected ticks and infected hosts. Expansion of existing endemic areas or to new geographic regions occurs when populations of competent vectors and reservoirs or the abundance of susceptible hosts increase both in total number and in geographic range.

Roe deer carry large number of ticks and moves over long distances (Vor et al., 2010) and may therefore add to the spread of the pathogen itself as well as by moving infected ticks to other areas (Overzier et al., 2013a). Factors contributing to a wider occurrence of suitable hosts such as WTD, white-footed mice, roe deer, field mice etc. may be landscape changes leading to an expansion in the distribution range as well as in the density of those hosts.

Landscape changes such as reforestation may also lead to an expansion of the anthropophilic ticks which are spread also when their primary feeding hosts expand (Sonenshine, 1993).

The increase and spread of *I. scapularis* in the Eastern US has lead to an increase in Lyme Borreliosis cases (Sonenshine, 1993) and may similarly contribute to the expansion of *A. phagocytophilum*. In Europe, the increasing geographic range of *I. ricinus* as well as the expansion to higher altitudes has recently been discussed by several authors (Materna et al., 2005; Jore et al., 2011; Jaenson et al., 2012; Medlock et al., 2013).

Table 8 | Detection of DNA of *Anaplasma phagocytophilum* in spleen/blood of vertebrate hosts in Asia and Africa*.

Group of animals	Animal species	Country	No. of investigated	Prevalence in %	Method	References
ASIA						
Wild ruminants	Sika deer (*Cervus nippon*)	Japan	22	46.0	nPCR[a]	Jilintai et al., 2009
			126	19.0	nPCR[a]	Kawahara et al., 2006
			32	15.6	nPCR[a]	Masuzawa et al., 2011
	Korean water deer (*Hydropotes inermis argyropus*)	Korea	66	63.6	nPCR[a]	Kang et al., 2011
	Wood mouse (*Apodemus sylvaticus*)	China	20	10.0	nPCR[a]	Zhan et al., 2008
			21	9.5	nPCR[a]	Zhan et al., 2009a
	Black-striped field mouse (*Apodemus agrarius*)	China	24	20.8	nPCR[a]	Cao et al., 2006
			142	9.9	nPCR[a]	Zhan et al., 2009a
			78	12.8	qPCR[b]	Zhan et al., 2010
			12	16.7	nPCR[a]	Yang et al., 2013
		Korea	358	5.6	nPCR[a]	Chae et al., 2008
			373	23.6[d]	nPCR[a]	Kim et al., 2006
	Korean field mouse (*Apodemus peninsulae*)	Russia	359	0.6[d]	nPCR[a]	Rar et al., 2011
		China	43	7.0	nPCR[a]	Cao et al., 2006
			74	5.4	nPCR[a]	Zhan et al., 2009a
			4	25.0	qPCR[b]	Zhan et al., 2010
	Bank vole (*M. glareolus*)	Russia	61[d]	6.6[d]	nPCR[a]	Rar et al., 2011
	Red-backed vole (*Myodes rutilus*)	Russia	189[d]	14.8[d]	nPCR[a]	Rar et al., 2011
	Red gray-backed vole (*Myodes rufocanus*)	Russia	776[d]	5.2[d]	nPCR[a]	Rar et al., 2011
		China	65	4.6	nPCR[a]	Zhan et al., 2009a
	East-European field vole *(Microtus rossiaemeridionalis)*	Russia	38[e]	2.6[d]	nPCR[a]	Rar et al., 2011
	Brown house rat (*Rattus norvegicus*)	China	9	55.5	qPCR[b]	Zhan et al., 2010
			9	33.3	nPCR[a]	Zhan et al., 2008
	Chinese white bellied rat (*Niviventer confucianus*)	China	48	12.5	nPCR[a]	Zhan et al., 2008
			115	5.2	nPCR[a]	Zhan et al., 2009a
	White-bellied giant rat (*Niviventer coxingi*)	China	4	25.0	nPCR[a]	Zhan et al., 2008
			4	25.0	nPCR[a]	Zhan et al., 2009a
	Lesser rice field rat (*Rattus losea*)	China	2	50.0	nPCR[a]	Zhan et al., 2008
			32	3.1	nPCR[a]	Zhan et al., 2009a
	Brown rat (*R. norvegicus*)	China	47	8.5	nPCR[a]	Zhan et al., 2009a
	Siberian chipmunk (*Tamias sibiricus*)	Russia	24	25.0[d]	nPCR[a]	Rar et al., 2011
		China	3	33.3	nPCR[a]	Cao et al., 2006
			18	5.6	nPCR[a]	Zhan et al., 2009a
	Great long-tailed hamster (*Tscherskia triton*)	China	65	9.2	qPCR[b]	Zhan et al., 2010
	Cricetulus sp.	China	39	5.1	nPCR[a]	Zhan et al., 2009a
	Gray hamster (*Cricetulus migratorius*)	China	3	33.3	qPCR[b]	Zhan et al., 2010
Small mammals (insectivores)	White-toothed shrew (*Crocidura lasiura*)	Korea	33	63.6[d]	nPCR[a]	Kim et al., 2006
	Common shrew (*Sorex araneus*)	Russia	137[d]	4.4[d]	nPCR[a]	Rar et al., 2011
Other	Chinese hare (*Lepus sinensis*)	China	54	1.9	nPCR[a]	Zhan et al., 2009b
	Wild boar (*Sus scrofa*)	Japan	56	3.6	nPCR[a]	Masuzawa et al., 2011
Domestic animals	Dog	China	101	10.9	nPCR[a]	Zhang et al., 2012a
	Cattle	Japan	15	80.0	PCR[a]	Ooshiro et al., 2008
			78	1.0	nPCR[a]	Jilintai et al., 2009
			1251	3.4	PCR[b]	Murase et al., 2011
			50	2.0	nPCR[c]	Ybañez et al., 2013

(Continued)

Table 8 | Continued

Group of animals	Animal species	Country	No. of investigated	Prevalence in %	Method	References
		China	71	23.9	nPCR[a]	Zhang et al., 2012a
			201	23.4	nPCR[a]	Zhang et al., 2012a
	Yaks	China	158	32.3	nPCR[a]	Yang et al., 2013
	Cattle-yaks	China	20	35.0	nPCR[a]	Yang et al., 2013
	Sheep	China	70[f]	7.1	qPCR[b]	Zhan et al., 2010
			49	42.9	nPCR[a]	Yang et al., 2013
	Goat	China	35[f]	5.7	qPCR[b]	Zhan et al., 2010
			91	38.5	nPCR[a]	Yang et al., 2013
			90	48.9	nPCR[a]	Zhang et al., 2012b
			472	26.7	nPCR[a]	Zhang et al., 2012a
			262	6.1	nPCR[a]	Liu et al., 2012
AFRICA						
Domestic animals	Dog	Tunisia	228	0.9[d]	PCR[a]	M'Ghirbi et al., 2009
	Horse	Tunisia	60	13	nPCR[a]	M'Ghirbi et al., 2012

**This table does not claim completeness. It does not include studies with 0% prevalence.*

nPCR, nested PCR; qPCR, real-time PCR.

[a] 16S rRNA gene as target.

[b] Msp2 gene as target.

[c] GroEL gene as target.

[d] Total prevalence not specified in the paper, prevalence was calculated by the authors of the present manuscript

[e] Microtus spp.

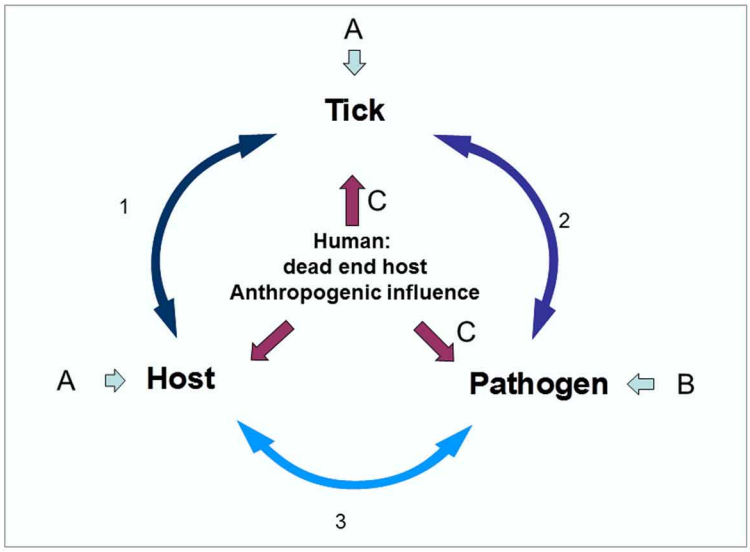
[f] Partially with symptoms.

Domestic animals including pet animals such as the dog and farm animals such as sheep and cattle may be transported to other areas, in-between countries, even continents, and can thus also add to the spread of infection. Ticks may be spread by birds over long distances and with them *A. phagocytophilum*–infected ticks. Studies from Europe indicate that migrating birds may be important in the dispersal of *A. phagocytophilum* infected *I. ricinus* (Alekseev et al., 2001b; Björsdorff et al., 2001). However, *A. phagocytophilum* DNA has sometimes been detected in ticks collected from birds at low prevalence, and it was questioned by some authors whether birds may really be involved in the spreading of the pathogen whereas other authors discussed their possible involvement (Daniels et al., 2002; Ogden et al., 2008; Franke et al., 2010; Hildebrandt et al., 2010a; Dubska et al., 2012; Palomar et al., 2012; Hornok et al., 2013; Kang et al., 2013). The involvement of birds and their ticks in the life cycle of *A. phagocytophilum* has also been tested in a transmission study in the US. For the two bird species [American robin (*Turdus migratorius*) and Gray catbird (*Dumetella carolinensis*)] involved, no significant role in the life cycle was found (Johnston et al., 2013). However, the establishment of ticks in a new habitat depends on the density of hosts in that area, the habitat structure, and the character of the local microclimate and its changes (Daniel, 1993). As an example of this complexity, **Figure 1** shows a summary of several direct and indirect factors which are influencing the occurrence and the spread of *A. phagocytophilum* to humans.

GEOGRAPHIC DISTRIBUTION AND GENETIC VARIATION

As already shown in **Tables 3–8**, *A. phagocytophilum* has a wide geographical distribution. However, there is a huge lack of knowledge on ecology, epidemiology and source attributions, vector biology and the clinical implication of different pathogenic strains, related to risk posed on animals and humans (Zhang et al., 2013). This intercepts with the development of effective prevention, control, and eradication strategies for *A. phagocytophilum*. As already mentioned, transovarial transmission does not seem to occur in tick species associated with infection of humans or animals and the dependence on reservoir animals for maintenance of infection in nature seems crucial (Ogden et al., 1998; Liz et al., 2002). Understanding the extent and mechanisms behind bacterial strain diversity, geographical distribution, and host-pathogen fitness on vector and animal level is increasingly important to give accurate estimates to veterinary and public health risks. Former and future developments in methodologies in molecular epidemiology and genetic fingerprinting like multi-locus sequence typing (MLST), pulse field gel electrophoresis (PFGE), high throughput genome sequencing, blood meal genetic analyses, and the study of microbiomes by for instance metagenomic analyses are powerful approaches to delineating bacterial population structures and the evolutionary processes that underlie these (Dumler et al., 2003; Bown et al., 2007; Dark et al., 2012).

A. phagocytophilum is currently viewed as a single bacterial species, seemingly capable of infecting a broad range of



A Availability of ticks and hosts:
✓Land structure
✓Land use and management
✓Habitat fragmentation
✓Climate and climate change
✓Survival rate of ticks
✓Spread through travel and trade

B Availability and persistence of the pathogen:
✓Spread through travel and trade
✓Development of resistence

C Encounter rate of humans and tick/pathogen:
✓ Recreational or work activities
✓ Demographic changes
✓ Self-protection

1. Tick-host-relationship
✓Availability and number of hosts
✓Questing behaviour of the tick
✓Tick-host-encounter rates
✓Duration of bloodmeal

2. Tick-pathogen-relationship
✓Duration of infection in the tick
✓Vector competence of the tick
✓Efficiency of transstadial transmission

3. Host-pathogen-relationship:
✓Susceptibility of the host
✓Length and level of infection

FIGURE 1 | Direct, indirect, and anthropogenic influences on the tick-host-pathogen relationship of *Anaplasma phagocytophilum*.

hosts based on *16S rRNA* gene analyses. The appearance of *16S rRNA* gene variants in ticks seems to be dependent on the habitat structure and therefore of the occurrence of specific potential reservoir hosts, which supports the theory of a host association of some variants (Overzier et al., 2013a,b). The situation appears to be even more complex and delicate in its partiality for certain hosts than previously foreseen, when high resolution methods are used to further delineate strains at host level. Strain variation with potential specific host tropism seems to be abundant in *A. phagocytophilum* and as such, this has to be taken into account when considering the spread of infection, and the contribution of wildlife such as wild ruminant species in infection cycles involving domestic animals and humans.

A. phagocytophilum is sometimes seen to circulate between hosts sharing similar ecological niches (Al-Khedery et al., 2012; Michalik et al., 2012). For example, phylogenetic investigations of the *groEL* gene have revealed a clustering of sequences into those from roe deer and those from others, as well as a clustering according to geographic origin (Alberti et al., 2005a,b; Silaghi et al., 2011c,d).

Investigations on several *A. phagocytophilum* strains from different hosts in California indicated that multiple unique strains of *A. phagocytophilum* with distinct host tropisms exist (Rejmanek et al., 2012). Furthermore, one study in the Western US showed no overlap in the endemic cycles found with variants from HGA cases and from the suggested wild-life reservoir, the dusky-footed wood rat (Foley et al., 2008a,b).

A. phagocytophilum 16S rRNA gene variants and possibly also *msp4*, *groEL* or *ankA* gene variants, may cycle differently in the blood of infected hosts, however, the epidemiological consequences of cyclic variation during persistent infection in different hosts are still unknown (Granquist et al., 2010c). The MSP4 is believed to be involved in the host-pathogen interaction and therefore may show host specific characteristics due to selective pressures exerted by the host immune systems, thus a high sequence heterogeneity is observed among *A. phagocytophilum* strains in this particular gene (Massung et al., 2003; de la Fuente et al., 2005a). Red deer for instance, previously shown to carry strains that show similarities with ovine strains in the *16S rRNA* (100%) and *ank* (99%) gene sequences (Stuen et al., 2001), have recently been shown to carry *msp4* genotypes that appear distinct from sheep variants (Stuen et al., 2013). This stands in contrast to earlier assumptions that red deer and occasionally roe deer may contribute to a natural transmission cycle in Europe, also involving livestock and humans (Alberdi et al., 2000; Rymaszewska,

2008). Characterization of variations in the *msp4* sequence, have shown similar structures of strains isolated from humans and dogs in the US (de la Fuente et al., 2005a). Homologous isolates from horse and donkey in California and Italy, respectively, and separate clustering in ruminants are additional examples of evolutionary aspects related to host susceptibility and geographical distribution of this organism (de la Fuente et al., 2005a). Similar patterns have been observed when comparing human, dog, and rodent strains with horse and ruminant strains based on components of the type IV secretion system (Al-Khedery et al., 2012). A German roe deer strain is different in the MSP4 by 23 amino acid changes, compared to the HZ-reference strain representing an outlier of the diversity within the species (de la Fuente et al., 2005a; Ladbury et al., 2008). The diversity of partial *msp4* gene in Norwegian sheep and Austrian wild ungulates have shown great variation in sequence types (Ladbury et al., 2008; Silaghi et al., 2011b), while little heterogeneity has been shown for this gene among isolates from horses (Silaghi et al., 2011b,d).

Investigations of the variable part of the *msp2 (p44)* gene have shown a clustering into variants obtained from ruminant species and those from dogs, horses, and humans, as well as a clustering into those from Europe and the US (Silaghi et al., 2011b,d).

The *ank* gene has also been used to assess the degree of phylogenetic relationship between strains of *A. phagocytophilum* as this gene is considered less conserved among strains and even more appropriate for high resolution phylogenetic studies (Massung et al., 2000; von Loewenich et al., 2003). In one study, *ankA* gene sequences were found to separate into four clearly distinct clusters. Sequences from dogs, humans, horses, and cats were found exclusively in cluster I, whereas samples from sheep, cows, European bison, and red deer were parts of clusters I and IV. Roe deer sequences were almost exclusively contained in clusters II and III. Based on these results, roe deer seems unlikely to be reservoir of human granulocytic anaplasmosis (Scharf et al., 2011), which supports the findings from studies mentioned earlier.

RESEARCH GOALS AND APPROACHES

Thus far, it is not clear if the differences in infection rates in vectors and hosts outlined above truly reflect differences in vector competency of the vector species and reservoir competency of the host species or whether they reflect differences in the opportunities to acquire the infections (i.e., encounter rates). Previous studies have indicated the existence of enzootic cycles of gene variants in relation to species of ticks and hosts. The knowledge about infection cycles are important for infection and disease control in domestic animals and humans. Future studies should therefore investigate the relationship between genetic strains of *A. phagocytophilum*, ticks and different hosts, by genetic fingerprinting and blood meal analysis in order to unravel the ecology and phylogeographic distribution of *A. phagocytophilum* in nature for evidence based risk assessment and risk management. Vector competence of different tick species should be studied, especially considering the potential niche cycles and great variety of strains and variations in the different geographic areas. Which hosts and vectors that competently can keep which variants in endemic cycles in nature should be unraveled.

Further studies should investigate pathogenesis and mechanisms of persistence in host infections. The complexity of cellular and humoral immune responses in rickettsial diseases may be important targets of prophylactic and metaphylactic treatment strategies to control and cure infections by *A. phagocytophilum* in animals and humans. Factors involve in pathogenicity of the different variants should therefore be elucidated.

Cell culturing and novel molecular tools allow for rapid sequencing and annotation of whole genome structure. Several comprehensive contributions on *A. phagocytophilum* proteomics from experimental studies in culture systems, tick- and mouse models have been provided (Lin et al., 2011; Troese et al., 2011; Mastronunzio et al., 2012; Kahlon et al., 2013). However, tick and ruminant host interactions with highly pathogenic strains of the bacterium, like the Norwegian Sheep variant 1 (Stuen et al., 2002), should be studied by use of proteomic approaches to reveal key elements for future control strategies in management of this intrusive disease in livestock production. Longitudinal studies to investigate antigenic variation on genomic levels during persistent infections may reveal hitherto unknown mechanisms of immune evasion and persistence, useful in development of diagnostic and therapeutic approaches. To achieve prophylaxis by vaccination further studies on mechanisms of immune evasion and infection strategies are required. The whole genome of several variants of the bacterium has to be sequenced in order to do comparative genomics and develop proper recombinant vaccine antigens for future cross-infection studies.

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Spatiotemporal dynamics of emerging pathogens in questing *Ixodes ricinus*

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Ixodes ricinus transmits *Borrelia burgdorferi* sensu lato, the etiological agent of Lyme disease. Previous studies have also detected *Rickettsia helvetica*, *Anaplasma phagocytophilum*, *Neoehrlichia mikurensis*, and several *Babesia* species in questing ticks in The Netherlands. In this study, we assessed the acarological risk of exposure to several tick-borne pathogens (TBPs), in The Netherlands. Questing ticks were collected monthly between 2006 and 2010 at 21 sites and between 2000 and 2009 at one other site. Nymphs and adults were analysed individually for the presence of TBPs using an array-approach. Collated data of this and previous studies were used to generate, for each pathogen, a presence/absence map and to further analyse their spatiotemporal variation. *R. helvetica* (31.1%) and *B. burgdorferi* sensu lato (11.8%) had the highest overall prevalence and were detected in all areas. *N. mikurensis* (5.6%), *A. phagocytophilum* (0.8%), and *Babesia spp.* (1.7%) were detected in most, but not all areas. The prevalences of pathogens varied among the study areas from 0 to 64%, while the density of questing ticks varied from 1 to 179/100 m^2. Overall, 37% of the ticks were infected with at least one pathogen and 6.3% with more than one pathogen. One-third of the *Borrelia*-positive ticks were infected with at least one other pathogen. Coinfection of *B. afzelii* with *N. mikurensis* and with *Babesia spp.* occurred significantly more often than single infections, indicating the existence of mutual reservoir hosts. Alternatively, coinfection of *R. helvetica* with either *B. afzelii* or *N. mikurensis* occurred significantly less frequent. The diversity of TBPs detected in *I. ricinus* in this study and the frequency of their coinfections with *B. burgdorferi* s.l., underline the need to consider them when evaluating the risks of infection and subsequently the risk of disease following a tick bite.

Keywords: vector-borne disease, *Borrelia burgdorferi, Candidatus Neoehrlichia mikurensis, Rickettsia helvetica, Rickettsia conorii, Anaplasma phagocytophilum, Babesia, Ixodes ricinus*

INTRODUCTION

In The Netherlands, the hard tick *Ixodes ricinus* is the main vector of a variety of human pathogens. The most prevalent tick-borne disease is Lyme borreliosis (Stanek et al., 2012). This multisystemic disorder is caused by several members of the *Borrelia burgdorferi* sensu lato complex. Of the 18 genospecies of this complex (Margos et al., 2011), *B. afzelii, B. garinii, B. spielmanii, B. bavariensis*, and *B. burgdorferi* sensu stricto have already been detected in The Netherlands, in both patients and questing ticks. *B. lusitaniae*, and *B. valaisiana* were detected in questing *I. ricinus*, but their public health significance is less clear (Collares-Pereira et al., 2004; Diza et al., 2004; De Carvalho et al., 2008; Coipan et al., 2013).

Over the last decades, the incidence of Lyme borreliosis has increased significantly in Europe (Smith and Takkinen, 2006). A long-term retrospective study among general practitioners in The Netherlands has shown a continuing increase in consultations for tick bites and *erythema migrans* in the last decade (Hofhuis et al., 2006). The incidence of *erythema migrans* patients increased from 39 per 100,000 inhabitants in 1994 to 134 per 100,000 inhabitants in 2009.

Previous studies in The Netherlands have identified the presence of other pathogens in questing *I. ricinus* as well. Human babesiosis is caused by the intraerythrocytic protozoa *Babesia divergens, B. venatorum (EU1)*, and *B. microti* (Vannier and Krause, 2012). A recent study reported these three *Babesia* species in approximately 1% of questing *I. ricinus* (Wielinga et al., 2009). The spotted fever syndrome is caused by at least 15 different *Rickettsia* species, some of which are transmitted by *I. ricinus* (Heyman et al., 2010). *Rickettsia conorii* and *R. monacensis* are probably the most common tick-borne *Rickettsiae* to cause disease in Europe (Heyman et al., 2010), whereas the pathogenicity of *R. helvetica* is still questionable (Svendsen, 2011). All three rickettsial species have been previously found in The Netherlands (Sprong et al., 2009) with local prevalences varying from <1% (*R. conorii*) to as high as 66% (*R. helvetica*). *Anaplasma phagocytophilum*, the etiologic agent of human granulocytotropic anaplasmosis (Sprong et al., 2009), has been detected

in Dutch ticks in several studies (Tijsse-Klasen et al., 2010, 2011b). *Neoehrlichia mikurensis* can be considered an emerging zoonosis, as more than eight human cases have been described in Europe since 2010, while previously it was considered non-pathogenic. Despite an overall prevalence of *N. mikurensis* in questing ticks of approximately 7% (Jahfari et al., 2012), no human cases have been reported in The Netherlands.

Autochthonous tick-borne diseases other than Lyme disease have not been reported, except for a single case of granulocytotropic anaplasmosis in 1999 (Van Dobbenburgh et al., 1999). This may be caused by lower pathogenicity, lack of overt symptoms, or lack of awareness of public and health professionals.

Multiple studies have reported coinfection with some of the tick-borne pathogens (TBPs) (Belongia, 2002; Ginsberg, 2008; Nieto and Foley, 2009; Reye et al., 2010; Burri et al., 2011; Lommano et al., 2012). It is known that the severity of Lyme disease may be affected by simultaneous infections with other TBPs (Belongia, 2002; Swanson et al., 2006). Some of them, such as *A. phagocytophilum*, modulate host immunity and increase susceptibility to various second pathogens, including *B. burgdorferi* (Thomas et al., 2001; Holden et al., 2005). Thus, coinfection might be partly responsible for the variability in clinical manifestations that are usually associated with Lyme borreliosis.

The acquirement of a tick-borne disease depends on many environmental, societal, and immunological factors, but it is always preceded by the bite of a tick infected with the causal agent. Previous studies have shown that the risk of infection of humans by TBPs depends mainly on the density of questing infected ticks—the acarological risk (Glass et al., 1994, 1995; Nicholson and Mather, 1996; Dister et al., 1997; Kitron and Kazmierczak, 1997). The study of mixed infections in questing ticks might, therefore, reveal patterns of coinfection of *B. burgdorferi* s.l. with two or more other pathogens, allowing us to generate hypotheses on the transmission cycle of some more obscure pathogens from the dynamics of better-known ones. The aim of this study was to assess the acarological risk of exposure to TBPs in The Netherlands by comparing the abundances of questing ticks infected with *B. burgdorferi* s.l. and with other TBPs.

METHODS

COLLECTION OF TICKS AND TICK DATA

All ticks were collected on a monthly basis between 2006 and 2010 in 21 sites. In Duin&Kruidberg field sampling was conducted between 2000 and 2009. The sites were spread all over The Netherlands and they have been selected based on Lyme borreliosis incidence in humans, and the availability of volunteers. The same sites were described in some previous studies regarding ticks and TBPs in The Netherlands (Wielinga et al., 2006, 2009; Sprong et al., 2009; Gassner et al., 2011; Tijsse-Klasen et al., 2011a). Sampling of ticks was done by blanket dragging, using a 1 m^2 cloth on a 100 m long transect. Based on morphological criteria, ticks were identified to species level, with stage and sex recorded. The density of ticks was estimated as the number of questing ticks *per* 100 m^2. Additionally, data on the presence of ticks and TBPs in other 39 areas were collected from some previous studies that have used the same sampling and analysis methodology (Schouls et al., 1999; Tijsse-Klasen et al., 2010, 2011b; Jahfari et al., 2012).

DNA EXTRACTION OF TICKS

All the collected ticks were immersed in 70% alcohol and stored at −20°C until the DNA extraction. DNA from questing ticks was extracted by alkaline lysis (Wielinga et al., 2006). Questing larvae were not taken into account as humans are generally bitten by either nymphs or adult *I. ricinus* (Hugli et al., 2009; Tijsse-Klasen et al., 2011b).

PCR DETECTION AND IDENTIFICATION OF PATHOGENS

The presence of the DNA of different TBPs (*Rickettsia spp., B. burgdorferi* s.l., *Ehrlichia/Anaplasma spp.,* and *Babesia spp.*) was determined by polymerase chain reaction (PCR) followed by reverse line blotting (RLB) as described before (Wielinga et al., 2006; Tijsse-Klasen et al., 2010). To minimize cross contamination and false-positive results, positive and negative controls were included in each batch tested by PCR and RLB assays. Furthermore, DNA extraction, PCR mix preparation, sample addition, and PCR analysis were performed in assigned separate labs. PCR products of some samples were sequenced by dideoxy-dye termination sequencing of both strands, and compared with sequences in GenBank (http://www.ncbi.nlm.nih.gov/), using BLAST (Altschul et al., 1990). The sequences were aligned and analysed using BioNumerics 6.6 (Applied Maths, Kortrijk, Belgium).

The prevalence of infection was calculated as the percentage of ticks infected with a certain microorganism.

ACAROLOGICAL RISK

To calculate the densities of questing ticks infected with each of the five pathogens' genera, we multiplied the prevalence of infection with the density of questing ticks in each of the investigated sites.

CORRELATION BETWEEN PREVALENCE AND TICK DENSITY

For some pathogens, we noticed that the prevalence might correlate with the density of questing ticks at the sampling locations. To test this possibility we fitted a binomial model to our data, by defining the prevalence of infection as an exponential function of the tick density (d) at each sampling location. Knowing that the number of infected ticks (k) out of the total number of ticks tested (n), is binomially distributed with a probability (p), we used the function $p = aExp[bd], 0 < a < 1$, to test an alternative model ($b < 0$) against a null model ($b = 0$) by a likelihood-ratio test. The alternative (decreasing exponential) model fitted significantly better to our data with p-value $P \leq 0.05$.

SEASONAL DYNAMICS

To test for the seasonal dynamics of infection in ticks, a binomial model for the probability of infection (p) was fitted to our data, in combination with the sampling days (t) and pooled over the sampling locations and development stages. The probability of infection was a logit function $p = \frac{e^y}{1+e^y}$ and we modeled seasonality by using the trigonometric function $y = a + b\cos(2\pi t/365) +$

$c\sin(2\pi t/365)$ to describe an oscillation with a period of 1 year and possible phase shift. For each pathogen, we tested the seasonal model against the non-seasonal model ($y = a$) based on a likelihood ratio test. The seasonal model fitted significantly better to our data with p-value $P \leq 0.05$. All the statistical analyses were performed using Wolfram Mathematica 9.

RESULTS

The mean density of questing nymphs and adult ticks varied greatly between sites, from as low as 1 (at Houtvesterijen Heide) up to 179/100 m^2 (at Duin&Kruidberg; **Table 2**), results that are consistent with previous Dutch studies (Wielinga et al., 2006).

PATHOGEN DETECTION AND IDENTIFICATION

A total of 5570 questing nymphs and adult *I. ricinus* from 22 different study areas were tested for the presence of TBPs by PCR amplification followed by RLB (**Table 1**). The recently identified *B. bavariensis* reacted consistently with our *B. garinii* probe (Margos et al., 2009), and therefore we grouped these two *Borrelia* genospecies. Five *Borrelia* genospecies were found in this study in all twenty-two study areas (**Table 1**), with the overall prevalence (11.8%) inscribed in the interval of average European prevalence (Rauter and Hartung, 2005), and comparable with previous studies in The Netherlands (Wielinga et al., 2006; Gassner et al., 2011). *B.afzelii* was predominant (6.7%), followed by *B. garinii/B. bavariensis* (1.5%), *B. valaisiana* (1.2%), and *B. burgdorferi* sensu stricto (0.2%). The remaining fraction of the *Borrelia* positive samples could not be further identified to the species level by RLB. Sequencing several of these samples revealed the presence of *B. spielmanii,* corroborating previous findings of this genospecies in The Netherlands (Wang et al., 1999). *B. lusitaniae*, which was recently found in The Netherlands (Tijsse-Klasen et al., 2010), was not detected in this study.

R. helvetica was most frequently detected in tick lysates, its 31.1% average prevalence (**Table 1**) being among one of the highest in Europe [range 1.5 to more than 40.6% (Christova et al., 2003; Severinsson et al., 2010)]. A previous study from our laboratory found *R. helvetica* not only in vertebrate hosts, but also in tick larvae at comparable prevalences as for the other tick stages, indicating a high efficiency of transovarial transmission (Sprong et al., 2009). Thirty-three *Rickettsia* isolates could not be identified up to the species level by RLB. Sequencing of these samples revealed the presence of *R. monacensis*, which was reported in The Netherlands before (Sprong et al., 2009). *Rickettsia conorii* was detected in only three questing ticks from one study area (Veldhoven). *A. phagocytophilum*-infected ticks were recorded with an overall prevalence of only 0.8% (**Table 1**). *N. mikurensis* DNA was found with a global prevalence of 5.6% (**Table 1**). *Ehrlichia canis* DNA was detected in only 5 tick lysates from four different study areas, which resulted in an overall prevalence of 0.1% (5/5343). Ninety-nine *Ehrlichia* isolates could not be identified to the species level neither by RLB nor by sequencing. *Babesia venatorum*, formerly also known as *B.EU1* (Duh et al., 2005), was present with a global prevalence of 1.0% (41/4238). The prevalence of *B. microti* in questing ticks was 0.4% (17/4238), and the

Table 1 | Presence of microorganisms in questing *I. ricinus* nymphs and adults.

Pathogen	Positive/tested *I. ricinus* (*n*)	Prevalence *I. ricinus* (%)	95% CL		Tested/%positive study areas
			LCL	UCL	
Borrelia burgdorferi s.l.	628 (5308)	11.8	11.0	12.7	22/100
–*Borrelia afzelii (and B.ruski)*	355 (5308)	6.7	6.0	7.4	22/100
–*Borrelia garinii/B.bavariensis*	79 (5308)	1.5	1.2	1.9	22/77.3
–*Borrelia valaisiana*	64 (5308)	1.2	0.9	1.5	22/81.8
–*Borrelia burgdorferi* ss.	10 (5308)	0.2	0.1	0.4	22/36.4
–Untypeable *Borrelia*	133 (5308)	2.5	2.1	3.0	22/90.9
Rickettsia helvetica	1265 (4061)	31.1	29.7	32.6	19/100
Rickettsia conorii	3 (4061)	0.1	0.0	0.2	19/5.3
Untypeable *Rickettsia*	33 (4061)	0.8	0.6	1.1	19/68.4
Anaplasma phagocytophilum	44 (5343)	0.8	0.6	1.1	22/63.6
Neoehrlichia mikurensis	300 (5343)	5.6	5.0	6.3	22/81.8
Ehrlichia canis	5 (5343)	0.1	0	0.2	22/18.2
Untypeable *Ehrlichia*	99 (5343)	1.9	1.5	2.3	22/72.7
Babesia microtii	17 (4238)	0.4	0.2	0.6	19/31.6
Babesia venatorum (EU1)	41 (4238)	1.0	0.7	1.3	19/73.7
Babesia divergens	1 (4238)	0.0	0.0	0.1	19/5.3
Untypeable *Babesia*	12 (4238)	0.3	0.2	0.5	19/63.2

Tick lysates were subjected to PCR followed by Reverse Line Blotting. PCR products that specifically reacted to the generic ("catch all") probes, but that could not be further specified to the (geno)species level were designated as Untypeable. Reverse Line Blot analysis could not distinguish between B. garinii and the recently identified B. bavariensis. Calculations of prevalence were based on all tick lysates that were analysed (n).

CL, confidence limits; LCL, lower confidence limit; UCL, upper confidence limit.

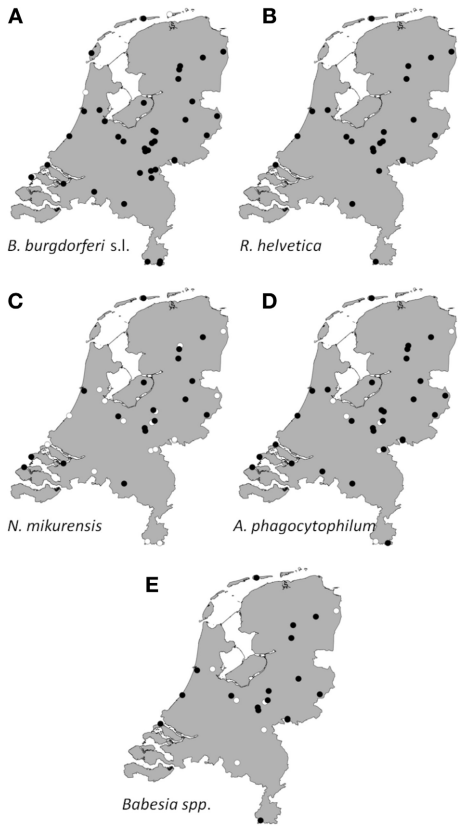

FIGURE 1 | Aggregated presence/absence map of questing *I. ricinus* nymphs/adults infected with *B. burgdorferi* s.l. (A), *R. helvetica* (B), *N. mikurensis* (C), *A. phagocytophilum* (D), *Babesia* species (E). The black dots represent presence of the microorganism; the white ones represent absence. Presence/absence points from previous studies were also incorporated.

protozoan was detected in 6 from 19 sites. Only one tick from the Duin&Kruidberg area contained the DNA of previously reported *B. divergens* (Wielinga et al., 2009). Twelve *Babesia sp*. could not be further identified by neither RLB, or sequencing. The average prevalence of *Babesia*-positive ticks in the study areas was 1.6% (**Table 1**).

SPATIAL DISTRIBUTION AND VARIATION

Collated data were used to generate presence/absence maps of the five major TBPs in The Netherlands (**Figure 1**). The presence/absence of *Borrelia spp., R. helvetica, A. phagocytophilum, N. mikurensis*, and *Babesia spp*. was assessed for 61, 24, 39, 39, and 25 locations, respectively. The presence of these pathogens was observed in 58, 24, 33, 20 and 18 areas, respectively, heterogeneously distributed across The Netherlands. The few absence points were scattered over The Netherlands as well, and did not cluster in any geographic region (**Figure 1**).

Borrelia prevalence was between 5% (Houtvesterijen Heide) and 50% (Bellingwedde; where only six ticks were tested), while for *R. helvetica* it varied even more, from 3% in some sites (Apeldoorn), to 64% in others (Duin&Kruidberg) (**Table 2**). Lower variations in prevalences were observed for *N. mikurensis*, *A. phagocytophilum* and *Babesia spp*. (**Table 2**). For *N. mikurensis*, the prevalence was on average of 5%, but some areas displayed values of over 10% (**Table 2**). *Babesia spp*. showed an overall prevalence of 1.7%, similarly to Germany and Luxembourg (Hartelt et al., 2004; Reye et al., 2010). *A. phagocytophilum* was the least prevalent pathogen in our study, with a mean prevalence of 0.8%—comparable with the 0.5–1% prevalence found in different European countries (Koci et al., 2007; Hildebrandt et al., 2010; Severinsson et al., 2010). However, one of the sites displayed a 10-fold higher prevalence than average (Bilthoven 8%, **Table 2**).

Identification of high risk-areas depends on both pathogen prevalence and density of questing ticks (nymphs and adults). The density of questing ticks varied between 1/100 m^2 (Houtvesterijen Heide) and 179/100 m^2 (Duin&Kruidberg; **Table 2**). The density of questing *Borrelia*-infected ticks varied between 0 and 19 ticks per 100 m^2 (**Figure 2**), whereas the maximum densities of *A. phagocytophilum, N. mikurensis* and *Babesia spp.* infected ticks were 3.0, 13, and 2.9 ticks per 100 m^2, respectively. The density of questing *R. helvetica*-infected ticks varied between 0 and 22 ticks per 100 m^2, with one notable exception: Duin&Kruidberg area had both a high tick density and an exceptionally high *R. helvetica* prevalence, which resulted in a density of questing *R. helvetica*-infected ticks of up to 119 ticks per 100 m^2. Considering that these are calculated as average values for an entire season, it is therefore inevitable that the densities of infected questing ticks are actually higher for peak months of tick activity [i.e., May-June (Gassner et al., 2011)].

Based on a likelihood ratio test, performed for a decreasing model and a constant one, we detected a significant negative correlation between the density of questing ticks and the infection prevalence with *B. burgdorferi* s.l. ($p = 3.6 \times 10^{-10}$) and *Babesia spp.* ($p = 4.9 \times 10^{-5}$) (**Figure 3**). On the other hand, there was no correlation found between these variables for *R. helvetica* ($p = 1.0$), *N. mikurensis* ($p = 1.0$) and *A. phagocytophilum* ($p = 0.69$) (**Figure 3**). Graphs for the density of infected questing ticks against the density of questing ticks revealed that the former is linearly increasing with the latter for *R. helvetica*, *N. mikurensis* and *A. phagocytophilum* (**Figure 4**). For the other two pathogens—*Babesia spp.* and *B. burgdorferi* s.l., the density of infected questing ticks reached the maximum values at densities of questing ticks of 119 and 268, respectively (**Figure 4**).

TEMPORAL VARIATION

To gain insight into long-term dynamics of ticks and their pathogens, we analysed the data obtained from Duin&Kruidberg, where a 10-year (2000–2009) tick-surveillance was performed. This area was selected at that time because of its unusual high tick density/activity. The prevalences of all pathogens were relatively stable over the past decade (*B. burgdorferi* s.l. 7.0%, *B. afzelii* 4.6%, *A. phagocytophilum* 0.7%, *R. helvetica* 65%, *Babesia spp.* 1.1%), except for *N. mikurensis*, whose prevalence increased from 3.5% (2000–2007) to 12% in the last 2-year interval (2008–2009). The average density/activity of adult ticks remained relatively low with 7–34 ticks per 100 m^2. The average density/activity of nymphal ticks was more pronounced

Table 2 | Prevalence (%) of the five major pathogens found in the 22 study areas.

Geographic location	*B. burgdorferi* s.l.			*R. helvetica*			*N. mikurensis*			*A. phagocytophylum*			*Babesia spp.*			Density (/100 m^2)	
	+	T	%	+	T	%	+	T	%	+	T	%	+	T	%	Nymphs	Adults
Apeldoorn	15	38	39	1	38	3	3	38	8	0	38	0	5	38	13	17	5
Appelscha	10	79	13	11	76	14	3	79	4	0	79	0	4	79	5	19	5
Bellingwedde	3	6	50	2	6	33	0	6	0	0	6	0	0	6	0	7	0.3
Bijlmerweide	34	330	10			ND	1	330	0.3	0	330	0			ND	12	1
Bilthoven	4	40	10	6	40	15	0	40	0	3	40	8	1	40	3	9	3
Duin& Kruidberg	123	1640	8	848	1327	64	113	1676	7	11	1676	1	12	1499	1	160	19
Ede	48	354	14	23	354	6	36	353	10	3	353	1	2	353	1	54	7
Eijsden	28	232	12	23	232	10	0	232	0	1	232	0.4	10	232	4	34	1
Gieten	10	136	7	31	136	23	6	136	4	2	136	1	2	136	1	59	5
Haaksbergen	9	105	9	11	105	10	1	105	1	4	105	4	2	105	2	77	2
Hoge Veluwe	2	8	25	2	8	25	0	8	0	0	8	0	0	8	0	25	1
Hoog Baarlo	28	311	9	24	311	8	2	311	1	4	311	1	9	311	3	34	2
Hoogeveen	47	163	29	48	163	29	11	163	7	0	163	0	5	163	3	63	3
Houtvest_Bos	49	510	10			ND	35	510	7	4	510	1			ND	32	2
Houtvest_Heide	4	88	5			ND	5	88	6	1	88	1			ND	1	0.2
Kwade Hoek	43	162	27	13	162	8	23	162	14	0	162	0	3	162	2	9	4
Montferland	18	1470	12	12	147	8	11	147	7	0	147	0	3	147	2	40	3
Nijverdal	24	127	19	13	127	10	18	127	14	1	127	1	8	127	6	34	2
Ruinen	25	94	27	30	94	32	2	94	2	2	94	2	1	94	1	18	1
Twiske	46	292	16	62	292	21	13	292	4	1	292	0.3	0	292	0	36	2
Veldhoven	25	242	10	14	239	6	13	242	5	6	242	2	1	242	0.4	47	17
Wassenaar	33	204	15	91	204	45	4	204	2	1	204	0	3	204	1	46	3
Total/Average	628	5308	11.8	1265	4061	31.1	300	5343	5.6	44	5343	0.8	71	4238	1.7	38	4

Tick density/activity of each study area was expressed as the average density/activity of the questing ticks collected from April to September from at least three consecutive years. Average prevalences of the study areas (n = 22) were calculated. +, positive samples; T, tested; ND, Not determined.

(102–410 ticks per 100 m^2) and peaked in 2004–2005 (**Figure 5**). The likelihood ratio test detected similar decreasing trends in the temporal relation between the prevalence and the tick density as for the spatial variation analysis (not shown). Despite the inverse relationship between the prevalence and the tick density, the peaks of density/activity of *infected* ticks coincided with the peak of high densities of questing ticks in 2004–2005 (**Figure 5**).

COINFECTION

Overall, 37% (2064/5570) of the ticks was infected with one or more pathogens and 6.3% (350/5570) with more than one pathogen of different genera. Furthermore, 37% (234/628) of the *Borrelia*-positive ticks were infected with at least one other pathogen of a different genus. Almost 5% (29/628) of the *Borrelia*-positive ticks were also positive for three or more other pathogens. One tick carried the DNA of *B.afzelii, R. helvetica, N. mikurensis*, and *B.microti*. Mixed infections, involving two or three *Borrelia* genospecies, occurred in only 0.3% (15/5308) of the tick lysates. Coinfection of *B. afzelii* with *N. mikurensis* or with *Babesia spp.* occurred significantly more than random, whereas infection of *R. helvetica* with either *B.afzelii* or *N. mikurensis* occurred significantly less frequent (**Table 3**).

SEASONAL DYNAMICS

Seasonality modeling of the prevalence indicated a different periodicity of the analysed pathogens (**Figure 6**). Thus, *B. afzelii, N. mikurensis* and *Babesia spp.* showed highest prevalences in ticks at time periods corresponding to October, while non-*afzelii B. burgdorferi* and *R. helvetica* had the highest prevalence around June. Annual prevalence of *A. phagocytophilum* was not seasonal.

DISCUSSION

In order to assess the acarological risk of acquiring a tick-borne infection in The Netherlands, the abundance of questing ticks infected with *B. burgdorferi* s.l. and four other genera of TBPs were compared.

Our study revealed the nationwide circulation of TBPs in enzootic cycles. Although the most common tick-borne infection is acknowledged to be Lyme borreliosis, our results showed that there are other pathogens present in questing ticks at prevalences comparable with *B. burgdorferi* (i.e., *R. helvetica*, **Table 2**). Due to the fact that our investigations only detected the DNA of the microorganisms under discussion, and not the viable cells, we cannot asseverate their infectiousness for other vertebrate hosts. However, previous studies implicate *Ixodes ricinus* ticks as vectors for these microorganisms (Barbour et al., 1983; Ackermann et al.,

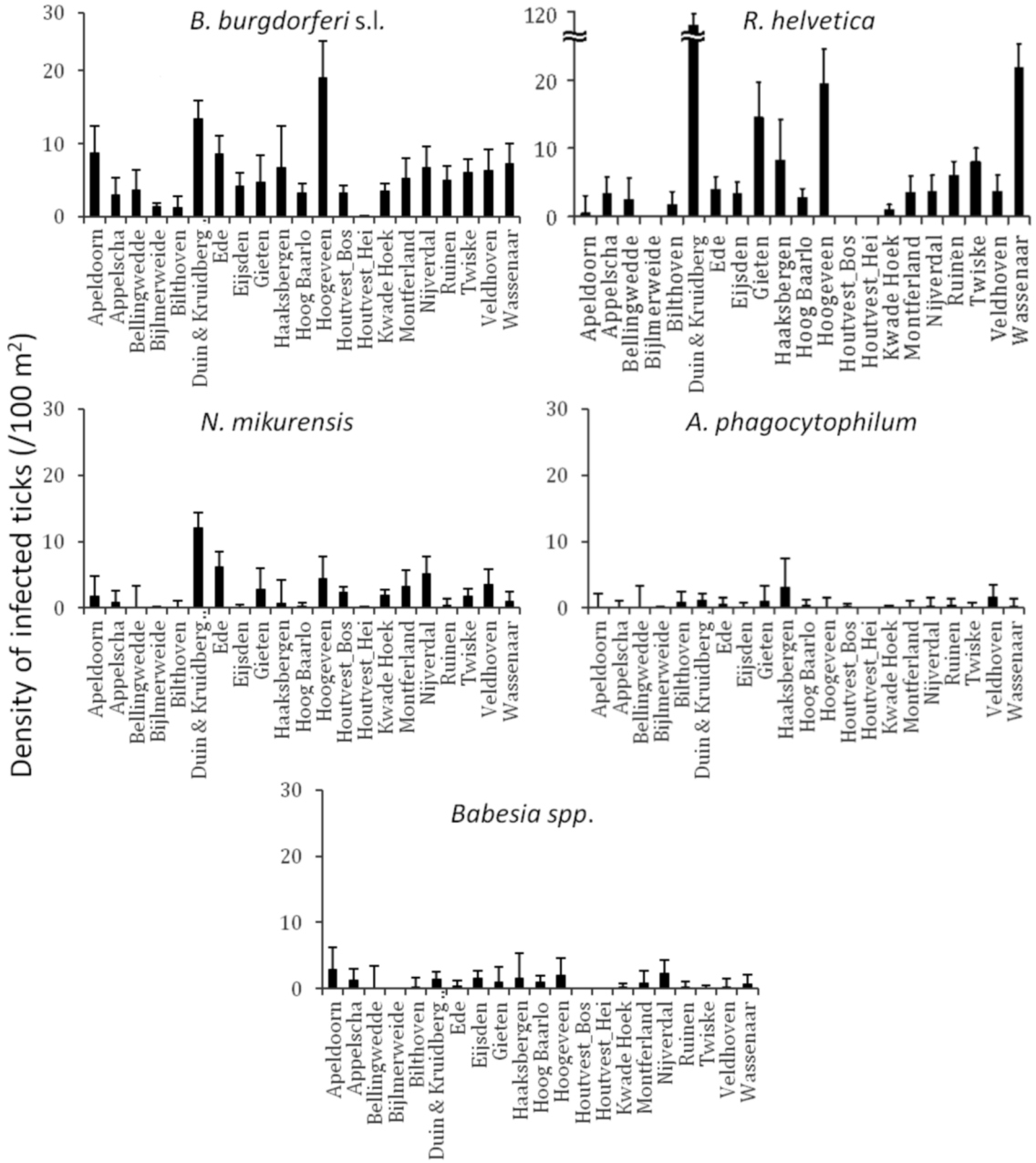

FIGURE 2 | Identification of high risk-areas depends on both prevalence and tick density/activity. Their calculated product defines the density/activity of infected ticks (nymphs and adults/100 m^2). The error bars depict the upper limit of the 95% confidence interval. Duin&Kruidberg's density of *R. helvetica* infected ticks reaches to 119/100 m^2.

1984; Lotric-Furlan et al., 1998; Rydkina et al., 1999; Kjemtrup and Conrad, 2000; Parola and Raoult, 2001; Gray et al., 2002; Bonnet et al., 2007; Sprong et al., 2009; Heyman et al., 2010; Jahfari et al., 2012), and therefore the risk for public health should not be neglected. Although no human disease with the organisms other than *B. burgdorferi* s.l. was reported so far in The Netherlands, it is known that infection with some of th0em (e.g., *Ehrlichia*) is generally either asymptomatic or mild, self-limiting diseases (Ismail et al., 2010).

SPATIAL DISTRIBUTION AND VARIATION

All the pathogens were observed in most of the areas in which investigations were conducted, regardless of the geographical position. The absence in certain areas might be explained by the relatively low number of ticks collected/tested (**Table 2**). The prevalences of infection in the ticks varied significantly between the areas investigated. The lack of a full perspective on the host community at each of the sites does not allow us to make a definite statement on why we see such a variation of the prevalence

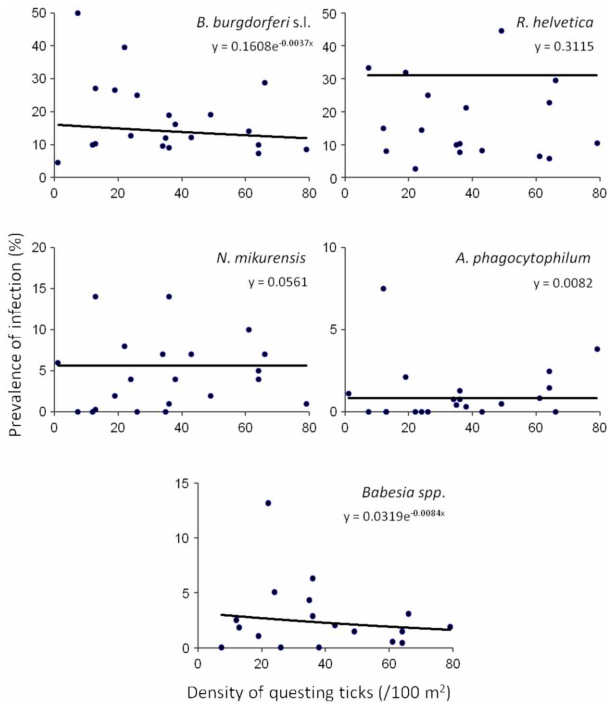

FIGURE 3 | Density and prevalence relations. Significant negative correlations between the density of questing ticks and the infection prevalence were found for *B. burgdorferi* s.l. ($p = 3.6 \times 10^{-10}$) and *Babesia* spp. ($p = 4.9 \times 10^{-5}$). On the other hand, there was no correlation found between these variables for *R. helvetica* ($p = 1.0$), *N. mikurensis* ($p = 1.0$), and *A. phagocytophilum* ($p = 0.69$). Note that due to the very small exponents, the curves look approximately linear, although they are in fact exponential, as explained in the text. The data set included all of the areas except for Duin&Kruidberg.

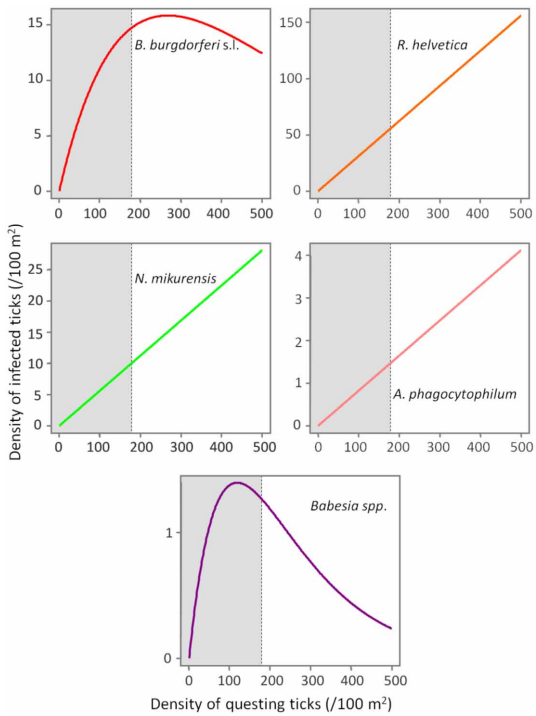

FIGURE 4 | Evolution of the density of infected ticks (y-axis) with the density of questing ticks (x-axis). The density of infected ticks is obtained by fitting a model ($p = aExp[bd]$) to a range of questing ticks densities. The numbers are expressed as ticks/100 m^2. The gray area marks the normal questing ticks densities (0–179/100 m^2) in The Netherlands.

of infection. We propose, however, that the extremely high local variability of the pathogens may be associated with the differences in host assemblages in the investigated habitats. As ticks can feed on many different animals and every host species has a unique reservoir competence [e.g., rodents being the most competent reservoirs of *B. afzelii* (Gern and Humair, 2002)], the presence of different hosts in different communities affects the prevalence of infection with various microorganisms.

In terms of the risk for public health, neither the density of questing ticks, nor the prevalence of infection alone, has any significance. Instead, it is their product—the density of infected questing ticks—that defines high or low risk areas (Glass et al., 1994, 1995; Nicholson and Mather, 1996; Dister et al., 1997; Kitron and Kazmierczak, 1997). We noticed that in some areas, where tick densities were highest, the mean prevalence of *Borrelia* infection had very low values (8% for Duin&Kruidberg; **Table 2**). Using a log-likelihood ratio statistics, we tested the hypothesis of a constant prevalence over the range of questing ticks density. The test confirmed the independence of the two variables but only for *R. helvetica*, *N. mikurensis*, and *A. phagocytophilum*, while for *B. burgdorferi* and *Babesia spp.* it indicated a slight negative correlation of the prevalence with the tick density (**Figure 3**). Thus, we would expect that the density of ticks infected with *B. burgdorferi* and *Babesia spp.* would decrease as the density of questing ticks increases. Plotting the density of infected questing ticks as an exponential function of the questing ticks' densities, however, revealed that over the usual range of questing ticks densities the density of infected ticks is also increasing, and the downward trend might be observed only for questing ticks densities of over 100 (for *Babesia spp.*) or 200/100 m^2 (for *B. burgdorferi*) (**Figure 4**). This observation is consistent with the finding made by Randolph (Randolph, 2001) that, in Europe the density of *Borrelia* infected ticks depends much more on the density of all ticks than on the infection prevalence, and that only in areas where the tick density is unusually high (100–450/100 m^2) is the infection prevalence consistently low.

TEMPORAL VARIATION

In terms of temporal variation, the longest series of data we had was for 10 successive years (Duin&Kruidberg, **Figure 5**). At this site, the density of questing ticks was highest in 2004–2005, and it was due to a steep increase in the number of questing nymphs. The variations in tick density might indicate yearly fluctuations in the composition and availability of reservoir hosts. For example, a mast year might have been responsible for the increment in small mammals' population size (i.e., rodents), with the upsurge of nymphs at a consequential rate. The trend line indicated the maintenance of relatively constant

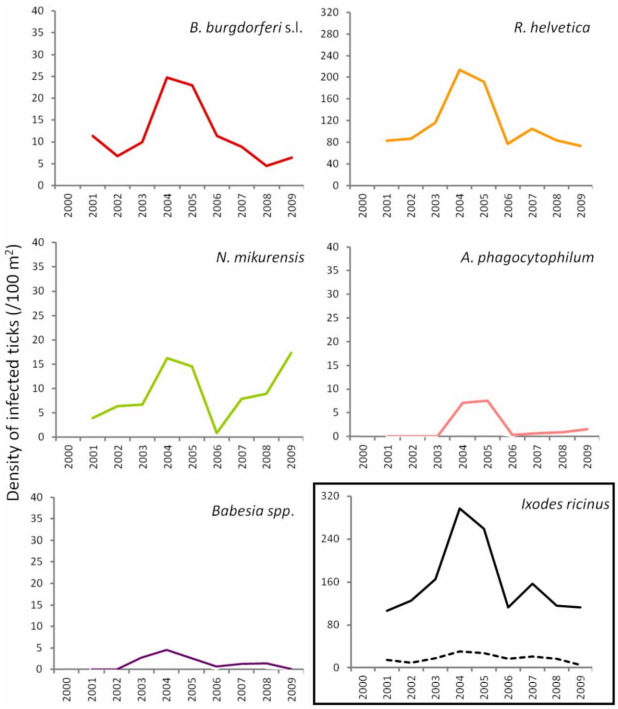

FIGURE 5 | Changing average (2 years) of density of infected ticks and tick density/activity in Duin&Kruidberg area. Density/activity of nymphs and adults are shown in the bottom right graph as continuous and dotted line, respectively.

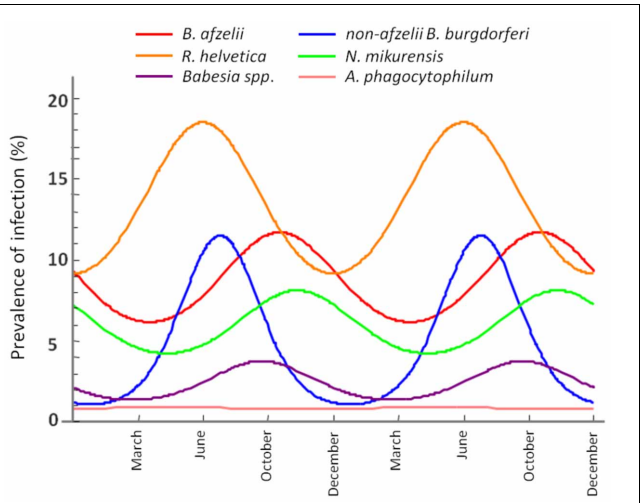

FIGURE 6 | Seasonal variation of the infection rate in ticks. The maximum infection rates of non-*afzelii B. burgdorferi*, and *R. helvetica* are in June, while the maximums of *B. afzelii, N. mikurensis*, and *Babesia spp.* overlap in October.

Table 3 | Observed and expected coinfections.

	R. helvetica	*A. phagocytophilum*	*N. mikurensis*	*Babesia spp.*
OBSERVED (%)				
Borrelia (all)	3.3	0.1	1.6	0.4
B. afzelii	1.8	0.0	1.3	0.3
R. helvetica		0.3	2.2	0.5
A. phagocytophilum			0.0	0.0
N. mikurensis				0.1
EXPECTED (%)				
Borrelia (all)	3.9	0.1	0.7	0.2
B. afzelii	2.2	0.1	0.4	0.1
R. helvetica		0.3	1.9	0.5
A. phagocytophilum			0.0	0.0
N. mikurensis				0.1
χ^2-TEST (*p*-VALUE)				
Borrelia (all)	**0.03****	0.30	**0.00***	**0.01***
B. afzelii	**0.03****	0.24	**0.00***	**0.00***
R. helvetica		0.80	**0.05****	0.77
A. phagocytophilum			0.10	0.42
N. mikurensis				0.66

*χ^2-tests were used to calculate the associations of several combinations of pathogens. *Significant positive associations and **significant negative associations ($p < 0.05$) are shown in bold.*

prevalences for *B. burgdorferi, A. phagocytophilum*, and *R. helvetica.Babesia* prevalence showed a slight decrease over time while, on the contrary, *N. mikurensis* showed a steep increase (almost 3-fold). The maintenance of relatively constant prevalences of infection in time implies that the acarological risk is predominantly dependent on the density/activity of ticks (**Figure 5**).

COINFECTION

One-third of the ticks infected with *Borrelia* were also infected with at least one other TBP. Recent studies in other European countries have shown that mixed infections of the TBPs do not represent an exception but more likely the rule.

A negative significant association was found between all *Borrelia* (and *B. afzelii* alone) and *R. helvetica*, as well as between *N. mikurensis* and *R. helvetica* (**Table 3**). On the other hand, significant positive associations were found between *Borrelia* (and particularly *B. afzelii*) and *N. mikurensis* and between *Borrelia* and *Babesia spp.* (**Table 3**). These findings lead us to the hypothesis that *B. afzelii, N. mikurensis*, and *Babesia* might share the same reservoir hosts, while *R. helvetica* is maintained in other enzootic cycles.

SEASONAL DYNAMICS

Further evidence for our hypothesis came from the seasonality modeling of the infection prevalence. This indicated a variation in the same phase for *B. afzelii, N. mikurensis* and *Babesia spp.* on the one side and for non-*afzelii B. burgdorferi* and *R. helvetica* on another (**Figure 6**). That means that the infection peak in questing ticks is different for different pathogens, further suggesting that they were acquired from the distinct vertebrate hosts. Scientific literature confirms this. Rodents are known to be competent transmission hosts for *B. afzelii* (Gern and Humair, 2002; Hanincova et al., 2003a) and *B. microti* (Gray et al., 2002), and

they have been designated as potential reservoirs for *N. mikurensis* (Ginsberg, 2008; Andersson and Raberg, 2011). On the other hand, non-*afzelii Borrelia*, like *B. garinii* and *B. valaisiana* have been shown to be associated with birds (Gern and Humair, 2002; Hanincova et al., 2003b), while a study of de la Fuente and co-workers (De La Fuente et al., 2005) found that *A. phagocytophilum* infections occurred in deer, cattle and various bird species, meaning that birds might serve as reservoirs for both these bacteria. *R. helvetica* was previously found at high rates in both rodents (29%) and roe deer (19%) (Sprong et al., 2009). The fact that *R. helvetica* was negatively associated with *B. afzelii*, although they might share the same hosts, is possibly due to that the former is transovarially transmitted in ticks which act thus as both vectors and reservoirs of the rickettsiae (Sprong et al., 2009); therefore, they alone can be responsible for the maintenance of the bacteria, without the intervention of a rodent host in the cycle. Hence, our findings are not coincidental, and indicate that certain coinfections are more likely to occur than the others, given particular combinations of vertebrate hosts.

Although previous meta-analyses indicate that coinfection and co-exposure for some of the TBPs appear to occur somewhat unpredictably across different areas and different hosts (Nieto and Foley, 2009), it is anticipated that future wildlife studies will help define geographical risks of coinfection and provide insight into the dynamics of infection within reservoir hosts.

CONCLUSION

We have shown that ticks and the five genera of TBPs have a ubiquitous distribution in The Netherlands, with the few absence point presumably determined by the small number of collected ticks. The pathogens were found in sites all over The Netherlands, encompassing a variety of habitats, from open areas such as dune and heather to deciduous or coniferous forests.

This study brings valuable information on the prevalence, geographic distribution and temporal variation of *B. burgdorferi* s.l., *R. helvetica, N. mikurensis, A. phagocytophilum* and *Babesia spp.* in questing *I. ricinus*. Due to their omnipresence, we underline the need to consider all of these pathogens when evaluating the risks of infection and subsequently of disease following a tick bite.

Whereas the incidence of Lyme disease is on the rise, other tick-borne diseases remain heavily unreported, and even knowledge on the human exposure to them is scarce. Our study suggests that there are pathogens positively associated with *Borrelia* (i.e., *N. mikurensis* and *Babesia spp.*) in questing ticks. This strengthens the idea of established enzootic cycles (common reservoir hosts) in which these microorganisms are maintained, and it is consequently possible that they might follow the same upward trend as the Lyme spirochetes. In the case of *N. mikurensis* we have in fact witnessed the beginning of what might be a following upward trend.

Human activity in any natural habitat is accordingly accompanied by an imminent risk of exposure to any of the pathogens. Although the risk, as measured by the density of infected ticks, may vary in time and space, its driving factor appears to be the tick density/activity. It is therefore possible that the risk of exposure to TBPs would be minimized by developing effective and sustainable methods for the control of *Ixodes ricinus* populations.

AUTHOR CONTRIBUTIONS

Hein Sprong, Willem Takken, and Joke van der Giessen designed parts of the study. Catharina B. Maassen and Willem Takken organized the collection of ticks and field data. Manoj Fonville performed lab tests and laboratory analyses. Elena Claudia Coipan and Setareh Jahfari analysed data and performed statistical analysis. Katsuhisa Takumi performed mathematical analyses. Hein Sprong, Joke van der Giessen, and Willem Takken supervised different parts of study. Elena Claudia Coipan and Hein Sprong wrote the final manuscript. All authors read and approved the final manuscript.

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Endogenous tick viruses and modulation of tick-borne pathogen growth

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Ticks transmit a wide range of viral, bacterial and protozoan pathogens, many of which can establish persistent infections of lifelong duration in the vector tick and in some cases are transmitted transovarially to the next generation. In addition many ixodid and argasid tick cell lines and, by inference the parent ticks from which they were derived, harbor endogenous viruses (ETV) of which almost nothing is known. In general, low level persistent infections with viral pathogens (arboviruses) are not known to have a deleterious effect on tick survival and fitness, suggesting that they can strike a balance with the tick innate immune response. This tolerance of arbovirus infection may be modulated by the permanent presence of ETV in the host cell. In mosquito cells, temporary or permanent silencing of the genes of an endogenous virus by RNA interference can result in changes in replication rate of a co-infecting arbovirus. We propose that tick cell lines offer a useful model system for *in vitro* investigation of the modulatory effect of ETV on superinfecting pathogen survival and replication in ticks, using the molecular manipulation techniques applied to insect cells.

Keywords: tick, endogenous virus, tick cell line, mosquito, pathogen, co-infection, dsRNA virus, St Croix River virus

BACKGROUND

Ticks are haematophagous parasitic arthropods that feed on a wide range of mammalian, avian, reptilian and amphibian hosts. There are nearly 900 known species of ticks of which 193 belong to the Argasidae (soft ticks) and 702 to the Ixodidae (hard ticks) (Guglielmone et al., 2010). As well as causing direct damage to the vertebrate host during feeding (including skin damage, blood loss, and in some cases immunosuppression), around 10% of tick species are known to be vectors of microorganisms causing disease in livestock, companion animals and humans (Jongejan and Uilenberg, 2004). Most of the arboviruses transmitted by ticks are RNA viruses in the families *Flaviviridae*, *Bunyaviridae*, and *Reoviridae*, including the causative agents of tick-borne encephalitis, Crimean-Congo hemorrhagic fever and Kemerovo tick-borne viral fever infections in humans, and louping ill and Nairobi sheep disease in domestic ruminants (Nuttall, 2009; Belhouchet et al., 2010). The only confirmed DNA arbovirus, African swine fever virus (*Asfarviridae*), is transmitted by ticks of the genus *Ornithodoros* (Plowright et al., 1969), and recent evidence suggests that ixodid ticks may play a role in transmission of the DNA poxvirus causing lumpy skin disease (Tuppurainen et al., 2011). Pathogenic bacteria transmitted by ticks include *Borrelia* spirochaetes and members of the obligately intracellular genera *Anaplasma*, *Ehrlichia* and *Rickettsia* (Jongejan and Uilenberg, 2004); ticks may also play a role in reservoir maintenance and/or transmission of *Bartonella*, *Coxiella* and *Francisella* species (Parola and Raoult, 2001; Reis et al., 2011). The life cycle and transmission of the protozoan pathogens *Babesia* and *Theileria* is intimately bound up with the life cycles and development of their tick vectors (Young and Leitch, 1980; Florin-Christensen and Schnittger, 2009).

That ticks harbor apparently endosymbiotic bacteria has been known for many years (Cowdry, 1925), but identification and characterization of many of these bacteria has only become possible with the advent of molecular phylogenetic techniques and development of suitable *in vitro* culture systems. These endosymbiotic bacteria persistently infect all life cycle stages of the ticks and are passed on to the next generation transovarially. It is unclear whether or not bacterial endosymbionts are transmitted to vertebrates during tick feeding; a recent study of humans bitten by *Ixodes* ticks suggests that salivary transmission of the intramitochondrial tick symbiont *Candidatus* Midichloria mitochondrii (Sassera et al., 2006) can occur to a level sufficient to induce production of specific antibodies (Mariconti et al., 2012). In contrast, *Rickettsia peacockii*, an endosymbiont of the tick *Dermacentor andersoni*, was not pathogenic for laboratory animals and failed to infect a range of mammalian cells *in vitro* (Kurtti et al., 2005). Many of these endosymbionts, including *Candidatus* M. mitochondrii and the *Francisella*-like endosymbionts of *Dermacentor* spp ticks, have only been detected by PCR amplification of gene fragments and/or microscopy (Noda et al., 1997; Scoles, 2004; Epis et al., 2013) but a small number have been isolated and propagated *in vitro* in tick cell lines (Kurtti et al., 1996; Simser et al., 2001, 2002; Mattila et al., 2007). Progress in this area is only limited by the number of researchers, their access to infected ticks, and the range of cell lines derived from appropriate tick species.

TICK CELL LINES AND ENDOSYMBIOTIC BACTERIA

The first continuous tick cell lines were established from developing adult *Rhipicephalus appendiculatus* ticks nearly 40 years ago (Varma et al., 1975). Thereafter, a combination of improvements

in culture methods and, more recently, greatly increased interest in tick cells as research tools, led to the present situation in which over 50 continuous tick cell lines, established from two argasid and fourteen ixodid tick species, are currently in existence (Bell-Sakyi et al., 2007, 2012). The majority of these can be obtained through the Tick Cell Biobank (http://tickcells.pirbright.ac.uk).

Tick cell lines have been applied in many areas of tick and tick-borne pathogen research, including biology, functional genomics, proteomics, antibiotic susceptibility, acaricide resistance and vaccine development (Bell-Sakyi et al., 2007, 2012). Partly due to the availability of a sequenced and partially annotated genome, around 80% of >150 studies published since 1995 have utilized either or both of two particular cell lines, IDE8 and ISE6 derived from embryonic *Ixodes scapularis* (Munderloh et al., 1994; Kurtti et al., 1996). These two cell lines support isolation and growth of numerous intracellular bacteria, including tick-borne pathogens such as *Anaplasma marginale*, *Anaplasma phagocytophilum*, *Ehrlichia ruminantium*, and *Ehrlichia chaffeensis* (reviewed by Bell-Sakyi et al., 2007).

Other tick cell lines have themselves been found to harbor originally endosymbiotic bacteria, which may eventually come to dominate the balance with their host cells, resulting in deleterious effects. When the *Dermacentor andersoni* cell line DAE100 was cured of chronic infection with *R. peacockii*, growth rate in the cured cells more than doubled compared to infected cells (Kurtti et al., 2005). Four out of five *Carios capensis* cell lines were found to harbor rickettsial endosymbionts which eventually interfered with cell survival (Mattila et al., 2007).

In two recent studies, bacterial DNA, possibly indicating persistent infections with endosymbionts, was detected in tick cell lines. Najm et al. (2012) intermittently detected a small fragment of a *Candidatus* M. mitochondrii gene in two tick cell lines derived from *Rhipicephalus* (*Boophilus*) *decoloratus* and *Ixodes ricinus*, but were unable to amplify a larger section of a different bacterial gene from the same samples. DNA sequences matching *Rickettsia africae* and *Francisella*-like endosymbionts were amplified from cell lines derived from, respectively, *Amblyomma variegatum* and three different *Dermacentor* species, although no actual bacteria were seen by light or electron microscopy (EM) (Alberdi et al., 2012). It is unclear whether, in the two above-mentioned cases, intact replicating bacteria are present in the tick cell lines or fragments of bacterial genome have become integrated into the host genome as described for *Wolbachia* in woodlice (Martin et al., 2010).

The phenomenon of microbial DNA becoming integrated into the host cell genome is well-recognized by virologists (Feschotte and Gilbert, 2012). In the past, the term endogenous virus has been used to refer to DNA copies of viral genomes integrated into the DNA genomes of hosts/vectors; these endogenous viruses are now generally known as endogenous virus elements (EVE). In practice, the term endogenous virus refers to replicating viruses which have been identified in cells by a variety of techniques including EM, virus isolation and/or molecular techniques. In arthropods some of these replicating viruses are strict arthropod-only viruses, while others can infect vertebrate cells and hence are regarded as actual or potential arboviruses.

ENDOGENOUS VIRUSES IN TICK CELL LINES

It has only recently been recognized that most, if not all, continuous tick cell lines harbor viruses that are presumed to be endogenous. The first of these, named St Croix River virus (SCRV), was detected in the *I. scapularis* cell line IDE2 over a decade ago (Attoui et al., 2001); to date, this orbivirus (*Reoviridae*) remains the only identified and sequenced "tick virus" (Nuttall, 2009; Belhouchet et al., 2010). Phylogenetic analysis indicates that SCRV represents a lineage ancestral to other known tick- and insect-borne orbiviruses (**Figure 1**). When other *I. scapularis* cell lines were screened by PCR for SCRV, the virus was found in IDE8 but not in IDE12, ISE6 or ISE18 (Alberdi et al., 2012). SCRV was also detected in two *R. appendiculatus* cell lines, RA243 and RA257 (Alberdi et al., 2012) that were established at least 15 years earlier than the *I. scapularis* lines in a different laboratory (Varma et al., 1975) and from ticks originating from a different continent. Although there is now some doubt about the geographic origin of SCRV (North America or Africa?), its "tick only" nature is strongly supported by failure to infect any non-tick cells with this virus including cell lines derived from the mosquitoes *Aedes albopictus*, *Aedes pseudoscutellaris* and *Aedes aegypti*, the amphibian *Xenopus laevis* (XTC cells) and mammalian Vero, BHK-21, BSR, BGM, KB, HELA, Hep2 cells (authors' unpublished results), indicating that it is highly unlikely to be an arbovirus. On the other hand, SCRV was passaged successfully alongside the bacterial pathogen *Ehrlichia ruminantium* from IDE8 (Bell-Sakyi et al., 2000) to ISE6 cells, passaged 5 weeks and several subcultures later from the ISE6 cells to the *Rhipicephalus sanguineus* cell line RSE8 (Kurtti et al., 1982), and virus was demonstrated in RSE8 cells 2 months later by PCR amplification of a portion of the SCRV segment 2 as described previously (Alberdi et al., 2012), indicating that SCRV can replicate in cells from at least three different tick species (authors' unpublished results).

Alberdi et al. (2012) examined 35 ixodid tick cell lines by EM and found that at least 25 of them, including IDE2 and IDE8, contained reovirus-like particles, while out of 47 ixodid and argasid cell lines screened by PCR for SCRV, only the four mentioned above were positive. The eight argasid cell lines included in that report were not examined by EM, but all have since been examined and found to harbor large numbers of icosahedral virus particles (**Figure 2**), the structure of many of which is reminiscent of reoviruses. In some lines, structures suggestive of bunyaviruses (**Figure 3**) have also been observed (authors' unpublished data). Putative novel nairovirus (*Bunyaviridae*) sequences were amplified by PCR from several of these argasid lines, as well as from nine of the ixodid cell lines (Alberdi et al., 2012). Thus, it appears that endogenous viruses are commonly present in tick cell lines and, by inference, in the parent ticks from which the cell lines were derived; certainly viruses other than SCRV present in the ~90% of cell lines that are derived from tick embryos must have been transovarially transmitted. However, to date no reports have been published of examination of whole ticks or tick tissues for presence of SCRV or any other putative endogenous virus; such a study involving both *I. scapularis* and *R. appendiculatus* ticks collected from the field might answer the question of the origin of SCRV. On the other hand, if the structures described as "glycogen

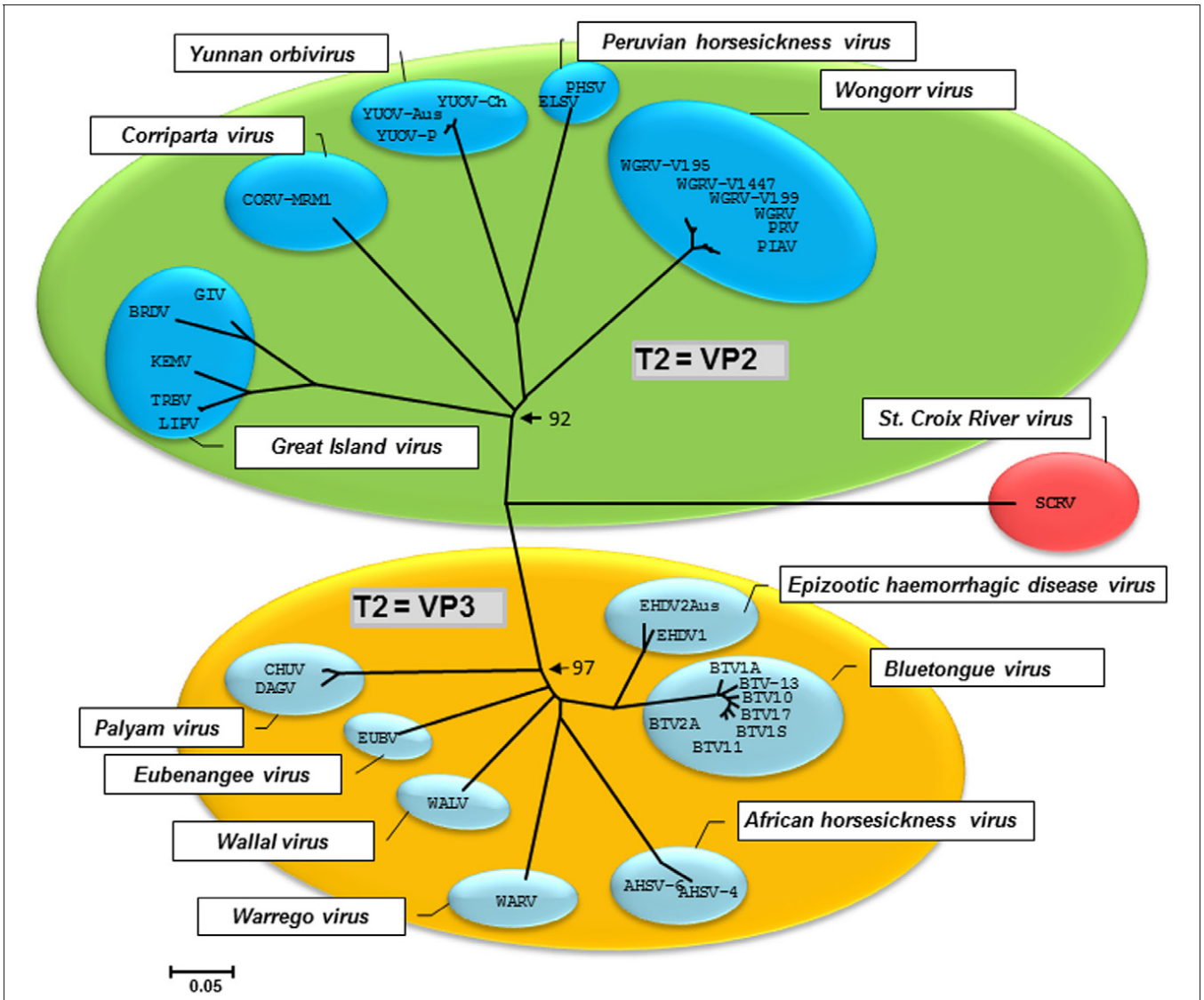

FIGURE 1 | Neighbor joining phylogenetic tree constructed using the subcore shell protein (T2 protein) of orbiviruses. The T2 protein of tick-borne and mosquito-borne orbiviruses (green group) is the VP2, while in midge-borne orbiviruses (yellow group) the T2 is VP3. SCRV (red) roots all known orbivirus T2 proteins and represents the ancestral lineage (Attoui et al., 2001). The tree was constructed using the *P*-distance algorithm implemented in the MEGA5 programme. The scale bar indicates the number of substitutions per site. Values at the nodes indicate bootstrap confidence.

granules" in early EM studies of tick tissues could actually have been endogenous viruses, as inferred by Alberdi et al. (2012), there exists visual evidence of their distribution in ixodid tick salivary glands (Fawcett et al., 1982), perineurial cells (Binnington and Lane, 1980) and midgut cells (Jaworski et al., 1983). Of particular interest are two EM studies of argasid tick Malpighian tubules in which accumulations of "glycogen particles" are seen both within the cytoplasm of pyramidal and cuboidal cells and packed into apical extensions apparently budding out from the surface of pyramidal cells (El Shoura, 1987, 1988). Their resemblance to the structures seen in *O. moubata* cell lines *in vitro* (**Figure 2**) is striking. Whether or not the endogenous viruses detected in tick cell lines are "tick only" can only be definitively determined by exhaustive *in vitro* infection studies using cell lines derived from other arthropods, other invertebrates and vertebrates, and *in vivo* inoculation of potential vertebrate hosts. However, partial sequence determination and comparison with other viruses within the same genus may yield clues as to the ability of these novel viruses to infect cells derived from different host taxa.

The genus *Orbivirus* encompasses arboviruses transmitted to vertebrate hosts by mosquitoes, midges and ticks, as well as the apparently "tick only" SCRV. Until recently, the genome of orbiviruses was believed to encode 7 structural proteins (VP1–VP7) and 3 non-structural proteins (NS1–NS3). Belhouchet et al. (2011) identified a novel non-structural protein (NS4) that is encoded by an alternate open reading frame (ORF) on genome segment 9. NS4 is a protein that is expressed

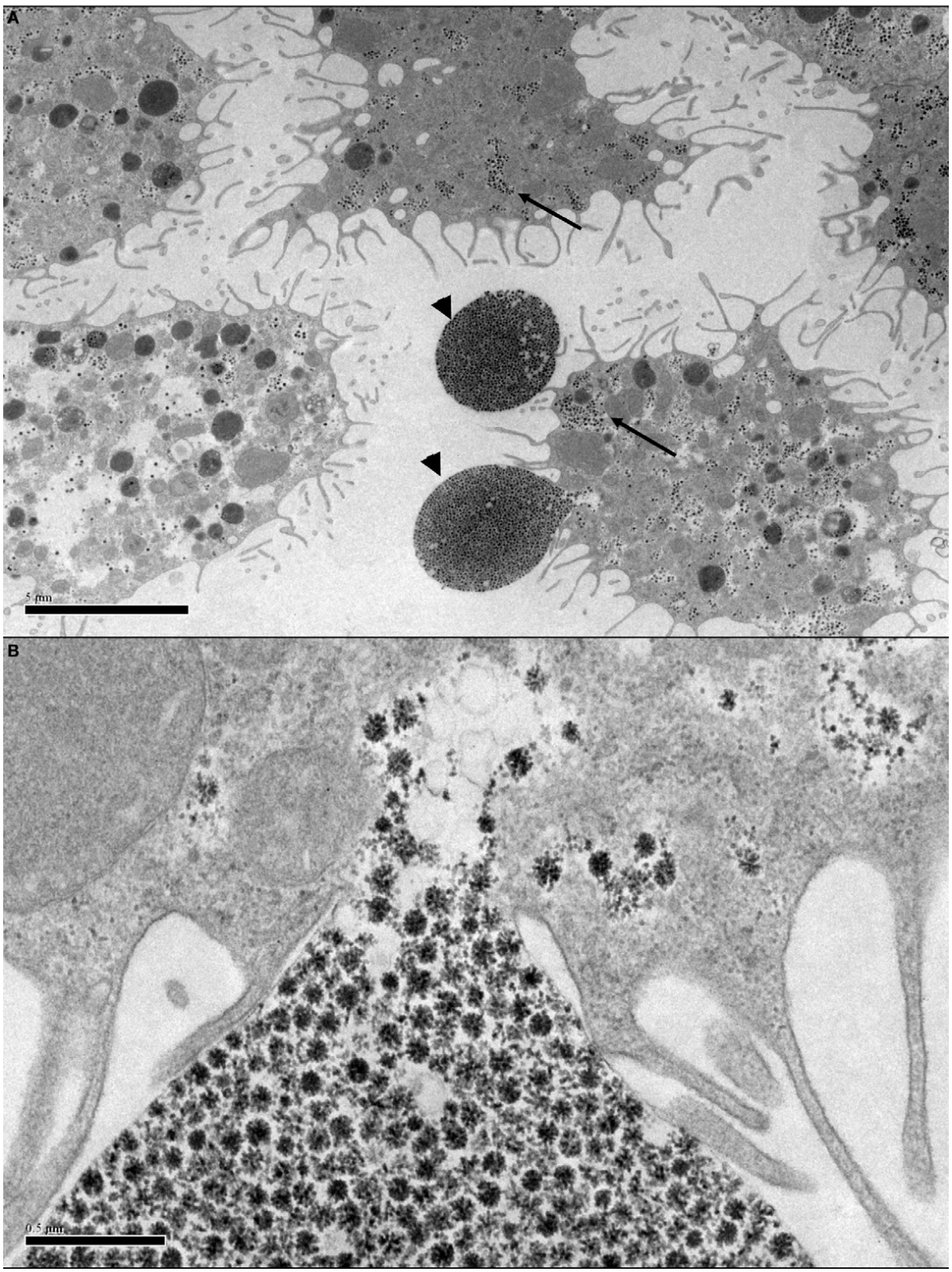

FIGURE 2 | Transmission electron micrographs of *Ornithodoros moubata* cell line OME/CTVM25 showing putative endogenous viruses. (A) Cells with "filopodia" extending from the cell surface. Reovirus-like particles (arrows) are abundant in the cytoplasm. The viruses appear to be using the filopodia to form vesicles (arrowheads) carrying a large number of virus particles with diameters of 75–80 nm that may be budding from the cells into the supernatant medium. Scale bar 5.0 μm. **(B)** Closer view of **(A)** showing the site of attachment of the virus vesicle to the cell membrane. Scale bar 0.5 μm.

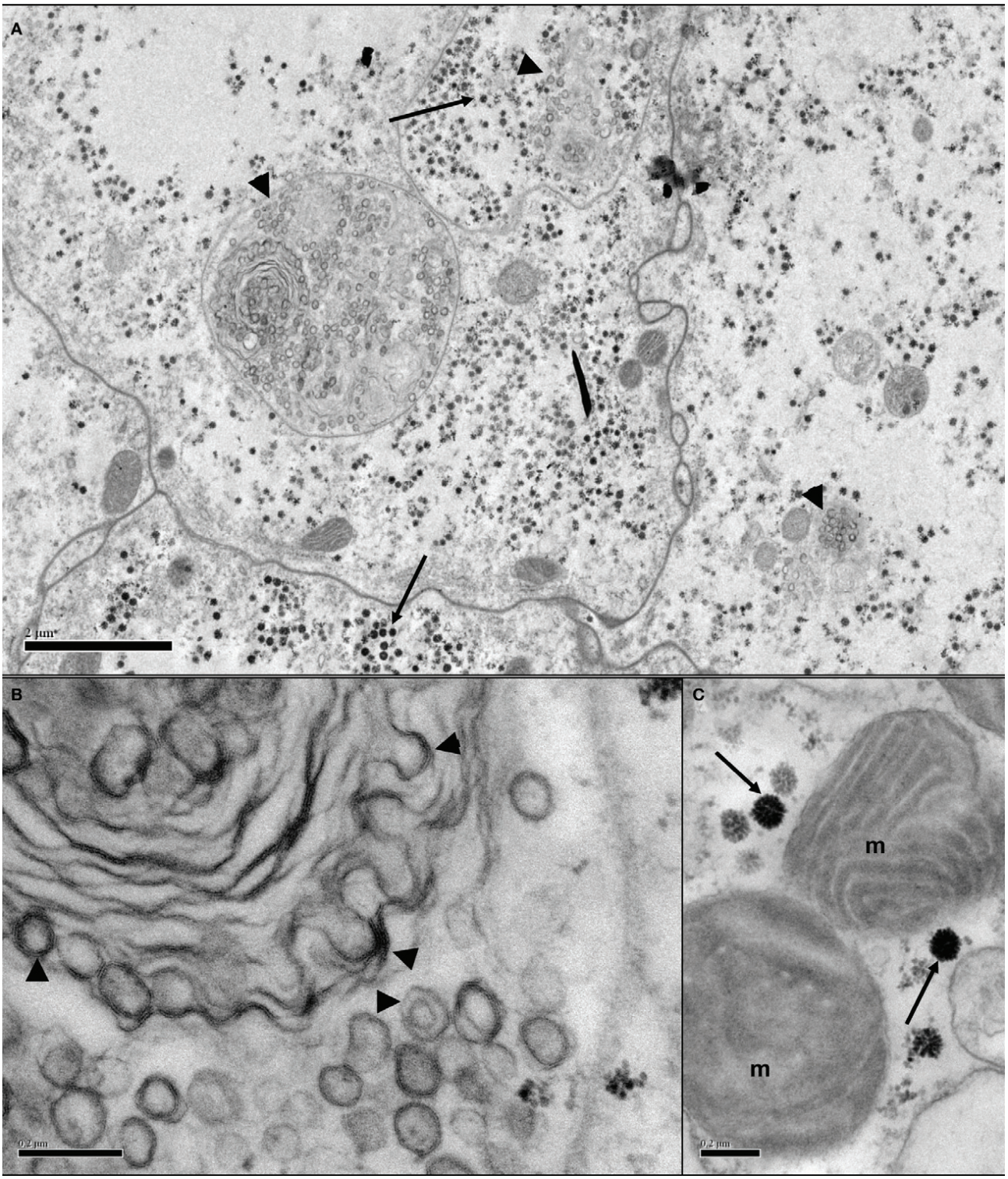

FIGURE 3 | Transmission electron micrographs of *Omithodoros moubata* cell line OME/CTVM26 showing putative endogenous viruses. (A) Several adjoining cells containing putative reovirus-like (arrows) and bunyavirus-like (arrowheads) particles in both individual and shared cellular compartments. Scale bar 2.0 µm. **(B)** Bi- and tri-layered bunyavirus-like particles (arrowheads) appear to be budding from intracellular membranes of unknown function. Scale bar 0.2 µm. **(C)** Icosahedral virus particles of around 100 nm diameter (arrows) in close proximity to mitochondria (m). Scale bar 0.2 µm.

as early as 4 h post-infection and localizes to both the cytoplasm and the nucleus, particularly the nucleolus. All orbiviruses sequenced to date have been shown to contain a fully functional NS4 ORF, except SCRV in which the NS4 ORF is interrupted by a stop codon (Belhouchet et al., 2011). Immunofluorescence analyses have shown the presence of NS4 in the cell membrane during late stages of infection in bluetongue virus-infected mammalian cells. NS4 associates with lipids and, like other viruses which make use of lipid pathways such as hepatitis C virus (Feld, 2012), NS4 is likely to be involved in virus exit and possibly re-infection. The defective NS4 ORF of SCRV may explain in part why this virus is unable to infect cell lines other than those derived from ticks (Belhouchet et al., 2011), though not how it can transfer from one tick cell line to another. However, four types of virus particles are known for orbiviruses. These include whole intact virus particles (having the two outer capsid proteins), infectious subviral particles (generated by the effect of proteases on whole particles), transiently-enveloped particles and core particles (which have lost the outer capsid proteins) (Mertens et al., 1987; Belhouchet et al., 2011). Core particles are known to be able to infect arthropod-derived cell lines, bypassing the requirement for the NS4 protein as part of the transiently enveloped particle (Mertens et al., 1987). Moreover, many tick cells *in vitro* have a tendency to be strongly phagocytic (authors' unpublished observations) and could actively take up virus particles.

Since tick cell lines are increasingly being used for isolating and propagating viruses and bacteria, the possible consequences of the presence of endogenous tick viruses (ETV) on co-infection with exogenous microorganisms must be considered.

CO-INFECTIONS IN TICKS AND TICK CELL LINES

In nature, ticks may be infected with more than one pathogen, but little is known about the interactions between different pathogens co-infecting the same tick, nor about the interactions between co-infecting pathogens and endosymbiotic bacteria (Ginsberg, 2008). If, as it seems likely, many ticks harbor one or more endogenous tick viruses (ETV), these might be expected to interact in some way with single or multiple pathogen infections within the host tick. Such interactions could be direct interactions between microorganisms, or indirect interactions at the cellular level through modulation by the ETV of the host cell response to the coinfecting pathogen.

Arboviruses are known to cause lifelong infections in ticks, and may be transmitted transovarially albeit at a low level (Labuda and Nuttall, 2004). Similarly, arbovirus infections in tick cell lines generally cause little or no cytopathic effect (CPE) and persist for extremely long periods (Leake et al., 1980; Leake, 1987; Bell-Sakyi et al., 2012). Tick-borne bacteria and protozoa may also persist within the host for extended periods—*Ehrlichia ruminantium* can survive within *A. variegatum* ticks for 15 months [Ilemobade 1976 cited by Camus et al. (1996)], and some species of *Babesia* are transovarially transmitted and can persist through multiple generations of ticks even when they are feeding on non-susceptible hosts (Chauvin et al., 2009). Such persistent infections, if heavy, may adversely affect tick longevity but generally do not prevent feeding or oviposition, whereas *in vitro* pathogenic bacteria of the genera *Anaplasma*, *Ehrlichia* and *Rickettsia* usually destroy the host cell. In contrast, the endosymbiont *R. peacockii* has little adverse effect on cells of its natural host *D. andersoni*, but when transferred to cells of different tick species, the bacterium can cause cell death (Kurtti et al., 2005). When the ETV SCRV was transferred from IDE8 cells to RSE8 cells via ISE6 cells, no CPE that could be ascribed to the virus was detected in either of the recipient cell lines. Similarly, the presence of SCRV in RA243 cells does not appear to have any adverse effect, as the cell line in our laboratory is in good health, can be subcultured at fortnightly intervals and is currently at passage 355 (authors' unpublished observations).

In order to begin to elucidate the possible role of ETV in modulating co-infections with arboviruses and intracellular bacteria at the cellular level, it is necessary to turn to research on EVE and endogenous viruses of other arthropods, in particular mosquitoes which share a haematophagous lifestyle with ticks, and *Drosophila*, the most-studied arthropod to date.

ENDOGENOUS VIRUSES MAY MODULATE ARBOVIRUS INFECTION IN INSECTS

EVE in arthropod genomes have been identified in both insects and ticks. Those found in insects belong to or are related to the genera *Nucleopolyhedrovirus* (dsDNA), *Nudivirus* (dsDNA), *Totivirus* (dsRNA), *Partitivirus* (dsRNA), *Seadornavirus* (dsRNA), *Cripavirus* (+ssRNA), *Flavivirus* (+ssRNA), *Phlebovirus* (−ssRNA), *Nairovirus* (−ssRNA), *Lyssavirus* (−ssRNA), and *Thogotovirus* (−ssRNA) (Crochu et al., 2004; Taylor and Bruenn, 2009; Katzourakis and Gifford, 2010; Liu et al., 2010; Feschotte and Gilbert, 2012). Once integrated into the host genome, the viral genome becomes a "fossil" DNA copy that is unlikely to give rise to a replicating virus.

Endogenous replicating viruses identified in arthropod-derived cell lines belong to a number of families. Examples of these include the C6/36 densovirus (*Parvoviridae*; *Densovirus*), cell fusing agent virus (CFAV) (*Flaviviridae*; *Flavivirus*) and *Aedes pseudoscutellaris* reovirus (ApRV) (*Reoviridae*; *Dinovernavirus*) in mosquito cell lines, SCRV (*Reoviridae*; *Orbivirus*) in tick cell lines, and *Drosophila melanogaster* totivirus (*Totiviridae*; *Artivirus*) in a *Drosophila* cell line (Attoui et al., 2001, 2005; Crochu et al., 2004; Wu et al., 2010; Zhai et al., 2010).

An endogenous arthropod virus may or may not be species-specific. For example, a densovirus (ssDNA) derived from *Armigeres* mosquitoes killed *Aedes albopictus* C6/36 cells (which are persistently infected with C6/36 densovirus), while many other densoviruses persistently infect specific mosquito cell lines (Zhai et al., 2008) in the absence of CPE. Replication of endogenous viruses in specific cell lines may significantly reduce replication of other viruses. CFAV, isolated from *Aedes aegypti* cells, interferes with replication of other flaviviruses during co-infection. When mosquito cells were infected by CFAV prior to co-infection with another flavivirus such as

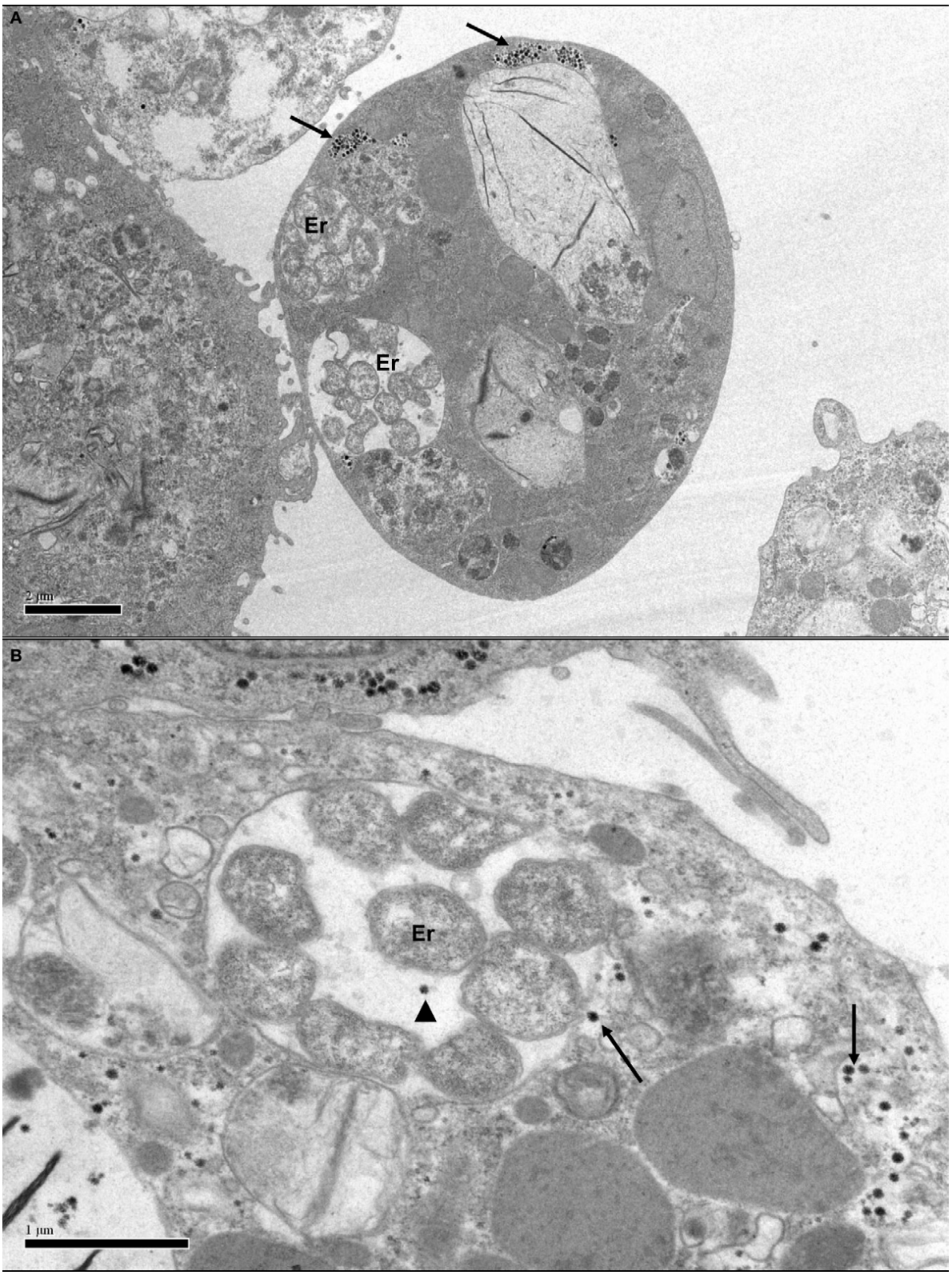

FIGURE 4 | Transmission electron micrographs of *Ixodes scapularis* cell line IDE8 infected with *Ehrlichia ruminantium*. (A) IDE8 cell showing membrane-bound intracytoplasmic morulae containing several *E. ruminantium* bacteria (Er) co-infecting the same cell as SCRV particles in aggregates (arrows). Scale bar 2.0 µm. **(B)** IDE8 cell with aggregated and single SCRV particles, apparently even sharing the same cellular compartment as *E. ruminantium* bacteria (arrowhead). Scale bar 1.0 µm.

yellow fever virus (YFV) 17D, relatively low copy numbers of YFV could be detected. Ultimately, YFV replication was "silenced" (authors' unpublished data). However, if the co-infection with both viruses was realized simultaneously, the effect of CFAV on YFV 17D was found to be minimal.

ApRV persistently infects the mosquito cell line AP61 (Attoui et al., 2005). Real-time PCR indicated that there were only ∼6 ApRV particles per cell. Treatment with an innate immune modulator such as 2-amino purine (kinase inhibitor) increased by 10-fold the number of ApRV particles per cell, indicating that the arthropod innate immune system controlled levels of virus replication, probably through the RNAi pathway. In contrast, when ApRV was used to infect C6/36 cells which have a defective RNAi pathway (Morazzani et al., 2012), virus titers reached $\sim 5 \times 10^4$ viral particles/cell. Moreover, an exogenous virus may have unexpected effects on replication of an endogenous virus as seen with infection of *Aedes albopictus* cell lines. Following Liaoning virus (LNV) infection of C6/36 cells, significantly large amounts (milligram quantities) of the C6/36 densovirus were generated alongside similar quantities of LNV (Attoui et al., 2006), suggesting that dsRNA viruses in particular possess viral silencing suppressor proteins which partly counteract the host antiviral RNA interference (RNAi) mechanism.

The presence of ApRV in AP61 cells may also explain why these cells may not support replication of particular viruses. When Saboya virus (SABV, genus *Flavivirus*) was used to infect AP61, relatively low copy numbers of SABV were detected (authors' unpublished observation). Like endogenous viruses, endosymbiotic bacteria may also interfere with the replication of an exogenous virus. Dengue virus replication, dissemination and transmission were suppressed in *Aedes aegypti* mosquitoes infected with *Wolbachia* (Eleftherianos et al., 2013). Higher levels of Cecropin and Defensin were depicted in the *Wolbachia*-infected mosquitoes. There was an up-regulation of various Toll pathway genes, which was linked to a prevention of oxidative stress in the mosquitoes. Up-regulation of the Toll pathway may therefore be a mechanism by which mosquitoes control particular virus infections (Bian et al., 2010; Pan et al., 2012). These findings contrasted with the situation in *Aedes albopictus*, where naturally-occurring *Wolbachia* infection did not protect mosquitoes from being infected with dengue virus, while specific exogenous *Wolbachia* strains inhibited virus transmission (Blagrove et al., 2012; Lu et al., 2012; Eleftherianos et al., 2013).

DO ENDOGENOUS VIRUSES PLAY A MODULATING ROLE IN INFECTION OF TICK CELLS WITH ARBOVIRUSES AND BACTERIA?

In order to study the possible involvement of an ETV in modulation of pathogen infection in tick cells, the nature of the ETV genome and its taxonomic status need to be known. This information is necessary to understand the replication cycle and interactions of the ETV with various signaling pathways. Initial insights may be gained from ultrastructural analyses using thin EM sections of tick cells and these may reveal morphological similarities to known groups of arboviruses. Universal molecular techniques have become an essential tool for the identification of naturally occurring arboviruses in biological samples. Of particular interest are those based on broad spectrum RT-PCR approaches. There are several conventional or real-time pan-generic PCR approaches for the identification of flaviviruses (Moureau et al., 2007), orbiviruses (Palacios et al., 2011) and bunyaviruses, particularly pan-nairovirus, pan-orthobunyavirus and pan-phlebovirus PCRs (Lambert and Lanciotti, 2009). Full-length characterizations may be realized using virus sequence-independent methodologies described for dsRNA including the single primer amplification technique (SPAT, Attoui et al., 2000) or the SPAT-derived full length amplification of complementary cDNA (FLAC, Maan et al., 2007), or for ssRNA such as that combining a FLAC/SMART (Switch Mechanism At the 5′ end of RNA Template) methodology (Attoui et al., 2007).

Ultimately, silencing replication of an endogenous virus may be a practical way to generate a virus-free cell line. The replication of ApRV in AP61 cells was silenced by continuously feeding AP61 cells with ApR specific siRNA derived, *in vitro*, from the entire virus genome by treatment with recombinant Dicer. An ApRV-free AP61 cell line was hence derived that permitted replication of higher titers of SABV (authors' unpublished results). It will be interesting to apply this methodology to tick cell lines and attempt to derive ETV-free tick cells. The well-characterized SCRV represents an ideal target given the availability of the entire genome sequence (Attoui et al., 2001); this ETV infects three tick cell lines, IDE2, IDE8 and RA243 (Attoui et al., 2001; Alberdi et al., 2012) that together support the growth of a wide range of viral and bacterial pathogens (Bell-Sakyi et al., 2007, 2012).

Comparison of ETV-infected and ETV-free tick cell lines would be an ideal model system to study the effect of the ETV on replication of various exogenous pathogens, for example SCRV and *E. ruminantium* in IDE8 cells (**Figure 4**), and the effect that ETV may have on up-regulating or down-regulating host cell pathways including those involved in innate immunity to microbial infection. Future work in our laboratory will focus on creation of ETV-free cell lines that will allow us to examine the role of ETV both in co-infections and potentially in cell line immortalization.

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Tick vaccines and the control of tick-borne pathogens

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Ticks are obligate hematophagous ectoparasites that transmit a wide variety of pathogens to humans and animals. The incidence of tick-borne diseases has increased worldwide in both humans and domestic animals over the past years resulting in greater interest in the study of tick-host-pathogen interactions. Advances in vector and pathogen genomics and proteomics have moved forward our knowledge of the vector-pathogen interactions that take place during the colonization and transmission of arthropod-borne microbes. Tick-borne pathogens adapt from the vector to the mammalian host by differential gene expression thus modulating host processes. In recent years, studies have shown that targeting tick proteins by vaccination can not only reduce tick feeding and reproduction, but also the infection and transmission of pathogens from the tick to the vertebrate host. In this article, we review the tick-protective antigens that have been identified for the formulation of tick vaccines and the effect of these vaccines on the control of tick-borne pathogens.

Keywords: tick-borne pathogens, vaccine, transmission-blocking, tick, vector

INTRODUCTION

Ticks are of great medical and veterinary importance as they can transmit a wide variety of infectious agents (de la Fuente et al., 2008a). The family Ixodidae comprises hard ticks of the *Amblyomma*, *Dermacentor*, *Rhipicephalus,* and *Ixodes spp.* that not only inflict direct damage to their host but also rank second to mosquitoes as vectors of disease. The *Ixodes ricinus* species alone transmits viruses, bacteria, and protozoa that cause in humans tick-borne encephalitis, Lyme disease, and babesiosis, respectively (de la Fuente et al., 2008a). In cattle, anaplasmosis caused by *Anaplasma spp.,* and babesiosis, caused by *Babesia spp.*, are two of the most important diseases transmitted by *Rhipicephalus spp.* ticks (Merino et al., 2011a).

Vector-borne diseases are on the increase and new infectious agents are also emerging leading to significant public health concerns as potential zoonotic disease threats (Parola and Raoult, 2001; de la Fuente and Estrada-Peña, 2012). Amongst other factors, climate change itself can have an adverse effect on the distribution of ticks and tick-borne diseases. It is predicted that more than 50% of tick species of the genus *Rhipicephalus* (*Boophilus*) could expand its range in Africa, with more than 70% of this range expansion linked to economically important tick species such as *R. appendiculatus, R. microplus*, or *R. decoloratus* (Estrada-Peña et al., 2006; Olwoch et al., 2007).

The ultimate goal of arthropod vector vaccines is the control of vector infestations and vector-borne diseases (VBD). The effect of vector vaccines on VBD could be obtained by (a) reducing vector populations and thus the exposure of susceptible hosts to vector-borne pathogens, (b) reducing the arthropod vector capacity for pathogen transmission, and, preferably, (c) a combination of these factors.

Herein, we review recent advances in tick vaccine development focused on discovery and characterization of tick protective antigens that impact on pathogen infection and transmission. Identification of molecules essential for both tick survival and pathogen infection and transmission will likely contribute to the discovery of novel vaccine strategies for the simultaneous control of ticks and tick-borne pathogens (**Figure 1**).

CONTROL METHODS FOR TICKS AND TICK-BORNE DISEASES

A major component of integrated tick control has been the application of acaricides. However, their use has had limited efficacy in reducing tick infestations and is often accompanied by serious drawbacks, including the selection of acaricide-resistant ticks, environmental contamination and contamination of milk and meat products with drug residues (Graf et al., 2004; Ghosh et al., 2007).

An alternative host-targeted method involves the elimination of ticks from the host using baits impregnated with different compounds such as acaricides or antibiotics. Immature *I. scapularis* ticks were eliminated from mice using bait boxes impregnated with fipronil, therefore reducing the subsequent populations of nymphs and adults and thus reducing the proportion of ticks infected with the Lyme disease agent, *Borrelia burgdorferi* (Sonenshine et al., 2006). Field trials by Dolan et al. (2011) have revealed that infections rates with *A. phagocytophilum* and *B. burgdorferi* can be significantly reduced in both rodent reservoirs and ticks *I. scapularis* using antibiotic-treated baits. Thus this method can successfully reduce tick infestations and may also help to reduce pathogen transmission but can also contribute to the selection of acaricide and/or antibiotic resistant ticks.

Ecological approaches to control diseases involve intervention in the natural cycle of disease agents vaccinating wild reservoirs

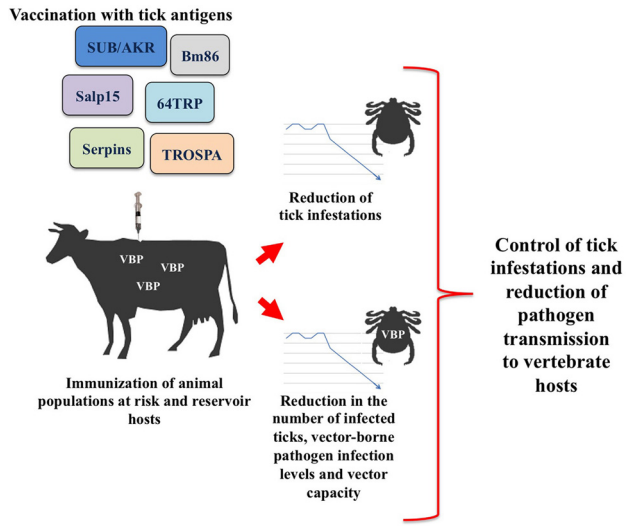

FIGURE 1 | Transmission blocking vaccines based on tick recombinant proteins aim to reduce vector infestations and the infection and transmission of vector-borne pathogens (VBP).

but the effects may be complex and hard to predict. For instance, Tsao et al. (2004) immunized white-footed mice, reservoir host for the Lyme disease agent, with a recombinant outer surface protein A (OspA). Even though vaccination significantly reduced the prevalence of *B. burgdorferi* in nymphal ticks, the results also indicated that non-mouse hosts played a larger than expected role in infection dynamics, suggesting the need to vaccinate additional hosts.

Entomopathogenic fungi, such as *Metarhizium anisopliae* and *Beauveria bassiana*, are active against a range of several economically important species of ticks under laboratory and field conditions, for example *R. annulatus* (Pirali-Kheirabadi et al., 2007), *I. scapularis* (Hornbostel et al., 2005), *R. appendiculatus* and *A. variegatum* (Kaaya et al., 1996). Despite the relative safety of this type of biocontrol method they haven't been successfully implemented as yet because of their environmental instability, and potential damage to non-target species.

Ticks can harbor a wide range of endosymbiotic bacteria including *Rickettsia*, *Francisella*, *Coxiella*, and *Arsenophonus*, amongst others (Alberdi et al., 2012). Tick control strategies could be devised based on interference with their endosymbionts for the control of these vectors and the pathogens they hold (Ghosh et al., 2007). For instance, *Wolbachia pipientis* when transfected into *Aedes aegypti* mosquitoes hinders the replication of Dengue and Chikungunya viruses (Iturbe-Ormaetxe et al., 2011).

Vaccination is an attractive alternative for the control of tick infestations and pathogen infections as it is a more environmentally friendly method. By targeting a common vector, several tick-borne diseases can be controlled simultaneously (Brossard, 1998; de la Fuente et al., 1998, 2007a,b, 2011; Rodríguez Valle et al., 2004; Almazán et al., 2005b). Since vector-borne pathogens exploit tick proteins to establish an infection, targeting the pathogen in the vector by blocking transmission is an innovative and promising method to control vector-borne infections (Lee and Opdebeeck, 1999; Havlíková et al., 2009). However, the selection of suitable antigens is a major constraint on vaccine development.

FINDING CANDIDATE TICK PROTECTIVE ANTIGENS

Candidate tick protective antigens have been identified using high throughput screening technologies allowing rapid, systematic and global antigen screening and providing a comprehensive approach for the selection of candidate vaccine antigens (Diatchenko et al., 1999; Almazán et al., 2003; Antunes et al., 2012). Other screening approaches include using RNA interference (RNAi) (de la Fuente et al., 2005, 2008b, 2010; Almazán et al., 2010; Kocan et al., 2011) and capillary feeding (Almazán et al., 2005a; Canales et al., 2009a; Gonsioroski et al., 2012; Rodriguez-Valle et al., 2012). Using a functional genomics approach, Antunes et al. (2012) identified differentially expressed genes in *B. bigemina*-infected *Riphicephalus* ticks. TROSPA and serum amyloid A in particular significantly reduced bacterial infection levels in the ticks. Other methods such as protein arrays (Manzano-Román et al., 2012) and yeast surface display (Schuijt et al., 2011b) have also been proposed for the identification and characterization of antigens that elicit tick immunity.

The tick protective antigen, Subolesin, was discovered by expression library immunization and evaluation of expressed sequence tags (Almazán et al., 2003). Ghosh et al. (2008) employed strategic methods for the isolation of targeted molecules using affinity purification of proteins showing reactivity with immunoglobulins of animals previously immunized with different sources of tick antigens. Rachinsky et al. (2008) investigated the differences in protein expression in midgut tissue of uninfected and *Babesia bovis*-infected *R. microplus* ticks to establish a proteome database containing proteins involved in pathogen transmission. As pathogen neutralization occurs within the feeding vector, the development of a successful transmission-blocking vaccine requires that the antigen induce high and long-lasting circulating antibody titers in immunized hosts.

Nano/microparticle technologies can be applied toward the development of transmission-blocking vaccines that target antigens expressed only inside the vector. Although not yet used in ticks, experiments by Dinglasan et al. (2013) showed that a single inoculation and controlled release of mosquito antigen in mice, elicited long-lasting protective antibody titers against malaria sexual stages. Conserved carbohydrate targets have been identified in the midgut of arthropod species (Dinglasan et al., 2005) and are a promising tool for the elaboration of transmission blocking vaccines that control a wide range of arthropod vectors.

SPECIFIC TICK ANTIGENS AND THEIR EFFECT ON PATHOGEN TRANSMISSION (SEE TABLE 1)

BM86-BASED VACCINES

Tick vaccines became commercially available in the early 1990's for the control of cattle tick infestations (Willadsen et al., 1995; Canales et al., 1997; de la Fuente et al., 1998, 2007b). TickGARD (in Australia) and Gavac (in Latin American countries) are both derived from *R. microplus* midgut membrane-bound recombinant protein BM86. The protective action of BM86-based vaccines in cattle is due to the positive correlation between

Table 1 | Overview of tick protective antigens and their effect on the control of tick-borne pathogens.

Vaccinated hosts (N)[a]	Recombinant tick antigen	Vector[b]	Pathogen[c]	Reduction in vector infection[d]	References
Cattle (>260,000)	Bm86	*R. microplus*	*Babesia sp.*	76%[e]	de la Fuente et al., 1998
Cattle (>260,000)	Bm86	*R. microplus*	*Anaplasma sp.*	No effect	de la Fuente et al., 1998
Cattle (5)	Ba86	*R. annulatus*	*Babesia sp.*	N/D	Canales et al., 2009a
Cattle (5)	Ba86	*R. annulatus*	*Anaplasma sp.*	N/D	Canales et al., 2009a
Rabbit (4)	Bm95	*R. microplus*	*Babesia sp.*	N/D	Canales et al., 2009b
Rabbit (4)	Bm95	*R. microplus*	*Anaplasma sp.*	N/D	Canales et al., 2009b
Cattle (5)	HGAg	*H. a. anatolicum*	*Theileria annulata*	10%	Das et al., 2005; Ghosh et al., 2008;
Cattle (5)	Haa86	*H. a. anatolicum*	*Theileria annulata*	3 calves survived lethal challenge	Jeyabal et al., 2010
Cattle (5)	Bm91	*R. microplus*	*Babesia,*	N/D	Willadsen et al., 1996
Cattle (5)	Bm91	*R. microplus*	*Anaplasma*	N/D	Willadsen et al., 1996
Mice (5)	SUB	*I. scapularis*	*A. phagocytophilum*	33%	de la Fuente et al., 2006b
Cattle (4)	SUB	*R. microplus*	*A. marginale*	98%	Merino et al., 2011b
Cattle (4)	SUB	*R. microplus*	*B. bigemina*	99%	Merino et al., 2011b
Mice (15)	SUB	*I. scapularis*	*B. burgdorferi*	40%	Bensaci et al., 2012
Mice (10)	64TPR	*I. ricinus*	TBEV	52%	Labuda et al., 2006
Rabbits (2); Cattle (4)	RmFER2	*I. ricinus, R. microplus, R. annulatus*	*Anaplasma sp., Babesia sp.*	N/D	Hajdusek et al., 2010
Mice (5)	Salp15	*I. scapularis*	*B. burgdorferi*	60%	Dai et al., 2009
Mice (5)	Salp25D	*I. scapularis*	*B. burgdorferi*	Three-fold	Narasimhan et al., 2007
Cattle (5)	RAS-3, RAS-4, RIM36 coktail	*R. appendiculatus*	*T. parva*	38%	Imamura et al., 2008
Mice (5)	TROSPA	*I. scapularis*	*B. burgdorferi*	75%	Pal et al., 2004
Mice (5)	tHRF	*I. scapularis*	*B. burgdorferi*	20–30% mice fully protected	Dai et al., 2010
Mice (3)	TSLPI	*I. scapularis*	*B. burgdorferi*	30%	Schuijt et al., 2011a

[a] *N, number of individuals per group.*

[b] *Arthropod vector species in which vaccine was tested.*

[c] *Pathogen species in which the effect of vaccination was tested.*

[d] *Reduction in vector infection was determined with respect to the control group vaccinated with adjuvant/saline.*

[e] *Overall reduction in the incidence of dead animals caused by infections with Babesia sp. after vaccination.*

Abbreviation: N/D, not determined.

antigen-specific antibodies and reduction of ticks infestations and fertility (Rodríguez et al., 1995; de la Fuente et al., 1998; Merino et al., 2011a). The mechanism by which BM86 immunization affects ticks involves antibody-antigen interaction that interferes with the still unknown BM86 biological function thus reducing the number, weight and reproductive capacity of engorging female ticks (de la Fuente et al., 1998, 1999). As a result, the prevalence of some tick-borne pathogens can indirectly be affected (de la Fuente et al., 2007b). Vaccine trials with BM86 resulted in a reduction in the incidence of babesiosis, as well as reduced tick infestations in vaccinated cattle herds, and these results were corroborated in extensive field trials (de la Fuente et al., 1998, 2007a; Rodríguez Valle et al., 2004). However, because *A. marginale* is also mechanically transmitted by blood-contaminated mouth parts of biting insects and fomites, BM86 antigen vaccination controlled the transmission of *A. marginale* only in regions where ticks are the main vectors (de la Fuente et al., 1998).

Despite the effectiveness of these commercial BM86-based vaccines for the control of cattle tick infestations, they show strain-to strain variation in efficacy and are effective against *Rhipicephalus* tick species mainly (de la Fuente and Kocan, 2003; Willadsen, 2006; de la Fuente et al., 2007a,b; Odongo et al., 2007) hence the need to develop improved vaccine formulations (Guerrero et al., 2012).

BM86 ORTHOLOGS AND HOMOLOGS

BA86 is a recombinant *R. annulatus* BM86 ortholog protein with over 90% similarity to BM86 (Canales et al., 2008). Experimental trials in cattle proved the efficacy of recombinant BA86 for the control of *R. annulatus* and *R. microplus* infestations, showing that the efficacy of both BM86 and BA86 is higher against *R. annulatus*. These results suggested that physiological differences between *R. microplus* and *R. annulatus* and those encoded in the sequence of BM86 orthologs

may be responsible for the differences in susceptibility of tick species to BM86 vaccines (Canales et al., 2009a; Jeyabal et al., 2010).

A BM86 ortholog of *Hyalomma anatolicum anatolicum*, HAA86, was cloned and expressed by Azhahianambi et al. (2009). Jeyabal et al. (2010) reported that vaccination of cattle with the recombinant HAA86 antigen did not only protect against homologous tick challenge but also reduced tick transmission of *Theileria annulata*, thus protecting the animals against lethal exposure.

The *R. microplus* BM95 glycoprotein is a BM86 homologue that protects cattle against infestations by South American cattle tick strains not protected by BM86 vaccination (Canales et al., 2009b). Studies with BM95 have shown it protects against a broader range of tick strain infestations suggesting BM95 could be a more universal antigen against infestations by *R. microplus* strains from different geographical areas (García-García et al., 2000; de la Fuente and Kocan, 2003).

The number of new upcoming promising targets that can affect both tick infestations and pathogen transmission is rising. Nijhof et al. (2010) have recently identified a novel protein from metastriate ticks with structural similarities to BM86, named ATAQ after a part of its signature peptide. Although its function is unknown, ATAQ is expressed in both midguts and Malpighian tubules, while BM86 is expressed only in midguts. The vaccine efficacy of recombinant ATAQ proteins against tick infestations has not been evaluated but it may constitute a good vaccine candidate with an increased cross-protective effect against heterologous ticks compared to BM86-based vaccines because ATAQ proteins are more conserved.

TICK SALIVARY PROTEINS

Arthropod vectors induce immunosuppression in the host during feeding and secrete pathogen transmission-enhancing factors that counteract host rejection responses. For example, the Lyme disease agent *B. burgdorferi* exploits tick salivary proteins (B-cell inhibitory protein BIP and Salp15 from *I. ricinus* and *I. scapularis*, respectively) to facilitate transmission to the mammalian host (Anguita et al., 2002, 2003; Hannier et al., 2004; Ramamoorthi et al., 2005). During feeding, tick salivary glands secrete a large variety of pharmacologically active molecules with immunosuppresive properties that facilitate pathogen transmission and are potential candidates for anti-tick vaccines that limit infestations and interfere with tick-borne pathogen transmission (Valenzuela, 2002; Ribeiro and Francischetti, 2003; Nuttall et al., 2006; Titus et al., 2006; Nuttall, 2009).

64TRP is a 15 kDa protein that resembles mammalian host skin proteins, identified in expression libraries as a putative tick cement protein involved in the attachment and feeding of *R. appendiculatus* (Trimnell et al., 2002; Havlíková et al., 2009). The protein derives from the cement cone that secures the tick's mouthparts in the host skin and, as a broad-spectrum vaccine antigen, is effective against adult and immature stages of several tick species, including *I. ricinus* (Trimnell et al., 2005). Recombinant forms of *R. appendiculatus* 64TRP induce potent humoral and delayed type hypersensitivity responses (Trimnell et al., 2002). In hamster, guinea pig and rabbit models this cement antigen acts as a dual-action vaccine by targeting the tick-feeding site (impairing attachment and feeding) and cross-reacting with "concealed" midgut antigens, resulting in death of engorged ticks (Trimnell et al., 2002, 2005; Havlíková et al., 2009). Histological and immunocytological studies have indicated that the key mode of action of 64TRP immunisation is the local cutaneous delayed type hypersensitivity response induced at the skin site of tick feeding (Labuda et al., 2006). Recent experiments have illustrated how vaccination with this antigen also affects tick vector capacity. Labuda et al. (2006) reported that vaccination of mice with 64TRPP antigen prevented transmission of tick-borne encephalitis virus (TBEV) by *I. ricinus* thus having a protective effect on pathogen transmission.

Salp15 is another secreted salivary protein with host immunosuppressive properties, inhibiting $CD4^+$ T-cell activation (Anguita et al., 2002), complement activity (Schuijt et al., 2008), and dendritic cell function (Hovius et al., 2008a). OspC is an outer surface protein produced by *B. burgdorferi*. When ticks take a blood meal, the spirochetes initiate its synthesis in the midguts of infected ticks. Salp15 physically binds to OspC on *B. burgdorferi* spirochetes surface during exit from the salivary glands, facilitating the survival of spirochetes, pathogen transmission and host infection (Ramamoorthi et al., 2005; Dai et al., 2009). Salp15-OspC interaction potentially conceals OspC from the host immune response protecting the spirochete (Ramamoorthi et al., 2005). Mice immunized with recombinant Salp15 and challenged with *B. burgdorferi* infected nymphs were significantly protected from infection (Dai et al., 2009). Antibodies directed against Salp15 may separate Salp15 away from OspC leaving it exposed to the immune responses, or, hypothetically, Salp15 antibodies could bind to Salp15-coated spirochetes and release the spirochetes more effectively to phagocytes (Dai et al., 2009). Immunization of murine hosts with a combination of Salp15 and OspA provide better protection from *B. burgdorferi* infection than either alone (Dai et al., 2009). Salp15 homologs have been identified in *I. ricinus* ticks, they also bind *B. garinii* and *B. afzelii* OspC to facilitate spirochete transmission (Hovius et al., 2008b).

Salp25D is expressed *by I. scapularis* salivary glands and midguts (Das et al., 2001) and has homology to peroxiredoxins antioxidants (Barr and Gedamu, 2003). Immunization of mice with rSalp25D reduces *Borrelia* acquisition by *I. scapularis* (Narasimhan et al., 2007) demonstrating it plays a critical role during tick feeding in the mammalian host, protecting the bacteria from reactive oxygen produced by neutrophils and facilitating *Borrelia* acquisition by ticks. Therefore it could be used to vaccinate reservoir hosts to interrupt the spirochete life cycle and reduce its prevalence in ticks in Lyme disease endemic areas. Interestingly, Salp25D does not influence transmission from the tick to the mammalian host (Narasimhan et al., 2007).

The **tick histamine release factor (tHRF)** from *I. scapularis* was characterized by Dai et al. (2010). tHRF is secreted in tick saliva, upregulated in *B. burgdorferi*-infected ticks and it appears to have a role in tick engorgement and efficient *B. burgdorferi* transmission (Dai et al., 2010). Silencing tHRF by RNAi

significantly impaired tick feeding and decreased *B. burgdorferi* infection levels in mice. Actively immunized mice with recombinant tHRF, or passively transferring tHRF antiserum, also markedly reduced the efficiency of tick feeding and *B. burgdorferi* infection in mice. Blocking tHRF might offer a viable strategy to develop vaccines that block tick feeding and therefore transmission of tick-borne pathogens.

The *I. scapularis* salivary protein **TSLPI (Tick Salivary Lectin Pathway Inhibitor)** identified by Schuijt et al. (2011a) protects *B. burgdorferi* from direct killing by the host complement system. Silencing *TSLPI* mRNA significantly reduces *Borrelia* loads in nymphs and also impairs transmission to mice. TSLPI plays a significant role in both transmission and acquisition of *Borrelia* (Schuijt et al., 2011a) but immunization against rTSLPI does not completely block bacterial transmission from the tick to the host, suggesting the need for a combination of tick proteins in future tick antigen-based vaccines to prevent Lyme disease (Schuijt et al., 2011b).

OTHER TICK PROTEINS

Ferritins are iron-storage proteins that play a pivotal role in the homeostasis of iron during tick feeding. A common heavy chain type ferritin 2 (Kopacek et al., 2003), without functional orthologs in vertebrates, has been recently characterized as a gut-specific protein secreted into the tick hemolymph, where it acts as an iron transporter (Hajdusek et al., 2009). Ferritin 2 (RmFER2) knockdown by RNAi and vaccination with the recombinant protein resulted in reduction of feeding, oviposition and fertility in *I. ricinus, R. microplus* and *R. annulatus* (Hajdusek et al., 2009, 2010) thus highlighting its potential use as a future dual action tick and tick-borne diseases protective antigen candidate.

TROSPA is a tick receptor for *B. burgdorferi* OspA that has been identified in the tick midgut (Pal et al., 2004; Antunes et al., 2012). Tick-borne pathogens can adapt from the vector to the mammalian host by differential gene expression. For example, outer surface proteins OspA and OspB are produced when Lyme disease spirochetes enter and reside in ticks (Pal and Fikrig, 2003) but they are downregulated during transmission to the host. Other genes that facilitate transmission from ticks and colonization of the host such as bba52 and OspC are upregulated. TROSPA expression is upregulated during *B. burgdorferi* infection and downregulated during tick engorgement. The receptor's physiological function is unknown but binding of OspA to TROSPA is essential for *B. burgdorferi* to colonize the tick gut, thus supporting bacterial infection in the vector (Pal et al., 2004). *B. burgdorferi* infection enhances expression of specific tick genes such as TROSPA and salp15 that can be targeted to prevent the transmission of *Borrelia* spirochetes and other tick-borne microbes (Hovius et al., 2007). Blocking TROSPA with TROSPA antisera or via RNA interference (RNAi) reduces *B. burgdorferi* adherence to the gut of *I. scapularis,* and as a result reduces bacterial colonization of the vector and, potentially, pathogen transmission to the host (Pal et al., 2004). Bacterial OspA has been used as a Lyme disease vaccine that blocks pathogen transmission as anti-OspA antibodies destroy the spirochetes in the tick gut before transmission to the host occurs (Pal et al., 2000). Studies by Tsao et al. (2001) suggested that vaccination of mice with OspA could reduce transmission of the bacteria to the tick vector regardless of whether the reservoir host was previously infected or not. A combination of OspA with TROSPA antigens may enhance vaccine protective efficacy against Lyme disease.

Serpins (serine protease inhibitors) are a large family of structurally related proteins found in a wide variety of organisms, including hematophagous arthropods. They are known to regulate many important functions such as blood coagulation, food digestion, inflammatory and immune responses (Mulenga et al., 2001) and therefore are attractive target antigens for tick vaccine development. Combining different serpins to vaccinate cattle results in a reduction of engorgement rates and increased mortality of *Haemaphysalis* and *Rhipicephalus* ticks (Imamura et al., 2005, 2006). Furthermore, immunization of cattle with a cocktail vaccine containing recombinant *R. appendiculatus* serpins RAS-3, RAS-4, and a 36 kDa immune-dominant protein RIM36, reduces tick infestations and also has an effect on the tick mortality rate of *Theileria parva*-infected ticks by increasing it from 10.8 to 48.5% in the vaccinated group (Imamura et al., 2008). Infection of cattle with *T. parva* could not be prevented by the vaccine although the presence of the pathogen in peripheral blood was delayed by a couple of days indicating the vaccine also had an effect on pathogen transmission to the mammalian host.

Tick Subolesin (SUB), the ortholog of insect and vertebrate akirins (AKR) (de la Fuente et al., 2006a; Goto et al., 2008; Canales et al., 2009c; Galindo et al., 2009; Macqueen and Johnston, 2009; Mangold et al., 2009), was discovered as a tick protective antigen in *I. scapularis* (Almazán et al., 2003). Most vertebrates have two closely related AKR homologues, AKR1 and AKR2 (Goto et al., 2008). Only one SUB/AKR gene has been identified in insects and ticks, which is evolutionary and functionally related to mammalian AKR2 (de la Fuente et al., 2006a; Goto et al., 2008; Galindo et al., 2009; Macqueen and Johnston, 2009). SUB has a role in tick immunity and other molecular pathways and has been shown to protect against tick infestations and infection by vector-borne pathogen such as *A. phagocytophilum*, *A. marginale*, *B. bigemina*, and *B. burgdorferi* (de la Fuente et al., 2006b; Merino et al., 2011b; Bensaci et al., 2012). RNAi experiments have demonstrated that SUB knockdown affects the expression of genes involved in multiple cellular pathways (de la Fuente et al., 2006c, 2008c). It also has an effect on pathogen infection by reducing tick innate immunity that results in higher infection levels but also indirectly by affecting tick tissue structure and function and the expression of genes required for pathogen infection, therefore interfering with pathogen infection and multiplication (Zivkovic et al., 2010; de la Fuente et al., 2011) (**Figure 2**). Vaccines containing conserved SUB/AKR protective epitopes have been shown to protect against tick, mosquito and sand fly infestations, thus suggesting the possibility of developing universal vaccines for the control of arthropod vector infestations (Moreno-Cid et al., 2013). However, the effects of SUB/AKR vaccines on vector-borne viruses showed no effect on tick-borne encephalitis virus infection and transmission (Havlíková et al., 2013).

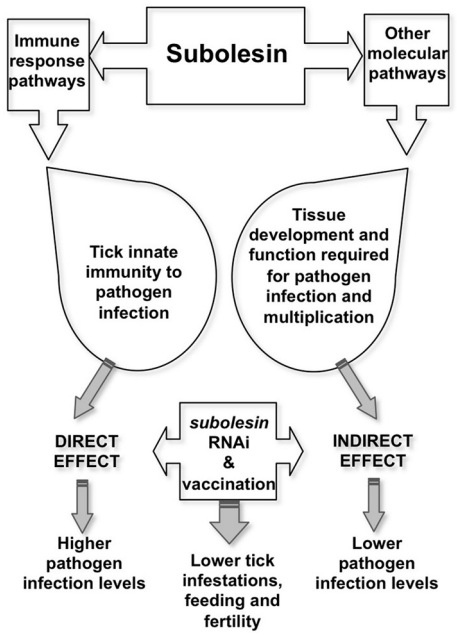

FIGURE 2 | Model for Subolesin role in pathogen infection. Targeting SUB by vaccination or RNAi reduces tick immunity, thereby increasing pathogen infection levels. However, lower pathogen infection levels may result from the effect on tissue structure and function and the expression of genes that are important for pathogen infection and multiplication. Both direct and indirect effects of targeting SUB results in lower tick infestations, feeding and fertility.

CONCLUSIONS AND FUTURE DIRECTIONS

This review has focused on studies showing the effects of tick antigens on the control of tick-borne pathogens by either decreasing the exposure of susceptible hosts to infected ticks (i.e., BM86) or by reducing tick vector capacity (i.e., TROSPA) (Kocan, 1995; de la Fuente and Kocan, 2003; Willadsen, 2006; de la Fuente et al., 2007a,b).

Tick-borne pathogens are maintained in a complex enzootic infection cycle involving ticks and vertebrate hosts (Wilson, 2002). Our understanding of the biology of vector-pathogen interactions, primarily involving model insects has advanced over the past decades. However, our knowledge of tick biology, especially the molecular interactions with the pathogens they maintain and transmit, and the mechanism by which the tick immune response influences invading pathogens, remains insufficient. The relative fitness of a pathogen within the vector can be a major determinant of pathogen prevalence within the vertebrate host population. For example, strains of the tick-borne rickettsia *A. marginale* differ markedly in their transmission efficiency (Ueti et al., 2009). These areas are understudied but important and warrant future investigation.

Transmission-blocking vaccines that interfere with specific aspects of tick physiology important for arthropod survival or development may prevent multiple infections that are often co-transmitted by a single tick species, an advantage over vaccines which only target particular pathogens. For example, immunization of hosts using SUB significantly inhibits tick infection with multiple pathogens such as *A. marginale* and *B. bigemina* (Merino et al., 2011b).

Progress in the development of transmission blocking vaccines has been slow. The limiting step in the development of vector vaccines has been the identification of new antigens that induce protective immune responses whilst preventing pathogen transmission (de la Fuente and Kocan, 2003). The number of proteins that may be of value as antigens has continued to increase quite rapidly over recent years but there have not been many reports of their actual assessment in vaccination trials (Willadsen, 2004; Guerrero et al., 2012). Very few antigens appear to be highly effective on their own suggesting the need for a multi-antigen or chimeric vaccine that incorporates critical tick and pathogen antigenic epitopes (Almazán et al., 2012; Parizi et al., 2012b; Moreno-Cid et al., 2013) to elicit synergistic anti-pathogen and anti-tick immune responses.

The selection of new vaccine antigens from the study of tick-pathogen interactions using systems biology requires the development of algorithm that allow the selection of the most effective targets to control tick infestations and pathogen transmission (de la Fuente, 2012).

Finally, identification of new protective antigens that are conserved across vector species, with similar structure and/or sequence motifs, may provide the opportunity to develop a universal and so more commercially viable vaccine for the control of multiple arthropod infestations and their associated pathogens (de la Fuente et al., 2011; Parizi et al., 2012a; Moreno-Cid et al., 2013).

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Transport of ixodid ticks and tick-borne pathogens by migratory birds

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Birds, particularly passerines, can be parasitized by Ixodid ticks, which may be infected with tick-borne pathogens, like *Borrelia* spp., *Babesia* spp., *Anaplasma, Rickettsia*/*Coxiella*, and tick-borne encephalitis virus. The prevalence of ticks on birds varies over years, season, locality and different bird species. The prevalence of ticks on different species depends mainly on the degree of feeding on the ground. In Europe, the *Turdus* spp., especially the blackbird, *Turdus merula*, appears to be most important for harboring ticks. Birds can easily cross barriers, like fences, mountains, glaciers, desserts and oceans, which would stop mammals, and they can move much faster than the wingless hosts. Birds can potentially transport tick-borne pathogens by transporting infected ticks, by being infected with tick-borne pathogens and transmit the pathogens to the ticks, and possibly act as hosts for transfer of pathogens between ticks through co-feeding. Knowledge of the bird migration routes and of the spatial distribution of tick species and tick-borne pathogens is crucial for understanding the possible impact of birds as spreaders of ticks and tick-borne pathogens. Successful colonization of new tick species or introduction of new tick-borne pathogens will depend on suitable climate, vegetation and hosts. Although it has never been demonstrated that a new tick species, or a new tick pathogen, actually has been established in a new locality after being seeded there by birds, evidence strongly suggests that this could occur.

Keywords: ticks, migratory birds, tick-borne pathogens, tick-borne encephalitis virus, borrelia, babesia

INTRODUCTION

There is ample evidence that birds, particularly passerines, can be parasitized by Ixodid ticks (Hoogstraal et al., 1961, 1963; Nuorteva and Hoogstraal, 1963; Anderson and Magnarelli, 1984; Mehl et al., 1984; Weisbrod and Johnson, 1989; Stafford et al., 1995; Olsen et al., 1995a; Nicholls and Callister, 1996; Smith et al., 1996; Ishiguro et al., 2000; Alekseev et al., 2001; Björsdorff et al., 2001; Scharf, 2004; Comstedt et al., 2006; Poupon et al., 2006; Ogden et al., 2008; Hasle et al., 2009). These ticks may be infected with tick-borne pathogens, like *Borrelia* spp. (Olsen et al., 1995a,b; Gylfe et al., 2000; Hanincova et al., 2003; Comstedt et al., 2006; Poupon et al., 2006; Ogden et al., 2008; Hasle et al., 2010; Kjelland et al., 2010; Franke et al., 2012; Socolovschi et al., 2012), *Anaplasma* spp. (Alekseev et al., 2001; Björsdorff et al., 2001; Daniels et al., 2002; Ogden et al., 2008; Franke et al., 2012), *Babesia* spp. (Hasle et al., 2011), *Rickettsia*/*Coxiella* (Elfving et al., 2010; Socolovschi et al., 2012) and Tick-borne encephalitis virus (TBEV) (Waldenström et al., 2007; Geller et al., 2013). The prevalence of ticks on birds varies between years, season, locality and different bird species. The prevalence of ticks on different species depends mainly on the degree of feeding on the ground (Mehl et al., 1984; Hasle et al., 2009; Marsot et al., 2012). In particular, thrushes, i.e., the *Turdus* spp. in Europe (Hasle et al., 2009), Russia (Alekseev et al., 2001) and Japan (Ishiguro et al., 2000), and *Catharus* spp. in North America (Smith et al., 1996), have a high prevalence of tick infestation. e.g., in our Norwegian material 31.5% of blackbirds, *Turdus merula*, and 25.1% of song thrushes, *T. philomelos*, were infested by ticks (Hasle et al., 2009). Blackbirds are often infected with several ticks on each bird. Five per cent of blackbirds ($N = 543$) had ten or more ticks, and one single blackbird had 66 nymphs of *Ixodes ricinus* (own data). Only one study has compared historic and new data concerning ticks on birds. Hasle et al. (2009) compared data from 2003 to 2005 with Mehl et al.'s (1984) data from 1965 to 1970 on two Norwegian bird observatories, and found an increase of the prevalence of tick infestation on passerine northward migrating birds from 4.2 to 6.9% ($p < 0.001$). Birds can easily cross barriers, like fences, mountains, glaciers, desserts and oceans, which would stop mammals, and they can move much faster than the wingless hosts. Therefore, birds have a potential of spreading ticks far beyond the home ranges of mammals and reptiles. Migrating bats can, like birds, cross barriers and move long distances in a short time, and could also have a potential of transporting ticks and tick-borne pathogens, like *Bartonella*, *Borrelia* spp. and members of the family Rickettsiales (Gill et al., 2008; Mühldorfer, 2013).

INTRODUCING NEW TICK SPECIES

Although it has never been demonstrated that a new tick species, or a new tick pathogen, actually has been established in a new locality after being seeded there by birds, the evidence strongly suggests that this could happen. Hasle et al. (2009) studied northward migrating birds on four bird observatories situated north of

the Skagerrak and Kattegat (**Figure 1**). From 9.768 passerine birds examined they found seven nymphs of *Hyalomma rufipes*. The *Hyalomma* species have a northern distribution limit in Southern Europe and North Africa (Estrada-Peña et al., 2004), and these nymphs would have had to be transported all the way from the Mediterranean to Norway, the last 130–150 km over open sea. Although many *Hyalomma* individuals may be brought to Norway every year, they would not settle in a cold temperate climate. Also, a larva of *Dermacentor* sp. was found on a willow warbler, *Phylloscopus trochilus* on Akerøya, Hvaler, an island off the Southern Norwegian coast. No *Dermacentor* sp. is endemic in Norway. These findings demonstrate that non-endemic tick species can be transported to new places, across geographic barriers. Considering an influx of 30–80 million passerines crossing the sea every spring, millions of ticks will be transported every year, and transport of exotic species would not be a rare event. The limiting factor will not be the ticks' dispersal ability, but the suitability of the area the ticks are released for the survival and reproduction of the ticks. A suitable climate is one of the prerequisites for establishing a tick species. For *I. ricinus*, which has its northern border of distribution in Norway and Sweden, the distribution correlates well with the isolines for the first frost night, number of days with snow cower, and the length of the growth season (Jaenson et al., 2009). As these isolines overlap we cannot from these data tell which climatic factor is the most important. As ticks are prone to desiccation (Daniel and Dusbábek, 1994), microclimatic conditions of sun, soil and vegetation cover will determine the individual ticks' chance of survival, much like in the Parable of the Sower (Mattew 13:5). *Dermacentor reticulatus*, which occurs in North Germany, is a vector for *Babesia canis*, *F. tularensis*, *Rickettsia slovaka* and *C. Burnetii*. It needs to complete the whole life cycle during one year (Dautel et al., 2006), which unlikely could take place during a normal year in Norway. However, future climate change could make Norway hospitable for this species. Conceivably, individual specimens of *D. reticultus* could transmit diseases after being transported to Norway by birds. An indirect evidence of this is a case of *Babesia canis* in a Norwegian dog that had not been abroad. *D. reticulatus* is the main vector for *B. canis* (Øines et al., 2010).

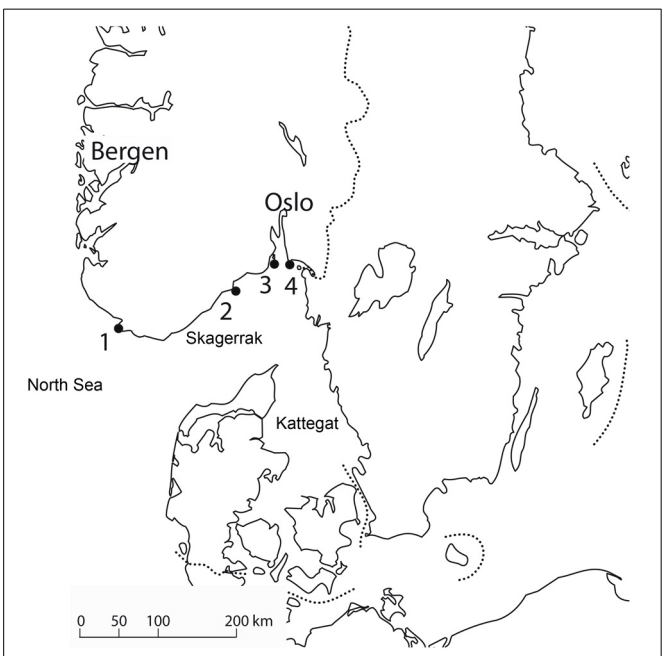

FIGURE 1 | Study area of Hasle et al. (2009). The participating bird observatories were: (1). Lista, (2). Jomfruland, (3). Store Færder, (4). Akerøya. Northward migrating birds would have to cross The North Sea, Skagerrak or Kattegat.

Apart from protection from frost and desiccation, a good tick habitat will depend on appropriate hosts. e.g., islands that harbor only small animals cannot support the full life cycle of *I. ricinus*, as the adults need hosts of a certain minimum size (Jaenson et al., 1994). Possibly, some tick species may be host specific, although this is controversial (Randolph, 2004).

For establishing a new population, an ecological *niche* must be available. According to the Competitive exclusion principle, two different species cannot sustainably occupy the same *niche* (Hardin, 1960). However, this is a theoretical consideration, and very similar species, like *I. ricinus* and *I. persulcatus* may have different climate requirements, e.g., *I. persulcatus* is much more cold-resistant than *I. ricinus* (Tokarevich et al., 2011). Therefore, their *niches* are not exactly identical. Furthermore, in a transient situation sympatric distribution can occur, as is shown in a recent publication showing a shift in the distribution range of *I. persulcatus* toward North and West in Karelen, Russia, into areas previously occupied by *I. ricinus* (Bugmyrin et al., 2013). It is shown for *I. persulcatus* (Filippova, 2002) as well as *I. scapularis* (Oliver et al., 1993) that they can readily mate and breed with *I. ricinus*, but the offspring is sterile. In a situation with coexistence, the reproductive success of the least abundant species would suffer much more than the most abundant species (Hasle, 2010). Ticks seeded in an area occupied by another species with which they can interbreed would have a very low chance of copulating with the same species.

Climate change, as well as changes in vegetation, is expected to influence the distribution of different tick species (Estrada-Peña, 2001; Gray et al., 2009). Global warming would be expected to push a Northern limit of a distribution range further north. Such changes take place over decades, and dispersal by mammalian tick-hosts would be sufficient to keep up with the changes within a continent. However, if a geographical barrier, e.g., a sea, like Skagerrak and Kattegat, separates two hospitable areas, one with a tick species, and one without that species, and if the ecological *niche* is not occupied by another tick species, transport by birds would be a possible mechanism.

TRANSPORT OF TICK-BORNE PATHOGENS

Birds can potentially transport tick-borne pathogens by transport of infected ticks. In addition, the birds may act as amplifying host by being infected with tick-borne pathogens that can infect their parasitizing ticks, which can transmit the pathogens to subsequent hosts, or by hosting co-feeding ticks, i.e., ticks that feed near each other on the same host, when pathogens may pass from one tick to the other.

TRANSPORT OF INFECTED TICKS

Transport of already infected ticks is an obvious mechanism of transporting tick-borne pathogens by birds, as most tick-borne pathogens survive transstadially, i.e., from larvae to nymphs, and from nymphs to adults. This is shown for *Anaplasma marginale* (Stich et al., 1989), *Babesia divergens* (Bonnet et al., 2007), *Borrelia burgdorferi* sensu lato (Bellet-Edimo et al., 2005) and TBEV, and is indeed a prerequisite for being a tick-borne pathogen (Randolph, 2011). However, the transstadial survival may not be 100%, as some data indicate that *B. afzelii* can disappear from *I. ricinus* (Matuschka and Spielman, 1992) and *B. burgdorferi* s.s. from *I. scapularis* (Ogden et al., 2008) that are feeding on birds in Europe and North America respectively. Corresponding to this, Hasle et al. (2010) found that engorged ticks had a lower prevalence, i.e., odds ratio 0.24 ($P = 0.004$), of *B. afzelii* than unengorged ticks from birds. To spread a pathogen, a later stadium of the tick would have to parasitize a host that can mediate further transmission. Adult *I. ricinus* parasitize animals of the size of cats and larger (Jaenson et al., 1994), while small rodents and birds are the main reservoirs for the *Borrelia* spp. most common in Europe (Kurtenbach et al., 2002). Therefore, when fully engorged, *Borrelia*-infected nymphs molt to adult stage, it is not obvious that they can spread the pathogen further. However, although not very efficient, transovarial transmission is described for Babesia (Bonnet et al., 2007), Borrelia (Bellet-Edimo et al., 2005) and TBEV (Danielová and Holubová, 1991), which means that infected larvae can be transported by birds, and then molt to infected nymphs. Likewise, adult ticks may produce infected offspring after arrival on a new place, by transovarial transmission.

Human pathogens can also be transported by ticks that normally don't bite humans, as is shown for *I. uriae* (Olsen et al., 1995b).

In the case of TBEV, the virus is thought to be maintained in nature mainly by transmission through co-feeding on small rodents, as infected nymphs spread the virus to co-feeding larvae (Labuda and Randolph, 1999). It is almost exclusively nymphs and larvae of *I. ricinus* that can be transported by birds (Jaenson et al., 1994; Hasle et al., 2009). As the bird host would be the first host for the feeding larvae, they would not carry TBEV before entering the bird unless the virus is transmitted transovarially. Infected nymphs would proceed to adult stage after feeding, and would not feed on small rodents. Therefore, the TBEV-infected ticks transported by birds would be a dead-end for maintaining the TBEV in nature, without transovarial transmission. Assuming transovarial transmission, the TBEV could be seeded by birds to new areas if the climate, vegetation and hosts are suitable for the natural TBEV-cycle. In Norway, the first cases of TBE were notified in Southern Norway in 1998. Since then there has been an increase in annual cases, to the present 10–15 cases per year. A very relevant question would be why this has not happened before. The answer may well be that it has happened before, but that the conditions necessary for maintaining the fragile cycle of TBEV (Randolph and Rogers, 2000) in the nature have not been present before. The distribution of TBEV in Scandinavia is discontinuous, with at least 300 km of land distance from the endemic areas in Sweden (Båhuslän) to the Agder counties in Norway, while crossing the Skagerrak or Kattegat sea is a three to five hours flight for small passerine birds. It is difficult to find another explanation for this discontinuous distribution than transport via birds. This is the closest we can come to a proof that birds have seeded a tick-borne infection to a new area.

Babesia venatorum is an emerging tick-borne human disease in Europe. The first findings of this parasite in Norway have been on four nymphs of *Ixodes ricinus* brought to Norway by birds (Hasle et al., 2011). *B. venatorum* is a primarily a roe deer parasite (Duh et al., 2005), and adult *I. ricinus* readily feed on roe deer. Therefore, birds could effectively import and spread this parasite.

BIRDS INFECTED BY TICK-BORNE PATHOGENS

Birds can be infected by different genuses of Anaplasmataceae, i.e., *Anaplasma phagocytophilum* (de la Fuente et al., 2005), *Rickettsia rickettsia* (Lundgren et al., 1966) and *Coxiella burneti* (Babudieri and Moscovici, 1952). We have not found any experimental data confirming that birds infected by the Anaplasmataceae can transfer these pathogens to ticks. The *Babesia* spp. appears to be host specific, at least to the class, and some of them to one species (Peirce, 2000), no mammal *Babesia* sp. has been found infecting birds. Some species of *Borrelia* can infect birds, notably *B. garinii*, *B. valaisiana*, *B. turdi* (Gylfe et al., 2000; Richter et al., 2000) and *B. burgdorferi* s.s. (Anderson and Magnarelli, 1984; Anderson et al., 1986), but probably not *B. afzelii* (Kurtenbach et al., 2002), although this notion has been challenged by the findings of Franke et al. (2010). Hasle et al. (2010) found an odds ratio for *Borrelia* infection of 4.3 for ticks parasitizing the *Turdus* spp., i.e. 3.5 for *B. garinii* and 30.3 for *B. valaisiana*. This indicates that the *Turdus* spp. are more susceptible for infection with these *Borrelia* genospecies than other birds, and that the ticks got the pathogen during feeding on the bird. Thus, the *Turdus* spp., apart from being likely to carry ticks, were more likely to carry *Borrelia*-infected ticks than other bird genera. Therefore, the *Turdus* spp., especially the blackbird, may be important in the spreading and hosting of *B. garinii*, which is the main agent for neuroborreliosis (van Dam et al., 1993; Balmelli and Piffaretti, 1995).

The *Borrelia* species causing tick-borne relapsing fever are transmitted by soft ticks, *Ornithodorus* spp., which have a different biology from the *I. ricinus*-like ticks in that they live in nests and burrows and have a feeding time of just a few minutes Johnson and Golighty (2000). These ticks would not be prone to long-range transport by birds, but the birds may contract the *Borrelia*, which can be transmitted further, as suggested for *Borrelia hermsii*, by (Schwan et al., 2007).

Transport of tick-borne pathogens from one endemic area to another could have an impact, even if the pathogens already occur there, by spreading new strains to new areas.

TRANSFER OF PATHOGENS THROUGH CO-FEEDING

Transmission of a tick-borne pathogen through co-feeding has been demonstrated for the tick-borne encephalitis-virus (TBEV) on *Myodes glareolus* and *Apodemus sylvaticus* (Labuda and Randolph, 1999), but no data exists for such transmission of TBEV on birds. In Waldenström et al.'s material (2007) they found

three TBEV-positive *I. ricinius*, one nymph and two larvae, on one individual European robin, *Erithacus rubecula*, which strongly suggests that the bird either was viremic or that transmission through co-feeding did occur.

Also, for *Borrelia* this mechanism has been suggested for mammals. Gern and Rais (1996) demonstrated transmission of *B. burgdorferi* between co-feeding *I. ricinus* on AKR/N mice in the laboratory. By using a generalized linear model (GLM) Hasle et al. (2010) found that ticks that were co-feeding on birds with ticks infected with one genospecies of *Borrelia* had an increased probability of being infected with the same genospecies. A tick co-feeding with another tick with *B. afzelii* had an odds ratio of 3.9. If the statement that birds cannot be infected with *B. afzelii* is correct, this is an indication that non-systemic transmission between co-feeding ticks has occurred. For *B. valaisiana* the odds ratio for ticks co-feeding with other ticks with *B. valaisiana* was 760, compared to the average prevalence. The latter could partly be explained by the effect of feeding on the *Turdus* spp., and by systemic infection of the bird. Geller et al. (2013) found the same strain of *B. afzelii* (PGau) on two *I. ricinus* nymphs that were feeding on the same Great tit (*Parus major*), a finding that suggests transmission through co-feeding.

The existing data support the hypothesis that all the three mechanisms of spreading tick-borne pathogens by birds may occur in the nature.

MIGRATION ROUTES

Knowledge of the bird migration routes is crucial for understanding the possible impact of birds as spreaders of ticks and tick-borne pathogens. For instance, the main direction of migration in Europe is NE/SW for the *Turdus* spp., *E. rubecula, Phoenicurus phoenicurus, Prunella modularis* and *Troglodytes troglodytes* (Gjershaug et al., 1994; Bruderer, 1997; Fransson and Hall-Karlsson, 2008), which are all species with a high prevalence of tick infestation (Hasle et al., 2009; Marsot et al., 2012). These species would be unlikely to transport the TBEV from Central to North-West Europe, but could possibly transport this virus from Central to South-West Europe. On the other hand, species that migrate along a more N/S direction across the Alps, like *Sylvia curruca, S. communis* and *S. atricapilla*, or NW/SE, like *Motacilla alba* and *Luscinia svecica* (Gjershaug et al., 1994; Fransson and Hall-Karlsson, 2008) could potentially cross the Skagerrak sea and transport ticks carrying TBEV from the TBE-endemic areas in Central Europe to Norway and Sweden. During NW migration *Motacilla alba* and *Luscinia svecica* may pass areas endemic for *Ixodes persulcatus* before reaching Sweden, and could, theoretically, introduce this tick species to Sweden, as the climate in Sweden would be suitable for *I. persulcatus*. However, the ecological *niche* is already occupied, by *I. ricinus*, and *I. persulcatus* would therefore be unlikely to colonize Sweden, even if they were brought there by birds. On the other hand, to bring this speculation further, if they can be transported past areas occupied by *I. ricinus* it could still remain a possibility for colonization of *I. persulcatus* in Sweden. *I. persulcatus* survives extreme winter temperatures in Siberia, and could possibly do that in Scandinavia as well. The findings of *I. persulcatus* and human cases with S-TBEV in Kokkola (N63°50′ E23°07′), Finland, several hundred kilometres from the known western distribution range of *I. persulcatus* (Jääskeläinen et al., 2006), suggests long distance transport by birds.

CONCLUSIONS

Transport of ticks by migratory birds, including tick species and tick-borne pathogens that are not endemic to a new area, is a common event. Establishment of a new tick species in an area will require favorable climate, vegetation and hosts, and an available ecological *niche*. New tick-borne pathogens could be spread if there are susceptible tick and vertebrates hosts present. It still remains to prove if this way of dispersal has happened.

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Host specialization in ticks and transmission of tick-borne diseases: a review

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Determining patterns of host use, and the frequency at which these patterns change, are of key importance if we are to understand tick population dynamics, the evolution of tick biodiversity, and the circulation and evolution of associated pathogens. The question of whether ticks are typically host specialists or host generalists has been subject to much debate over the last half-century. Indeed, early research proposed that morphological diversity in ticks was linked to host specific adaptations and that most ticks were specialists. Later work disputed this idea and suggested that ticks are largely limited by biogeographic conditions and tend to use all locally available host species. The work presented in this review suggests that the actual answer likely lies somewhere between these two extremes. Although recent observational studies support the view that phylogenetically diverse host species share ticks when found on similar ecological ranges, theory on host range evolution predicts that host specialization should evolve in ticks given their life history characteristics. Contemporary work employing population genetic tools to examine host-associated population structure in several tick systems support this prediction and show that simple species records are not enough to determine whether a parasite is a true host generalist; host specialization does evolve in ticks at local scales, but may not always lead to speciation. Ticks therefore seem to follow a pattern of being global generalists, local specialists. Given this, the notion of host range needs to be modified from an evolutionary perspective, where one simply counts the number of hosts used across the geographic distribution, to a more ecological view, where one considers host use at a local scale, if we are to better understand the circulation of tick-borne pathogens and exposure risks for humans and livestock.

Keywords: adaptation, Argasidae, *Borrelia burgdorferi* sensu lato, community diversity, epidemiology, Ixodidae, population genetic structure, transmission

INTRODUCTION

The host range of a parasite, that is, whether it is a host generalist or host specialist, is a vital life history trait that will affect both a parasite's population dynamics and its evolutionary trajectory. Specialist parasites may better track host responses to infection and thus be better able to exploit a host than generalist parasites experiencing diffuse host selection pressures (Whitlock, 1996; Lajeunesse and Forbes, 2002), whereas generalists may be better adapted to cope with environmental variation that affects host community stability (e.g., Kassen, 2002). Host range can also have direct impacts on interacting organisms. For example, generalist parasites can play an important role in host competitive interactions; if one host type suffers more strongly from infection than another, and if those host species compete for resources, infection may tip the balance in favor of the more resistant or tolerant host type (apparent competition; Park, 1948; Holt and Lawton, 1994). When a parasite is also a vector, host range takes on an entirely new dimension because the ability of a vector to exploit one vs. several hosts will not only affect its own population dynamics and evolutionary trajectory, but also that of the associated microparasites.

Ticks are particularly interesting organisms to consider in terms of host range evolution and its consequences. These macroparasites have strong direct effects on host reproductive success and population dynamics, particularly when infestation intensities are high (e.g., Feare, 1976; Duffy, 1983; Boulinier and Danchin, 1996). In tropical zones, they can have significant impacts on livestock production and are a major focus of control efforts (Frisch, 1999; Jonsson, 2006). Ticks also transmit the greatest diversity of pathogenic agents among vector organisms; many of these microparasites are widespread and of considerable medical and veterinary interest (Parola and Raoult, 2001). Understanding the links between host biodiversity, tick host preference and performance, and pathogen transmission are therefore essential for predicting both tick population dynamics and the epidemiology of tick-borne diseases.

Theoretical studies suggest a direct relationship between host diversity and disease risk, but not always in the same direction (Begon, 2008; Johnson and Thieltges, 2010). At a global scale, we expect that increased host biodiversity should lead to increased parasite diversity and exposure (Jones et al., 2008). However, at more local scales, the effect of adding biodiversity can be more variable (Salkeld et al., 2013). With the addition of highly competent hosts for either the pathogen or the vector, or by increasing vector biodiversity, transmission risk may increase (Ogden and Tsao, 2009; Roche et al., 2013). However, the reverse may also be true. In particular, the dilution effect model which predicts reductions in disease risk with increases in host biodiversity, has gained significant popularity over the last decade (Keesing et al., 2006; Randolph and Dobson, 2012). Under this model, different host species within a community vary in their disease reservoir competence; by increasing local biodiversity, competent hosts are diluted among non-competent hosts and the overall density of infected individuals is reduced (Ostfeld and Keesing, 2000). Some empirical evidence for this effect has been found (e.g., Logiudice et al., 2003; Ezenwa et al., 2006; Haas et al., 2011; Johnson et al., 2013), but criticisms to its universality are numerous. For example, it often remains difficult to distinguish between true dilution and an simple reduction in reservoir density when additional species are added to a community (Begon, 2008). Likewise, this model assumes that vector abundance remains the same with increases in host biodiversity (i.e., there is no vector amplification) (Randolph and Dobson, 2012); when vector abundance increases, the number of infected vectors may remain the same, even if infection prevalence is reduced. Finally, and most importantly for this review, the dilution model assumes that vectors are host generalists and exploit both competent and non-competent hosts within a community (Ostfeld and Keesing, 2000). Surprisingly, we have little information on this last aspect, even in well-studied systems. If strong host preferences occur within a community, particularly to the point where vectors have evolved into host races, this model is no longer applicable because, regardless of the diversity of local host species, vectors may tend to use the preferred host. Indeed, host feeding preferences in mosquitoes have been suggested to explain the lack of evidence for the dilution model in some mosquito—West Nile virus systems (e.g., Kilpatrick et al., 2006) and it is becoming increasingly apparent that specific local interactions between reservoir hosts and vectors likely dictate disease risk more than host diversity *per se* (Salkeld et al., 2013).

The aim of the present review is to outline what we currently know about host specialization in ticks and to discuss how this process may affect pathogen circulation. To give proper weight to current ideas, we start with a discussion of the historical notions associated with host range evolution in ticks. We then outline expectations of host use in ticks with respect to previous theoretical work. We review recent studies that have addressed this question using a population genetic approach and show that simple species records are not enough to determine whether or not a parasite is a true host generalist. We then provide a direct example of how host specialization in a tick vector can impact pathogen circulation using Lyme disease bacteria transmission within its marine cycle as a case study. We finish with a discussion of why ticks are exciting organisms to consider in terms of their ability to shift hosts and how the evolution of local host specialization may greatly alter our ability to make reasonable predictions on exposure risk and disease epidemiology.

HISTORICAL VIEWS ON HOST SPECIALIZATION IN TICKS

Many ticks, both hard (Ixodidae) and soft (Argasidae), exploit their hosts for only a short period of time (hours to days) during the bloodmeal and their survival therefore depends strongly on their ability to cope with the conditions of the abiotic environment for the long off-host periods of their life cycle. This dual life style has led to some debate about the relative roles of host and habitat factors in determining both the limits to tick distributional ranges and their evolution.

Based on consideration of specific morphological adaptations, Hoogstraal and colleagues felt that the evolution of tick biodiversity was closely linked to that of their hosts (Hoogstraal and Aeschlimann, 1982; Hoogstraal and Kim, 1985). Hoogstraal and Aeschlimann (1982) considered that at least 700 of the 800 described species of the superfamily Ixodoidea were strict, or relatively strict, host specialists and that this characteristic limited the geographical distribution and population density of most tick species. They suggested that anomalies in recorded data had contributed to an incorrect or ambiguous view of host specificity in this group of parasites. As an example, they note that a tick will secondarily attach to any host type if dislodged during the bloodmeal because its discriminatory senses are dulled or lost and that this behavior may frequently lead to erroneous host records. They do, however, concede that host specificity in ticks is also tightly linked to the ecological characteristics of the host species themselves; hosts that form breeding aggregations have, for example, more specialized ticks, whereas wandering hosts have ticks with either modified life cycles (1 or 2 host ticks) or moderate to low host specificity. These authors further suggested that domestication likely improved conditions for these moderate to low host specific ticks and that their exploitation of livestock has focused our attention on these examples. For them, ticks placed in the "non-particular specificity" category required more intensive investigation, but still remained the exception to the rule.

Klompen et al. (1996) called into question the notion that tick evolution was closely linked to that of their hosts and notably by criticizing the idea that current observations of host-associations in ticks supported host specificity and co-speciation in this group. The authors compiled data from the published literature and found strong positive correlations between the degree of host specificity and sampling effort (number of collections). They felt that this analysis, along with numerous examples of ticks that exploit diverse taxa sharing the same ecological habitat, demonstrated that perceived host specificity in ticks was largely an artifact of incomplete sampling. They suggest that most ticks are not limited by host use, but rather by biogeography; abiotic conditions during the long off-host period of the life cycle. Interestingly, they also remark that recorded host specificity is not a measure of host adaptation *per se*. Although seemingly meant to support the notion that perceived host specialists may not be specifically adapted for the host they are using, the statement can also be interpreted to the contrary that is, perceived host

generalists may in fact show specific adaptations to particular host types that are undetected in typical collecting studies.

The view put forth by Klompen and colleagues was supported by a later study that examined ecological ranges of ticks and their hosts. In particular, Cumming (1999) compared the recorded range of different African tick species compiled in a database of published collection records to that of known host species. He found that ∼50% of the 229 examined tick species had more restricted ranges than their hosts and that records for the other 50% of species were not complete enough to make any strong conclusions. Only one tick species *Amblyomma rhinocerotis*, a specialist tick of rhinoceros, seemed to conform to the hypothesis that range limits in ticks are determined by their hosts. However, records used in the study did not differentiate between successful and failed host use attempts and did not explicitly consider the relative abundance of ticks on a host or the frequency of records for a given host type. This means that for some species, recorded host ranges may be larger than in reality. Likewise, no consideration was given to local host densities; in some areas of Africa, host densities may be too low to support viable tick populations.

More recently, Nava and Guglielmone (2013) published a meta-analysis of host specificity in Neotropical ticks where they explicitly considered uneven host use and the phylogenetic relatedness of recorded host species [notably by the incorporation of Poulin and Mouillot's (2005) specificity index]. Like Klompen et al. (1996), they found a significant correlation between host range and sampling effort. This relationship was less obvious for the specificity index, supporting the use of this index as a more reliable measure of specificity. Based on their results, no Neotropical tick species was limited to a single host species, and most species used an array of hosts belonging to different families or orders. The index tended to show lower host specificity for immature stages compared to adult stages, a result the authors attribute to host-size constraints that may limit the number of available hosts for adult ticks. More generally, this study concludes that host ecological similarities are more important than host phylogeny in shaping host-parasite relationships in ticks and that ticks tend to be host generalists.

Although these meta-surveys all agree on the primary importance of the abiotic habitat in determining host range and the geographic distribution of ticks, they also all share the same potential pitfalls. First, these studies all assume that tick species are correctly identified using standard morphological characters. However, recent work has shown that the notion of a tick species can be complex (e.g., Estrada-Peña et al., 2012) and many ticks remain poorly described (e.g., Dantas-Torres et al., 2012). Soft ticks are notoriously difficult to identify (Estrada-Peña et al., 2010) and call into question many host records. In addition, many recent revisions have been made that incorporate genetic-based identifications (Guglielmone et al., 2010), but such methods were not employed in historical survey data. Even host range studies that make tick identifications based on conserved genetic markers or traditional morphological characters may miss recently evolved divergences or more cryptic phenotypic changes. More detailed analyses at both genetic and morphological levels can reveal such divergence events (see below). Finally, host specialization in these studies is largely considered from a simple quantitative perception (i.e., number of hosts used). A qualitative framework (i.e., differential performance on different hosts) may provide a more realistic picture of natural interactions and may help us better predict host use and disease risk.

HOW SPECIALIZED SHOULD WE EXPECT TICKS TO BE?

The specialist-generalist dilemma is founded on the notion "a jack of all trades is master of none." That is, there is an assumed cost of being adapted to a particular host in that it limits fitness on alternative hosts. This cost and the optimal host range for a parasite will depend on several factors related to the intrinsic characteristics of the parasite, those of the host, and the conditions of the local environment. For example, host availability and predictability is thought to be of prime importance (Jaenike, 1990; Combes, 2001); when hosts are found in high abundance and temporally predictable, parasites should specialize to maximize fitness. Host availability will, of course, depend on both local host abundance, a parameter of the host population, and the ability of a parasite to reach the host through passive or active dispersal, an inherent trait of the parasite. The relative intimacy of the interaction may also dictate optimal specialization; when parasites require a suite of specific traits to overcome physical and physiological barriers to host exploitation, we expect that successful exploitation of a large range of hosts will be difficult (i.e., specialization should evolve). The outcome of ecological specialization can also be modified by local interactions that may favor use of one host over another, such as exposure to predators that alters host–parasite encounter rates (Forister et al., 2012). The mechanistic basis for the presumed cost of adaptation was initially considered to take the form of simple genetic trade-offs; a parasite carrying a certain allele to optimally exploit one host, should suffer lower fitness on alternative hosts (Futuyma and Moreno, 1988). However, the natural world appears more complex and host adaptation likely depends on the genetic architecture underlying a series of traits involved in host use and the potential consequences of gains or losses of particular gene functions (Forister et al., 2012). Both theoretical and field-based studies have suggested that the joint evolution of host preference and performance can greatly favor the evolution of host specialization by restricting the homogenizing effects of gene flow (e.g., De Meeûs et al., 1995; Ravigné et al., 2009; Forister et al., 2012). Gene duplications have also been shown to facilitate expanded resource breadth (e.g., Makino and Kawata, 2012) and may play a role in initiating the evolution of host specificity after a host shift. Finally, these different factors will be conditioned by the relative evolutionary potential of the host and the parasite that is, their respective reproductive rates and generation times (Gandon and Michalakis, 2002).

Several features of ticks may favor the evolution of host specialization (Magalhães et al., 2007). First, ticks engage in a deeply intimate exchange when they exploit their host, particularly in the case of hard ticks where bloodmeals last several days. To maintain blood flow, reduce detection and evade vertebrate immune responses, ticks inject a plethora of bioactive molecules into the host with the saliva (Brossard and Wikel, 2004; Francischetti et al., 2009). This is necessary because innate and acquired immunity in vertebrates can strongly limit tick success (Sonenshine, 1993).

For example, Anderson et al. (2013) found a negative association between innate immunity (measured as host blood bacterial killing ability) and the abundance of *Amblyomma hebraeum* and *Rhipicephalus evertsi evertsi* in free ranging African buffalo. Similarly, the development of acquired immunity has been shown to vary significantly among different host species of *Ixodes ricinus* (e.g., Randolph, 1994). The complexity of the tick-host interface should, in principal, limit host range and select for specificity. Similarly, ticks generally have low active dispersal rates and must rely on host movements for among population dispersal (Falco and Fish, 1991; Balashov, 2010). They also frequently emit aggregation hormones that can favor site fidelity (Sonenshine, 1991). These traits will both increase encounter rates with the same host type and limit gene flow between populations that may be undergoing diffuse selection pressures. At the extreme, certain tick species may remain on a single host individual for the entire life cycle (one-host tick), dropping off the host only to lay eggs (e.g., *Rhipicephalus microplus*); in such cases, one may expect strong selection for host specialization (see below). Reproductive potential should also be greater in ticks than in their hosts; ticks can lay anywhere between 500 and 20,000 eggs after repletion, with hard ticks laying all eggs in a single bout and soft ticks spreading laying out over several short bloodmeals (Sonenshine, 1991). Generation times likewise vary between 1 and 6 years (Sonenshine, 1993), and although long for a parasite, they will frequently be shorter than for the associated vertebrate hosts. The last factor, related to the underlying genetic architecture of adaptation in ticks, is more complicated and we are only in the very initial stages of understanding genetic evolution and adaptation in ticks. In general, tick genomes are large (one-third to two times the human genome) with a high percentage of repeated elements (Nene, 2009; Meyer et al., 2010). The presence of high quantities of mobile elements may, on the one hand, generate high background genetic variation on which selection for specialization may act, but they may also break apart adaptive gene complexes that favor adaptation to specific hosts (e.g., Sunter et al., 2008; Casacuberta and Gonzalez, 2013). Results of genome sequencing projects on focal tick species should improve our understanding of how genetic architecture modulates the evolution of host specialization in ticks (Hill and Wikel, 2005; Guerrero et al., 2006).

INTEGRATION OF POPULATION GENETICS TO STUDY HOST USE

When considering the evolutionary ecology of ticks and their associated pathogens, the notion of host range has to be modified from an evolutionary perspective, where one considers counts of the number of hosts used across the geographic distribution, to a more ecological perspective where one considers host use at a local scale and its implications in terms of co-evolutionary interactions and pathogen circulation. Indeed, field observations suggest that even broad host generalists tend to feed on only a few main hosts locally, with these hosts changing across different areas of the distribution (e.g., Balashov, 2010). This type of local thinking can provide key information on the relative ease of host switching in these species and how this may modify disease risk. With the development of genetic and genomic tools, we can now have more ready access to information on host use and specialization in vector-borne disease systems at local scales, going beyond simple records of observed host use. In particular, population genetic approaches that employ neutral genetic markers, in combination with information on host use, can enable us to determine whether ticks have locally diverged into reproductively isolated units that exploit specific host types (McCoy, 2008). Even host use information can be obtained indirectly via modern molecular methods (e.g., amplification and identification of bloodmeal traces; Kent, 2009) in cases where direct host sampling is not possible. This information can then be related to pathogen prevalence estimates in both hosts and vectors to infer transmission pathways and disease risk. Only a few such studies on ticks have been conducted to date, but all have revealed significant patterns of local host-associated genetic structure in ticks. We briefly review these examples in the following paragraphs.

The cosmopolitan seabird tick *Ixodes uriae* was the first tick system studied to test for the presence of host-associated population genetic structure. This hard tick exploits nesting colonial seabirds in the circumpolar regions of both hemispheres and was considered to be a seabird generalist, with more than 60 different host species recorded (Dietrich et al., 2011). *I. uriae* is a nidicolous (i.e., nest-inhabiting) tick and is only associated with its seabird host for the bloodmeal which it takes once in each of its three life stages (larva—nymph—adult; adult males do not feed) for a period of between 3 and 12 days, the length increasing with successive life stages (Eveleigh and Threlfall, 1974; Frenot et al., 2001). The remainder of its life is spent in the substrate surrounding the host nesting area, often in aggregates of several hundred individuals (Benoit et al., 2007). This tick reproduces sexually and a female will lay several hundred eggs in a single bout before dying (Eveleigh and Threlfall, 1974). A diverse array of viruses and bacteria circulate in seabirds via *I. uriae*. Some are clearly pathogenic for humans, like the bacteria of the *Borrelia burgdorferi* sensu lato (Bbsl) complex responsible for Lyme disease (see below), whereas others are largely limited to seabirds with unknown effects for these hosts or for humans (Dietrich et al., 2011). After observing asynchrony in the timing of tick exploitation on different sympatric seabird species, McCoy et al. (1999) hypothesized that *I. uriae* populations may consist of a series of local host-specific groups. They tested the predictions of this hypothesis using a population genetic approach with specifically-developed microsatellite markers (McCoy and Tirard, 2000) and found that throughout the different zones of its global distribution, this tick had indeed formed host-specific genetic groups (or host races) and that these host races had evolved independently in different isolated regions (McCoy et al., 2001, 2005, 2012; Dietrich et al., 2012). This divergence has been suggested to be relatively recent (Kempf et al., 2009a), but has been accompanied by both phenotypic changes in body morphology (Dietrich et al., 2013) and host-associated variation in performance on different host species (Dietrich, 2011). An example of local patterns of host-associated divergence in this tick is shown in **Figure 1**. For this analysis, ticks were sampled from three different seabird hosts breeding within four large colonies and were genotyped at a series of microsatellite markers. All tick individuals were included in a global clustering analyses (see figure legend for details) and the main pattern found

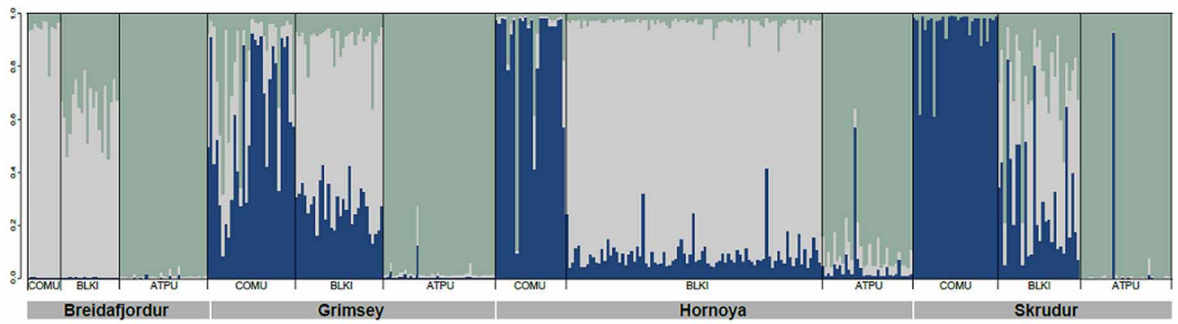

FIGURE 1 | Host-associated genetic structure in the seabird tick *Ixodes uriae* from four North Atlantic mixed colonies. The data for 8 microsatellite markers are re-analysed from Kempf et al. (2009a). The number of genetically distinct pools of individuals present within the sampled populations was determined using the Bayesian clustering approach implemented in STRUCTURE v.2.3.1 (Pritchard et al., 2000). The number of potential clusters (K) was set from 1 to 13, with 5 independent runs. Computations were run under the admixture model with correlated allelic frequencies and sampling location as a prior. Simulations were carried out using a burn-in of 100,000 iterations, followed by a run length of 100,000 iterations. The most probable number of populations was 3. Each tick individual is represented by a thin vertical bar composed of K segments whose length is proportional to the probability that the tick belongs to each genetic pool (here, respectively blue, light gray, and green for the 3 groups). The seabird hosts present in each colony are shown below the colony name and are abbreviated as in **Table 1**. In all colonies, there is significant genetic structure among the 3 tick groups (Kempf et al., 2009a) with the appearance of a well-defined genetic race for ATPU ticks. For COMU ticks, the Breidafjordur colony showed a different pattern of group membership than the other 3 colonies, with COMU ticks being more closely related to BLKT ticks of the other colonies. In this colony, the BLKT ticks are an admixture of the other two races.

shows that ticks using different local seabird host species tend to be genetically distinct; there are clear patterns of within-colony structure among host-associated tick groups, with some evidence of occasional admixture between groups and large scale dispersal among locations.

Rhipicephalus (*Boophilus*) *microplus* is a widely distributed, one-host tick that exploits livestock (mainly *Bos indicus, B. taurus*, and *Equus caballus*) and wild ruminants in subtropical and tropical regions. This tick originated in Asia, but during the second half of the 19th century spread via cattle transportation to Australia, Madagascar, South Africa, Latin America, Mexico, and the USA (Cumming, 1999). Since this time, it has continued to spread across the different invaded continents (e.g., detected in West Africa in 2007; Madder et al., 2007). This tick causes significant damage to livestock production across its distribution (Frisch, 1999), including the transmission of numerous diseases of medical and veterinary importance such as bovine and equine babesiosis (Apicomplexa: *Babesia bigemina, B. bovis, B. caballi*) and anaplasmosis (Proteobacteria: *Anaplasma marginale*). However, the degree to which *R. microplus* uses native wildlife and the potential reservoir status of these alternative host species is unclear. Chevillon and colleagues recently tracked the invasion of this tick in New Caledonia (Koffi et al., 2006) and showed that despite a strong initial bottleneck and, after only ∼240 generations since its arrival on the island, the tick had diverged into two well-defined host-specific groups with little to no genetic exchange, a race that exploits cattle and another that exploits the rusa deer (*Rusa timorensis*) (De Meeûs et al., 2010). However, the general tendency for host-associated divergence across the vast distribution of this tick is still unknown. Indeed, in New Caledonia, acaricide use is extremely high and may have favored the rapid divergence of host-associated groups (Chevillon et al., 2007). In other areas of the world, acaricide use may be more or less restricted, but local host-related structure has never been tested. What is clear from this initial study is that, despite its recent colonization history and the continued presence of its ancestral host species in newly invaded zones, *R. microplus* can rapidly evolve host specificity to novel host species and this specificity may greatly alter its population dynamics and the transmission of pathogens between livestock and wildlife.

The third example comes from *Ixodes ricinus*, the principal European vector of Lyme disease and other major pathogens of human interest (*Babesia* spp, Tick-borne encephalitis virus, *Anaplasma phagocytophilum*, etc.). This tick has a wide distribution across western Europe and, due to global change, is currently expanding its range northward and to higher altitudes (see review of Léger et al., 2013). *I. ricinus* is found in deciduous woodlands and mixed forests, where it is highly sensitive to temperature and humidity, and where it is typically active from spring to autumn (Gray, 1998). The general life cycle is similar to *I. uriae*, but this tick actively quests on the vegetation for its host. It is commonly recognized that host specificity of the different life stages of *I. ricinus*, and related tick species, is linked to host size constraints; larvae and nymphs parasitize almost all vertebrate size classes, whereas female adults only feed on larger mammals (e.g., Eisen and Lane, 2002). Indeed, this hard tick is touted as the example *par excellence* of a host generalist, parasitizing a vast range of terrestrial vertebrates including mammals, birds, and reptiles (Sonenshine, 1991, 1993). Recent work on the population genetics of *I. ricinus* nonetheless suggests that some host specificity may evolve within local communities, other than that related to size constraints. Previous studies have indicated a lack of population genetic structure at large spatial scales (Delaye et al., 1997; De Meeûs et al., 2002; Casati et al., 2008; Noureddine et al., 2011), but strong patterns of heterozygote deficits within populations, a potential indicator of local substructure (De Meeûs et al., 2002;

Kempf et al., 2010). Indeed, a reanalysis of data from De Meeûs et al. (2002) that took local substructure into account showed significant patterns of isolation by distance among Swiss populations of *I. ricinus* (De Meeûs, 2012). Likewise, patterns of mate choice suggest the presence of assortative mate pairing in some populations (Kempf et al., 2009b). Kempf et al. (2011) measured genetic variation at microsatellite markers in a large sample of ticks collected directly from sympatric host types (birds, rodents, lizards, wild boars, and deer) in several European locations and observed significant genetic structure among ticks from different host types, but only in certain populations. This suggests that host choice is not random for *I. ricinus* and that host preferences may evolve in local populations and be linked to mate choice. However, as different areas of the distribution seem to show different degrees of host-related divergence and different tick life stages have host-associated feeding constraints, these observations require further investigation in order to fully understand the specialization process in this species. The evolution of host associated divergence may vary, for example, with the history and composition of local host communities (Kempf et al., 2011). Under this hypothesis, we could predict that longer established and/or more stable host communities should show stronger patterns of host-associated divergence than more recently colonized or perturbed host communities (**Figure 2**). The formation of specialized host races in *I. ricinus* would not only profoundly alter our understanding of how populations of this tick function under natural conditions, but would also represent a significant transmission constraint for the different pathogens it vectors. The existence of such patterns would thus require explicit consideration in epidemiological models of tick-borne disease.

Finally, an initial population genetic study of a group of soft ticks that exploit marine birds, ticks of the *Ornithodoros capensis* complex, has also suggested that local host specialization may evolve in ticks (Gómez-Díaz et al., 2012). As mentioned above, soft ticks have fundamentally different life cycles than hard ticks and may be less intimately associated with the host due to their numerous, but brief, encounters for the bloodmeal. The *O. capensis* system is interesting in that it parallels the *Ixodes uriae* system in terms of host characteristics, but has a complementary geographic distribution, covering temperate to tropical regions of both hemispheres, rather than polar zones (Dietrich et al., 2011). A detailed study based on ticks collected from different seabird species breeding sympatrically in colonies of the Cape Verde Archipelago and typed at a conserved mitochondrial genetic marker (16S) and a single nuclear marker (18S) showed initial indications of host-associated divergence. Indeed, five sympatric lineages of ticks were found within the island archipelago, many well-outside their described geographic distribution, suggesting wide-scale dispersal of these ectoparasites. However, a detailed analysis of haplotype structure within a single lineage revealed patterns of divergence among ticks exploiting different sympatrically breeding seabird host species (Gómez-Díaz et al., 2012).

Interestingly, observational and experimental results in North American ticks may also support the presence of local tick specialization. For example, the relative infestation prevalence of *Ixodes scapularis*, the main vector of Lyme disease in the eastern US, on rodent and lizard hosts shifts from north (on rodents) to south (on lizards), potentially in relation to relative host abundance (e.g., Apperson et al., 1993; Durden et al., 2002). In the western US, Swei et al. (2011) carried out a removal experiment where the main host of *Ixodes pacificus*, the western fence lizard (*Sceloporus occidentalis*), became unavailable to questing larvae. Rather than immediately switch to alternative hosts, a higher than average number of questing larvae was maintained in the population over the course of the season. There was likewise no obvious increase in larval infestation rates on surveyed deer mice and only about a 5% increase on female wood rats. This could suggest that larvae continued questing for lizard hosts after their removal. In the following year, the authors observed a significantly lower density of questing nymphs, supporting the hypothesis that questing larvae never found appropriate hosts when lizards were unavailable. Population genetic studies of both of these systems could be particularly revealing to understand whether observed host use represents a highly plastic behavior,

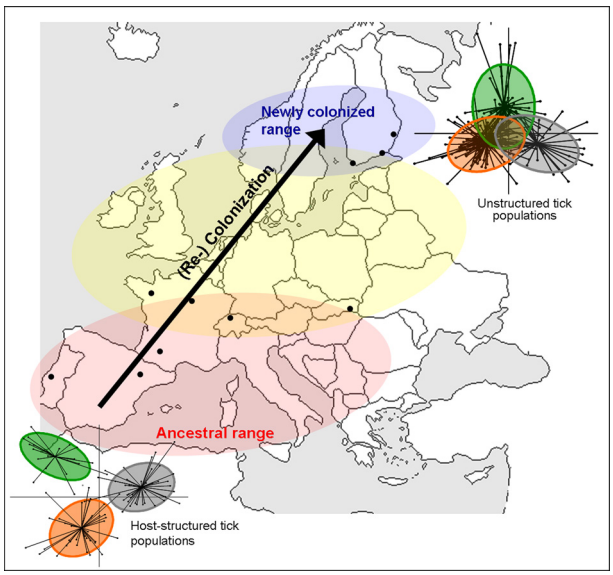

FIGURE 2 | Schematic representation of population expansion of *Ixodes ricinus* (arrow) and hypothesized consequences for host-associated genetic structure. The red zone represents well-established *I. ricinus* populations that persisted in both Southern and Central Europe during the last glacial phase (Porretta et al., 2013). The blue area represents the recently colonized zone for *I. ricinus* and the yellow zone, a transition area where population age and history may be variable (Léger et al., 2013; Porretta et al., 2013). Under the hypothesis that the evolutionary age of a tick population may affect the evolution of host specialization, we would expect a strong pattern of specialization in the red zone, and no host specialization in the blue zone because ticks have only been exposed to local hosts for a few generations. In the yellow zone, patterns of host specialization may be more variable. On the figure, the degree of host-associated population structure of *I. ricinus* in the extreme zones are represented by a between-group analysis of neutral genetic variation, where each dot represents an individual tick and the color indicates different host-associated tick populations. Greater separation of groups indicates stronger genetic divergence. The relative evolutionary age of tick populations could be changed to some other habitat-based factor that may affect the evolution of host specialization.

or whether genetic divergence has occurred among ticks that exploit different local hosts. Indeed, an initial genetic study in *Dermacentor albipictus*, a one-host tick of North American ungulates, suggests the evolution of host specialization among different sympatric host species, despite the large dispersal potential of both wild and domestic hosts (Leo, 2012).

CONSEQUENCES FOR PATHOGEN CIRCULATION

Many pathogens that cause human or livestock disease are maintained in natural foci which then spillover to humans or their domesticated animals (Balashov, 2010). As vertebrate species may vary greatly in their reservoir competence for different pathogens, host specificity of vectors becomes an essential issue for developing predictive models of disease risk (such as the dilution effect model mentioned above). As the host specificity of vectors may be more of a local scale phenomenon, an ecological view of this aspect is the most appropriate to incorporate into studies of pathogen circulation. **Figure 3** illustrates how changes in local host use can alter patterns of pathogen transmission and exposure risks for humans. In particular, if ticks form naturally-occurring host-associated groups, human exposure risk will depend both on the degree of vector specificity for each host type and on the cues that different tick groups use in order to select their particular host. Humans, as incidental hosts, may only be exposed to host groups that share similar cues for questing ticks (such as mice in **Figure 3**). Under these conditions, estimates of infection prevalence derived from the overall tick population, collected by flagging for example, may significantly under- or over-estimate exposure risk.

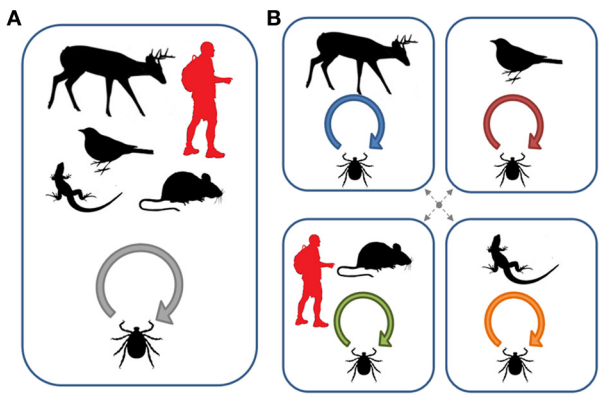

FIGURE 3 | Pathogen circulation within host communities depending on vector specialization. (A) Several host species are available locally, but all are equally used by the vector (i.e., the vector is a true generalist). The local transmission and evolution of the microparasite will therefore depend on the abundance of each host species and the relative selection pressures they impose. Under this scenario, humans are exposed to the general vector population and surveys of disease prevalence in this general population will be a reasonable measure of risk. **(B)** Each vertebrate host species is exploited by a distinct vector population resulting in the presence of locally independent or semi-independent disease cycles. Dotted arrows refer to incomplete host-associated isolation. The evolutionary outcome of these interactions will depend on the degree of specificity of each vector population and the ability of the microparasite to exploit alternative hosts after transmission. Human exposure in this type of system will also depend on vector specificity and the cues used for host selection (in the diagram, humans are primarily exposed to ticks that exploit small mammals). Under these conditions, a survey of infection prevalence from the overall tick population may significantly under- or over-estimate exposure risk (Figure after McCoy, 2008).

An illustration of the consequences of vector specialization on pathogen circulation can be found if we return to the *Ixodes uriae* system and examine patterns of prevalence and diversity in Lyme disease bacteria (*Borrelia burgdorferi* sensu lato—Bbsl) circulating among different host-associated tick populations within seabird colonies. Detailed analyses of tick infections by Bbsl indicated significant differences in local prevalence estimates among different sympatric tick populations (Gómez-Díaz et al., 2010; **Table 1**). While little variation exists among host groups in terms of the presence of different Bbsl genospecies (**Table 1**), significant genetic structure is evident if one looks at strain variation within a given genospecies (**Table 2**). *B. garinii* is the dominant genospecies found in the marine system and, although geographic structure is low among circulating strains (Gómez-Díaz et al., 2011), host-associated structure is high within most colonies (**Table 2**) and demonstrates that the evolution of host specificity can be a major barrier to local transmission. Results from the *Ixodes uriae* system may have limited direct effects for Lyme disease epidemiology *per se* (although some *B. garinii* strains are exchanged with terrestrial systems; Gómez-Díaz et al., 2011), but similar patterns occurring in terrestrial vector-borne disease systems may call into question our current thinking on the transmission ecology of specific pathogens. For example, in terrestrial systems, different genospecies of the Bbsl complex are associated with different host types, a pattern that is thought to be maintained by host complement responses to the bacteria (Kurtenbach et al., 2002, 2006). However, as pathogen transmission depends both on the ability of the host to carry the infection and on host use and infectivity of the vector, it is possible that vector specialization may have favored the evolution of these associations, or at least, may play a role in their maintenance. Recent theory also predicts a significant role of vector diversity in driving epidemiological patterns of associated disease. Roche et al. (2013) used a theoretical reservoir-vector-pathogen framework to study the transmission consequences of increasing host reservoir and/or vector species richness within the context of large community assemblages. They found that increasing vector diversity, regardless of the variance in infectivity among these vectors, could increase overall disease transmission in the system because increases in vector richness tend to lead to a greater overall abundance of potential vectors. However, this tendancy is modified if vectors specialise on different host types. Clearly, it appears that if we are to better understand the evolution and epidemiology of vector-borne diseases, we need to explicitly test whether perceived generalist vectors are true generalists or rather composed of a diverse assemblage of cryptic host specialists.

CONCLUSIONS AND PERSPECTIVES

Our goal in this review was to clarify our current thinking on the evolution of host specialization in ticks and to consider how this process may alter patterns of tick-borne disease transmission. Historical notions of host specialization in ticks have been

Table 1 | Borrelia prevalence and distribution among *Ixodes uriae* ticks from different seabird species breeding in four seabird colonies of the North Atlantic.

Colony (coordinates)	Host species (nb sampled)	Nb. Ticks (Ad, Ny)	% Prev*	Borrelia species**				
				Bg	Bl	Bbss	Ba	Co
Hornøya, Norway (70°22′N, 31°10′E)	BLKI (259)	380 (265, 115)	10.3	22	0	7	1	0
	ATPU (62)	92 (67, 25)	31.8	17	0	5	3	0
	COMU (52)	107 (105, 2)	17.8	14	0	1	1	0
Skrudur, Iceland (64°54′N,13°38′W)	BLKI (17)	28 (25, 3)	17.9	2	2	0	0	1
	ATPU (19)	31 (26, 5)	22.6	7	0	0	0	0
	COMU (27)	32 (23, 0)	18.8	4	2	0	0	0
Grimsey, Iceland (66°33′N, 18°00′W)	BLKI (24)	30 (16, 14)	33.3	5	2	0	0	3
	ATPU (28)	39 (29, 10)	33.3	11	2	0	0	0
	COMU (23)	30 (25, 5)	33.3	5	3	0	0	2
Breidafjordur, Iceland (65°23′N, 22°54′W)	BLKI (11)	20 (2, 18)	45.0	6	2	0	0	1
	ATPU (25)	30 (14, 16)	63.3	13	0	0	0	2
	COMU (7)	12 (12, 0)	25.0	1	0	0	0	1

Host species include Black-legged kittiwakes Rissa tridactyla (BLKI), Atlantic puffins Fratercula arctica (ATPU), and Common murres Uria aalge (COMU). Bacteria of the Borrelia burgdorferi sensu lato complex in I. uriae were characterized using a nested PCR procedure followed by direct sequencing of a FlaB gene fragment. The species found include B. garinii (Bg), B. lusitaniae (Bl), B. burgdorferi sensu stricto (Bbss), and B. afzelii (Ba). The species could not be clearly identified in the case of co-infections (Co). See Duneau et al. (2008) for details on the molecular analysis.

**May be slightly overestimated because some ticks are from the same host individual.*

***Not all positive amplifications could be successfully sequenced for species identification.*

Table 2 | Analysis of Molecular Variance (AMOVA) results for *B. garinii* isolated from *Ixodes uriae* ticks sampled from three different seabird host species in four mixed species breeding colonies in the North Atlantic (see Table 1 for details).

Component	*df*	% Variation	Φ-statistic	*P*-value
Among colonies	3	6.31	0.06307	0.16227
Among host races within colonies	8	14.01	0.14955	**<0.001**
Within host races	94	79.68	0.20319	**<0.001**

The analysis specifically tests for genetic structure among isolates both among tick host races within colonies and among colonies. The Φ-statistic provides an estimate of the genetic structure among isolates at each hierarchical scale and takes into account both the molecular distance among sequences and their frequencies (Excoffier and Lischer, 2010). P-values are based on permutation procedures specific to each hierarchical level and indicate the significance of each component (significant values in bold). Analyses are based on a 308 bp fragment of the FlaB gene (see Duneau et al., 2008, for details). An alternative grouping with colonies nested within tick races provided similar results.

contradictory in terms of whether ticks tend to be host specialists or host generalists. However, by re-evaluating the spatial and temporal scales considered in analyses of host use, both views can be supported.

At the scale of the global geographic distribution of a species, ticks tend to be host generalists. Most species have large repertoires of potential host species and can exploit phylogenetically diverse host species that share the same ecological habitats. Studies in other ectoparasites likewise suggest that the ecological similarity of the host environment may be more important than host phylogenetic similarities in determining a parasite's host range (Krasnov et al., 2010). Ecological fitting, the ability of an organism to colonize and form novel associations, therefore seems to be an appropriate framework for understanding tick host use at large spatial scales (Agosta and Klemens, 2008). However, given the complexity of the tick-host interface and the life history characteristics of these ectoparasites, the ability to exploit a highly diversified group of hosts seems counter-intuitive. How can one account for the high plasticity in host use that seems to be maintained by ticks? The genetic architecture of tick genomes may provide some clues to this apparent flexibility; its relatively large size and repetitive nature may provide ticks with the diversity and/or redundancy required to rapidly exploit novel hosts (Sunter et al., 2008; Nene, 2009). Detailed analyses from current tick genome projects should provide some data to examine this question more directly (Hill and Wikel, 2005; Guerrero et al., 2006).

At a more local scale, host specialization seems to be the norm in ticks, at least in the tick systems studied in detail so far. These species are able to rapidly form distinct host-associated populations within local communities and these different populations can show differences in both host preference and performance. In this sense, simple records of host species observations are not sufficient to determine whether a tick species is a specialist or a generalist; detailed information on patterns of host use is required. The local scale is also the appropriate one to consider if we want to better predict pathogen circulation and exposure

risk to humans and their domestic animals, because it is at these spatial scales that transmission takes place. In order to explicitly take into account this contrast in host use between local and global scales, a more suitable measure of host specialization in tick species would be to estimate turnover (or beta diversity) in host use across the geographic range (Krasnov et al., 2011).

The question remains as to the fate of local specialist, global generalist ticks. Do ticks which diverge into local host-associated genetic groups continue toward speciation or is perceived adaptation based simply on phenotypic plasticity and ecological fitting? The evolution of specialization is considered to be an essential step toward speciation (e.g., Maynard Smith, 1966; Schluter, 2000). Analyses in phytophagous insects have often shown positive correlations between the number of distinct species within taxa and the relative degree of plant specialization (e.g., Dyer et al., 2007; Forister et al., 2012). If ticks frequently evolve host specialization and this leads to speciation, we should also expect higher species richness in those genera that tend to show more stringent host preferences, even if host shifts frequently occur. We should also expect that sympatric host races should become increasingly isolated over evolutionary time to the point of becoming distinct entities (e.g., evolve sustainable adaptive diversity; De Meeûs et al., 2003). Current data on these aspects is greatly lacking. A partial analysis of the African tick database compiled by Cumming (1998) suggests a positive correlation may exist between the number of tick species within a given genera and their recorded number of hosts (e.g., for mammalian host types; $r = 0.78$, $P < 0.001$). However, in the *Ixodes uriae* system, seabird host races are not more divergent in the ancestral range of the species compared to more recently colonized regions (McCoy et al., 2005; Dietrich et al., 2012) suggesting that the race state may be maintained over long time periods. Field-based studies on a larger range of tick species will be required to examine these hypotheses in more detail. Genetic studies that examine host and vector co-structures would also reveal the potential role of on-going gene flow in the long-term maintenance of host-associated genetic races.

The question of host specialization in ticks is an important one for understanding their evolution, their population dynamics and the circulation and diversification of the parasites that they transmit. Ticks can be particularly challenging parasites to work with because they have relatively long life cycles, are often difficult to maintain under laboratory conditions and have complicated genetic architectures. However, their complex life histories, remarkable adaptations for host exploitation, and importance for the transmission of a diverse range of pathogens renders them exciting models for addressing such questions. With increasing advances in genetic technologies, data to address many of questions raised in this review should become available. With a better understanding of the evolution of host specialization over ecological and evolutionary time, the planning of effective tick control programs and our understanding of tick-borne disease circulation and emergence should greatly improve.

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Tick salivary compounds: their role in modulation of host defences and pathogen transmission

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Ticks require blood meal to complete development and reproduction. Multifunctional tick salivary glands play a pivotal role in tick feeding and transmission of pathogens. Tick salivary molecules injected into the host modulate host defence responses to the benefit of the feeding ticks. To colonize tick organs, tick-borne microorganisms must overcome several barriers, i.e., tick gut membrane, tick immunity, and moulting. Tick-borne pathogens co-evolved with their vectors and hosts and developed molecular adaptations to avoid adverse effects of tick and host defences. Large gaps exist in the knowledge of survival strategies of tick-borne microorganisms and on the molecular mechanisms of tick-host-pathogen interactions. Prior to transmission to a host, the microorganisms penetrate and multiply in tick salivary glands. As soon as the tick is attached to a host, gene expression and production of salivary molecules is upregulated, primarily to facilitate feeding and avoid tick rejection by the host. Pathogens exploit tick salivary molecules for their survival and multiplication in the vector and transmission to and establishment in the hosts. Promotion of pathogen transmission by bioactive molecules in tick saliva was described as saliva-assisted transmission (SAT). SAT candidates comprise compounds with anti-haemostatic, anti-inflammatory and immunomodulatory functions, but the molecular mechanisms by which they mediate pathogen transmission are largely unknown. To date only a few tick salivary molecules associated with specific pathogen transmission have been identified and their functions partially elucidated. Advanced molecular techniques are applied in studying tick-host-pathogen interactions and provide information on expression of vector and pathogen genes during pathogen acquisition, establishment and transmission. Understanding the molecular events on the tick-host-pathogen interface may lead to development of new strategies to control tick-borne diseases.

Keywords: ticks, saliva, immunomodulation, pathogen, transmission

INTRODUCTION

Ticks are obligate blood feeding ectoparasites of a wide range of vertebrates (amphibians, reptiles, birds, mammals). To acquire a blood meal, ticks insert their highly specialized mouthparts through the host skin and, depending on the species, anchor them in the skin by attachment cement (Sonenshine, 1991). Fast feeding soft ticks (Argasidae) feed repeatedly and rapidly with deep penetration of the host skin, causing considerable damage to the host (Binnington and Kemp, 1980), whereas hard ticks (Ixodidae) feed only once in each developmental stage for a prolonged period and penetrate the host epidermis either superficially (Metastriata, e.g., *Dermacentor* spp., *Rhipicephalus* spp.), or more deeply (Prostriata, e.g., *Ixodes* spp., Metastriata, e.g., *Amblyomma* spp.) (Sonenshine, 1991; Bowman et al., 1997a). Ticks are pool feeders; during the process of penetration of the host skin and probing for blood, capillaries and small blood vessels are injured and an extensive haemorrhagic pool forms at the feeding lesion in the host dermis. Hard ticks may require several days to weeks to complete their blood meal. The volume of ingested blood and the duration of feeding are developmental stage- and species-specific, whereby tick females may ingest more blood than 100-times their initial body weight (e.g., Sauer et al., 1995).

A host would normally react to damage of the skin and the presence of the feeding tick by the formation of a haemostatic plug, activation of the coagulation cascade, vasoconstriction, inflammatory responses leading to wound healing and tissue remodeling, all of which would disrupt tick feeding and cause rejection of the tick, with detrimental effects on tick viability and reproduction. However, ticks succeed in completing their blood meal due to the presence of a large number of biologically active molecules in their salivary glands, displaying anticoagulation, antiplatelet, vasodilatory, anti-inflammatory, and immunomodulatory activities. These molecules have developed during the host-parasite co-evolution and are crucial to overcoming haemostatic and immune responses of the host, enabling ticks to complete feeding and development (Wikel, 1996; Bowman et al., 1997a; Brossard and Wikel, 2008; Nuttall and Labuda, 2008;

Francischetti et al., 2009; Mans, 2010; Fontaine et al., 2011). Tick saliva composition is complex and in many cases redundant, reflecting the complex and redundant host defence responses. Some of the tick salivary compounds have been characterized and their functions identified, but the functions remain unknown for most of the molecules (Andrade et al., 2005; Steen et al., 2005; Ribeiro et al., 2006; Brossard and Wikel, 2008; Francischetti et al., 2009; Fontaine et al., 2011) (**Figure 1**).

In addition to blood feeding, ticks are vectors of a large number of pathogenic microorganisms (viruses, bacteria, protozoa) causing diseases in humans and animals. The common route of a pathogen within the vector is ingestion via infected host blood, migration through the gut to the haemocoel and the penetration of salivary glands. For many pathogens, salivary glands are the organs where they develop and multiply. Thus, tick salivary glands are suggested to play a key role in pathogen transmission to the vertebrate host. However, transmission of pathogens via tick saliva is not a simple mechanistic process, instead pathogens exploit tick salivary molecules for their survival and multiplication in the vector and for transmission to and establishment in the hosts (Bowman et al., 1997a; Ramamoorthi et al., 2005; Brossard and Wikel, 2008; Nuttall and Labuda, 2008).

The phenomenon of promotion of pathogen transmission via arthropod saliva (saliva-assisted transmission, SAT) has been reported in a number of blood-feeding arthropods, including ticks, however, the molecular mechanisms of these processes are largely unknown (Nuttall and Labuda, 2008). Although SAT has been reported for several tick-pathogen associations, only a limited number of tick molecules associated with pathogen transmission have been identified (Ramamoorthi et al., 2005; Hovius et al., 2008b). Therefore, understanding the physiology of tick salivary glands is important for the elucidation of their role in both the modulation of host defences and pathogen transmission.

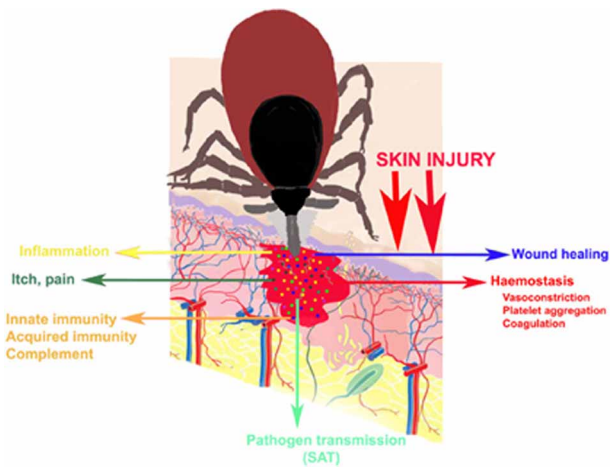

FIGURE 1 | Hard ticks (Ixodidae) insert their mouthparts into the skin of their hosts and cause tissue injury. Tick mouthparts are anchored in the host skin by a cement cone. At the tick attachment site, a haemorrhagic pool is created. During the prolonged blood-meal ticks secrete a rich cocktail of bioactive salivary molecules to the host and modulate host defence responses (itch, pain, haemostasis, inflammation, immune reactions) to their benefit. The tick salivary cocktail contains molecules (SAT factors) that facilitate pathogen transmission and infection of the host.

The molecular background of tick-host associations (e.g., Brossard and Wikel, 2008; Francischetti et al., 2009), their significance in transmission of tick-borne pathogens (e.g., Nuttall and Labuda, 2008) and the natural ecology of tick-host-pathogen interactions with consequences for epidemiology of tick-borne infections in humans (Randolph, 2009; Estrada-Peña et al., 2012) have been extensively reviewed. The present survey summarizes the current knowledge on main tick strategies to overcome host defence responses and the assistance of tick saliva in the transmission of tick-borne pathogens. Although intensive research on tick salivary gland transcriptomes and proteomes is in progress, there are indications that the range of biologically active compounds in tick salivary glands is much wider and gaps exist in understanding their complexity and interactions during the process of feeding and pathogen transmission.

TICK SALIVARY GLANDS

Tick salivary glands are multifunctional complex organs (Sonenshine, 1991; Sauer et al., 1995, 2000; Bowman and Sauer, 2004; Bowman et al., 2008). In fasting ticks, salivary glands assist in the absorption of water vapor from unsaturated air. They consist of an anterior region of acini (generally agranular and primarily involved in osmo-regulation) attached directly to the main duct. The acini are arranged more caudally in lobules connected by intralobular and interlobular ducts to the main salivary duct. The caudal acini increase greatly in size during feeding and are involved in the production and secretion of salivary bioactive components. The main salivary ducts pass antecranially into the salivarium which fuses with the pharynx and forms the oral cavity. Salivary glands enable the feeding ticks to concentrate blood nutrients by returning excess water and ions via saliva to the host as the ingested host tissues and tick saliva flow in alternate directions through the common buccal canal. The regulation of salivary gland development, degeneration and fluid secretion are under neuro-hormonal control (Bowman and Sauer, 2004; Bowman et al., 2008).

Almost all ixodid ticks produce cement proteins that enable attachment of the tick to the host and seal the area around the mouthparts at the wound site. After a tick attaches to a host, expression of a series of genes and synthesis of proteins is initiated in their salivary glands, which reflect the stages of the feeding process. As feeding progresses, the amount of secreted saliva increases and salivary glands undergo a remarkable and rapid structural reorganization. At the peak of the feeding process, the glands can increase as much as 25-fold in size and content. Once the tick is engorged and detaches, the glands degenerate through a process of cell apoptosis (Bowman et al., 2008).

The composition of tick saliva is complex and redundant in many cases and reflects complex and redundant host defence responses. Tick saliva contains a large number of various non-proteinaceous substances and secreted proteins which are differentially produced during feeding and comprise inhibitors of blood coagulation and platelet aggregation, vasodilatory and immunomodulatory substances as well as compounds preventing itching and pain (Ribeiro et al., 1985; Wikel and Alarcon-Chaidez,

2001; Andrade et al., 2005; Steen et al., 2005; Brossard and Wikel, 2008; Francischetti et al., 2009). The blood-feeding strategy of ticks and, on the other hand, the pool and mode of action of the pharmacologically active compounds contained in their saliva and salivary glands are mostly species-specific. The activity, mechanisms of action and characteristics of these compounds have been studied more intensively during the last two decades and a number of novel molecules have been identified. Some of the tick salivary molecules have pleiotropic effects as they interfere with different arms of the defence responses of the vertebrate hosts. (e.g., Ribeiro and Francischetti, 2003; Francischetti et al., 2009).

Most of the efforts to identify bioactive molecules from ticks are aimed at preparing the active compounds in recombinant form, with their prospective use as pharmaceuticals. In addition, elucidation of the molecular mechanisms of interaction between the ectoparasites and their hosts and of the mechanisms of exploitation of tick molecules by pathogens to invade ticks and hosts can lead to the discovery of new vaccine targets against ticks and the pathogens that ticks transmit (e.g., Willadsen, 2004; Titus et al., 2006; Maritz-Olivier et al., 2007; Hovius et al., 2008b).

TICK SALIVARY COMPOUNDS AND HOST HAEMOSTASIS

Haemostasis is a complex and efficient mechanism that controls blood loss after vascular injury through a series of physiological events leading to termination of blood loss from damaged blood vessels (vasoconstriction), formation of a platelet plug, fibrin clot formation and fibrinolysis (Hoffman et al., 2009).

Research into the mechanisms by which ticks inhibit host haemostasis has led to the discovery and characterization of a variety of compounds with diverse biological activities and potential use in development of novel pharmaceuticals (Kazimírová, 2007; Francischetti et al., 2009; Koh and Kini, 2009; Chmelar et al., 2012). Differences in the anti-haemostatic repertoires suggest that anti-haemostatic mechanisms in hard and soft ticks evolved independently (Mans et al., 2008; Mans, 2010). Saliva of the same tick species simultaneously contain a number of anti-haemostatic molecules, inhibiting different arms of the haemostatic system, or in contrast, the same compounds can display multiple functions (Bowman et al., 1997a; Mans and Neitz, 2004; Valenzuela, 2004; Steen et al., 2005; Maritz-Olivier et al., 2007; Francischetti et al., 2009) (**Table 1**). However, it is important to note that the cocktail of anti-haemostatic compounds in tick saliva differs between species and in fact, there is no tick species whose complete anti-haemostatic capacities have been fully explored. In addition to discrovery of new sources of drug candidates, studies on tick anti-haemostatics contribute to our understanding of the mechanisms of interactions between ticks and their hosts in the process of feeding and pathogen transmission.

VASODILATORS

Following probing and injury of blood vessels by tick mouthparts, arachidonic acid is released by activated platelets and is converted into thromboxane A_2, a platelet-aggregating, platelet-degranulating, and vasoconstricting substance. Activated platelets release serotonin which, together with thromboxane A_2, is responsible for early vasoconstriction in local inflammation caused by tissue injury. To antagonize vasoconstrictors produced by the host at the site of tissue injury, vasodilators are secreted by ticks to the feeding pool. To date, only non-proteinaceous vasodilatory compounds have been identified in tick saliva. These include lipid derivatives such as prostacyclin and prostaglandins (Ribeiro et al., 1988, 1992; Bowman et al., 1996). However, a tick histamine release factor (tHRF), secreted in *Ixodes scapularis* saliva (Dai et al., 2010) and a novel *Ixodes ricinus* serine proteinase inhibitor (serpin) named IRS-2, which inhibits cathepsin G and chymase (Chmelar et al., 2011), probably also act as modulators of vascular permeability (Chmelar et al., 2012).

INHIBITORS OF PLATELET AGGREGATION

Platelet aggregation represents the initial and most immediate stage of haemostasis. Following vascular injury, platelets adhere to the subendothelial tissue and become activated by agonists such as collagen, thrombin, adenosine diphosphate (ADP), and thromboxane A_2. Agonists bind to specific receptors on the surface of platelets and initiate a long and highly complex chain of intracellular chemical reactions that lead to platelet aggregation and the formation of a heamostatic plug. The platelet aggregation cascade is targeted by ticks at several stages (Francischetti, 2010). A strategy used by a number of ticks is targeting ADP, an agonist important for completion of platelet aggregation, via salivary apyrase. Apyrase, an adenosine triphosphate (ATP)-diphosphohydrolase enzyme, hydrolyses the phosphodiester bonds of ATP and ADP. Apyrase activity has been demonstrated in the salivary glands and saliva of both soft ticks (Ribeiro et al., 1991; Mans et al., 1998a,b, 2002a) and hard ticks (e.g., *I. scapularis;* Ribeiro et al., 1985). Apyrase from *Rhipicephalus (Boophilus) microplus* belongs to the 5'-nucleotidase family (Liyou et al., 1999). On the other hand, apyrase activity has not been detected in the saliva of, e.g., *Amblyomma americanum* (Ribeiro et al., 1992), but increased prostaglandin levels in the saliva of this tick inhibit platelet aggregation by preventing ADP secretion during platelet activation (Ribeiro et al., 1992; Bowman et al., 1995).

Some of the tick-derived platelet aggregation inhibitors interfere with the interaction of collagen with platelet receptors. Activation of platelets by collagen is prevented, e.g., by Moubatin, a specific inhibitor of collagen stimulated platelet activation from *Ornithodoros moubata*, while tick adhesion inhibitor (TAI) identified in the same tick species inhibits the adhesion of platelets to matrix collagen (Waxman and Connolly, 1993; Karczewski et al., 1995). Moubatin belongs to the family of lipocalins and probably prevents platelet aggregation caused by ADP released from collagen-activated platelets (Valenzuela, 2004). Longicornin, another inhibitor of collagen-mediated platelet aggregation, was isolated from the hard tick *Haemapysalis longicornis* (Cheng et al., 1999). However, Longicornin does not bind directly to collagen fibers and does not affect platelet adhesion to collagen, indicating that the inhibitor, similarly to Moubatin, shares a common receptor with collagen.

Thrombin is a key enzyme in thrombosis and haemostasis. In addition to its main role in the formation of the fibrin clot, it induces platelet aggregation. Three functional

Table 1 | Examples of tick salivary molecules that modulate host defence reactions.

Tick species	Molecule	Target and/or function	References
VASODILATION			
Ixodes scapularis	Prostacyclin	Vasodilation	Ribeiro et al., 1988
I. scapularis	tHRF	Vasodilation	Dai et al., 2010
Ixodes ricinus	IRS-2	Cathepsin G, chymase	Chmelar et al., 2011, 2012
Amblyomma americanum	Prostaglandins	Vasodilation	Bowman et al., 1995
PLATELET AGGREGATION INHIBITORS			
Soft ticks (Argasidae)	Apyrase	ATP, ADP	Mans et al., 1998a,b
Ornithodoros moubata	Moubatin	Collagen receptor	Waxman and Connolly, 1993
O. moubata	Disaggregin	Integrin antagonist	Karczewski et al., 1994
Ornithodoros savignyi	Savignygrin	Integrin antagonist	Mans et al., 2002b
I. scapularis	Apyrase	ATP, ADP	Ribeiro et al., 1985
I. scapularis, I. pacificus	Ixodegrin	Integrin antagonist	Francischetti et al., 2005b
I. ricinus	IRS-2	Thrombin	Chmelar et al., 2011
Haemaphysalis longicornis	Longicomin	Collagen receptor	Cheng et al., 1999
Dermacentor variabilis	Variabilin	Integrin antagonist	Wang et al., 1996
ANTICOAGULATION AND FIBRINOLYSIS			
O. moubata	Ornithodorin	Thrombin	Van de Locht et al., 1996
O. moubata	TAP	FXa	Waxman et al., 1990
O. savignyi	Savignin	Thrombin	Nienaber et al., 1999
O. savignyi	TAP-like protein	FXa	Joubert et al., 1998
I. scapularis	Ixolaris	Tissue factor (TF) pathway inhibitor	Francischetti et al., 2002
I. scapularis	Salp 14	Intrinsic pathway	Narasimhan et al., 2002
I. scapularis	TIX-5	Inhibitor FXa-mediated FV activation	Schuijt et al., 2013
I. ricinus	Ir-CPI	Intrinsic pathway, fibrinolysis	Decrem et al., 2009
Amblyomma variegatum	Variegin	Thrombin	Koh et al., 2007
Amblyomma cajennense	Amblyomin-X	FXa	Batista et al., 2010
H. longicornis	Madanin-1; Madanin-2	Thrombin	Iwanaga et al., 2003
H. longicornis	Haemaphysalin	FXII/XIIa	Kato et al., 2005
H. longicornis	Longistatin	Fibrinolysis	Anisuzzaman et al., 2011
Rhipicephalus appendiculatus	65 kDa protein	Prothrombinase complex	Limo et al., 1991
Rhipicephalus (Boophilus) microplus	BmAP	Thrombin	Horn et al., 2000
	Boophilin	Thrombin, trypsin, plasmin	Macedo-Ribeiro et al., 2008
	Microphilin	Thrombin	Ciprandi et al., 2006
Boophilus calcaratus	Calcaratin	Thrombin	Motoyashiki et al., 2003
COMPLEMENT INHIBITORS			
O. moubata	OmCI	C5, prevention of interaction of C5 with C5 convertase	Nunn et al., 2005
I. scapularis	Isac	Alternative complement pathway, interacts with C3 convertase	Valenzuela et al., 2000
I. scapularis	Salp 20	C3 convertase	Tyson et al., 2007
I. ricinus	IRAC I, II, Isac paralogues	Alternative complement pathway, interacts with C3 convertase	Daix et al., 2007
IMMUNOSUPPRESSANTS/IMMUNOMODULATORS			
I. scapularis	Salp15	Impairs IL-2 production and T cell proliferation; binds *B. burgdorferi* OspC, protects the spirochaete from antibody-mediated killing	Anguita et al., 2002; Ramamoorthi et al., 2005
I. scapularis	IL-2 binding protein	Inhibits proliferation of human T cells and CTLL-2 cells	Gillespie et al., 2001
I. scapularis	ISL 929 and ISL 1373	Impair adherence of polymorphonuclear leukocytes	Guo et al., 2009
I. scapularis	Sialostatin L, L2	Inhibits cathepsin L activity	Kotsyfakis et al., 2006
I. ricinus	Iris	Modulates T lymphocyte and macrophage responsiveness, induces Th2 type responses	Leboulle et al., 2002; Prevot et al., 2006

(Continued)

Table 1 | Continued

Tick species	Molecule	Target and/or function	References
I. ricinus	BIP	Inhibitor of B cell proliferation	Hannier et al., 2004
	Ir-LBP	Impairs neutrophil functions	Beaufays et al., 2008
Dermacentor andersoni	P36	T cell inhibitor	Bergman et al., 2000
Hyalomma asiaticum	BIF	Inhibits LPS-induced proliferation of B cells	Yu et al., 2006
	Hyalomin A, B	Supresses host inflammatory responses (modulation of cytokine secretion, detoxification of free radicals)	Wu et al., 2010
R. appendiculatus	Japanin	Reprogrammes DC responses	Preston et al., 2013
Dermacentor reticulatus	SHBP	Histamin and serotonin binding protein	Sangamnatdej et al., 2002
R. appendiculatus	RaHBP(M), RaHBP(F)	Histamin binding proteins	Paesen et al., 1999
R. appendiculatus	TdPI	Tryptase inhibitor	Paesen et al., 2007
A. americanum	MIF	Inhibitor of macrophage migration	Jaworski et al., 2001
R. sanguineus	Ado, PGE_2	Modulate host inflammatory responses	Oliveira et al., 2011
CHEMOKINE BINDING			
Rhipicephalus sanguineus	Evasin-1	Chemokines CCL3, CCL4, CCL18	Frauenschuh et al., 2007; Déruaz et al., 2008
	Evasin-3	Chemokines CXCL8 and CXCL1	
	Evasin 4	Chemokines CCL5 and CCL11	
WOUND HEALING, ANGIOGENESIS			
I. scapularis	Metalloprotease	Inhibits angiogenesis	Francischetti et al., 2005b
I. ricinus	Metalloproteases	Involvement in tissue remodeling or disruption through digestion of structural components	Decrem et al., 2008
H. longicornis	Haemangin	Ihibitits angiogenesis	Islam et al., 2009
	HLTnI; troponin I-like molecule	Ihibitits angiogenesis	Fukumoto et al., 2006

Abbreviatons: tHRF, tick histamine release factor; IRS, I. ricinus serpin; TAP, tick anticoagulant peptide; TIX-5, tick inhibitor of factor Xa toward factor V; Ir-CPI, coagulation contact phase inhibitor from I. ricinus; BmAP, B. microplus anticoagulant protein; SHBP, serotonin- and histamine-binding protein; TdPI, tick-derived peptidase inhibitor; MIF, macrophage migration inhibitory factor; OmCI, O. moubata complement inhibitor; Isac, I. scapularis salivary anticomplement; Irac, I. ricinus anticomplement; Salp, salivary protein; ISL 929 and ISL 1373, I. scapularis salivary proteins 929 and 1373; Iris, I. ricinus immunosuppressor; BIP, B-cell inhibitory protein; P36, 36-kDa immunosuppressant protein; BIF, B-cell inhibitory factor; Ado, adenosine; PGE_2, prostaglandin E_2.

sites have been recognized in thrombin—the active site, the anion-binding exosite I that mediates binding of thrombin to fibrinogen, the platelet receptor and thrombomodulin, and the anion-binding exosite II, which is the heparin-binding site. Salivary antithrombins detected in both soft ticks and hard ticks that are involved in the inhibition of the coagulation cascade, also inhibit thrombin-induced platelet aggregation (Hoffmann et al., 1991; Nienaber et al., 1999; Kazimírová et al., 2002). The serpin IRS-2 from *I. ricinus* inhibits both cathepsin G- and thrombin-induced platelet aggregation (Chmelar et al., 2011).

Post-activation inhibitors of platelet aggregation target the platelet fibrinogen receptor. Activated platelets express surface adhesion receptor proteins, known as integrins that enable cell-cell and cell-matrix interactions. As the platelets are activated by platelet agonists (thrombin, collagen, and ADP), the ligands [fibronectin, vitronectin, von Willebrand factor, which have the common Arg—Gly—Asp (RGD) sequence, and fibrinogen] bind to glycoprotein (GP)IIb-IIIa via their RGD motif. Interaction between the fibrinogen and the GPIIb-IIIa complex is the important final step in platelet aggregation. Non-RGD disintegrins block the binding of fibrinogen to the integrin αIIbβ3, which is a fibrinogen receptor on surface of activated platelets. The αIIbβ3 antagonists can displace fibrinogen from its receptor thereby allowing disaggregation. Tick-derived disintegrin-like peptides such as Savignygrin (Mans et al., 2002b) and Variabilin (Wang et al., 1996) contain the integrin recognition motif RGD used for binding to GPIIb-IIIa and can inhibit platelet aggregation by preventing the binding of other ligands to the platelet receptor. In contrast, Disaggregin, a fibrinogen receptor antagonist from the soft tick *O. moubata*, is a GPIIb-IIIa antagonist, which lacks the RGD motif and inhibits platelet aggregation by preventing binding to ligands by distinct mechanisms from disintegrin-like peptides (Karczewski et al., 1994). Ixodegrins from *Ixodes pacificus* and *I. scapularis* display sequence similarity to Variabilin, with two additional cysteines in the RGD position (Francischetti et al., 2005b), but their disintegrin activity has yet to be confirmed (Francischetti, 2010).

Disaggregation of platelet aggregates is considered an important back-up mechanism that ticks can use if first-line defence mechanisms fail to inhibit platelet aggregation (Mans and Neitz, 2004). Aggregated platelets may be disaggregated by the removal of fibrinogen from the fibrinogen receptor by competitive binding of an antagonist to the fibrinogen receptor. Proteolysis of fibrinogen can also lead to platelet disaggregation. Apyrase from the soft

tick *O. moubata* displays a disaggregation effect on aggregated platelets (Mans et al., 2000), whereas the GPIIb-IIIa antagonist Savignygrin from *O. savignyi* can displace fibrinogen from its receptor and lead to disaggregation (Mans et al., 2002c).

INHIBITORS OF THE BLOOD-COAGULATION CASCADE

Blood coagulation involves a series of enzymatic reactions where an inactive proenzyme (coagulation factor) is converted to an active form, which then activates the next proenzyme in the cascade. Thrombin is involved in the final common pathway of the coagulation cascade and converts fibrinogen into fibrin clots, but also regulates the activity of blood coagulation factors and stimulates platelet reactions. Ticks have evolved powerful tools to prevent or prolong blood coagulation throughout their extended blood meal. A number of inhibitors of serine proteinases that are involved in the coagulation cascade have been identified and characterized from ticks. The majority of inhibitors identified so far are proteins that display a variety of molecular masses, targets and inhibitory mechanisms (Koh and Kini, 2009; Kazimírová et al., 2010). Based on the mechanism of action, anticoagulants from ticks can be classified as: thrombin inhibitors; inhibitors of activated factor X (FXa); inhibitors of the extrinsic tenase complex (ETC); contact system protein inhibitors (Koh and Kini, 2009), with thrombin and FXa being the most common targets.

Inhibitors of thrombin

Thrombin inhibitors derived from tick saliva belong to at least seven structural classes and target the enzyme at different sites and via different mechanisms (Koh and Kini, 2009). They comprise mainly the Kunitz-type proteinase inhibitors, Ornithodorin (Van de Locht et al., 1996), Savignin (Nienaber et al., 1999), Boophilin (Macedo-Ribeiro et al., 2008), and Rhipilin (Gao et al., 2011), antithrombins of the hirudin-like/Madanin/Variegin superfamily—Madanin I and II (Iwanaga et al., 2003) and Variegin (Koh et al., 2007), as well as various other peptides not ranked in any of the previous groups, e.g., Microphilin (Ciprandi et al., 2006), *Boophilus microplus* anticoagulant protein (BmAP) (Horn et al., 2000), or Calcaratin (Motoyashiki et al., 2003).

Inhibitors of factor Xa

The tick anticoagulant peptide (TAP) from saliva of the soft tick *O. moubata* has been the most intensively studied tick anticoagulant. TAP has some homology with Kunitz type inhibitors, but is a highly specific, reversible competitive inhibitor of FXa (Waxman et al., 1990). The soft tick *O. savignyi* also contains an FXa inhibitor with 46% identity to TAP (Joubert et al., 1998). The recombinant protein Amblyomin-X derived from an *Amblyomma cajennense* transcript encoding a protein containing an N-terminal Kunitz-type domain and a C-terminus with no homology to any known sequences was also found to inhibit FXa (Batista et al., 2010). Salp14, a protein belonging to the salivary protein (Salp) family was identified in saliva of *I. scapularis* and specifically inhibits the FXa active site (Narasimhan et al., 2002, 2004). An unnamed anticoagulant from *Rhipicephalus appendiculatus* saliva probably targets components of the prothrombinase complex, however, its mechanism of action has not been elucidated (Limo et al., 1991).

Inhibitors of the extrinsic tenase complex (ETC)

Ixolaris, a two domain Kunitz-type inhibitor of ETC and penthalaris, containing five Kunitz domains, both with homology to the tissue factor (TF) pathway inhibitor, were detected in *I. scapularis* (Francischetti et al., 2002, 2004). Recombinant ixolaris and penthalaris bind to FXa or FX and inhibit the TF/FVIIa complex. Inhibition of factor V and factor VII has been described for salivary gland extracts (SGE) of *Dermacentor andersoni* (Gordon and Allen, 1991), but the compound(s) have not been further characterized.

By screening a yeast surface display library prepared from salivary gands of nymphal *I. scapularis*, a salivary antigen named P23 was identified. Recombinant P23 (rP23) was found to delay the TF initiated thrombin generation (Schuijt et al., 2011b). Further analysis of rP23 (renamed TIX-5, tick inhibitor of factor Xa toward factor V) showed that the protein prolonged activation of the coagulation system by specifically inhibiting the factor Xa-mediated activation of factor V (Schuijt et al., 2013). This study revealed a unique molecular mechanism by which ticks inhibit the coagulation system of heir hosts and, in addition, the results brought new understanding on early activation of blood coagulation. Moreover, immunization with TIX-5 impaired tick feeding, indicating that inhibition of TIX-5 prevents the host anticoagulation mechanism needed for optimal tick feeding.

Contact system protein inhibitors

The tick-derived inhibitors of the contact phase described so far belong to the Kunitz-type proteinase inhibitor family. BmTI-A (*B. microplus* trypsin inhibitor-A), inhibits kallikrein and elastase and is present in *B. microplus* larvae (Tanaka et al., 1999). A plasma kallikrein-kinin system inhibitor named Haemaphysalin was identified in *H. longicornis* (Kato et al., 2005). A contact phase inhibitor (Ir-CPI) present in *I. ricinus* salivary glands inhibits the intrinsic coagulation pathway and, to a much lesser extent, fibrinolysis *in vitro* (Decrem et al., 2009).

Additional anti-haemostatic activities

Except for antiplatelet factors and anticoagulants, other biological activities which may be related to host haemostasis have been described in the saliva of ticks. Fibrinolytic activity due to the presence of a metalloprotease was detected in saliva of *I. scapularis*. The role of salivary metalloproteinases in tick feeding appears to be related to their antifibrinogen- and antifibrin-specific activities (Francischetti et al., 2003). Kunitz-type serine proteinase inhibitors (RsTI—*Rhipicephalus sanguineus* trypsin inhibitors) were isolated from larvae of *Rhipicephalus sanguineus* (Sant Anna Azzolini et al., 2003). They target plasmin and neutrophil elastase and their role in haemostasis is predicted to be similar to that of serine proteinase inhibitors such as those found, e.g., in *R. (B.) microplus* (see Tanaka et al., 1999). Longistatin, a plasminogen activator identified recently in *H. longicornis* was found to hydrolyse fibrinogen and delay fibrin clot formation (Anisuzzaman et al., 2011).

Several serine protease inhibitors with similarity to proteins of the serpin family were discovered in ticks (Mulenga et al., 2001, 2003). Tick serpins might also interact with host defence responses, including haemostasis. Iris, an immunomodulatory

serpin identified in the salivary glands of *I. ricinus* was the first ectoparasite serpin that was reported to both interfere with host haemostasis and the immune response and increase platelet adhesion, the contact phase-activated pathway of coagulation and fibrinolysis (Prevot et al., 2006).

Calcium-binding proteins with sequence homology to the calreticulin family are also present in tick saliva. Tick calreticulins may play a modulating role in host haemostasis through binding calcium ions which are required as coagulation enzyme cofactors (Jaworski et al., 1995). Phospholipase A2, detected in *A. americanum* (Bowman et al., 1997b), is probably responsible for the haemolytic activity of tick saliva.

TICK SALIVARY COMPOUNDS AND HOST IMMUNE RESPONSES

Host cellular innate immune responses and the complement system are the first lines of defence against invading pathogens. Complement comprises a group of serum proteins that can be activated by different pathways. Activation of the complement system leads to the generation of molecules with various biological activities in inflammation and opsonization and lysis of invading pathogens. Adaptive immune response is triggered when activated antigen-presenting cells migrate to lymphoid tissues where they present antigens to T cells, which play a central role in cellular immune responses at the site of infection or assist in the activation of B cells and the generation of an antigen-specific humoral response (Janeway et al., 1999).

Ticks have evolved varius strategies to modulate both innate and acquired immunity of their hosts in order to protect themselves from host immune responses to tick infestation and avoid impaired feeding and/or rejection (Gillespie et al., 2000; Leboulle et al., 2002; Valenzuela, 2004; Brossard and Wikel, 2008) (**Table 1**). The complex tick-host molecular interactions are considered as a competition between host defences against the ectoparasite and tick evasion strategies. Some hosts develop resistance to repeated tick infestation, while others develop no protective immunity, whereby host resistance or susceptibility depend on the tick-host association and can most likely be explained by tick-induced modulation of the host cytokine network (Andrade et al., 2005; Hajnická et al., 2005).

The *in vitro* effects of saliva and SGE derived from different tick species on functions of host immune effector cells, like granulocytes, macrophages, natural killer (NK) cells, T and B cells, have been extensively documented (e.g., Ramachandra and Wikel, 1992; Kubeš et al., 1994; Ferreira and Silva, 1998; Schoeler et al., 2000a; Gwakisa et al., 2001; Mejri et al., 2002; Hannier et al., 2003). SGE as well as repeated tick infestations are known to suppress the production of pro-inflammatory cytokines and the secretion of Th1 cytokines, whereas they upregulate Th2 cytokines, indicating a Th2 polarization of the host immune response by ticks (e.g., Ferreira and Silva, 1999; Mejri et al., 2001). Tick-mediated suppression of the Th1 lymphocyte reactivity may inhibit the expansion of antigen-specific T cell clones, differentiation of B cells, activation of macrophages and the NK cell activity. The tick-induced Th2 cytokine profile seems to be advantageous for the survival of the tick because of the anti-inflammatory effect of Th2 cytokines. In addition, the anti-inflammatory mechanisms may also enhance the transmission of tick-borne pathogens (Schoeler and Wikel, 2001; Wikel and Alarcon-Chaidez, 2001).

Despite a relatively broad knowledge of tick-induced host immunomodulation (Brossard and Wikel, 2008), only a limited number of immunomodulatory molecules have been identified and characterized in tick salivary glands (see **Table 1**). However, deeper understanding of the molecular basis of the strategies used by ticks to evade host resistance and immune mechanisms will probably open new possibilities to design vaccines for tick control and control of the transmission of tick-borne pathogens (Wikel and Alarcon-Chaidez, 2001; Brossard and Wikel, 2008).

INNATE IMMUNE RESPONSES AND COMPLEMENT

Innate immune responses represent the first line of immune defence of the hosts to local injury and involve complement, acute phase proteins, neutrophils, macrophages, mast cells, basophils, eosinophils, dendritic cells (DCs) and NK cells. Complement components, prostaglandins, leukotrienes, chemokines, and cytokines contribute to the recruitment of inflammatory cells to the site of injury (e.g., Andrade et al., 2005). Normally, the consequences of prolonged feeding of an ectoparasite would be local inflammation and rejection. However, ticks produce compounds that inhibit or modulate the pro-inflammatory functions of most cell types infiltrating the attachment site, e.g., neutrophils (Ribeiro et al., 1990; Guo et al., 2009), NK cells (Kubeš et al., 1994), macrophages (Kopecký and Kuthejlová, 1998; Kramer et al., 2011), T cells (e.g., Ramachandra and Wikel, 1992; Bergman et al., 2000) and DCs (Cavassani et al., 2005; Skallová et al., 2008).

The skin is the main organ of the tick-host interface, playing a crucial role in the response of the host to tick infestation as well as in pathogen transmission by the vector. Local modulation of cutaneous immune responses at the tick bite site occurs soon after tick attachment and is characterized by modulation of responses in resident cells that merge into a neutrophil-driven immune response a few hours post-attachment (Heinze et al., 2012a). Ir-LBP, a lipocalin present in *I. ricinus* was shown to inhibit neutrophil chemotaxis *in vitro* and host inflammatory response *in vivo* by decreasing the numbers and activation of neutrophils located at the tick bite site, impairing neutrophil function in inflammation (Beaufays et al., 2008). It was also demonstrated that due to an antialarmin effect on human primary keratinocytes, saliva of *I. ricinus* inhibits cutaneous innate immunity and migration of immune cells to the tick bite site, threreby creating favorable conditions for tick-borne pathogens that are transmitted to and multiply in the host skin (Marchal et al., 2009, 2011).

Recruitment of specific leukocyte populations during the inflammatory response is triggered by chemokines that are key mediators of the inflammatory response against parasites. Ticks have evolved various strategies to manipulate the host cytokine network. The chemokine CXCL8 [interleukin(IL)-8] is a chemoattractant for neutrophils. Anti-IL-8 activity impairing neutrophil functions was reported from the saliva of various hard ticks (Hajnická et al., 2001). Moreover, tick saliva contains a variety

of inhibitory activities directed against other pro-inflammatory cytokines such as IL-2 and the chemokines CCL2 (MCP-1), CCL3 (MIP-1α), CCL5 (RANTES), and CCL11 (eotaxin) (Hajnická et al., 2005). These activities are tick species-, developmental stage-, sex- and feeding stage-specific (Vančová et al., 2010b), but the anti-cytokine factors have not been identified. In contrast, Evasins, a family of novel chemokine binding proteins have been detected in salivary glands of *R. sanguineus* ticks (Frauenschuh et al., 2007). Evasins show selectivity to different chemokines: Evasin-1 binds to CCL3, CCL4, and CCL18; Evasin-3 binds to CXCL8 and CXCL1; and Evasin-4 binds to CCL5 and CCL11 (Frauenschuh et al., 2007; Déruaz et al., 2008). Evasin-3-like activities were also demonstrated for other metastriate tick species, providing further evidence that ticks control host neutrophil functions during feeding (Vančová et al., 2010a). *Hyalomma asiaticum asiaticum* ticks evade host immune reactions by modulating cytokine secretion and detoxification of free radicals (Wu et al., 2010). Two families of immunoregulatory peptides, Hyalomin-A and -B, identified in salivary glands of this species, suppress host inflammatory responses either by inhibiting secretion of tumor necrosis factor (TNF)-alpha, monocyte chemotactic protein-1 (MCP-1), and interferon (IFN)-gamma or by increasing the secretion of the immunosuppressant cytokine IL-10.

Modulation of wound healing and angiogenesis seems to be another strategy used by ticks to suppress host inflammatory responses and succeed in prolonged blood feeding (Francischetti et al., 2009; Hajnická et al., 2011). Tick salivary compounds have been shown to bind the transforming growth factor (TGF)-β1, the platelet-derived growth factor (PDGF), the fibroblast growth factor (FGF)-2 and the hepatocyte growth factor (HGF) in a species-specific manner (Hajnická et al., 2011). *Dermacentor variabilis* saliva suppresses basal and PDGF-stimulated fibroblast migration and reduces extracellular signal-regulated kinase (ERK) activity stimulated with PDGF, suggesting that ticks ensure prolonged maintenance of the feeding lesion in the host skin also by the suppression of ERK activation and fibroblast migration, i.e., important events in wound healing (Kramer et al., 2008).

In addition to growth-factor binding capacities, distinct tick salivary molecules with similarities to disintegrin metalloproteases and thrombospondin are involved in cell-matrix interactions and/or the inhibition of angiogenesis (Valenzuela et al., 2002; Francischetti et al., 2005a; Fukumoto et al., 2006; Harnnoi et al., 2007). The proteins ISL 929 and ISL 1373 with homology to the cysteine-rich domain of disintegrin metalloproteinases which were derived from the sialome of *I. scapularis*, reduce the expression of β2 integrins and impair the adherence of polymorphonuclear leukocytes (PMNs) (Guo et al., 2009). Inhibition of microvascular endothelial cell proliferation by the saliva of *I. scapularis* (Francischetti et al., 2005a) suggests that a metalloprotease is responsible for this activity. In addition to its anticoagulation properties, the Kunitz-like serine proteinase inhibitor Ixolaris from *I. scapularis* downregulates the vascular endothelial growth factor and reduces vessel density in tumours (Carneiro-Lobo et al., 2009). A troponin I-like molecule (HLTnI) present in varius organs, including salivary glands of *H. longicornis*, also inhibits capillary formation of human vascular endothelial cells (Fukumoto et al., 2006) and Haemangin, a Kunitz-type protein from the salivary glands of the same species, displays similar effects on angiogenesis and wound healing (Islam et al., 2009). These results indicate that tick anti-angiogenic factors, in addition to their inhibitory effects on angiogenesis, may also play an important role in controlling tick attachment and pathogen transmission.

Bradykinin and histamine are important mediators of itching and pain and could stimulate host grooming and removal of the feeding ticks. However, ticks developed efficient countermeasures to these host reactions. Tick salivary kininases have been shown to hydrolyse circulating kinins (e.g., bradykinin). For example, a dipeptidyl carboxypeptidase activity was found to account for the kininase activity of *I. scapularis* saliva (Ribeiro and Mather, 1998). In addition, amine-binding proteins of the lipocalin family that suppress host responses to local inflammation are produced by hard ticks. A male-specific histamine-binding salivary protein [RaHBP(M)] and two female-specific histamine-binding salivary proteins [RaHBP(F)-1,2] were isolated from the saliva of *R. appendiculatus* (Ra) (Paesen et al., 1999) and the gene for a protein that binds both serotonin and histamine (SHBP) was identified in *Dermacentor reticulatus* (Sangamnatdej et al., 2002). Recently, a tick derived protease inhibitor (TdPI) has been described and characterized from *R. appendiculatus* (Paesen et al., 2007). TdPI suppresses the activity of human β-tryptases, i.e., mast cell-specific serine proteases with roles in inflammation and tissue remodeling.

Another category of compounds produced by ticks to evade host immune responses are proteins that mimic host proteins. Tick macrophage migration inhibitory factor (MIF) is a peptide detected in salivary glands of the hard tick *Amblyomma americanum* (Jaworski et al., 2001). This peptide inhibits the migration of macrophages and probably protects the feeding ticks from macrophage attack.

Non-proteinaceous substances, like purine nucleoside adenosine (Ado) and prostaglandin PGE_2 present in saliva of *R. sanguineus*, are also involved in the modulation of host inflammatory and immune responses. These compounds inhibit the production of pro-inflammatory IL-12p40 and TNF-alpha and stimulate the production of anti-inflammatory IL-10 by murine DCs (Oliveira et al., 2011).

The complement system links the innate and adaptive responses of the host immune system and is activated via three main pathways (alternative, classical, and lectin pathway), whereby the alternative pathway is the major line of defence against invading pathogens and is also involved in resistance to ticks. SGE of ixodid ticks were found to inhibit complement activity of vertebrates, whereby the anti-complement activities correlated to the reported host range of the tested tick species (Lawrie et al., 1999). Subsequently, several molecules with anti-complement activities were identified in tick salivary glands. Isac, Salp 20 and Isac-1 from *I. scapularis* (Valenzuela et al., 2000; Tyson et al., 2007) and the Isac paralogues IRAC I and II from *I. ricinus* (Daix et al., 2007; Couvreur et al., 2008) specifically inhibit formation of the C3 convertase of the alternative pathway by blocking binding of complement factor B to complement C3b. On the other hand, OmCI (*Ornithodoros moubata* complement

inhibitor) belonging to proteins of the lipocalin family has been the first natural complement inhibitor isolated from a soft tick that specifically targets the C5 activation step in the complement cascade (Nunn et al., 2005).

ACQUIRED IMMUNE RESPONSES

During the first exposure to ticks, immunoglobulin and T cell-mediated immune responses are induced in the hosts. Salivary immunogens are processed by Langerhans cells located in the epidermis and presented to immunocompetent lymphocytes (Schoeler and Wikel, 2001; Andrade et al., 2005). Antigen presenting cells can also transport immunogens to draining lymph nodes and promote antibody- and cell-mediated responses. Delayed type hypersensitivity response characteristic of influx of lymphocytes and macrophages, basophils and eosinophils is often observed at the tick feeding site. Homocytotropic antibodies are produced and memory B and T lymphocytes are generated.

Immune resistance to ticks is important in protection from infestation with these ectoparasites and consequently also contributes to the reduction in pathogen transmission from infected ticks to the hosts (Wikel et al., 1997), although specific antigenic and functional components of tick saliva have not been well characterized. In resistant hosts (e.g., rabbits, guinea pigs), the presence of reactive antibodies and effector T lymphocytes assures a rapid response to infestation and can impair tick feeding, whereas ticks have evolved to overcome host immune responses in natural tick-host associations (Ribeiro, 1995). *I. scapularis* salivary antigens that elicit antibodies in resistant hosts have been determined based on screening of salivary gland cDNA expression library with tick-immune mice sera. Using this procedure, the presence of Salp25D, a protein which neutralizes the effect of reactive oxygen species produced by activated neutrophils, has been detected (Das et al., 2001; Narasimhan et al., 2007b). In contrast to resistant hosts, mice generally do not develop acquired resistance to repeated tick feeding (e.g., Schoeler et al., 1999); however, during secondary tick infestation, their cytokine response displays a mixed Th1/Th2 profile and enhanced activity of regulatory T cells (Heinze et al., 2012b).

A variety of tick species have been found to suppress the *in vitro* proliferation of lymphocytes induced with T and/or B cell mitogens. Tick-induced immunosuppression of the host is also characterized by decreased primary antibody responses to T cell-dependent antigens. Moreover, ticks have evolved ways to alter the production of T lymphocyte cytokines. Generally, it has been reported that tick saliva polarizes the host immune response toward a Th2 type profile characterized by the down-regulation of pro-inflammatory Th1 cytokines (IL-2, IFN-gamma) and enhanced production of Th2 cytokines (IL-4, IL-5, IL-6, IL-10, IL-13) (see Gillespie et al., 2000; Schoeler and Wikel, 2001; Wikel and Alarcon-Chaidez, 2001; Brossard and Wikel, 2008, and references therein). It has been suggested that inhibition of T cell responsiveness to mitogens could result from the direct effect of salivary gland proteins on lymphocytes or from their production of IL-10, while up-regulation of IL-4 and IL-10 probably leads to the development of a Th2 response (Ramachandra and Wikel, 1992; Wikel, 1999; Schoeler and Wikel, 2001; Wikel and Alarcon-Chaidez, 2001).

Several T cell inhibitors have been identified in ticks. A 36 kDa protein (P36) suppressing T cell proliferation is present in the saliva of feeding *D. andersoni* (Bergman et al., 2000). Iris was detected in the salivary glands of *I. ricinus* females (Leboulle et al., 2002). The production of Iris is induced in the tick salivary glands during the feeding process and the protein is secreted into the tick saliva. It suppresses T cell proliferation, induces a Th2 type immune response and inhibits the production of pro-inflammatory cytokines IL-6 and TNF-alpha. Salp15, a 15 kDa salivary gland protein from *I. scapularis* is another feeding-induced protein that inhibits the activation of T cells. Salp15 specifically binds to the CD4 molecules on the surface of CD4+ T (helper) cells, which results in inhibition of T cell receptor-mediated signaling, leading to reduced IL-2 production and impaired T cell proliferation (Anguita et al., 2002; Garg et al., 2006). In addition, Salp15 impairs DCs functions by inhibiting Toll-like receptor- and *Borrelia burgdorferi*-induced production of pro-inflammatory cytokines by DCs and DC-induced T cell activation (Hovius et al., 2008a). Evidence was also provided that the pathogen *B. burgdorferi* in *I. scapularis* exploits Salp15 during transmission to a vertebrate host, as it specifically interacts with *B. burgdorferi* outer surface protein C (OspC) and the binding of Salp15 protects *B. burgdorferi* from antibody-mediated killing *in vitro* (Ramamoorthi et al., 2005). Salp 15-like sequences encoding proteins of the Salp family have also been identified in salivary glands of *Ixodes pacificus*, *I. ricinus*, and *I. persulcatus*, which are other major vectors of disease agents in the USA and Eurasia (Hovius et al., 2007; Hojgaard et al., 2009; Mori et al., 2010). The results suggest that the Salp15 homologues can be involved in host immunomodulation and transmission of *Borrelia* species in the above regions.

Other immunomodulatory proteins facilitating tick feeding and pathogen transmission were also detected in the saliva of *I. scapularis*: a secreted IL-2 binding protein that suppresses T cell proliferation and the activity of immune effector cells responsive to IL-2 stimulation (Gillespie et al., 2001), and the salivary cysteine protease inhibitors sialostatin L and sialostatin L2, with inhibitory activity against cathepsin L (Kotsyfakis et al., 2006). Sialostatin L displays anti-inflammatory properties and inhibits proliferation of cytotoxic T lymphocytes and LPS-induced maturation of DCs, whereas sialostatin L2 does not modulate functions of antigen presenting cells, but is probably important for successful tick feeding (Kotsyfakis et al., 2006; Sá-Nunes et al., 2009). In addition, sialostatin L2 stimulates the growth of *B. burgdorferi* in murine skin, however, the mechanism of this growth stimulation has not been revealed (Kotsyfakis et al., 2010).

Ticks can also benefit from the suppression of B cell responses of vertebrate hosts by inhibiting the production of specific anti-tick antibody responses that could cause rejection of feeding ticks by the host. B cell inhibitory proteins (BIP and BIF) have been identified in *I. ricinus* and *H. asiaticum asiaticum,* respectively (Hannier et al., 2004; Yu et al., 2006). Along with feeding ticks, tick-borne pathogens like *B. burgdorferi* might also benefit from BIP-mediated B cell suppression in their vertebrate hosts (Hannier et al., 2004).

In addition to substances modulating the host immune responses, ticks produce immunoglobulin (IgG)-binding proteins

that bind ingested host IgGs and excrete them by salivation. This mechanism protects the ticks primarily from ingested host immunoglobulin's and facilitates their feeding (Wang and Nuttall, 1999).

A novel mechanism of tick-induced modulation of host adaptive immunity which may facilitate pathogen transmission has been discovered recently (Preston et al., 2013). Japanin, a salivary gland protein from *R. appendiculatus* belonging to a new clade of lipocalins from metastriate ticks, was found to target DCs. Japanin specifically reprogramms responses of DCs to a wide variety of stimuli *in vitro*, altering their expression of co-stimulatory and co-inhibitory transmembrane molecules and secretion of pro-inflammatory, anti-inflammatory and T cell polarizing cytokines and it also inhibits the differentiation of DCs from monocytes.

TICK SALIVA AND ITS INVOLVEMENT IN PATHOGEN TRANSMISSION

Tick-borne microorganisms are known to exploit tick salivary molecules to increase their pathogenicity and transmission to the vertebrate host, mainly by circumventing host defence responses (Nuttall and Labuda, 2008; Hovius et al., 2008b). In addition, by modulating skin immune reactions, tick saliva enhances non-systemic pathogen transmission between infected and uninfected co-feeding ticks (Labuda et al., 1996).

Exploitation of the highly modified skin site by molecules secreted in tick saliva by tick-borne pathogens has been referred to as SAT, previously saliva-activated transmission, i.e., promotion of transmission of pathogens by vector saliva (Nuttall and Labuda, 2004, 2008).

SALIVA-ASSISTED TRANSMISSION

The phenomenon of SAT was first described for the Thogoto virus (THOV)—*R. appendiculatus* association. Increased THOV transmission to uninfected *R. appendiculatus* nymphs was observed when the nymphs fed on animals inoculated with a mixture of the virus and SGE of tick females compared to nymphs feeding on animals inoculated with virus only (Jones et al., 1989). Enhanced infection of ticks feeding on animals experimentally inoculated with pathogens and tick saliva (or SGE), i.e., direct evidence of SAT (Nuttall and Labuda, 2008), has subsequently been reported for a few other pathogens, e.g., tick-borne encephalitis virus (TBEV) (Labuda et al., 1993a), *B. burgdorferi* s.l. (Pechová et al., 2002; Zeidner et al., 2002; Macháčková et al., 2006; Horká et al., 2009) and *Francisella tularensis* (Kročová et al., 2003) (see **Table 2**).

In studies involving *Borrelia* spirochaetes, injection of borreliae together with *I. ricinus* or *I. scapularis* SGE increased the level of bacteraemia in the murine host, enhanced the transmission of spirochaetes to feeding ticks and suppressed the production of pro-inflammatory cytokines in draining lymph nodes of mice (Pechová et al., 2002; Zeidner et al., 2002). Moreover, SGE of *I. ricinus* inhibited killing of *B. garinii* by murine macrophages and reduced the production of two major defence molecules of phagocytosis—superoxide and nitric oxide (Kuthejlová et al., 2001). Saliva of *I. scapularis* reduced PMN adhesion via downregulation of beta2-integrins and decreased the efficiency of PMN in the uptake and killing of spirochaetes, thus facilitating the transmission and initial survival of spirochaetes (Montgomery et al., 2004). The SAT compounds responsible for the described effects have not been identified, but they probably depend on vector competence of individual tick species for the pathogen and can vary with different pathogens (Nuttall and Labuda, 2008).

NON-VIRAEMIC TRANSMISSION

Studies on non-viraemic transmission (NVT) of pathogens from infected to non-infected ticks co-feeding on the same host provide indirect evidence of SAT (Nuttall and Labuda, 2008). By mimicking the natural conditions when infected and non-infected ticks feed in aggregates on a vertebrate host, Jones et al., 1987 demonstrated that transmission of THOV from infected to non-uninfected *R. appendiculatus* ticks co-feeding on non-viraemic guinea pigs was more efficient than transmission on higly viraemic hamsters, and suggested a novel mode of arthropod-borne virus transmission. NVT, independent of a systemic infection of a host, has subsequently been shown for other tick-pathogen associations, mainly TBEV and other arthropod-borne viruses (see Nuttall and Labuda, 2008). Because NVT of TBEV occurs on both susceptible and non-susceptible hosts and can also occur in the presence of virus-specific neutralizing antibodies, it is considered one of the main mechanisms of the maintenance of TBEV in natural foci (Labuda et al., 1993a; Randolph et al., 1999; Randolph, 2011).

Immunomodulation of the tick attachment site by tick salivary compounds is suggested to play a crucial role in the process of NVT as the local skin site of tick attachment is an important focus of viral replication early after transmission (Labuda et al., 1996). It was clearly shown that transmission of TBEV from infected to non-infected *I. ricinus* ticks feeding together on mice was correlated with infection in the skin. The virus was recruited in migratory Langerhans cells and neutrophils preferentially to the site in which ticks were feeding compared with uninfested skin sites and migratory monocyte/macrophages produced infectious virus.

Although the maintenance of the *B. burgdorferi* s.l. spirochaetes depends largely on systemic transmission, transmission of the bacteria between co-feeding ticks has also been demonstrated (Gern and Rais, 1996; Ogden et al., 1997). In laboratory models, duration of infectivity, density and distance between co-feeding ticks have been established as important factors affecting efficiency of transmission of the spirochaetes (Piesman and Happ, 2001; Richter et al., 2002). However, such models seldom mimic the situation in nature. On the other hand, field studies revealed that although sheep do not support systemic infections of *B. burgdorferi*, in the absence of alternative hosts, they can transmit localized infections from infected to uninfected ticks co-feeding at the same skin site (Ogden et al., 1997). Due to the presence of various *Borrelia* genospecies and their associations to certain groups of reservoir hosts, the extent and importance of non-systemic transmission in the ecology of Lyme borreliosis needs to be further explored (Randolph et al., 1996; Ogden et al., 1997; Randolph, 2011).

Table 2 | Examples of saliva-assisted transmission of tick-borne pathogens.

Pathogen	Tick species	SAT factor, effect	References
THOV	*R. appendiculatus*	SGE, enhanced transmission and infectivity	Jones et al., 1989
TBEV	*I. ricinus*	SGE, enhanced transmission and infectivity	Labuda et al., 1993a
Borrelia afzelii	*I. ricinus*	SGE, accelerating effect on spirochaete proliferation in the host, suppression of proinflammatory cytokines	Pechová et al., 2002
Borrelia burgdorferi s.s.	*I. ricinus*	SGE, accelerating effect on spirochaete proliferation in the host	Macháčková et al., 2006
B. burgdorferi s.s.	*I. ricinus*	Saliva, increased spirochaete load in host skin, increased transmission to ticks	Horká et al., 2009
Borrelia lusitaniae	*I. ricinus*	SG lysate, increase of spirochaete loads in target organs	Zeidner et al., 2002
B. burgdorferi s.s.	*I. scapularis*	SG lysate, increase of spirochaete loads in target organs	Zeidner et al., 2002
Francisella tularensis	*I. ricinus*	SGE, accelerates proliferation of the bacteria in the host	Kročová et al., 2003
THOV	*R. appendiculatus*	Non-viraemic transmission	Jones et al., 1987
TBEV	*I. ricinus*	Non-viraemic transmission	Labuda et al., 1993b
B. afzelii	*I. ricinus*	Co-feeding transmission	Richter et al., 2002
B. burgdorferi s.s.	*I. ricinus*	Co-feeding transmission	Gern and Rais, 1996
B. burgdorferi s.s.	*I. scapularis*	Co-feeding transmission	Piesman and Happ, 2001
TBEV	*I. ricinus*	Saliva, *in vitro* modulation of infection rate of DCs and production of cytokines	Fialová et al., 2010
B. afzelii	*I. ricinus*	SGE, anti-inflammatory activities	Severinová et al., 2005
B. afzelii	*I. ricinus*	SGE, impairment of signal pathways in DCs	Lieskovská and Kopecký, 2012a,b
		SGE, impairment of DCs functions	Slámová et al., 2011
B. burgdorferi	*I. ricinus*	Tick feeding, modulation of skin innate immunity	Kern et al., 2011
	I. ricinus	BIP, inhibition of B lymphocyte proliferation induced by the *B. burgdorferi* lipoproteins OspA and OspC	Hannier et al., 2003
B. burgdorferi	*I. ricinus*	Salp15 Iric-1, a Salp15 homologue, binds to OspC of *B. burgdorferi* s.s., *B. garinii,* and *B. afzelii*	Hovius et al., 2008c
B. burgdorferi	*I. scapularis*	Salp15, immunosuppressive functions, binds to OspC of *B. burgdorferi*, protects the spirochaete from antibody-mediated killing, facilitates transmission and replication of the spirochaete	Ramamoorthi et al., 2005
		Salp25D, antioxidant, facilitates the acquisition of spirochaetes by the vector from an infected mammalian host	Narasimhan et al., 2007b
		Salp20, inhibits complement, facilitates pathogen survival	Tyson et al., 2007
		P8, lectin complement pathway inhibitor, facilitates pathogen transmission	Schuijt et al., 2011a
A. phagocytophilum	*I. scapularis*	Salp16, facilitates migration of the pathogen to salivary glands	Sukumaran et al., 2006

HOST IMMUNOMODULATION

Immunomodulatory capacities of tick saliva are considered key factors in pathogen transmission. It has been demonstrated that the local skin site infested with ticks and modulated by tick saliva is an important focus of virus replication early after TBEV transmission by ticks. Cellular infiltration and cell migration at the tick attachment site may also facilitate pathogen transmission between infected and uninfected co-feeding ticks

(Labuda et al., 1996). These findings were supported by a study where the effects of TBEV infection on DCs and their modulation by *I. ricinus* saliva were demonstrated *in vitro*, showing that treatment of the cells with tick saliva increased the proportion of virus-infected cells and decreased the virus-induced production of TNF-alpha and IL-6 and reduced virus-induced apoptosis (Fialová et al., 2010).

There is increasing evidence that the host immune reactions to *B. burgdorferi* and consequently the outcome of *Borrelia* infection in the host and its infectivity for ticks depend on the presence of the vector. It was demonstrated that mice infected with *B. burgdorferi* via *I. ricinus* were more infective for subsequently attached ticks than those experimentally inoculated with the spirochaete (Gern et al., 1993). BALB/c mice developed a Th2 immune response against *B. burgdorferi* after tick inoculation and a mixed Th1/Th2 response after syringe inoculation. Moreover, in comparison with syringe inoculation of *B. burgdorferi*, IL-4 produced in host draining lymph nodes following tick bites greatly inhibited the production of anti-borrelial IgG2a antibodies (Christe et al., 2000). These findings were further supported by experiments in which a B cell inhibitory protein (BIP) from *I. ricinus* salivary glands suppressed B lymphocyte proliferation induced by the *B. burgdorferi* OspC, suggesting that BIP may play an important role in enhancing *B. burgdorferi* transmission by the tick (Hannier et al., 2003).

Immunomodulation by tick saliva resulting in down-regulation of cytokines, chemokines and antimicrobial peptides in vertebrate hosts was shown to facilitate transmission and infection by *Borrelia*. The significance of the anti-inflammatory properties of *I. ricinus* SGE was demonstrated experimentally in transmission of *B. garinii* to mice when the bacteria were used to stimulate inflammation (Severinová et al., 2005). In these experiments, tick saliva injected together with spirochaetes reduced the numbers of leukocytes and T lymphocytes in the infected murine epidermis at early time-points post infection and decreased the total cell count in draining lymph nodes. Maturation of DCs (Skallová et al., 2008) as well as interactions of *B. garinii* and murine DCs were also impaired by *I. ricinus* saliva through the inhibition of proliferation and IL-2 production by specific CD4+ T cells and decreased production of Th1 and Th2 cytokines by DCs (Slámová et al., 2011). In addition, *I. ricinus* saliva modulated IFN-gamma signaling pathways in DCs (Lieskovská and Kopecký, 2012a) and pathways activated by Toll-like receptor (TLR-2) ligand in *Borrelia*-stimulated DCs (Lieskovská and Kopecký, 2012b).

The involvement of tick salivary compounds in the modulation of skin innate immunity mediated by antimicrobial peptides of the cathelicidin and defensin families in the course of *Borrelia* infection was also demonstrated. When spirochaetes were inoculated to mice, *Borrelia* triggered skin inflammation with induction of the cathelin-related antimicrobial peptide, the mouse cathelicidin and TNF-alpha. However, after natural transmission of the spirochaetes via feeding *I. ricinus*, the inflammatory genes were supressed, suggesting that saliva of the vector tick facilitate *Borrelia* establishment in the host skin (Kern et al., 2011).

Generally, repeated infestation of mice with pathogen-free *Ixodes* ticks results in a polarization of the host immune response toward the Th2 anti-inflammatory cytokine pattern, with a corresponding down-regulation of Th1 responses (Schoeler et al., 1999, 2000b; Mejri et al., 2001). Consequently, down-regulation of pro-inflammatory factors promotes the initial establishment and dissemination of spirochetal infection, but reconstitution of cytokines down-regulated by tick infestation provides protection against tick-transmitted *B. burgdorferi* (Zeidner et al., 1996, 1997). This was demonstrated *in vivo* when mice or Guinea pigs repeatedly infested with pathogen-free *I. scapularis* nymphs were protected against infection with *B. burgdorferi* transmitted via infected ticks, suggesting that immunity against the tick interferes with transmission of the spirochatete (Wikel et al., 1997; Nazario et al., 1998). Moreover, immunization of Guinea pigs with *I. scapularis* salivary gland proteins produced within the first day of tick attachment impaired *B. burgdorferi* transmission from ticks to hosts, probably by evoking acquired immunity against tick feeding (Narasimhan et al., 2007a).

EXPLOITATION OF TICK MOLECULES BY PATHOGENS

Borrelia burgdorferi s.l. displays distinct phenotypic plasticity (Radolf et al., 2012) and exploits a number of tick proteins (**Table 2**) that support colonization and persistence of the pathogen in the vector and its transmission to the vertebrate host (Kung et al., 2013). Within an infected tick, spirochaetes express OspA and bind to the midgut wall of the tick by using a tick expressed protein (TROSPA) (Pal et al., 2004). After attachment of the tick to a host and onset of feeding, the spirochaetes start to express OspC and move from the midgut through the haemolymph to the salivary glands. In the tick salivary glands, spirochaetes bind to the secreted salivary protein, Salp15, which protects the spirochaetes from antibody-mediated killing and facilitates their transmission and replication in the host skin (Ramamoorthi et al., 2005). The spirochaetes are transmitted to the host with tick saliva containing various salivary molecules which modulate T cells (Salp15), complement (ISAC, Salp20), macrophages, neutrophils, and B cell activities (BIP) and other components of the host immune system (see above) and help *Borrelia* to infect and disseminate in the mammalian host.

Salp15 is the first tick SAT molecule and was identified in salivary glands of *I. scapularis*. Except for immunosuppressive functions, Salp15 is an immunoprotective antigen, because antiserum against the protein protects mice from *Borrelia* infection (Dai et al., 2009).

Salp15 Iric-1, a Salp15 homologue, was identified in *I. ricinus*, the vector of European *Borrelia* species. The protein was found to differentially protect *B. burgdorferi* s.s., *B. garinii,* and *B. afzelii* from antibody-mediated killing in the host (Hovius et al., 2008c).

Salp25D is another immunodominant salivary protein present in *I. scapularis*, which is important during tick acquisition of *B. burgdorferi* and acts as an antioxidant that facilitates pathogen survival (Das et al., 2001; Narasimhan et al., 2007b).

Salp20 protects *B. burgdorferi* from *in vitro* lysis and probably from components of the complement pathway during transmission from an infected tick to the host (Tyson et al., 2007).

A tHRF is up-regulated in *I. scapularis* salivary glands during the rapid feeding phase and probably facilitates tick engorgement and *B. burgdorferi* infection by modulation of vascular permeability and increasing blood flow to the tick bite-site (Dai et al., 2010). Immunization of mice with the recombinant protein interfered with tick feeding and decreased the spirochaete burden.

The Tick Salivary Lectin Pathway Inhibitor (TSLP, formerly P8) from salivary glands of *I. scapularis* was found to interfere with the lectin complement pathway and impair neutrophil phagocytosis and chemotaxis, and protects *Borrelia* from killing by those (Schuijt et al., 2011a).

Tick proteins were identified to be involved also in colonization of the vector and transmission of the intracellular bacterium *Anaplasma phagocytophilum* to vertebrate hosts. Generally, strategies of survival of these bacteria in the vector and transmission to vertebrate hosts are less explored than for *Borrelia. A. phagocytophilum* was found to induce expression of the *I. scapularis* salp16 gene in tick salivary glands during feeding. It was shown that RNA interference-mediated silencing of the salp16 gene expression diminished migration of the bacteria ingested via host blood meal to tick salivary glands, which demonstrates the specific requirement of the pathogen for a tick salivary protein to persist within the vector (Sukumaran et al., 2006). During the transmission of *A. phagocytophilum* to the vertebrate host, *I. scapularis* saliva probably modulates host inflammatory responses by inhibition of the production of inflammatory cytokines by macrophages during stimulation of Toll-like (TLR) and Nod-like receptor (NLR) signaling pathways (Chen et al., 2012).

CONCLUSIONS

Ticks have adapted to blood-feeding by counteracting host defence reactions such as haemostasis and immune responses. Ticks modulate host responses at the site of their attachment to the hosts by a wide range of salivary molecules (anti-haemostatics, anti-inflammatory compounds and immunomodulators) and, as a result, they create an environment which is favorable for both the feeding ticks as well as transmission of the microorganisms that ticks may carry. In spite of increasing knowledge on tick salivary compounds primarily involved in modulation of host defences and secondarily in acquisition, survival and transmission of tick-borne microorganisms, large gaps still exist in the identification of the bioactive molecules and characterization of their single or multiple biological functions. In general, there is a large redundancy in tick salivary molecules and, on the other hand, many such molecules can display multiple functions, responding to redundancy in vertebrate defence reactions.

Tick-borne pathogens co-evolved with their vectors and hosts and survive, multiply and circulate due to their adaptation to these different biological systems. Tick salivary molecules, due to their properties, serve as excellent immunomodulators of host immune reactions and as such create a favorable environment to the pathogens that are injected to the host's skin together with tick saliva during tick feeding. A wide range of events connected with pathogen transmission to vertebrate hosts facilitated by factors in tick saliva have been described and possible mechanisms of host immunomodulation by tick salivary molecules have been designed. However, the number of identified and characterized tick molecules exploited by pathogens is still limited. Advanced molecular techniques such as DNA microarrays, gene silencing RNA interference, next generation sequencing, *in vitro* studies using tick and host cell cultures, etc. are widely applied in studying tick-host-pathogen interactions. They provide information on the expression of vector and pathogen genes during pathogen acquisition and explain mechanisms of host reactions to the feeding tick and invading microorganisms. Consequently, a deeper understanding of events occurring on the tick-host-pathogen interface may lead to the development of new strategies in the control of tick-borne diseases.

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Influence of laboratory animal hosts on the life cycle of *Hyalomma marginatum* and implications for an *in vivo* transmission model for Crimean-Congo hemorrhagic fever virus

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Crimean-Congo hemorrhagic fever virus (CCHFV) is one of the most geographically widespread arboviruses and causes a severe hemorrhagic syndrome in humans. The virus circulates in nature in a vertebrate-tick cycle and ticks of the genus *Hyalomma* are the main vectors and reservoirs. Although the tick vector plays a central role in the maintenance and transmission of CCHFV in nature, comparatively little is known of CCHFV-tick interactions. This is mostly due to the fact that establishing tick colonies is laborious, and working with CCHFV requires a biosafety level 4 laboratory (BSL4) in many countries. Nonetheless, an *in vivo* transmission model is essential to understand the epidemiology of the transmission cycle of CCHFV. In addition, important parameters such as vectorial capacity of tick species, levels of infection in the host necessary to infect the tick, and aspects of virus transmission by tick bite including the influence of tick saliva, cannot be investigated any other way. Here, we evaluate the influence of different laboratory animal species as hosts supporting the life cycle of *Hyalomma marginatum*, a two-host tick. Rabbits were considered the host of choice for the maintenance of the uninfected colonies due to high larval attachment rates, shorter larval-nymphal feeding times, higher nymphal molting rates, high egg hatching rates, and higher conversion efficiency index (CEI). Furthermore, we describe the successful establishment of an *in vivo* transmission model for CCHFV in a BSL4 biocontainment setting using interferon knockout mice. This will give us a new tool to study the transmission and interaction of CCHFV with its tick vector.

Keywords: Crimean-Congo hemorrhagic fever, *Hyalomma marginatum*, BSL4, transmission, Crimean-Congo hemorrhagic fever virus, bunyavirus, tick, tick-borne virus

INTRODUCTION

Crimean-Congo hemorrhagic fever (CCHF) is an acute, tick-borne zoonosis caused by Crimean-Congo hemorrhagic fever virus (CCHFV), which belongs to the genus Nairovirus in the family *Bunyaviridae*. Since 2000, the incidence and geographic range of confirmed CCHF cases have markedly increased, with the disease being reported for the first time in Turkey, Iran, India, Greece, the Republic of Georgia, and some Balkan countries, and the detection of viral RNA in *Hyalomma* ticks recovered from deer in Spain. The virus circulates in nature in a vertebrate-tick cycle with ticks of the genus *Hyalomma* being the main vectors and reservoirs. Although the tick vector plays a crucial role in the virus life cycle, most of the work has focused on case studies, epidemiological investigations, and field studies of ticks collected from animal hosts. Despite the need to study the interaction between ticks and the virus in a controlled laboratory setting in order to understand how the virus is transmitted and maintained, little has been done (Shepherd et al., 1989; Gonzalez et al., 1991, 1992; Wilson et al., 1991; Dickson and Turell, 1992; Dohm et al., 1996). This is mostly because establishing and maintaining tick colonies is time consuming, and working with ticks in a biosafety level 4 (BSL4) biocontainment setting is challenging. Ideally, a transmission and infection model utilizes laboratory hosts that both support tick development and virus transmission.

Ticks belonging to the genus *Hyalomma* (*H.*) are considered the main vectors of CCHFV. After the first description of CCHF in 1944 in the agricultural steppe areas of Crimea, *Hyalomma marginatum* was immediately suspected when local inhabitants described unusually high numbers of this species as compared with the previous years (Grashchenkov, 1945). Subsequent outbreaks in Bulgaria, China, Yugoslavia, Pakistan, United Arab Emirates, Iraq, and other areas of Russia in the following decades reaffirmed that species of the genus *Hyalomma* were predominant [thoroughly reviewed by Hoogstraal (1979)]. Details of vector competence were tested for the ability to transmit the virus to the next stage or to eggs from infected females and *H. anatolicum, H. marginatum, H. rufipes, H. turanicum,* and *H. truncatum* are reported the ability to acquire the virus in nature and to pass the

virus to the eggs or to the next stage in its life cycle (Hoogstraal, 1979).

H. marginatum is the most important vector for CCHFV in southern Europe as well as parts of the Middle East and Central Asia (**Figure 1**). Studies on the life cycle and biology have been published for only 11 of the approximately 27 known *Hyalomma* species (Guglielmone et al., 2010). These studies involve various animal host species in the laboratory maintenance and biology of: *H. aegyptium* (Sweatman, 1968; Siroky et al., 2011). *H. anatolicum* (Snow, 1969; Ghosh and Azhahianambi, 2007; Ahmed et al., 2011), *H. dromedarii* (Bassal and Hefnawy, 1972), *H. excavatum* (Rechav, 1968, 1970; Hadani and Rechav, 1970), *H. impeltatum* (Logan et al., 1989b), *H. isaaci* (Rau, 1963; Das and Subramanian, 1972), *H. lusitanicum* (Hueli et al., 1984a; Ouhelli and Pandey, 1984), *H. rufipes* (Knight et al., 1978; Magano et al., 2000; Chen et al., 2012), and *H. truncatum* (Rechav and Fielden, 1997; Magano et al., 2000).

In nature *H.marginatum* is considered to have a two-host life cycle. This means that larval feeding, molting to nymphal stage, and nymphal feeding will occur on the same host (typically smaller mammals and ground-dwelling birds). Following this, the engorged nymphs drop off from the host, molt to their adult stage in the environment and attach to a second host (typically larger mammals such as ungulates) (Apanaskevich, 2004). Nevertheless, the biology of *H. marginatum* has been poorly investigated (Hueli et al., 1984b; Ouhelli, 1994; Yukari et al., 2011) and limited studies have investigated the life cycle involving domestic animals such as rabbits, cattle, and chickens. There is an urgent need to study the interaction of CCHFV with its natural tick vector in a controlled laboratory setting. Hitherto, studies with ticks at BSL4 have not been reported. Safe experimentation with ticks at this level requires appropriate facilities, extensively trained investigators, and thoroughly tested protocols.

Here, we report our evaluation of three common laboratory species as hosts for *H. marginatum* and their influence on the biology of the tick. Furthermore, we describe for the first time the use of ticks in a BSL4 setting and describe the development of protocols to safely carry out this work.

FIGURE 1 | Geographic distribution of *Hyalomma marginatum* based on (Hoogstraal, 1979), www.kolonin.org and http://www.efsa.europa.eu/en/efsajournal/pub/1723.htm (both accessed April 2013).

MATERIALS AND METHODS

TICKS

Unfed female and male *H. marginatum* ticks were collected by Dr. Zati Vatansever in Yozgat region of Turkey and species identification was confirmed based on the latest taxonomical key (Estrada-Peña et al., 2004). These ticks were fed on a rabbit, the female tick engorged, dropped off and laid eggs. Female carcass, as well as a sample of the larval offspring, tested negative for CCHFV by RT-PCR (Midilli et al., 2007). Subsequently, larval and nymphal offspring from this female were fed on rabbits and a portion of the emergent adult ticks were sent to the Insectary Services Division of the Galveston National Laboratory, UTMB, Galveston, Texas USA to initiate a colony. Adult ticks were fed on rabbits and first generation larvae hatched from a single female were used for the subsequent experimental studies. Ticks were kept in sterile clear plastic sample vials. Six 2 mm holes cut into the lightweight plastic lid, coupled with a piece of fine mesh approximately 4 cm^2 in size, served as a secure tick barrier while allowing sufficient air exchange. A folded narrow strip of autoclaved filter paper was maintained inside the vial to absorb excess moisture and allow the ticks to crawl upwards after molting. Ticks were kept under constant conditions (27°C ± 1°C, 80% ± 5% relative humidity, and 12/12 h light/darkness cycle) in the plastic vials within plastic desiccators containing saturated salt solution in the desiccator basin to provide correct humidity levels in environmental growth chambers with temperature and light control. Engorgement of the larvae, nymphs, and adult stages in the following experiments were monitored and ticks that dropped off were collected and weighed daily. Pre-oviposition and oviposition times were recorded for each engorged female. Oviposited egg masses were weighed daily. After the oviposition ended, total egg mass weight for each female and carcasses weight were recorded. Conversion efficiency indexes (CEI = g eggs/g female), which indicates the successful conversion of engorged body weight in to egg mass, were calculated according to Drummond and Whetstone (1970). Unfed nymphs used in the *in vivo* transmission models were generated by pulling off the engorged larvae from the host and then maintaining them under the above described conditions until they molted. All values were compared statistically by ANOVA (GraphPad Prism). Unfed nymphs used in the *in vivo* feeding models were generated by pulling off the engorged larvae from the first host and then maintaining them under the above described conditions until they molted.

EVALUATION OF TICK KILLING WITH DISINFECTANTS

Disinfectant solutions commonly used in biocontainment laboratories such as bleach (sodium hypochloride, 5%; Clorox, Oakland, CA), neutral-buffered formalin (10%; Fisher Scientific, MI), ethanol (70%; Sigma-Aldrich, St. Louis, MO), CaviCide® (Metrex Research Coorperation, Romulus, MI), and Microchem Plus® (National Chemical Laboratories, Philadelphia, PA) were tested for their ability to kill adult ticks. Ticks, 8 in each test group, were submerged in 50 ml of each solution and monitored at 15 min intervals. To confirm death, non-motile individuals were taken out of the solution, rinsed with distilled water, wiped down, and placed in the incubator at 27°C, 80% RH for 72 h.

Individuals showing any movement after 72 h were recorded as still viable.

ANIMALS

Female BALB/c mice, 8–12 weeks of age and >20 grams, female Hartley guinea pigs, >65 g, and male New Zealand white rabbits, >1.5 kg, were purchased from a commercial breeder (Charles River, Wilmington, MA) and used to feed instars of *H. marginatum*. None of the animals had been exposed to ticks previously. Mice were housed in isolator cages (Tecniplast, Buguggiate, Italy) with commercial diet and water *ad libitum* and exposed to ticks by either whole body infestation or using feeding capsules (see below). Rabbits and guinea pigs were housed in Allentown isolator cages with commercial diet and water provided *ad libitum*. Rabbits were infested with ticks using ear-bags while guinea pigs were infested with ticks in a feeding capsule (see below). All animal work was reviewed and approved by UTMB's Institutional Animal Care and Use Committee and Institutional Biosafety Committee.

TICK INFESTATIONS

(a) Whole Body Infestation of Mice: Three mice were placed individually into a restrainer made of metal mesh cloth that restricts the mouse from grooming. Three hundred larvae were allowed to attach to the restrained mouse during a 2 h period. After infestation, each mouse was placed into a micro-isolator cage, one mouse per isolator. The micro-isolator cage contained an inner floor consisting of a stainless steel grid above water, allowing the mouse to stay dry and move and feed normally while engorged ticks drop into the water where they were recovered. Water in the bottom of the cage was inspected for ticks and changed daily.

(b) Feeding Capsule Infestation of Mice: Mice were sedated by isoflurane inhalation (2–4%) and their eyes treated with ointment to prevent drying, and placed on a Delta warming pad (BrainTree sciences). A feeding capsule was fashioned from the top half of a 5 ml cryotube (Sarstedt, Germany). Kamar adhesive (Kamar Products, Zionville, IN) cement (an adhesive commonly used in the livestock industry for maximum adhesion of heat detector pads and tags) applied to the capsule rim. The capsule was attached to a shaved area on the back of the mouse caudal of the shoulder blades. Ticks were placed into the capsule on the following day after the integrity the capsule's attachment was assured by inspection. Adults and nymphs were directly hand-loaded into the capsule using forceps. Larvae were first counted onto to a small piece of gauze or a gelatin capsule, which was then transferred into the capsule. Infestations were done on three mice with 25 larvae in a single capsule, or on five mice with one male-female adult pair in a single capsule. The capsules were inspected daily for engorgement of the feeding instars, and engorged or detached ticks were removed.

(c) Feeding Capsule Infestation of Guinea Pigs: Guinea pigs were infested with ticks enclosed within a tightly woven cotton cloth fabric feeding capsule. The seam of the bags where the fabric edges join together was sewed tightly using a sewing machine (7 stitches/cm). Animals were sedated by isoflurane gas anesthesia (2–4%). The dorsum was shaved and then a fabric capsule was glued to the skin caudal of the shoulder blades using Kamar glue. Ticks were introduced to the capsule the following day after inspection of the capsule's integrity. The open end of the fabric capsule was folded twice laterally, and then longitudinally (2–3 cm) with the open end secured and was wrapped with multiple layers of adhesive bandages. Three guinea pigs were infested with 200 larvae and another three guinea pigs with three pairs of adults on each animal.

(d) Ear bag Infestation of Rabbits: Similar to feeding capsules utilized for guinea pigs, ear bags for the rabbit were fabricated using tightly woven cotton cloth. Bags were rectangular in shape with openings at both ends. One open end was attached to the shaved base of the ear with a double layered adhesive bandage and Kamar adhesive. The portion of the bag that extended beyond the length of the ear was folded three times laterally and then longitudinally (2–3 cm), and was wrapped with two layers of adhesive bandages. An Elizabethan collar (Lomir, Biomedical Inc., Malone, NY) was placed on the rabbit and the tips of both ear bags were taped together behind the collar to keep them out of reach and to prevent scratching. Rabbits were sedated with isoflurane gas anesthesia (2–4%) during the application of the ear bags. Eyes were treated with ointment to prevent drying and the rabbit was placed onto a warming pad. Ticks were placed into the ear bags the following day after inspection of the ear bag's integrity. Rabbits were infested with three male-female pairs of adults, or with 300 larvae per ear bag.

TICK WORK AT BSL4

The development of standard operating procedures (SOPs) for tick work at BSL4 was based on existing SOPs for arthropod containment level (ACL) 2/3 at UTMB as well as on U.S. guidelines (2003; Scott, 2005; Tabachnick, 2006; Prevention, 2009). All work involving infectious material was done in strict compliance with UTMB Environmental Health and Safety guidelines, and Institutional Biosafety Committee and Institutional Animal Care and Use Committee approved guidelines and protocols. For tick work, a simple, minimalist approach was adopted. A room within the BSL4 of the Galveston National Laboratory was designated for tick research and contained only items required for these studies. The room has no sink or floor drain and is equipped with sticky mats on work surfaces, within the glove box, and in front of the door. As described above for colony rearing methods, ticks were contained in capped rearing vials which were housed in sealed desiccators containing appropriate saturated salt solutions to maintain relative humidity, and the desiccators are in turn kept within climate-controlled environmental growth chambers. The researcher must know exactly how many ticks are taken into the facility and account for the numbers at various stages of the experiment through to termination. Accurate counts were maintained throughout the experiments such that records and contents of the rearing vials verified the final state of each tick. Live ticks and ticks feeding on animals were handled within a non-ventilated glove box lined with sticky tape. Since

working with arthropods often requires the use of small instruments and considerable dexterity, investigators practiced tick work extensively in the National Biocontainment Training Center mock BSL4 training laboratory while wearing positive pressure suits.

All steps involving the handling of ticks feeding on mice were conducted in a non-ventilated Plexiglas glove box within the BSL4 tick room and animals were sedated using isoflurane inhalation anesthesia. Mice with capsules glued onto the skin were housed individually in green line IVC micro-isolator rat cages (Tecniplast, Buguggiate, Italy), with nestles (Ancare, Bellmore, NY) as enrichment and bright-white Diamond Dry cellulose bedding (Harlan Laboratories, Indianapolis, IN) to easily visualize potentially escaped ticks. Other enrichment items were avoided so that the capsule would not be dislodged by routine contact. As additional layers of containment, a ring of double-sided sticky tape was positioned along the inner perimeter of the cages one inch below the cage top and a mesh cap was placed over the cage air supply ventilation ports to prevent any tick escape from the cage. Ticks in feeding capsules were inspected daily in the glove box and collected from the capsule after detachment and stored in photoperiod controlled environmental chambers for molting to the next stage.

In vivo TRANSMISSION MODEL

Uninfected *H. marginatum* nymphs or adults were obtained from our established colony and up to five nymphs or up to two adults (male and female) were put into a feeding capsule that had been applied to 4–8 weeks-old female STAT-1 knockout mice (129S6/SvEv-*Stat1*^{tm1Rds}; Taconic, Germantown, NY). This mouse strain carries a homozygous disruption of the Signal Transducers and Activators of Transcription 1 gene that eliminates the intracellular mechanism by which cells respond to interferons, resulting in a mouse model with extreme susceptibility to CCHFV (Bente et al., 2010). Animals were moved from Animal Biosafety Level (ABSL) 2 to the ABSL4 3 days after ticks attached and were subsequently challenged intraperitoneally with 100 plaque forming units of CCHFV IbAr 10200 as previously described (Bente et al., 2010). Mice were humanely euthanized on day 4 post challenge when becoming moribund, which allowed sufficient time for all nymphs to complete feeding. Engorged nymphs were allowed to molt to the next stage in plastic vials within plastic desiccators in climate-controlled environmental growth chambers (27°C ± 1°C, 80% ± 5% relative humidity, and 12/12 h light/darkness cycle) within the BSL4 tick room. Before dissection, ticks were euthanized by placing them in a freezer for 30 min. Salivary glands were removed from newly emerged adults under a stereomicroscope. Total RNA was extracted from salivary glands and the remaining tick body by phenol/chloroform extraction with Trizol (Invitrogen, Carlsbad, CA) for RT-PCR testing of CCHFV. Semi-engorged adults were removed 3 days after virus challenge, euthanized, and dissected under the stereomicroscope. Salivary glands, midgut, and ovaries were removed and total RNA isolated from tissues using phenol/chloroform extraction (see above). All samples were tested for CCHFV and amounts calculated as genome equivalence by qRT-PCR using a recombinant RNA standard as previously described (Wolfel et al., 2007) using Qiagen's Quantitect Fast probe kit and were run on a CFX 96 real-time detection system (BioRad, Hercules, CA).

RESULTS

HOST COMPARISON

In the first part of this study, we evaluated three common laboratory animal species (mice, guinea pigs, and rabbits) as hosts for *H. marginatum* and their influence on the life cycle of the tick. All infestations were done with life stages coming from the same batch and were performed simultaneously on the three hosts to ensure that all ticks were the same age. As seen in nature, *H. marginatum* exhibited a two-host feeding behavior on all three host species (mice, guinea pigs, rabbits) tested with the larval and nymphal stages feeding on the same host. Animals were checked daily after the larval infestation; none dropped off and no crawling engorged larva were observed. The duration of feeding and molting for ticks fed on each host is summarized in **Figure 2**. The longest time span for the total life cycle was observed for ticks fed on guinea pigs with 112 days and the shortest life cycle was seen in ticks from mice with 73 days. A major difference was seen in adult feeding time among the species tested extending up to 14 days for ticks fed on guinea pigs although the duration of feeding of immature stages appeared similar.

Whole body infestation of mice was the least successful feeding technique among the larval feedings with an attachment rate of 1.55% whereas the larval attachment rates in capsules on mice and guinea pigs and in ear bags on rabbits were 40–60, 51–82, and 80–95%, respectively. As a result of the low attachment success rate during whole body infestation, subsequent studies on mice were conducted using capsule infestations to determine the biological features of *H. marginatum* in mice. Ticks were left on the host molting between larval and nymphal feeding. Larval molting was usually very quick in all tested hosts (2–4 days), and occurred while larvae were still anchored in the host's skin by their mouthparts (**Figure 3**). The larval exoskeleton ruptured and the newly emerged nymph attached in close proximity to its larval feeding site and then commenced feeding (**Figure 3**). Mean weights of engorged nymphs that dropped from mice, guinea pigs and rabbits were 20.9 ± 4.6, 13.5 ± 3.9, and 15.25 ± 6.15 mg, respectively and the differences between groups were statistically significant ($p < 0.05$). Molting success rates of engorged nymphs that had fed on either mice, guinea pigs, or rabbits were 85, 92, and 97%, respectively. Molting periods are listed in **Figure 2**. After molting to the adult stage, male and female ticks were fed on the same host species. Switching host species between different life stages was not attempted in this study. Adult pre-oviposition and oviposition feeding times are shown in **Figure 2**. Mean engorged weights were 568.20 ± 150.79, 231.88 ± 40.84, and 435.32 ± 83.50 mg for the females that were fed on mice, guinea pigs, and rabbits, respectively and the differences were statistically significant between groups ($p < 0.05$). Oviposited egg masses (mg) of female ticks, representing the total number of eggs laid by an individual female in a 24 h period, feeding on mice, rabbits and guinea pigs were measured daily as depicted in **Figures 4A–C**. For females fed on mice, the largest daily egg mass weights were deposited early in the oviposition phase with declining weights over a 10–12

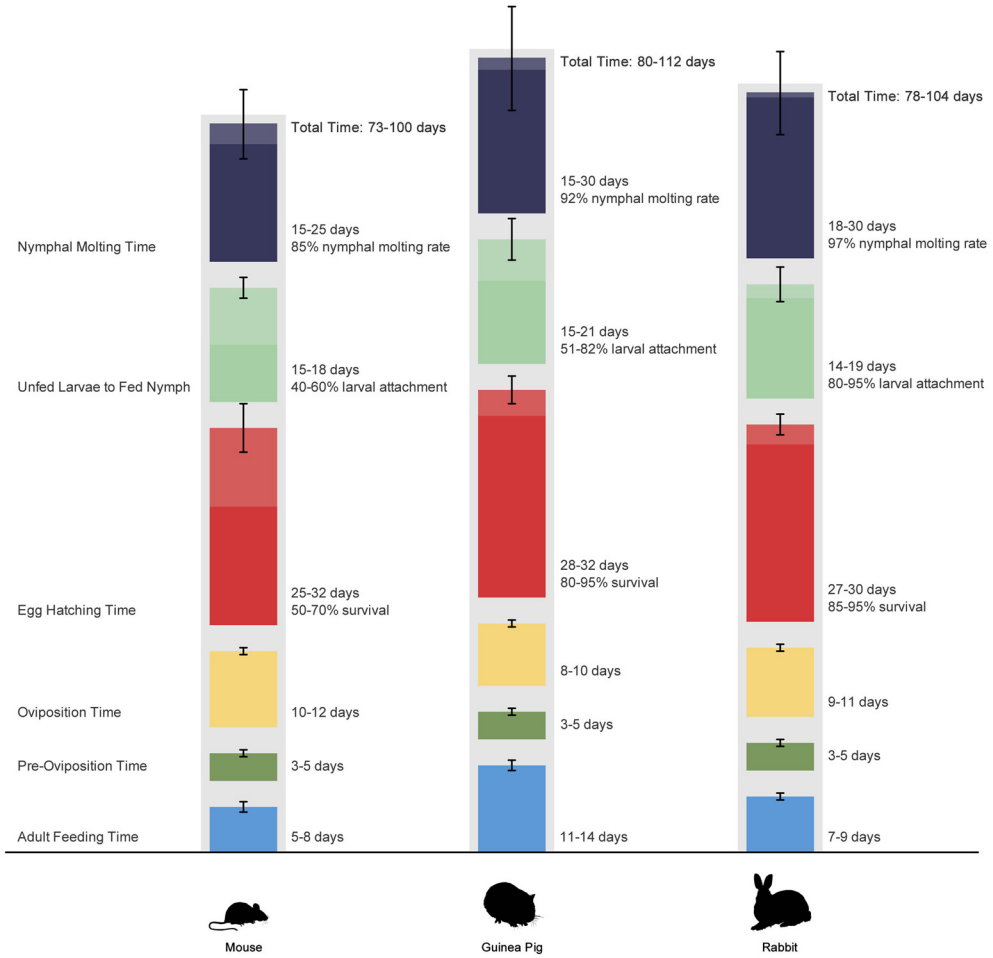

FIGURE 2 | Life cycle of *Hyalomma marginatum* under laboratory conditions when feeding on mice, guinea pigs, and rabbits. Colored segments represent time periods required for different life stages. Bars represent range of values. Values were not compared statistically.

day period; whereas for females fed on guinea pigs and rabbits, oviposition started with lower egg mass weights. Subsequently, the egg mass increased to highest weights at the second and third days and were maintained for 9–11 days in rabbits, and 8–10 days in guinea pigs. Mean total egg mass weights were 396.6 ± 88.84 mg in mice, 98.44 ± 20.38 mg in guinea pig and 248.88 ± 64.93 mg in rabbit. Egg masses were estimated to contain between 2303 and 9288 eggs. The CEI vs. weight can be found in **Figures 4D**,**E**. Comparison of the engorged weights, total egg mass weights and weight gains (%) of the females fed on the three different hosts is provided in the **Figure 4F**. Maximum weight gains were observed in the female *H. marginatum* ticks feeding on mice. Adult *H. marginatum* ticks remove a significant amount of blood during feeding, which could lead potentially lead to anemia in mice, and needs to be taken into consideration when using mice as host model for adult *H. marginatum* ticks. Larvae hatching times are given in **Figure 2**. Larval hatching rates were between 50–70, 85–95, and 70–90% for the egg batches oviposited by females fed on mice, rabbits, and guinea pigs, respectively.

TICK CONTROL

As part of the establishment of the tick work at BSL4, adult *H. marginatum* ticks were submerged in commonly used disinfectants in order to evaluate the ability to kill ticks (**Table 1**). Ethanol was fast acting; it killed 100% of the ticks within 1.5 h, followed by formalin and bleach (2 h). Interestingly, Microchem, commonly used as a disinfectant in chemical showers of the BSL4 laboratories, took up to 120 h post exposure to kill 100% of the ticks.

TRANSMISSION STUDIES

A total of five non-infected adult *H. marginatum* ticks were fed on three STAT-1 KO mice challenged with 100 PFU of CCHFV IbAr 10200 and removed 3 days post virus challenge. Ticks were dissected, RNA extracted from both salivary gland pairs, midgut and ovaries, and were tested were for CCHFV by QRT-PCR. CCHFV RNA was found in pairs of salivary glands (1.01×10^7 to 2.31×10^9 genome equivalents), total midgut (3.74×10^4 to 1.67×10^7 genome equivalents) and in the ovaries (8.54×10^2 to 6.12×10^3 genome equivalents) of all five ticks. Subsequently, a

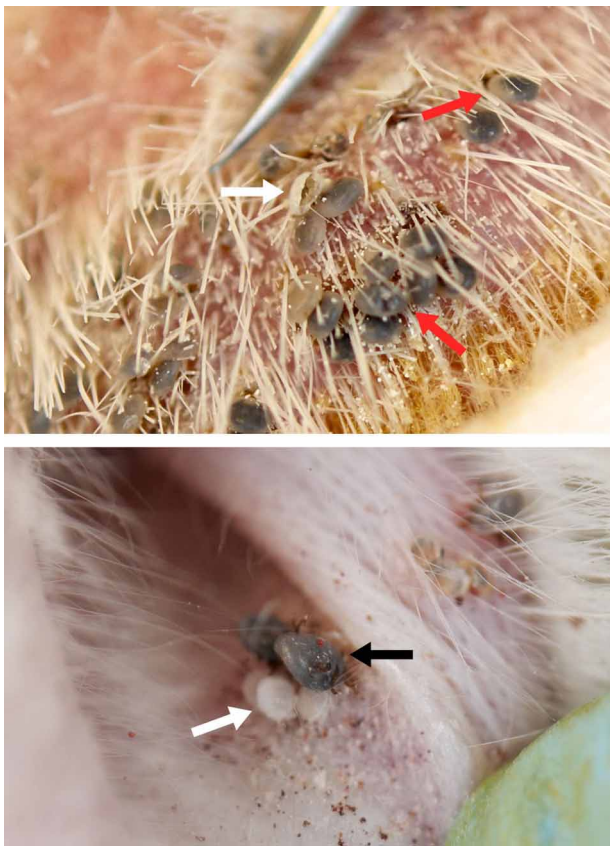

FIGURE 3 | Molting larvae and newly emerged nymphs feeding on guinea pig (top) and rabbit (bottom). Red arrows indicate molting larvae. White arrows show ruptured cuticles left from molted and emerged larvae still attached to the skin. Newly emerged nymphs feeding in close proximity to where they fed as larvae (black arrow).

total of 8 CCHFV-negative *H. marginatum* nymphs were fed on 3 STAT-1 KO mice that had been challenged with 100 PFU of CCHFV IbAr 10200. After molting to the adult stage was completed, ticks were dissected and salivary glands were removed. RNA was extracted from salivary glands and remaining tick body. All eight adult ticks were positive for CCHFV by QRT-PCR. The CCHFV genome equivalents values ranges from 8.68×10^5 to 3.05×10^8 in the body and 4.74×10^4 to 6.49×10^8 in the pairs of salivary glands.

DISCUSSION

H. marginatum is considered to be the main vector for CCHFV in southern Europe, parts of the Middle East, Africa, and Central Asia (**Figure 1**). Only three studies have characterized the life cycle of this tick species in a laboratory setting (Hueli et al., 1984b; Ouhelli, 1994; Yukari et al., 2011) and no studies describe the life cycle of this species when fed on laboratory animals. Studies on the transmission of CCHFV with this tick species are also very limited and involve only the natural hosts (Zgurskaya et al., 1971; Levi and Vasilenko, 1972; Blagoveshchenskaya et al., 1975; Kondratenko, 1976; Zarubinsky et al., 1976). Here we characterized three different laboratory animal species as experimental hosts for *H. marginatum*. The genus *Hyalomma* includes one, two or three host tick species. Some species such as *H. anatolicum, H. scupense*, and *H. dromedarii* can complete their life cycle using either one or two hosts. Another member of the genus, *H. excavatum* is known to follow two or three host biology depending on the hosts (Apanaskevich, 2004). *H. marginatum* is known as a two host tick species, feeding on birds and small mammals as immatures, and feeding as adults on large mammals primarily on artiodactyls (Hoogstraal, 1979; Apanaskevich, 2004). Yukari et al. (2011) reported that *H. marginatum* is a strict two host tick within their experimental design using rabbits and cattle for larval and adult feeding, respectively. Conversely, Ouhelli (1994) suggested that *H. marginatum* is able to switch to a three host life cycle as seen with *H. scupense* and *H. excavatum*. However, only data of a two host life cycle were presented. Furthermore, Ouhelli describes *H. marginatum* adults as not completing their engorgement on the rabbit host. We observed however that *H. marginatum* adults completed feeding successfully on rabbits, confirming the report of Yukari et al. studies (2011). More importantly, we also demonstrated that *H. marginatum* shows a strict two-host life cycle with all three host species: larvae engorged and molted to the nymphal stage onsite and attached to the same host on all three host types.

Capsule feeding is desirable in a biocontainment setting to better control the ticks especially before feeding commences. We expected that the feeding capsule would result in a generally lower attachment rates since a feeding capsule does not give the tick the full opportunity to quest for an appropriate feeding spot. Nonetheless, attachment success rates during capsule feeding were generally high and varied between 40 and 95% depending on the host. Interestingly, whole body infestation rates of larvae on mice was far lower (1.55% success rates) compared to capsule feeding (>40%). Although the lower level of attachment rates might have been due to the removal of ticks during grooming, Hoogstraal proposed that rodents are generally not an appropriate host for immature stages of *H. marginatum* (Hoogstraal, 1979). *H. isaaci*, previously grouped as a subspecies of *H. marginatum* (formerly *H. marginatum isaaci*), was also reported to not parasitize rats and mice experimentally (Das and Subramanian, 1972). In another experimental study, *H. rufipes* (formerly *H. m. rufipes*), was also reported to attach poorly to field mice (Magano et al., 2000). Most rodents live in burrows in nests with considerably higher humidity, which does not fit the environmental needs of *H. marginatum* and other former subspecies. Also, these rodents have a social grooming behavior which is likely to remove a large proportion of instars. All of these factors may have played a role in the development of an incompatibility between these tick species and host species.

Das and Subramanian (1972) reported that 91.5% of *H. isaaci* larvae attached to rabbit and dropped as engorged nymphs in 15–18 days, while very few larvae attached and engorged on guinea pigs. In our study, similar attachment results were seen for *H. marginatum* on rabbits; however, attachment rates on guinea pig were far greater (51–82%).

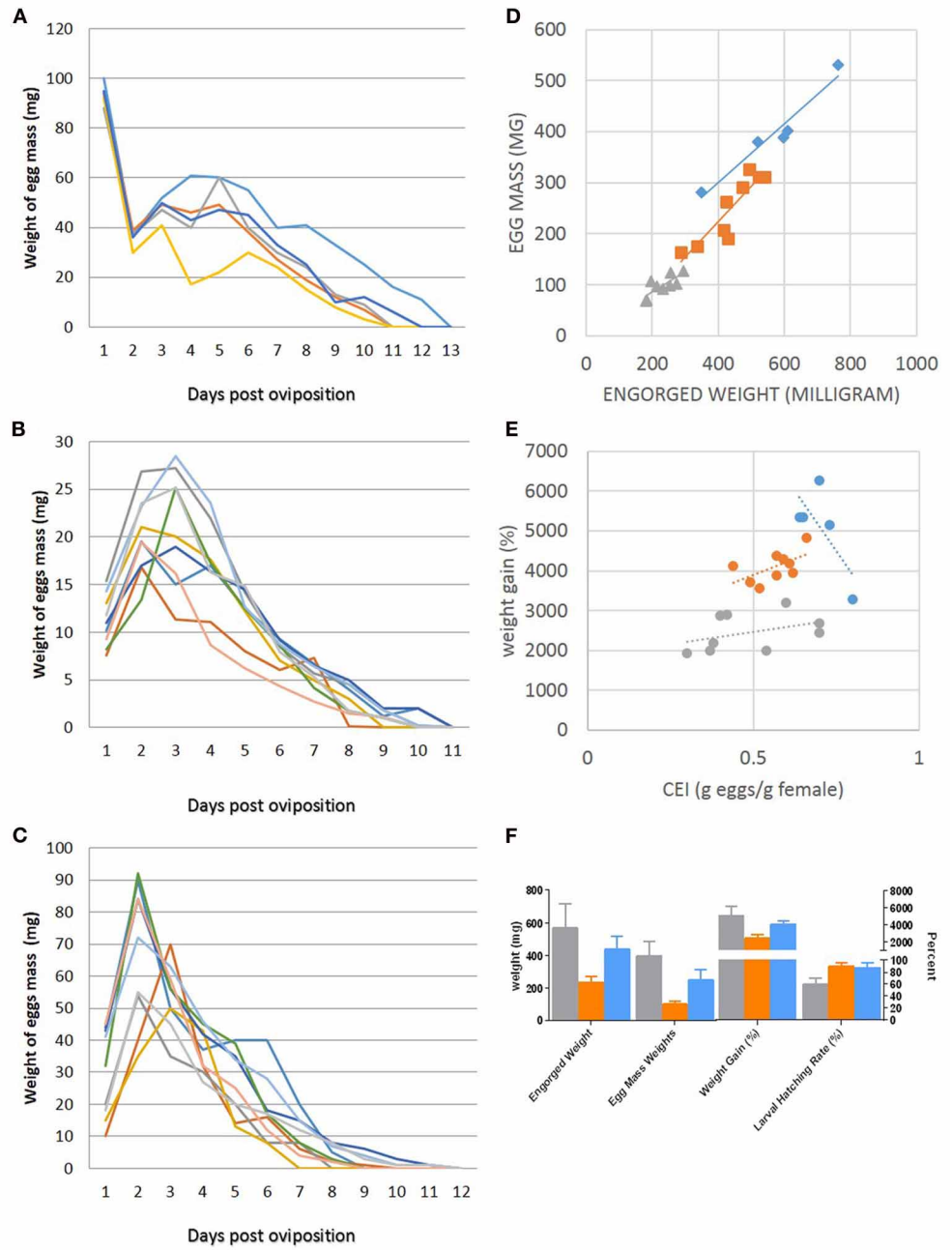

FIGURE 4 | Summary of oviposition data (A–F). Newly deposited egg mass weights measured daily of the females fed on mice **(A)**, guinea pigs **(B)**, and rabbits **(C)**. Each color represents an individual female. Comparisons of egg mass to engorged weight **(D)** and weight gain to conversion efficiency index (CEI) in **(E). (F)** Comparison of the engorged weights, total egg mass weights, and weight gains (%) of the females fed on the three different hosts. For **D–F**, data are color-coded in gray for mice, orange for guinea pigs, and blue for rabbits.

Molting times for nymphs in our study were similar for all three species studied, and correlate with what has been reported in nature (Petrova-Piontkovskaya, 1947) and in laboratory conditions (Yukari et al., 2011).

Mean engorged weights of nymphs were measured as 20.9 ± 4.6, 15.25 ± 6.15 and 13.5 ± 3.9 mg for those fed on mice, rabbits, and guinea pigs, respectively. Significantly, higher mean engorgement weights were recorded on nymphs of *H. truncatum* which fed on the field mice as well and the authors explained this finding with the feeding on non-natural and natural hosts of the ticks (Magano et al., 2000). Molting rates were 85, 92, and 97% for the nymphs that dropped off from mice, guinea pigs, and rabbits, respectively. Similar molting rates were reported with rabbits (Hueli et al., 1984b). For *H. rufipes* nymphs, higher molting

Table 1 | Killing times of *Hyalomma marginatum* using different disinfectants.

Time	Cavicide	Microchem 5%	Formalin 10%	Bleach 5%	Ethanol 70%
15 min	0	0	0	0	0
30 min	0	0	0	0	25
45 min	0	0	0	0	50
60 min	0	0	0	25	75
1.5 h	0	0	75	75	100
2 h	0	0	100	100	
3 h	0	0			
4 h	25	0			
6 h	75	0			
8 h	100	0			
12 h		0			
24 h		0			
36 h		0			
48 h		25			
72 h		50			
96 h		75			
120 h		100			

Table depicts the percent of ticks that are killed when submerged in disinfectant for a particular amount of time. Experiments were done with eight ticks in each group. Ticks were rinsed with deionized water after exposure, wiped clean, and kept in the incubator for 72 h at 27° C with 80% RH to determine if any ticks become active.

success was reported when engorged on guinea pigs (100%) and striped mice (*Rhabdomys pumilio*) (99.9%). Considering that *H. rufipes* has shown three-host feeding pattern on mice, which is not seen in *H. marginatum* biology, this elevated molting success could have arisen due to the adjustment of this species to hosts of a wider range (Magano et al., 2000).

Adult feeding times (for female ticks) were considerably longer for those fed on guinea pigs (11–14 days) as compared to rabbits (7–9 days), or mice (5–8 days). Feeding times on rabbits were reported to be up to 21 (Yukari et al., 2011) and 13 days (Hueli et al., 1984b) previously. On the other hand, higher engorgement weights were seen in females fed on mice (556 ± 207 mg), although feeding times for this stage on this host were considerably shorter (5–8 days) in our study. Differences seen in feeding time and engorgement weights on various hosts could be explained by factors of feeding on natural and non-natural hosts, in a similar way to that reported for nymphal stages (Magano et al., 2000); however, questions such as why different stages engorge with different maximum weights on different hosts, requires further detailed studies involving immunological aspects of the hosts and feeding patterns.

Daily oviposition by the females fed on different hosts showed distinct patterns as seen in **Figures 4 A–C**. When fed on guinea pigs and rabbits, oviposition started with lower egg mass weights, subsequently, the egg mass increased to the highest weights at 2nd–3rd day as described similarly by Ouhelli (1994) who fed the female ticks on rabbits and cattle. On the contrary, when fed on mice, oviposition began with the highest daily egg mass weights with subsequent egg masses weighing less each day. Considering the cattle and rabbits are the natural hosts for *H. marginatum*, females fed on guinea pigs could be regarded as showing similar egg laying pattern to natural biology.

Here, we also describe our work with ticks at BSL4, which to our knowledge not been reported before. The construction of a BSL4 facility and the arrangement of a tick facility within it, low humidity maintained in the facility and required operating procedures all are sufficient to guarantee that no escaped tick life stage could survive for an extended time. Nevertheless, it remains a fundamental responsibility for those working with ticks in the BSL4 laboratory to ensure that all ticks are safely contained. Arthropod security is based on multiple layers of containment. As demonstrated here, ticks are very resistant to being submerged or covered in disinfectant solution of any kind. Although a tick is most likely to be washed off the positive pressure protective suit during the mandatory chemical decontamination shower when exiting the BSL4 facility—and would ultimately be destroyed during the effluent waste sterilization process—this method of killing the ticks would be considered the worst case scenario. The most critical objective is to take every possible precaution to ensure that all ticks remain accounted for in the dedicated room within the BSL4. We demonstrated in our study that all tick stages can be successfully fed within a feeding capsule fastened to the animal's back, which further facilitates controlling the tick escape. Although the use of a capsule denies the tick's innate behavior to quest for a suitable feeding site, the success rates demonstrated in our studies were high.

In order to study the interaction of CCHFV with its tick host, a technique that reliably infects ticks with the virus is desirable. Parental virus inoculation such as oral or anal are difficult to conduct in instar stages of ticks whereas intracolemic inoculation is not useful for vector competence studies (Randolph and Nuttall, 1994). Furthermore, all above mentioned techniques are difficult to conduct in a BSL4 setting. A host feeding model which mimics the natural infection is therefore desirable. Previous studies looked at CCHFV transmission from host animals challenged with the virus to different species of *Hyalomma* ticks (Logan et al., 1989a; Shepherd et al., 1989; Dickson and Turell, 1992; Gordon et al., 1993; Dohm et al., 1996). Generally speaking, the transmission rates are fairly low. For example, Logan et al. noted an 4.4% tick infection rate when feeding on suckling mice challenged with CCHFV (Logan et al., 1989a). Transmission rates were 13% for *H. impeltatum* nymphs feeding on CCHFV-challenged guinea pigs (Dohm et al., 1996). This is most likely due to the fact that the utilized host animals have no or very low level viremia compared to some of their natural hosts. Interferon response knockout mice have recently been described as an animal model that mimics human disease (Bente et al., 2010; Bereczky et al., 2010; Zivcec et al., 2013). It was demonstrated that CCHFV replication results in viremia levels of up to 10^{10} genome equivalents per ml (Bente et al., 2010) or $> 10^3$ $TCID_{50}/\mu l$ (Zivcec et al., 2013). We hypothesized that feeding *H. marginatum* nymphs on STAT-1 KO mice will ensure that the tick is exposed to high levels of CCHFV during feeding, and therefore, result in a reliable infection of the tick with the virus. As hypothesized, the high viremia level resulted in

100% infection of *H. marginatum* nymphs during feeding as well as 100% infection after molting, which demonstrates transstadial transmission. Therefore, the *in vivo* feeding model on STAT-1 KO mice is a means to reliably infect *H. marginatum* ticks with CCHFV. Furthermore, the CCHFV-infected adults that are generated in this model can then be used to study the transmission of CCHFV from the tick to the mammalian host. Despite the limitation of only be able to use small tick numbers per experiment, this is a valuable tool to study the transmission of the virus and vector competence for CCHFV of various other tick species.

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Interaction of the tick immune system with transmitted pathogens

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Ticks are hematophagous arachnids transmitting a wide variety of pathogens including viruses, bacteria, and protozoans to their vertebrate hosts. The tick vector competence has to be intimately linked to the ability of transmitted pathogens to evade tick defense mechanisms encountered on their route through the tick body comprising midgut, hemolymph, salivary glands or ovaries. Tick innate immunity is, like in other invertebrates, based on an orchestrated action of humoral and cellular immune responses. The direct antimicrobial defense in ticks is accomplished by a variety of small molecules such as defensins, lysozymes or by tick-specific antimicrobial compounds such as microplusin/hebraein or 5.3-kDa family proteins. Phagocytosis of the invading microbes by tick hemocytes is likely mediated by the primordial complement-like system composed of thioester-containing proteins, fibrinogen-related lectins and convertase-like factors. Moreover, an important role in survival of the ingested microbes seems to be played by host proteins and redox balance maintenance in the tick midgut. Here, we summarize recent knowledge about the major components of tick immune system and focus on their interaction with the relevant tick-transmitted pathogens, represented by spirochetes (*Borrelia)*, rickettsiae (*Anaplasma)*, and protozoans (*Babesia)*. Availability of the tick genomic database and feasibility of functional genomics based on RNA interference greatly contribute to the understanding of molecular and cellular interplay at the tick-pathogen interface and may provide new targets for blocking the transmission of tick pathogens.

Keywords: tick, tick-borne diseases, innate immunity, phagocytosis, antimicrobial peptides, *Borrelia, Anaplasma, Babesia*

TICK-PATHOGEN INTERFACE: GENERAL CONSIDERATIONS

Ticks are the most versatile arthropod diseases vectors capable to transmit the broadest spectrum of pathogens comprising viruses, bacteria, protozoa, fungi and nematodes to their vertebrate hosts (Jongejan and Uilenberg, 2004). The tick-borne diseases, such as Lyme disease, tick-borne encephalitis, rickettsiosis (spotted fever), ehrlichiosis or human granulocytic anaplasmosis, are of great concern in human health and their serious threat discourage people from outdoor work or leisure activities. No less important are tick-transmitted zoonoses, such as anaplasmosis, babesiosis, theileriosis and African swine fever that cause substantial economic losses to the livestock production worldwide.

The success rate of pathogens transmitted by ticks is mainly given by the favorable aspects of tick physiology arising from their adaptation to the relatively long-lasting blood feeding. The modulation of host immune and inflammatory responses by various bioactive molecules present in the tick saliva (Francischetti et al., 2009) facilitates pathogen acquisition and transmission. Furthermore, the long-term persistence of ingested microbes in the midgut lumen is facilitated by the absence of extracellular digestive enzymatic apparatus, which is in ticks located inside the digestive vesicles of midgut cells (Sonenshine, 1991; Sojka et al., 2013). Nevertheless, ticks possess defense mechanisms that allow them to maintain the pathogens and commensal microbes at the level, which does not impair their fitness and further development. The long lasting co-evolution of ticks with pathogens resulted in the mutual tolerance, apparently adapted to the tick physiological differences (Mans, 2011). Therefore, the detailed knowledge of tick physiology and behavior is crucial to understand the fate of pathogens within the tick vector. For instance, the length of feeding, that strikingly differs between the hard and soft ticks (days vs. minutes, respectively), definitely shape the course of pathogen transmission. Pathogens transmitted by the hard ticks (*Ixodidae*) usually undergo several days of development until they infect the host. On the contrary, pathogens transmitted by the soft ticks (*Argasidae*) are ready for transmission immediately after the feeding starts. A good example here

is difference in the time of transmission between the *Borrelia* spirochetes causing Lyme disease (transmission several days after attachment) and relapsing fever (transmission several minutes after attachment) vectored by the hard and soft ticks, respectively (Sonenshine, 1991). Another important aspect that should be taken into consideration is the tick feeding strategy, the differences between one- and multi-host ticks in terms of transovarial and transstadial transmission.

The transmitting pathogen acquired from the infected host has to overcome several tissue barriers within the tick body comprising midgut, hemocoel and salivary glands or ovary (in case of transovarial transmission). Each of these compartments may play a decisive role in the tick vector competence for a certain microbe. The tick midgut is probably the most important tissue for survival and proliferation of the pathogens since many of them have to persist here until the molting and subsequent feeding. On their route from the midgut to the peripheral tissues, the pathogens are facing cellular and humoral defense mechanisms functioning within the tick hemolymph. Therefore, the abilities to cope with or avoid the tick immune responses are crucial for the pathogen transmission. During the last two decades, our knowledge about the invertebrate immunity has rapidly expanded, mainly given by the research on the model organisms such as fruit fly *Drosophila melanogaster* (Ferrandon et al., 2007), horseshoe crab, crayfish or ascidians (Iwanaga and Lee, 2005; Söderhäll, 2010). A substantial progress has been also made in the field of blood feeders, such as mosquitoes (Osta et al., 2004; Hillyer, 2010) and tsetse flies (Lehane et al., 2004). The information on the tick innate immunity is rather fragmentary and allows only approximate comparison with other invertebrates (Sonenshine and Hynes, 2008; Kopacek et al., 2010). Nevertheless, even these scattered data indicate that ticks possess defense mechanisms protecting them against microbial infection (**Figure 1**). At the cellular level, they comprise phagocytosis, encapsulation and nodulation of foreign elements. The humoral defense is based on a variety of pattern-recognition proteins and effector molecules such as lectins, complement-related molecules and a broad spectrum of common as well as specific antimicrobial peptides (AMPs) (Kopacek et al., 2010). In addition, possibly important but rather unexplored role in the tick defense system is played by the immune molecules of the host origin.

In this review, we will follow the transmission routes of pathogens and subsequently enumerate the potential obstacles they have to evade in the tick body. The general features of tick immunity will be further discussed in relation to our current knowledge of tick interaction with the three most intensively studied agents of tick-borne diseases, represented here by *Borrelia* spirochete, intracellular rickettsia *Anaplasma*, and malaria-like protozoa *Babesia*.

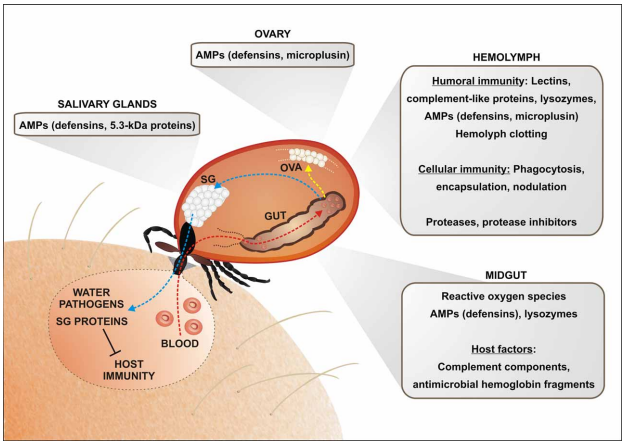

FIGURE 1 | An overview of the tick immune mechanisms and molecules constituting potential barriers for the pathogen transmission. The pathogen transmission is tightly linked with physiology of blood feeding and tick innate immunity. Ingested blood meal is accumulated in the midgut content (red arrow; only one caecum shown). Hemoglobin and other proteins are taken up by the tick midgut cells and digested intracellularly in the lysosome-like digestive vesicles (Sojka et al., 2013). Liberated amino acids and other compounds are transported to the peripheral tissues and ovaries, supplying mainly egg development (yellow arrow). Importantly, the blood meal is concentrated by reabsorption of excessive water, which is spitted back into the wound by the action of salivary glands (blue arrow). Tick saliva contains a great variety of anti-coagulant, immunomodulatory and anti-inflammatory molecules that facilitate pathogen acquisition and transmission. The ingested pathogens have to survive the period between detachment and subsequent feeding of the next tick developmental stage and overcome several obstacles on its route through the tick body. In the midgut, tick may utilize some of the host immune molecules (e.g., complement system) for its own defense against intestinal inhabitants. Hemoglobin fragments, derived from the host hemoglobin, are secreted into the midgut lumen and exert strong antimicrobial activity. Tick midgut tissue also expresses a variety of endogenous AMPs, which sustain the midgut microbes at a tolerable level. An important, but still poorly understood, role is most likely played by the maintenance of the redox homeostasis in the tick midgut. Pathogens intruding into the tick hemocoel can be phagocytosed by tick hemocytes or destroyed by effector molecules of the humoral defense system, comprising AMPs, components of the primordial complement system (thioester-containing proteins (TEPs), convertase-like factors and fibrinogen-related lectins (FREPs). Ticks probably possess a mechanism of hemolymph clotting, but genes/proteins putatively involved in the activation of prophenoloxidase cascade leading to melanization have not yet been identified in any tick species. Tick salivary glands express also a variety of AMPs, which may impair pathogen acquisition and persistence in the tick, as demonstrated for the 5.3-kDa antimicrobial peptides and their role in the defense against *Anaplasma* infection (Liu et al., 2012). Abbreviations: GUT, midgut; OVA, Ovary; SG, salivary glands.

TICK IMMUNE SYSTEM

TICK MIDGUT—THE PRIMARY SITE OF TICK-PATHOGEN INTERACTIONS

Although the midgut of arthropod disease vectors is most likely the principle organ that determines their vector competence, the general knowledge of the mutual interplay between ingested pathogen, commensal microflora and tick itself is still inadequate. Unlike in mosquitoes and other insect blood feeders, the microbes ingested by ticks are not in direct contact with digestive proteases secreted into the lumen and the highly nutritious broth of concentrated blood proteins, neutral pH and long-term storage present an ideal environment for microbial proliferation. Therefore, ticks have to possess efficient defense mechanisms which maintain the intestinal microflora at tolerable level.

Two recent high-throughput mapping projects of the microflora (microbiome) by the next generation sequencing were carried out in two tick species, *R. microplus* (Andreotti et al., 2011) and *Ixodes ricinus* (Carpi et al., 2011). These studies revealed an extreme diversity of the bacterial community (more than hundred different organisms identified in one tick species), which apparently reflects tick geographical and environmental origin as well as developmental stage. However, encounter with a microbe, which a tick hardly meets in nature, could have a fatal consequence because of the lack of effective defense. A good example is the artificial infection of soft tick, *Ornithodoros moubata*, with the Gram (−) bacterium, *Chryseobacterium indologenes* (Buresova et al., 2006), which resulted in rapid tick death. Although this soft tick secretes into the midgut lumen at least two kinds of antimicrobial compounds protecting against Gram (+) bacteria, lysozyme (Kopacek et al., 1999; Grunclova et al., 2003) and defensins (Nakajima et al., 2001, 2002), these molecules apparently fail to protect the ticks against some Gram (−) bacteria. Defensins have been also frequently reported to be expressed in the midgut tissues of hard ticks (Hynes et al., 2005; Rudenko et al., 2005; Zhou et al., 2007), but their secretion and antimicrobial activity in the midgut lumen has not yet been unambiguously demonstrated. A defensin-related molecule named longicin, expressed in the midgut of *Haemaphysalis longicornis*, was reported to be active against a variety of microbes including Gram (+) and Gram (−) bacteria, fungi and various *Babesia* species (Tsuji et al., 2007) (see also below).

A specific role of the midgut defense against Gram (+) and some fungi is played by the antimicrobial activity of large peptides derived from the host hemoglobin (hemocidins). The antibacterial hemoglobin fragments were initially isolated from the midgut contents of the cattle tick *R. microplus* (Fogaca et al., 1999) and later also identified in the midgut of other soft and hard tick species (Nakajima et al., 2003; Sonenshine et al., 2005). The generation of antimicrobial hemoglobin fragments most likely occurs in the digestive cells during the initial phase of hemoglobin digestion by the synergic action of cathepsin D-type and cathepsin L-type aspartic and cysteine peptidases, respectively (Horn et al., 2009; Cruz et al., 2010).

Hemoglobin digestion and the concomitant process of heme detoxification via hemosome formation (Lara et al., 2003) is necessarily associated with the maintenance of the redox homeostasis in the tick midgut. Although this process is virtually unknown in ticks, the paradigm to follow is the recent seminal finding on the importance of redox balance in the mosquito midgut epithelial immunity. In the malaria vector *Anopheles gambiae,* a tandem of heme peroxidase and dual oxidase (Duox) catalyzes formation of dityrosine network between the midgut epithelium and lumen. This network prevents delivery of the epithelial immunity elicitors and ultimately results in up-regulation of intestinal microflora and *Plasmodium* infection in the lumen (Kumar et al., 2010). Heme peroxidase and NADPH oxidase 5 (Nox5) were further shown to mediate the epithelial nitration of *Plasmodium* ookinetes and hereby their opsonization for subsequent lysis by the complement-like action of thioester-containing protein TEP1 (Oliveira Gde et al., 2012). The redox situation may also indirectly affect the pathogen transmission by changing its balance with other microflora present in the midgut. An example, how the midgut microflora determines the competence of *A. gambiae* and malaria parasites was reported recently, showing that ROS produced by the mosquito midgut dweller *Enterobacter* sp. interfere with *Plasmodium* development (Cirimotich et al., 2011). The interrelationship between the redox balance and intestinal microflora could be quite complex, as demonstrated using sugar vs. blood fed mosquitoes *Aedes aegypti* (Oliveira et al., 2011). The presence of heme in the mosquito diet caused a significant decrease of ROS levels, resulting in consequent expansion of midgut bacteria. This phenomenon was interpreted as a result of the mosquito adaptation against the high oxidative stress potentially caused by reaction of pro-oxidative heme with high levels of continuously produced ROS (Oliveira et al., 2011).

By contrast, very little is known about the maintenance of redox homeostasis in the tick midgut except for one report showing the role of catalase in the regulation of the oxidative stress in the cattle tick *R. microplus* (Citelli et al., 2007) and the seminal work on the heme-detoxification pathway described in the same species (Lara et al., 2003, 2005). Nevertheless, the genomic and transcriptomics data from other tick species suggest that ticks do maintain the redox homeostasis in their midguts as they possess ROS-generating enzymes, such as NOX5 or DUOX, and arsenal of antioxidant enzymes and radical scavengers comprising catalases, glutathione- and thioredoxin peroxidases, glutathione S-transferases, and selenoproteins (Anderson et al., 2008; Megy et al., 2012). Thus, the framework of redox balance and its direct or indirect impact on the persistence of pathogens in the tick midgut offers almost unlimited inspiration for the further research.

IMMUNE REACTIONS WITHIN THE TICK HEMOLYMPH

The major portion of our knowledge on the tick innate immunity is associated with cellular and humoral immune responses within the tick hemocoel. The volume of tick hemolymph increases linearly during the tick feeding from about 2–3 μl in unfed to almost 150 μl in fully engorged females, as demonstrated for *Dermacentor andersoni* (Kaufman and Phillips, 1973). At least three types of hemocytes, namely plasmatocytes, granulocytes I and granulocytes II, have been recognized in the hard and soft ticks, out of which the former two are phagocytic (Sonenshine, 1991; Borovickova and Hypsa, 2005). Several studies demonstrated the capability of hemocytes from different tick species to engulf foreign material and different microbes (Inoue et al., 2001; Loosova et al., 2001; Buresova et al., 2006). In addition, it was demonstrated that phagocytosis of microbes by the tick hemocytes is associated with humoral defense mechanisms, such as the production of ROS (Pereira et al., 2001) or complement-like molecules (Buresova et al., 2009, 2011). The process of hemocytic encapsulation of artificial implants, possibly linked with hemolymph coagulation and cellular response against *Escherichia coli* resembling nodulation, was reported to occur in the hemocoel of *Dermacentor variabilis* (Eggenberger et al., 1990; Ceraul et al., 2002).

Of special interest is the phagocytosis of tick-transmitted pathogens, such as *Borrelia* spirochetes, which seem to be

engulfed at least in part by the process of "coiling" phagocytosis (Rittig et al., 1996). A comparison of the phagocytic and borreliacidal activity against *B. burgdorferi* injected into the hemocoel of natural vector, *I. scapularis* and a refractory tick *D. variabilis*, revealed much stronger immune response against the spirochetes in the latter immunocompetent tick species (Johns et al., 2000, 2001a). On the other hand, it was recently shown that infection of *I. scapularis* hemocytes by *A. phagocytophilum* is mediated by the protein named P11 and is required for successful migration of the pathogen from the midgut to salivary glands (Liu et al., 2011), meaning that phagocytosis or engulfment of the pathogen by tick hemocytes does not necessarily cause its elimination. This rise an interesting question whether at least some of the tick-transmitted pathogens may take an advantage of being engulfed by tick hemocytes to hide from the attack of humoral immune responses in the hemocoel.

Effector molecules of several types have been described in the tick hemolymph, out of which reports on tick defensins are the most frequent since they have been identified in a number of hard and soft tick species (Chrudimska et al., 2010; Kopacek et al., 2010). Moreover, the recent analysis of *I. scapularis* genome revealed an extensive expansion of genes encoding for defensins and defensin-like peptides divided into two multi-gene families referred to as scapularisins and scasins, respectively (Wang and Zhu, 2011). Typical mature defensins are ∼4 kDa cationic peptides with a conserved pattern of six paired cysteins, derived by C-terminal cleavage after the furin (RVVR) motif from ∼8 kDa pre-prodefensin. Tick defensins are usually active against Gram (+) bacteria and their interactions with transmitted pathogens [except for the above mentioned longicin (Tsuji et al., 2007)] have not been yet unequivocally demonstrated. Varisin, a defensin isolated from the hemolymph of *D. variabilis*, exerted a borreliacidal effect in combination with lysozyme (but not alone), which may in part explain the incompetence of this species to sustain *B. burgdorferi* spirochetes (Johns et al., 2001b). Interestingly, depletion of varisin from the *D. variabilis* hemolymph using RNA interference resulted in the significant reduction of *Anaplasma marginale* infection, indicating that the impact of defense mechanisms on a certain pathogen might be quite complex and not always predictable (Kocan et al., 2008b, 2009).

In addition to defensins, ticks possess a specific class of histidine- and cysteine-rich antimicrobial peptides of size about 10 kDa, namely hebraein identified in *Amblyoma hebraeum* (Lai et al., 2004) and microplusin isolated from the hemolymph of *R. microplus* (Fogaca et al., 2004). Unlike defensins, which kill bacteria in a detergent-like manner by disruption of bacterial membranes, the bacteriostatic effect of microplusin is based on its capacity to sequester copper required mainly for bacterial respiration (Silva et al., 2009). Another cysteine-rich antimicrobial peptide, unrelated to microplusin and referred to as ixodidin, was isolated from *R. microplus* hemocytes and its antibacterial activity was proposed to be linked to the inhibitory activity against serine proteases by yet unknown mechanism (Fogaca et al., 2006).

The process of self/nonself recognition within the tick hemolymph is believed to involve the interaction of tick lectins and carbohydrates associated with the invading microbes (PAMPs or pathogen-associated molecular patterns). The activity of lectins/hemagglutinins with preferential binding specificity for N-acetyl-D-hexosamines, sialic acids and glycoconjugates have been identified in the hemolymph of several hard and soft tick species (Grubhoffer et al., 2008; Sterba et al., 2011) and is mainly attributed to the presence of fibrinogen-related proteins (FREPs) related to Dorin M, isolated and characterized from the soft tick *O. moubata* (Kovar et al., 2000; Rego et al., 2006). In contrast to mammalian ficolins, Dorin M lacks the N-terminal collagen-like domain and is closely related to the lectins of tachylectin-5 type known to function as pattern recognition molecules in the horseshoe crab immune system (Gokudan et al., 1999; Kawabata and Tsuda, 2002; Ng et al., 2007). The genomes of *I. scapularis* and *I. ricinus* contain genes encoding for a variety of FREPs named Ixoderins that can be phylogenetically divided into three major groups (Rego et al., 2005; Kopacek et al., 2010). Although the role of FREP family members in the invertebrate immunity may be multifunctional, as recently suggested for gastropod mollusk (Hanington and Zhang, 2011), we hypothesize that at least some tick FREPs play a role in activation of tick complement system, components of which have been identified in ticks (Kopacek et al., 2012).

Ticks are unique among other invertebrates in that they possess representatives of all major classes of thioester-containing proteins (TEP) known in vertebrates and arthropods: (1) molecules related to α2-macroglobulins, (2) C3-components of complement system and (3) insect TEPs and (4) macroglobulin complement-related proteins (MCR) (Buresova et al., 2006). The pan protease inhibitors of α_2-macroglobulin type were reported to be present in the hemolymph of soft and hard ticks (Kopacek et al., 2000; Saravanan et al., 2003; Buresova et al., 2009), where they presumably protect the ticks against undesired proteolytic attack of endogenous as well as exogenous proteases including those of invading microbes. The inhibition of metalloproteases secreted by the Gram (−) bacterium *C. indologenes* was shown to be functionally linked with phagocytosis of this bacteria by the tick hemocytes (Buresova et al., 2009). Further functional study of the tick TEPs suggested that phagocytosis of different bacteria by the tick hemocytes depends on non-redundant involvement of various tick TEPs with a central role of C3-like molecules (Buresova et al., 2011). Although nothing is known about interaction of the tick TEPs with tick-transmitted pathogens, the paradigm of *A. gambiae* TEP1 as a complement-like molecule, which determines the mosquito competence to *Plasmodium* parasites (Blandin et al., 2004, 2008), should stimulate further research in this area. In addition to the TEP family, genome of *I. scapularis* contains genes encoding for putative C3 convertases (Kopacek et al., 2012) having the multi-domain architecture similar to that of factor C2/Bf and LPS-sensitive Factor C activating the ancient complement-like system in the horseshoe crab (Zhu et al., 2005; Ariki et al., 2008). These preliminary results suggest that ticks possess features of a primitive complement system, which evolved on Earth at least one billion years ago (Nonaka and Kimura, 2006).

The existence of tick molecule related to the horseshoe crab Factor C, which primarily serves to trigger the limulus clotting cascade upon recognition of bacterial endotoxins (Kawabata,

2010), may suggest that ticks possess also a system for hemolymph coagulation. This suggestion was in part corroborated by the high throughput screening of immune-responsive genes in *D. variabilis* challenged with different bacteria. The most inducible immune gene found among others was transglutaminase, which acts as a crosslinking enzyme in the terminal phase of clotting mesh formation (Jaworski et al., 2010). However, with a possible exception of the previous observation, where a fibrous matrix was formed around the Epon-Araldite particles implanted under *D. variabilis* cuticle (Eggenberger et al., 1990), a convincing evidence of hemolymph clotting in ticks is still missing.

In contrast to other arthropods, ticks most likely lack the prophenoloxidase (PPO) activation system leading to melanization, because no PPO-related gene has been yet identified neither in the genome of *I. scapularis* (Megy et al., 2012) nor within the extensive EST datasets from other tick species (Kopacek et al., 2010).

IMMUNE REACTIONS WITHIN THE SALIVARY GLANDS

The tick salivary glands and components of tick saliva have been investigated foremost for their indispensable role in the modulation of host hemostasis, inflammation and immune response at the tick-host interface (Francischetti et al., 2009). The increasing number of salivary glands transcriptomes (sialomes) from the hard and soft ticks revealed the expression of a various AMPs, such as defensins, microplusin/hebraein and lysozymes, in this tissue (Mans et al., 2008; Karim et al., 2011). A defensin-like peptide named longicornisin was purified from the salivary glands of *H. longicornis* (Lu et al., 2010) and two different antimicrobial peptides unrelated to any known AMPs designated as Ixosin and Ixosin B were isolated from the salivary glands of *Ixodes sinensis* (Yu et al., 2006; Liu et al., 2008). However, it still has not been demonstrated whether these salivary glands AMPs are secreted into the tick saliva or hemolymph and if they directly interact with pathogens. The only exception is the 5.3-kDa antimicrobial protein, referred to as ISAMP and isolated from the saliva of *I. scapularis*, which exerts activity against Gram (−) and Gram (+) bacteria (Pichu et al., 2009). The transcripts encoding the family of secreted 5.3-kDa proteins were previously described to be significantly enriched in the transcriptome of *I. scapularis* nymphs infected with *B. burgdorferi* (Ribeiro et al., 2006). More recently, it was demonstrated that the 5.3-kDa family members were markedly upregulated in the salivary glands and hemocytes during *A. phagocytophilum* infection and were involved in the *I. scapularis* defense against this pathogen. Intriguingly, they were also shown to be effector molecules regulated by the JAK-STAT pathway (Liu et al., 2012) and although the *I. scapularis* genome contains also components of the putative Toll and Imd immune signaling pathways (Megy et al., 2012; Severo et al., 2013), the 5.3-kDa family regulation by JAK/STAT is the only so far described case of tick antimicrobial response controlled by a signaling pathway.

RNA INTERFERENCE—AN ANTIVIRAL DEFENSE IN TICKS

The RNA interference (RNAi) is an ancient mechanism evolved for the inhibition of foreign genetic elements and precise regulation of the endogenous genes during organism development (Myers and Ferrell, 2005). The RNAi seems to work very well in the tick tissues (De La Fuente et al., 2007b) and the genome of *I. scapularis* contains all components important for the endogenous and exogenous RNAi machinery including dicers, argonauts, dsRNA binding proteins, exonucleases and surprisingly also RNA-dependent RNA polymerases (Kurscheid et al., 2009). The discovery that plant viruses encoded suppressors of the gene silencing machinery provided a strong support for RNAi function as a natural defense mechanism against viruses (Lindbo et al., 1993; Ratcliff et al., 1999). It was shown that viral proteins identified as suppressors in plants and insect cells were able to abrogate RNA silencing also in the tick cells (Garcia et al., 2006). In the context of tick immunity, we can speculate that RNAi could interfere directly with the viral infection or regulate production of antimicrobial peptides through the expression of microRNAs.

TICK INTERACTIONS WITH TRANSMITTED PATHOGENS

BORRELIA

Lyme disease is an emerging human tick-borne disease of temperate climates with a concurrent distribution spanning North America and Eurasia. It is caused by *Borrelia* spirochetes related to *Treponema* and *Leptospira*, mainly by *Borrelia burgdorferi* sensu stricto in the US. and *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* in Europe (Radolf and Samuels, 2010). Borreliosis in humans affects multiple body systems, producing a range of potential symptoms (Burgdorfer et al., 1989). The classical sign of early infection is circular, expanding, skin rash at the tick bite site called *erythema migrans*. Treatment with antibiotics is effective at this stage of infection. When left untreated, the spirochetes disseminate throughout the body and are associated with arthritis (*B. burgdorferi* sensu stricto), neurological symptoms (*B. garinii*) or dermatitis (*B. afzelii*) (Stanek et al., 2012). Although Lyme disease is intensively studied, an effective vaccine is still not available and annual incidence in many countries continues leading over other human vector-borne diseases (Bacon et al., 2008).

Borrelia spirochetes survive in an enzootic cycle involving three-host *Ixodes* ticks and small animals like rodents, birds and lizards (Steere et al., 2004). The spirochetes are usually not detected in larger mammals, which are essential in the tick life cycle as a source of sufficient amount of blood for feeding females (adult female of *Ixodes* ticks can take in total about one milliliter of blood Balashov, 1972), but complement system of the vertebrate innate immunity lyses most of the bacteria (De Taeye et al., 2013). Humans are not able to efficiently kill the pathogens and often get infected. However, they are mostly dead-end hosts for both the ticks and the pathogens. Transovarial transmission of *Borrelia* in ticks is not likely (Rollend et al., 2013) and people can contract Lyme disease only by feeding of infected nymphs or adults, where nymphs play a key role in the epidemiology of disease because of their small size and relatively short feeding time.

The interplay between tick proteins, *Borrelia* spirochetes and hosts has been mapped by transcriptomics and proteomics studies (Narasimhan et al., 2007a), antibody-screening assays (Das et al., 2001) and yeast-surface displays (Schuijt et al., 2011b). Several tick genes have been identified as crucial for acquisition of the infection in ticks, *Borrelia* persistence in the midgut

and transmission into the next host during subsequent feeding (**Figure 2** and **Table 1**). *Borrelia* colonization of the tick midgut lumen and their persistence until the next feeding is crucial process for the successful transmission of the parasite. *Borrelia* outer surface protein A (OspA) (De Silva et al., 1996), which is expressed predominantly inside the tick vector, is essential for pathogen adherence to the midgut cells during the acquisition phase and plays a significant role in the pathogen persistence. During the subsequent feeding, OspA expression is suppressed, but upregulated expression of OspC facilitates invasion of the tick salivary glands and transmission to the new host (Schwan et al., 1995). Tick protein called TROSPA (tick receptor for OspA) has been found to be implicated in the binding of OspA (Pal et al., 2004). TROSPA is specifically expressed in the midgut and its mRNA levels increase following the spirochete infection and decrease in response to engorgement. Importantly, interference with TROSPA expression by RNAi or its saturation by TROSPA antisera reduces *Borrelia* adherence to the midgut surface, preventing pathogen colonization of the vector and reducing its transmission (Pal et al., 2004).

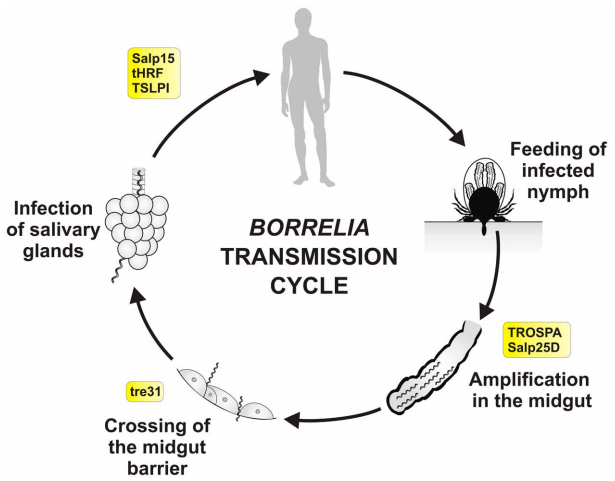

FIGURE 2 | Tick molecules involved in *Borrelia* transmission. Schematic diagram representing general stages of *B. burgdorferi* sensu lato infection and transmission in the *Ixodes* nymph, the most important developmental stage for human infection. Tick molecules, for which interference with *Borrelia* acquisition, persistence and/or transmission have been proved by genetic tests, are shown in rectangles (see text and the **Table 1** for their function and references). *Borrelia* spirochetes, that persisted in the tick midgut after the previous feeding (transstadial transmission), multiply rapidly within the newly engorged blood. They remain immobile and attached to the midgut cells. Around 53–72 h after the placement spirochetes become motile and swiftly cross the midgut barrier (between the cells), enter the hemolymph, salivary glands and via the saliva they infect new host (Ohnishi et al., 2001; Dunham-Ems et al., 2009). Transovarial transmission of *Borrelia* does not occur and newly hatched larvae are not infectious.

An antibody-screening assay performed on rabbit sera with acquired resistance to the tick bites after *I. scapularis* infestation identified salivary gland protein called Salp25D (Das et al., 2001). Salp25D encodes for a glutathione peroxidase, is upregulated upon feeding, and silencing of this gene by RNAi or immunization of mice with the recombinant protein impairs spirochete acquisition by ticks (Narasimhan et al., 2007b). Thus, Salp25D is most likely important for quenching the reactive oxygen species released from the activated neutrophils and hereby protects *Borrelia* during acquisition and colonization of the tick midgut.

During the tick feeding, *Borrelia* spirochetes, which multiplied previously in the midgut content, cross midgut barrier (between the cells) to get into the hemolymph and salivary glands (De Silva and Fikrig, 1995; Hojgaard et al., 2008; Dunham-Ems et al., 2009). *Borrelia* enolase, an enzyme found on the surface of spirochetes, was shown to bind host plasminogen and facilitate dissemination of *Borrelia* in the ticks and host (Coleman et al., 1995, 1997). In the later study, yeast surface display approach identified that *Borrelia* outer-surface lipoprotein BBE31 interacted with the tick protein called tre31 (Zhang et al., 2011). Expression of tre31 is induced in the midgut upon *Borrelia* infection and silencing of tre31 by RNAi or blocking of BBE31 using mice antibodies decreases spirochete burden in the hemolymph and salivary glands of feeding ticks.

It has been shown before that proteins contained in the tick saliva had strong pharmacological properties, targeting coagulation, platelet aggregation, vasoconstriction (Chmelar et al., 2012) and complement system (De Taeye et al., 2013). Salp15 is a salivary gland protein with remarkable immunosuppressive properties, which is bound by *Borrelia* OspC surface protein during host invasion and protects the spirochetes from antibody-mediated killing (Ramamoorthi et al., 2005). Its expression is upregulated in salivary glands upon *Borrelia* infection and silencing of Salp15 by RNAi dramatically reduces the capacity of spirochetes to infect mice. Moreover, antibodies raised against tick Salp15 protect

Table 1 | Tick molecules interfering with *Borrelia* acquisition, persistence and/or transmission.

Name	Supposed function	RNAi effect on the pathogen	References
Tick receptor for OspA (TROSPA)	Unknown	Reduced acquisition	Pal et al., 2004
Salivary protein 15 (Salp15)	Inhibition of the host complement system	Decreased transmission	Ramamoorthi et al., 2005
Salivary protein 25D (Salp25D)	Glutathione peroxidase	Decreased acquisition	Narasimhan et al., 2007b
Tick histamine release factor (tHFR)	Stimulation of histamine release	Decreased transmission	Dai et al., 2010
Tick receptor of BBE31 (tre31)	Unknown	Reduced persistence	Zhang et al., 2011
Tick salivary lectin pathway inhibitor (TSLPI, P8)	Inhibition of the host complement system	Decreased persistence and transmission	Schuijt et al., 2011a

mice from the infection (Dai et al., 2009). Tick salivary lectin pathway inhibitor TSLPI, previously identified by yeast surface display assay as P8 protein with ability to reduce complement killing of *Borrelia* (Schuijt et al., 2011b), interferes with lectin complement pathway, resulting in impaired neutrophil phagocytosis and chemotaxis (Schuijt et al., 2011a). Silencing of this protein by RNAi or exposure of ticks to TSLPI-immunized mice decreases persistence of *Borrelia* in nymphs and hampers their transmission, respectively. Tick histamine-release factor tHRF is a saliva protein able to bind host basophils and stimulate histamine release (Dai et al., 2010). This property can be exploited by *Borrelia* spirochetes for host infection. Expression of tHRF is upregulated in *Borrelia*-infected ticks and silencing of this gene by RNAi or tHRF blocking by antibodies reduce tick feeding and decrease spirochete burden in mice. The last molecule that should be mention is tick salivary protein named Salp20, which is an inhibitor of alternative complement pathway and partially protects serum sensitive species of *Borrelia* from lysis (Tyson et al., 2007) by displacing properdin from C3 convertase (Tyson et al., 2008). However, functional genetic studies are needed to prove its role *in vivo*.

ANAPLASMA

Anaplasmosis is considered as one of the most important vector-borne diseases of livestock (Kocan et al., 2010). The genus *Anaplasma* (Rickettsiales: Anaplasmataceae) includes six species of obligate intracellular bacteria, closely related to *Ehrlichia*, *Wohlbachia,* and *Neorickettsia*, occurring within the membrane-bound vacuoles called colonies in the host cytoplasm (Dumler et al., 2001; Kocan et al., 2008a). The *Anaplasma* rickettsiae preferably infect vertebrate red blood cells, however *A. phagocytophilum* attacks host neutrophils.

A. phagocytophilum infects a wide range of animals. It is responsible for the human granulocytic anaplasmosis (HGA), an emerging disease in the US, Europe and Asia, tick-borne fever in ruminants and equine and canine anaplasmosis (Woldehiwet, 2010). Three *Anaplasma* species exclusively infect ruminants: *A. marginale, A. centrale,* and *A. ovis*. *A. centrale* is used as life cattle vaccine in some regions, because infection with this parasite results only in mild clinical symptoms and could leave cattle persistently infected but immune against *A. marginale*, the causative agent of bovine anaplasmosis, which causes economic losses to the cattle industry worldwide. *A. ovis* is infective for sheep and wild ruminants, but infections are usually asymptomatic (Kocan et al., 2010). Also included in the genus *Anaplasma* are *A. bovis* and *A. platys*, which infect cattle and dogs, respectively.

All *Anaplasma* species are transmitted by Ixodid ticks, although tick transmissibility of *A. centrale* has been recently questioned (Shkap et al., 2009). The transmission cycle has been most extensively studied for *A. marginale* (Kocan et al., 1986, 1992a,b, 2008a). The developmental cycle in ticks is well coordinated with feeding and two *Anaplasma* morphotypes, reticulate (cell-dividing form) and dense core (infective form), can be found at each site of development (Kocan et al., 2010). Transovarial transmission of *Anaplasma* spp. from female ticks to their progeny does not occur. Therefore, ticks must acquire infection during blood feeding and the transmission cycles of these bacteria in nature are dependent upon the presence of infected reservoir hosts. Transmission by one-host ticks is probably accomplished by males, which can feed repeatedly and transfer between hosts (Sonenshine, 1991).

It has been shown that *Anaplasma* spp. modulate gene expression in ticks (De La Fuente et al., 2007a; Kocan et al., 2008a; Zivkovic et al., 2009; Sultana et al., 2010; Villar et al., 2010a,b), although differences may exist between species (Zivkovic et al., 2009). Functional studies of tick-*Anaplasma* interactions have shown how tick genes may affect bacterial infection (**Figure 3** and **Table 2**). Four differentially regulated genes/proteins, glutathione S-transferase (GST), salivary selenoprotein M (SelM), vATPase, and ubiquitin have been identified by suppression-subtractive hybridization and differential in-gel electrophoresis analyses using tick IDE8 cells infected with *A. marginale* (De La Fuente et al., 2007a). Glutathione S-transferases are intracellular enzymes with various functions, mostly accompanying cellular detoxification, but also signaling (Oakley, 2011). Selenoproteins are selenocysteine-containing proteins and important antioxidants (Reeves and Hoffmann, 2009). Vacuolar H^+ ATPases are membrane proteins acidifying a wide array of intracellular organelles by pumping protons across the plasma membranes (Nelson, 2003). Finally, ubiquitins are small regulatory proteins, involved

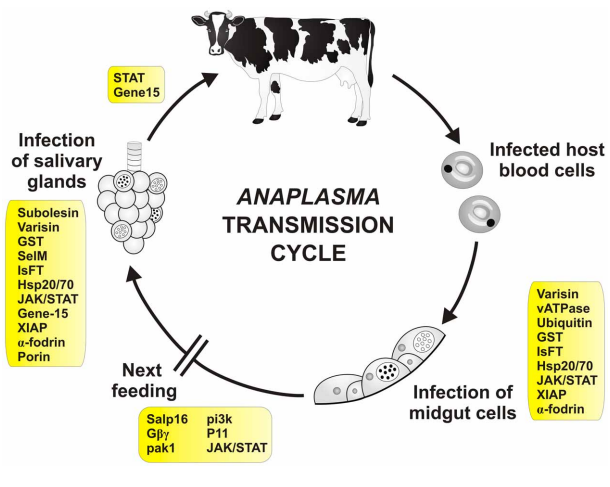

FIGURE 3 | Tick molecules involved in *Anaplasma* transmission. Schematic diagram representing general stages of *Anaplasma* infection and transmission (Kocan et al., 2008a). Tick genes, for which interference with *Anaplasma* acquisition and/or transmission have been proved by genetic tests, are shown in rectangles (see text and the **Table 2** for their function and references). Infected red blood cells (neutrophils for *A. phagocytophilum*) are engorged by the tick during blood meal. The released bacteria infect tick midgut cells and develop reticulate (cell-dividing; open circle) and dense core (infective; filled circle) forms of colonies inside the cells. During the next feeding, bacteria are released from the cells and infect other tissues including salivary glands. Here, they multiply inside the cells and are released into the saliva and transferred into the new host. Infection of tick hemocytes is required for the pathogen migration from the midgut to the salivary glands (Liu et al., 2011). Transovarial transmission of *Anaplasma* does not seem to occur. Transmission in one-host ticks is probably accompanied by tick males, which can feed repeatedly and transfer between hosts.

Table 2 | Tick molecules interfering with *Anaplasma* acquisition and/or transmission.

Name	Supposed function	RNAi effect on the pathogen	References
Salivary protein 16 (Salp16)	Unknown	Decreased acquisition	Sukumaran et al., 2006
Subolesin (SUB)	Component of the immune signaling pathways	Decreased acquisition	De La Fuente et al., 2006, 2008; Merino et al., 2011; Busby et al., 2012
Varisin	Defensin	Decreased acquisition	Kocan et al., 2008b
Vacuolar H^+ ATPase (vATPase)	Acidification of vesicles	Decreased acquisition	De La Fuente et al., 2007a; Kocan et al., 2009
Ubiquitin	Protein degradation	Decreased acquisition	De La Fuente et al., 2007a; Kocan et al., 2009
Glutathione S-transferase (GST)	Detoxification and signaling	Decreased acquisition	De La Fuente et al., 2007a; Kocan et al., 2009
Salivary selenoprotein M (SelM)	Protection against oxidative stress	Decreased acquisition	De La Fuente et al., 2007a; Kocan et al., 2009
α-1,3 fucosyltransferase (IsFT)	Glycosylation of proteins	Decreased acquisition	Pedra et al., 2010
G protein-coupled receptor Gβγ subunits (Gβγ)	Signal transduction	Decreased acquisition	Sultana et al., 2010
Phosphoinositide 3-kinase (pak1)	Cytoskeletal reorganization and signaling	Decreased acquisition	Sultana et al., 2010
p21-activated kinase (pi3k)	Cytoskeletal reorganization and signaling	Decreased acquisition	Sultana et al., 2010
Protein 11 (P11)	Unknown	Decreased acquisition	Liu et al., 2011
Heat-shock protein 20 (Hsp20)	Cellular stress response	Increased acquisition	Busby et al., 2012
Heat-shock protein 70 (Hsp70)	Cellular stress response	Decreased acquisition	Busby et al., 2012
Janus kinase (JAK)	Component of JAK/STAT signaling pathway	Increased acquisition	Liu et al., 2012
Signal transducer and activator of transcription (STAT)	Component of JAK/STAT signaling pathway	Increased acquisition and transmission	Liu et al., 2012
Gene-15	Antimicrobial peptide	Increased acquisition and transmission	Liu et al., 2012
X-linked inhibitor of apoptosis protein (XIAP)	E3 ubiquitin ligase	Increased acquisition	Severo et al., 2013
α-fodrin (CG8)	Spectrin α-chain	Decreased acquisition	Ayllón et al., 2013
Porin (T2)	Mitochondrial voltage-dependent anion-selective channel	Decreased acquisition	Ayllón et al., 2013

in an intracellular destruction and recycling of proteins in the proteasome, which is an important process also for the regulation of arthropod immune pathways (Ferrandon et al., 2007). Silencing of GST, vATPase or ubiquitin by RNAi decreases midgut *Anaplasma* acquisition in *D. variabilis* males fed on *A. marginale* infected cows, while silencing of GST or SelM decreases pathogen infection in the salivary glands of infected ticks fed on naïve sheep (De La Fuente et al., 2007a; Kocan et al., 2009). As previously mentioned, silencing of *D. variabilis* defensin named varisin that was shown to be expressed primarily in hemocytes, but also in midgut and other tissues (Hynes et al., 2008), decreased midgut pathogen acquisition in *D. variabilis* males fed on *A. marginale* infected cows and decreased infection in salivary glands of infected ticks fed on naïve sheep with obvious morphological abnormalities in bacterial colonies (Kocan et al., 2008b). Moreover, silencing of E3 ubiquitin ligase named x-linked inhibitor of apoptosis (XIAP) increases colonization of *I. scapularis* midgut cells and salivary glands by *A. phagocytophilum*, attracting even more attention to the ubiquitination process in ticks (Severo et al., 2013).

Fucosylation, which participates in many pathological processes in eukaryotes, has been shown to be modulated in ticks during *Anaplasma* infection (Pedra et al., 2010). *A. phagocytophilum* modulates expression of *I. scapularis* α-1,3 fucosyltransferase (IsFT) and uses α-1,3-fucosylation process to colonize the tick vector. Silencing of IsFT by RNAi reduces acquisition but not transmission of *A. phagocytophilum* in ticks.

The arthropod immune responses are generally regulated by Toll, Imd and JAK/STAT pathways (Ferrandon et al., 2007). Janus kinase (JAK)/signaling transducer activator of transcription (STAT) pathway has been shown to play a critical role in the tick defense against *Anaplasma* (Liu et al., 2012). Silencing of JAK/STAT genes by RNAi in *I. scapularis* causes burden of *A. phagocytophilum* in midgut, hemolymph and SG. The gene-15 of the salivary glands family encoding a member of 5.3-kDa antimicrobial peptide family is highly induced upon *Anaplasma* infection and regulated by JAK/STAT pathway. Silencing of gene-15 (and also STAT) by RNAi causes increased infection in salivary glands and transmission to the mammalian host.

Salivary protein 16 (Salp16) is an antigen recognized by tick-exposed host sera. Silencing of Salp16 by RNAi does not influence *A. phagocytophilum* acquisition in *I. scapularis* midgut, but the pathogen is not able to successfully infect the salivary glands (Sukumaran et al., 2006). Furthermore, expression of Salp16 in

the tick salivary glands is upregulated upon *Anaplasma* infection. It has been elegantly shown that Salp16 upregulation is not part of the tick defense mechanisms, but that *Anaplasma* selectively alter Salp16 expression for its benefit (Sultana et al., 2010). *A. phagocytophilum* infection induces actin phosphorylation, which is dependent on tick p21-activated kinase (ipak1)-mediated signaling. Activity of ipak1 is stimulated via G protein-coupled Gβγ receptor subunits (Gβγ), which in turn mediate phosphoinositide 3-kinase (pi3k) activation. In association with RNA polymerase II (RNAPII) and TATA box-binding protein, expression of Salp16 is selectively promoted. Silencing of ipak1, Gβγ or pi3k by RNAi reduces actin phosphorylation and *Anaplasma* acquisition by ticks (Sultana et al., 2010).

Recently, α-fodrin (spectrin α-chain) and mitochondrial porin (voltage-dependent anion-selective channel) were shown to be involved in *A. phagocytophilum* infection/multiplication and the tick cell response to infection in *I. scapularis* (Ayllón et al., 2013). The pathogen presence decreases expression of α-fodrin in the tick salivary glands and porin in both the midgut and salivary glands to inhibit apoptosis, subvert host cell defenses and increase infection. In the midgut, α-fodrin upregulation was used by the pathogen to increase infection due to cytoskeleton rearrangement that is required for pathogen infection. These results demonstrated that the pathogen uses similar strategies to establish infection in both vertebrate and invertebrate hosts.

After the initial infection of midgut cells, *Anaplasma* spread to other tick organs. However, the exact mechanism mediating migration to and infection of different tick organs is still not well known. Secreted *I. scapularis* protein 11 (P11), induced upon *A. phagocytophilum* infection, was shown to be important for *Anaplasma* migration from the midgut to the salivary glands, while being engulfed and hidden in the tick hemocytes (Liu et al., 2011). Silencing of P11 by RNAi or blocking the P11 with anti-sera or inhibition of hemocyte phagocytosis by injection of polystyrene beads into the tick hemolymph resulted in decreased *Anaplasma* infection of the tick salivary glands (Liu et al., 2011).

Tick subolesin (SUB), an ortholog of insect and vertebrate akirins, is possibly involved in several pathways, including innate immune responses, through a regulatory network involving cross-regulation between NF-κB (Relish) and SUB and SUB auto-regulation (Naranjo et al., 2013). SUB is down-regulated during *A. phagocytophilum* infection of tick nymphs, but up-regulated in female midguts and salivary glands infected with *A. marginale* or *A. phagocytophilum* (De La Fuente et al., 2006; Galindo et al., 2009; Zivkovic et al., 2010; Merino et al., 2011; Busby et al., 2012). Silencing of SUB by RNAi has strong effect on tick mortality and feeding and causes degeneration of midgut, salivary glands and reproductive organs (De La Fuente et al., 2008). After SUB knockdown, infection with *A. marginale* is significantly reduced in *D. variabilis* male salivary glands, but has only little effect on infection with *A. phagocytophilum* (De La Fuente et al., 2006; Ayllón et al., 2013). Subolesin has been used for vaccination against tick infestations and pathogen infection (De La Fuente et al., 2011). Although limited success has been obtained in this area, ongoing efforts are focused on the characterization of the *Anaplasma*-tick interface to develop vaccines for the control of tick infestations and pathogen transmission (De La Fuente, 2012).

BABESIA

Babesiosis is a tick-borne malaria-like disease affecting health of many animals and reducing cattle production in tropical and subtropical regions worldwide. Moreover, human babesiosis increasingly raises public health concern (Florin-Christensen and Schnittger, 2009). *Babesia*, the causative agent of babesiosis, is an apicomplexan parasite, which is together with *Theileria* referred to as piroplasm because of its pear-shape intra-erythrocytic stage. The genus *Babesia* constitutes paraphyletic group of parasites (described in various hosts with discrepancies in developmental cycles), which can be only distinguished by an appropriate molecular methods (Allsopp and Allsopp, 2006). They multiply in vertebrate erythrocytes (asexual stage) and cause severe symptoms related to their destruction. In the tick (hard tick), the parasite undergo sexual development in the midgut content, multiply in midgut cells and spread to different tissues including the salivary glands and ovary. Most of *Babesia*, unlike *Theileria*, are capable of transovarial transmission and newly hatched larvae are infectious to the hosts (Chauvin et al., 2009; Florin-Christensen and Schnittger, 2009).

It has been shown that infection of ticks with *Babesia* parasite pose negative effect on the tick development (Cen-Aguilar et al., 1998), thus ticks are supposed to evolve defense mechanisms to control *Babesia* infection and to regulate their mutual interaction. Although genomic sequences of *Babesia* and tick are available (Pagel Van Zee et al., 2007; Cornillot et al., 2012) and several projects have identified tick genes differently expressed upon *Babesia* infection (Rachinsky et al., 2007, 2008; Antunes et al., 2012; Heekin et al., 2012), only few tick genes have been shown to be directly implicated in the vector-pathogen interaction (**Figure 4** and **Table 3**). First of them, called longicin (Tsuji and Fujisaki, 2007), is defensin-like protein of *H. longicornis* exerting anti-microbial and anti-fungal activity. Recombinant longicin was reported to inhibit proliferation of *Babesia* (*Theileria*) *equi* merozoites in *in vitro* cultures and to reduce parasitemia of mice experimentally infected with *B. microti*. Moreover, silencing of this gene by RNAi increased number of *B. gibsoni* in the tick midgut content, ovary and laid eggs, pointing to longicin role in the regulation of *H. longicornis* vectorial capacity (Tsuji et al., 2007).

Longipain (Tsuji et al., 2008) is midgut-specific cysteine protease of *H. longicornis*, whose expression is upregulated upon blood feeding. Similarly as for longicin, recombinant protein inhibited proliferation of *Babesia* (*Theileria*) *equi* merozoites in *in-vitro* cultures and silencing of this gene by RNAi resulted in increased number of parasites in the midgut lumen, ovary, and hatched larvae. In general, inhibition of tick and parasite proteases is of interest as both the tick and the parasite genomes encode for several cysteine proteases important for blood digestion (Sojka et al., 2008) and host invasion (Florin-Christensen and Schnittger, 2009), respectivelly. Addition of various cysteine protease inhibitors into the *B. bovis* culture resulted in parasite growth inhibition (Okubo et al., 2007). The cysteine proteases inhibitor called cystatin-2 (Hlcyst2) from *H. longicornis* (Zhou et al., 2006) was overexpressed in midgut and hemocytes after *Babesia* infection. Recombinant HlCyst2 had slight effect on *B. bovis* growth in *in vitro* assays, but

its role in the tick infection has never been experimentally examined.

Three tick genes, namely TROSPA, serum amyloid A and calreticulin has been recently identified by cDNA screen as genes upregulated after the tick infection with *B. bigemina* (Antunes et al., 2012). TROSPA is a midgut receptor with unknown function, which is used by *Borrelia* spirochete as a docking protein for midgut colonization and spirochete persistence (Pal et al., 2004). Serum amyloid A is a homolog of vertebrate acute phase protein reacting to inflammation (Urieli-Shoval et al., 2000). Calreticulin is an intracellular protein with many functions, including calcium binding, protein folding and immune signaling (Wang et al., 2012). Involvement of these genes in *Babesia* infection has been confirmed by RNAi, where silencing significantly reduced *B. bigemina* numbers in *Rhipicephalus annulatus* and *R. microplus* (Antunes et al., 2012). Furthermore, RNAi silencing of subolesin (the previously mentioned ortholog of mammalian akirin) or vaccination with recombinant SUB strongly reduced acquisition of *B. bigemina* by *R. microplus* fed on an infected cattle (Merino et al., 2011).

Vitellogenin serves as a storage protein and source of amino acids during embryogenesis and its uptake is achieved by a specific vitellogenin receptor, which was identified from *H. longicornis* and shown by RNAi to be indispensable for egg development (Boldbaatar et al., 2008). Interestingly, *Babesia* DNA was not detected in eggs lays from ticks with silenced vitellogenin receptor previously fed on dogs infected with *B. gibsoni*. This suggests that impairing the vitellogenin uptake interrupt the parasite transovarial transmission.

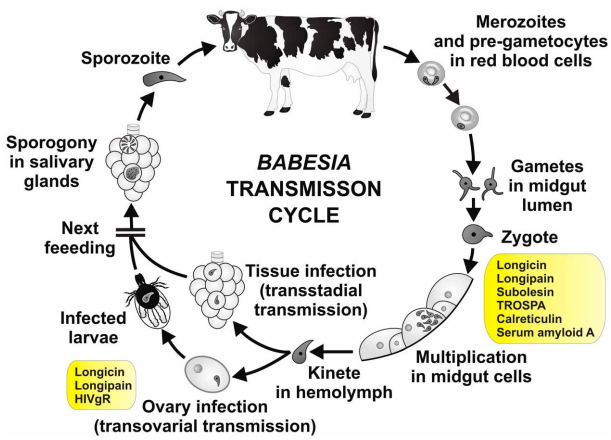

FIGURE 4 | Tick molecules involved in *Babesia* transmission. Schematic diagram representing general stages of *Babesia* infection and transmission (Zintl et al., 2003; Chauvin et al., 2009; Florin-Christensen and Schnittger, 2009). Tick genes, for which interference with *Babesia* acquisition and/or transmission have been proved by genetic tests, are shown in rectangles (see text and the **Table 3** for their function and references).
Pre-gametocytes in red blood cells, taken up within the blood meal, develop in the tick midgut content into matured gametocytes and gametes (ray bodies, Strahlenkörper) with distinctive spine-like projections. They fuse and give raise to the spherical spiked zygotes, which invade midgut cells. Inside the midgut cells, the zygotes transform, undergo meiosis and differentiate into motile prolonged kinetes (ookinetes). The kinetes escape the midgut cells, enter the hemolymph and invade other tick tissues, including ovary (transstadial and transovarial transmission, respectively). Here they undergo asexual reproduction and produce sporokinetes, which further spread the infection inside the tick or newly emerged larvae. During the next feeding, the kinetes that invaded salivary glands undergo a final cycle of multiplication (sporogony) to produce numerous sporozoites, host-invasive stages of the parasite. The sporozoites enter the tick saliva and infect host.

CONCLUSION—FUTURE PERSPECTIVES

The overall knowledge of tick innate immunity still lags far beyond the model invertebrate organisms and arthropod disease vectors. However, the availability of *I. scapularis* genome database (Megy et al., 2012), feasibility of functional genomics based on RNAi (De La Fuente et al., 2007b) and extensive number of tissue transcriptomes obtained from a variety of tick species promise to counterbalance experimental difficulties associated with tick handling and manipulation. Furthermore, introduction of the artificial membrane feeding (Krober and Guerin, 2007) extends our possibilities how to simulate the natural infections of ticks without the need of using laboratory animal models. These favorable conditions offer almost unlimited perspectives for the advanced research of the tick immune system and its impact on pathogen transmission. Among others, we can enumerate several high-priority topics, which can significantly aid to our understanding of the tick-pathogen relationship: (1) the role of epithelial immunity and maintenance of the redox balance for the pathogen persistence in the tick midgut; (2) interactions between the pathogens and commensal microflora; (3) tick antimicrobial peptides and their regulation via the Toll, Imd and JAK-STAT signaling pathways; (4) the role of tick primordial

Table 3 | Tick molecules interfering with *Babesia* acquisition and/or transmission.

Name	Supposed function	RNAi effect on the pathogen	References
Longicin	Defensin	Increased acquisition and transovarial transmission	Tsuji et al., 2007
Longipain	Cysteine protease	Increased acquisition and transovarial transmission	Tsuji et al., 2008
H. longicornis vitellogenin receptor (HIVgR)	Uptake of vitellogenin	Decreased transovarial transmission	Boldbaatar et al., 2008
Subolesin (SUB)	Component of the immune signaling pathways	Decreased acquisition	Merino et al., 2011
Tick receptor for OspA (TROSPA)	Unknown	Decreased acquisition	Antunes et al., 2012
Calreticulin	Protein folding and signaling	Decreased acquisition	Antunes et al., 2012
Serum amyloid A	Response to inflammation	Decreased acquisition	Antunes et al., 2012

complement system in the immune response against transmitted pathogens; (5) tick molecules involved in the pathogen acquisition, persistence or transmission as vaccine candidates; (6) the detailed description of the pathogen transmission cycles within the tick vector. Taken together, focused research in these areas can lead to the ultimate goal of efficient control of tick-borne diseases.

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Research on the ecology of ticks and tick-borne pathogens—methodological principles and caveats

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Interest in tick-transmitted pathogens has experienced an upsurge in the past few decades. Routine application of tools for the detection of fragments of foreign DNA in ticks, together with a high degree of interest in the quantification of disease risk for humans, has led to a marked increase in the number of reports on the eco-epidemiology of tick-borne diseases. However, procedural errors continue to accumulate in the scientific literature, resulting in misleading information. For example, unreliable identification of ticks and pathogens, erroneous interpretations of short-term field studies, and the hasty acceptance of some tick species as vectors have led to ambiguities regarding the vector role of these arthropods. In this review, we focus on the ecological features driving the life cycle of ticks and the resulting effects on the eco-epidemiology of tick-transmitted pathogens. We review the factors affecting field collections of ticks, and we describe the biologically and ecologically appropriate procedures for describing tick host-seeking activity and its correlation with environmental traits. We detail the climatic variables that have biological importance on ticks and explain how they should be properly measured and analyzed. We also provide evidence to critically reject the use of some environmental traits that are being increasingly reported as the drivers of the behavior of ticks. With the aim of standardization, we propose unambiguous definitions of the status of hosts and ticks regarding their ability to maintain and spread a given pathogen. We also describe laboratory procedures and standards for evaluating the vectorial capacity of a tick or the reservoir role of a host. This approach should provide a coherent framework for the reporting of research findings concerning ticks and tick-borne diseases.

Keywords: ticks, tick-transmitted pathogens, methodological gaps, protocols, interpretations

INTRODUCTION

Ticks are fascinating vectors of many pathogens affecting human and animal health, and research on this topic was boosted by the emergence of the zoonotic tick-borne disease, Lyme borreliosis, three decades ago (Ostfeld et al., 2005). The discovery of formerly unknown mechanisms of pathogen transmission, such as the non-viremic transmission of TBE virus (Labuda et al., 1993), and the re-emergence of certain tick-borne diseases, such as the ongoing epidemic of Crimean-Congo hemorrhagic fever in Turkey, likewise have created a wealth of research interest (Gale et al., 2010).

The adoption of DNA detection as a general tool during the 1990s to investigate the relationships between ticks, tick-transmitted pathogens and their vertebrate hosts further accelerated interest in the field. However, this has resulted in much misinterpretation of the vectorial capacity of ticks and the reservoir competence of vertebrates. A review of digital bibliographical databases for the period 2000–2010 revealed 512 papers dealing with ticks and transmitted pathogens in Europe alone (Estrada-Peña et al., 2013). Of these, 311 reported associations and relationships between tick and pathogens, at different scales (local, regional, or national). Further, 109 reports in that period dealt with newly determined associations between pathogens and ticks collected while feeding on a host. As noted by Kahl et al. (2002) "partially or fully-fed ticks removed from hosts that contain [pathogens] may or may not be vectors because nearly all hematophagous arthropods feeding on reservoir hosts are likely to ingest some microorganisms with the blood meal." Such reporting of crude associations between pathogens and engorged ticks has only served to confuse our understanding of the relationships between ticks, tick hosts and tick-transmitted pathogens.

A later surge in interest in ticks and tick-borne pathogens has been inspired by recent claims about the impact of forecasted climate change on the spatial distribution of ticks and associated pathogens (Brownstein et al., 2003; Ostfeld et al., 2005; Diuk-Wasser et al., 2006; Ogden et al., 2008; Jaenson et al., 2009). However, this research has been fraught with difficulty from the outset because of insufficient knowledge about the nature of many tick-pathogen associations (Randolph, 2009; Franke et al., 2013; Medlock et al., 2013). A proper understanding of how

abiotic factors shape the transmission cycles of tick-transmitted disease agents awaits a more rigorous analysis that is often limited by the current availability of data and the many indirect mechanisms that bear on them (Kahl et al., 2002; Eisen, 2008).

We assume that the many procedural and analytical errors in current tick and tick-borne zoonotic disease research are often a consequence of a lack of knowledge or of suitable training. This paper is not intended as an exhaustive review on tick biology and behavioral ecology, but a summary of existing knowledge regarding the processes involved in planning surveys, the collection of field data, and the relationships of ticks with abiotic variables. We have paid special attention to the variables regulating the activity of the ticks, and how these should be recorded and interpreted. Further, we discuss the need to standardize the eco-epidemiological terminology of ticks, hosts, and pathogens, as already discussed by Kahl et al. (2002) among others. We focus on exophilic ixodid ticks, which are those that develop on the ground, without needing to shelter in burrows or nests of their vertebrate hosts (such ticks are referred to as endophilic).

OVERVIEW

Figure 1 summarizes the flow of information and critical points in the collection of ticks, and the determinations and interpretations of relationships between ticks and microorganisms, which we will address in this review. The three key elements in this ecological system, namely the ticks, the hosts, and the tick-transmitted pathogens are impacted directly or indirectly by abiotic and biotic factors. We will review what specific combinations of abiotic factors activate ticks, and how to collect questing ticks to minimize sampling bias and maximize the robustness of the resultant data. We further review how to correlate weather variables with empirical data on ticks. At each stage of the surveys, accurate identification of ticks, their vertebrate hosts and associated microorganisms is critical, because errors would lead to reports of unusual associations of vertebrates, pathogens and ticks. Every tick specimen should be identified with reliable keys and compared with voucher reference specimens when necessary, before extracting their DNA for molecular analyses. In the same way, the lack of reliable identification of the hosts, or the erroneous detection of host DNA in the blood-meal remnants in the tick, might prevent accurate determination of the associations between the three partners. In the case of pathogen detection, polymerase chain reaction (PCR) and other molecular assays must be done in strict accordance with good laboratory practices (GLPs) and must include positive and negative controls with each run.

The importance of laboratory work is also shown in **Figure 1**. While the prevalence of a given pathogen may be calculated from field collections of unfed ticks, the vector status of the tick cannot be determined from such data alone. Additional vector and reservoir competence studies must be carried out in the laboratory before it can be concluded that a particular tick is a vector, or a host is a reservoir of a pathogen. We elaborate on these concepts in a later section of this review.

THE COLLECTION OF QUESTING TICKS

A critical initial step in studies on ticks and associated pathogens is the collection of the ticks, which, for the production of reliable results, should adhere to a series of principles. The abundance of ticks can be estimated either by surveying the hosts on which ticks feed or by sampling low vegetation or leaf-litter areas at defined intervals and periods of time. A rough estimation of the density of questing ticks can be obtained by sampling questing ticks from the vegetation and from the leaf litter (Tälleklint-Eisen and Lane, 2000b). Additionally, this approach can also be used to procure ticks for testing for zoonotic agents. Questing is the process in the life cycle of ticks in which they actively seek a host. The span and the intensity of the periods of questing,

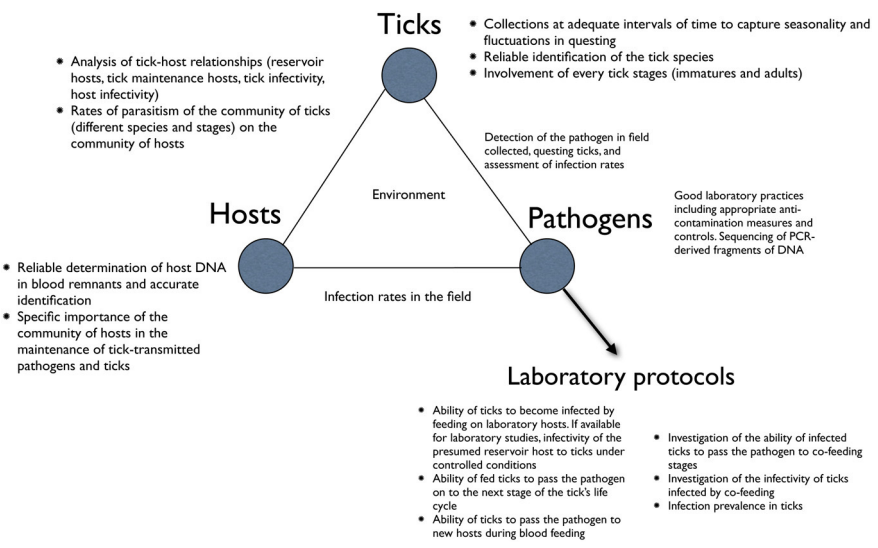

FIGURE 1 | A general description of the main points to address when studying the relationships between ticks, host and pathogens. The chart is aimed to pinpoint the key steps in these relationships and to outline the most common procedural errors detected in the scientific literature.

and even the simultaneous questing of different stages of the same species, may have profound epidemiological implications for the epidemiology of particular tick-transmitted pathogens (Randolph, 2000). By adequately recording the changing abundance of questing ticks, the response of a species to weather traits can be estimated, and their geographical or seasonal changes, tracked.

The common procedure for collections of questing ticks is by dragging a piece of fabric such as flannelette or blanket across the vegetation surface or over the leaf litter. These can either be large pieces of fabric dragged behind the operator, or more commonly are relatively small pieces (1 m^2) mounted on poles and brushed over the vegetation, a procedure known as "flagging." Many operators compromise between these two approaches. The ticks adhering to the fabric are collected at varying intervals, but it should be borne in mind that prolonged dragging distances result in a loss of ticks (see below). Flagging (or dragging) can provide an estimate of the rates of tick activity, which are characterized by several cycles of ascending and descending movements of the ticks in the vegetation. It is necessary to follow some basic rules in order to obtain an accurate representation of the questing activity, so that comparisons may be made with data from different sites, periods of time and from other studies, and to associate the observed questing rates with abiotic features (e.g., weather). Sampling must be conducted over homogeneous patches of vegetation, but a stratified sampling approach may be adopted to obtain a reliable representation of questing ticks in a given area. Vegetation cover affects the efficiency of the flagging or dragging method and heterogeneously modifies the micro-climate at the level where the ticks quest, thereby affecting their behavior and their observed abundance (Tälleklint-Eisen and Lane, 2000b). It also affects the abundance of hosts, and this may influence the perceived abundance of ticks, at the scale of small patches of vegetation. Tick abundance may vary significantly by aspect (exposure, such as south-facing) in hilly or mountainous areas (Lane et al., 1985).

To determine tick density (abundance per unit area) it is necessary to walk slowly over a defined distance. In some sites it is impossible to obtain sufficient uniformity for area-based dragging, and a time-based approach is indicated (e.g., number per hour) with adequate standardization. There are no rules regarding the overall length of a transect, but small ones (<100 m) may adversely affect the reliability of the results, since the spatial distribution of ticks is usually highly aggregated, even at sites where weather conditions and vegetation appear to be homogeneous (Wilson, 1998; Tälleklint-Eisen and Lane, 1999; Randolph, 2000). For each transect the operator must stop frequently enough (e.g., every 10 m) to remove ticks from the flag or drag otherwise too many ticks will be dislodged by the substrate. Sites with a high density of ticks demand more frequent removal of ticks from the fabric. The results should preferably be expressed as the number of ticks per unit area, but occasionally sites are too difficult to sample in this manner and in these cases a standardized time-based approach may be used (Gray et al., 1992). It also should be borne in mind that only a relatively small proportion of the total population of ticks in a given area may be questing simultaneously.

The questing activity of some ticks cannot be determined by dragging. For example, the immatures of some species of *Dermacentor,Rhipicephalus*, and *Hyalomma* are mainly endophilic, living inside the burrows of their hosts (Salman and Estrada-Peña, 2013). This also applies to certain species in the genus *Ixodes* that are nidicolous (Filippova, 1977; Lane et al., 1999). The only way to evaluate the seasonality of endophilic ticks is by direct sampling of the nesting materials or the burrows of their hosts or the primary hosts themselves (Vial, 2009). Moreover, the adults of the genera *Amblyomma* and *Hyalomma* are not collected homogeneously by dragging, because they have a "hunter" strategy to find a host. These ticks tend to shelter in litter areas to escape from desiccating microclimate conditions, and although they may be collected by dragging, this method is inefficient for such species. A survey should involve at least two or three consecutive years of sampling at relatively short intervals of time to be meaningful, preferable at 7–10 day intervals. Budgetary or other constraints may render this difficult, but relatively infrequent sampling (e.g., monthly intervals) fail to capture the seasonality of most tick species as well as the influence of abiotic factors on questing.

Quantitative terms regarding questing ticks have been reviewed previously (Wilson, 1994; Kahl et al., 2002). The *abundance* of questing ticks is a dimensionless measure, whereas *density* is a description of the number of ticks collected per unit area or time (Kahl et al., 2002). It is a mistake to interpret the number of questing ticks collected in field surveys as a measure of the total number of ticks in an area (Tälleklint-Eisen and Lane, 2000a,b).

Another method to obtain estimates of relative tick abundance is sampling from hosts. Ideally sentinel domestic animals such as sheep should be used in defined areas, but this is not often possible and most data sets consist of samples from domestic animals in relatively uncontrolled circumstances, from trapped rodents or birds and from wild ungulates at hunting points. Complete data sets are usually not possible because of the preferences of different tick stages for certain host species. Another drawback of this approach may be the fluctuations of host abundance, usually outside the control of the investigator that affect the intensity of tick infestations. However, compared with the sampling of ticks from the vegetation and leaf litter, data from animals are not so affected by short-term variables such as weather or from factors that can distort the data such as the structure of vegetation. When ticks are collected from hosts, the *prevalence of infestation* describes the percentage of hosts examined and found infested. The *mean density* of ticks per host is calculated as the total number of ticks obtained, divided by the total number of hosts examined. The *intensity of infestation* is the total number of ticks observed divided by the number of infested hosts (Kahl et al., 2002).

RELATING THE PHENOLOGY OF TICKS TO ENVIRONMENTAL (ABIOTIC) FEATURES

Tick researchers have been interested in discovering what abiotic factors drive the biology of ticks. The potential exists of evaluating seasonal patterns of activity resulting from various combinations of climatic conditions and in thereby obtaining an estimation of "risk" in a climate change scenario (Mannelli et al., 2003).

Many reports have correlated environmental traits with empirical data concerning the life cycle of several species of ticks. Results obtained so far confirm that the development and questing of *I. ricinus* (Lees and Milne, 1951; Gray, 1982, 1991; Perret et al., 2000, 2003, 2004; Randolph, 2002; Randolph et al., 2002; Estrada-Peña et al., 2004), *I. scapularis* (Daniels et al., 1996; Lindsay et al., 1999; Ogden et al., 2008), *I. pacificus* (Padgett and Lane, 2001), *A. americanum* (Schulze et al., 2001), *R. appendiculatus* (Randolph, 1993, 1997), *Hyalomma* spp. and *Rhipicephalus* spp. (Petney et al., 1987; Pegram and Banda, 1990), among others, are regulated by weather. It is, however, necessary to understand how the ticks become active before constructing guidelines for evaluating their patterns of activity.

Most ixodid ticks are inactive in the lowest layers of vegetation or in the leaf litter or soil before they begin to quest. A notable exception are the subadults of *I. scapularis*, which nocturnally can locate and attach to lizards sleeping in soil (Lane et al., 1995). The combination of a set of suitable conditions, which normally involves an activation temperature in the spring, triggers the activity causing the ticks to climb to the top of the vegetation to quest for hosts. During questing, ticks may lose water (Lees, 1946) that they normally regain by descending at intervals into the litter zone (Lees and Milne, 1951) where the ticks actively reabsorb water vapor from the atmosphere (Rudolph and Knülle, 1974; Kahl and Alidousti, 1997). After the ticks are rehydrated, they are ready to ascend the vegetation. Ticks vary in their ability to retain or to gain water (Kahl and Knülle, 1988) and there is an interspecific variability in the management of their water balance. Extrinsically, tick water balance is affected by the saturation deficit of water in the air (affecting water loss) and by relative humidity (affecting the possibility of water gain by active water vapor uptake), and, intrinsically, among other points, by the capability of ticks to find places with a favorable microclimate even when the weather is warm and dry, e.g., in the leaf litter. The energy reserves of the tick plus its ability to maintain an acceptable level of body water are the factors mainly regulating the short-term questing behavior of ticks. Host stimuli may also affect tick activity.

Tick questing activity in temperate regions follows a seasonal pattern with some short-term fluctuation largely depending on weather. Some species of ticks may have an almost uninterrupted process of parasitic and questing periods in tropical regions, because of the stable weather conditions (Belozerov and Kvitko, 1965). Besides weather or microclimate, photoperiod regulates tick seasonal periodicity by prevention (*behavioral diapause* in unfed ticks) or stimulation of questing activity in unfed specimens, or by interrupting development (*morphogenetic* or *developmental diapause* in engorged ticks). In both cases the inducing stimulus is the photoperiod, the day-night relative duration (or its change), which is perceived by ticks (Belozerov, 1982). These diapause mechanisms optimize the fate of a given population by enabling the ticks to find a host, to feed and to enter the molting period before the onset of adverse weather conditions such as winter. Although many studies and reviews have discussed how diapause determines the phenology of ticks (Gardiner and Gray, 1986; Pegram et al., 1988; Gray, 1991; Norval et al., 1991; Madder and Berkvens, 1997; Randolph, 1997), photoperiod is often ignored in studies on the seasonality of ticks.

Temperature is one of the main regulators of tick phenology. Only the temperature recorded between the litter and the height where ticks quest should be associated with the empirical data from field plots or dragging surveys. Other series of data, such as those recorded at stations used for climate recording, commonly placed at 2 m above the ground, are not suitable because they do not reflect the actual climate to which the ticks in the field site are exposed. There tends to be smaller variation of temperature and humidity at 2 m above the ground level than in the vegetation layer. The subtle variations of the microclimate experienced by the ticks will almost surely be lost in data series obtained from meteorological stations and possible correlations between weather and tick activity patterns are likely to be obscured. Ideally portable micro-loggers should be placed on the ground litter to record the weather at the sites where ticks develop and quest. A series of micro-loggers can also be placed at different heights above ground level to track changing conditions in microenvironments where the different tick stages quest.

Since parts of the seasonal tick activity pattern can be explained by responses to water loss, variables related to air humidity would probably show a regulatory action on the recorded phenology of questing ticks. It was demonstrated that the questing duration of nymphal *I. ricinus* is inversely related to the saturation deficit, whereas the duration of quiescence is not (Perret et al., 2003). The air water saturation deficit is the "drying power" of the air and is correlated with the losses of water by living tissues (Anderson, 1936). When environmental conditions are less desiccating, ticks will quest for longer periods. Abrupt declines in the proportion of questing ticks have been shown to coincide with abrupt increases in saturation deficit at field sites in Switzerland (Perret et al., 2000), the United Kingdom (Randolph et al., 2002) and Spain (Estrada-Peña et al., 2004). It is quite possible that other tick species exhibit similar behavior. It is important to note that relative humidity has a critical effect on the ability of ticks to absorb water. There is a critical equilibrium humidity above which ticks can actively absorb water and maintain their water balance (Knülle and Wharton, 1964) and survive for long time periods irrespective of the saturation deficit. Below this threshold of relative humidity, ticks cannot actively absorb water. Therefore, under conditions of low relative humidity, ticks cannot rehydrate even if the saturation deficit is low. However, under conditions of high relative humidity (e.g., >85% in *I. ricinus*) ticks can actively take up water from air and saturation deficit may have a lesser role in the regulation of questing. This is a complex process because temperature, saturation deficit and relative humidity change on a diurnal basis.

Saturation deficit and relative humidity are not the same as rainfall, which is the trait commonly used to explain the course of tick questing activity or the mortality of questing or developing ticks. Patterns of precipitation undoubtedly have an effect on the relative humidity at the regional scale (Rao et al., 1996; Thornton et al., 2000). However, the effects are not the same in different biomes. There is no universal relationship between air humidity (or saturation deficit) and rainfall patterns. It is likely that analysis of rainfall as the only effective mechanism for the mortality or activity of ticks will produce poor correlations and erroneous conclusions.

CONFOUNDING FACTORS AND PROCEDURAL GAPS IN STUDIES OF TICK PHENOLOGY

Multiple factors may affect the phenology of ticks, even if collections are carried out under a harmonized protocol as outlined above. Studies on the seasonality of ticks often correlate questing rates with the weather factors. However, an increase in questing rates may result from independent factors such as recruitment of newly molted ticks or resumption of activity during favorable weather conditions. A variable number of individuals may delay their onset of questing as a result of behavioral diapause (Gray, 1991), and older individuals may survive from previous periods of questing and overlap with later molted specimens. This could confound the correlation of the observed levels of activity with environmental factors. There is a lack of adequate studies regarding the effects of photoperiod on the induction of diapause and thus on tick seasonality (Gray, 2008). In fact, most recent research on seasonal activity has addressed only the presumed actions of either temperature or relative humidity.

One of the common ways to summarize tick questing activity is the clustering of observations by habitat type or plant alliances. The classification of the alliances of plant species into ecological categories is an old discipline that provides a homogeneous classification of the world biomes, which can then be correlated with weather traits and the characteristics of the soil (Menzel et al., 2006). Categories of vegetation are built on rules that summarize the dynamics of the plants and their response to the prevailing features of climate. These associations are in any case coarse in scale and the pattern emerges at national or continental scales. The association of the distribution patterns of ticks throughout the dominant biomes in large regions has been widely used to deal with otherwise confusing information about the distribution of ticks (Gilot et al., 1992, 1996). However, such correlations might give the false impression that the finely-tuned local processes of tick phenology are consistently correlated with simple categories of vegetation. Analyses of life cycle processes require linking the scales at which variation is measured to the scales at which the processes operate (Huston, 1999): The dominant vegetation influences occurrence, development and activity of ticks because it modulates the microclimate and because it affects the abundance of hosts. The actual driver of the processes is the microclimate, and the vegetation is only an indicator (Randolph and Storey, 1999). We advocate the description of patterns of presence and abundance of ticks as related to the vegetation only after understanding how *local* vegetation affects *local* microclimate and thus its indirect effects on the processes of tick life cycles. Otherwise, considerable information is lost in the process of clustering into categories of vegetation.

IDENTIFICATION OF TICKS

The importance of the correct identification of ticks is commonly underestimated. Without accurate identifications, every conclusion drawn from such material would be erroneous. Although ticks have attracted much interest in the past few years, this interest has not necessarily been accompanied by an adequate taxonomic expertise. The taxonomy of ticks is a rather complex topic, which is still the subject of debate. The identification of ticks is still mainly (if not only) based on the morphology of the specimens and on individual expertise, with the support of a variety of keys that describe the morphology of the stages and the differences between closely related species. Publications that cover every species in a particular area or country are seldom available, with a few notable exceptions (Filippova, 1977, 1997; Keirans and Clifford, 1978; Matthysse and Colbo, 1987; Durden and Keirans, 1996; Walker et al., 2000). A literature search on the epidemiology of tick-transmitted pathogens published in the past few decades suggested that few of the authors had taxonomic expertise, and there is usually no indication of how the ticks in their studies were identified. Contemporary researchers tend to use convenient, though not always relevant, reprints (available for example in their institute library or on the internet). This approach reveals a lack of criticism in a critical step of their research. It has become customary to identify only the adult stage of ticks, probably because it is easily collected from domestic hosts and easier to identify that the immatures. However, this provides an unreliable overview of the diversity of ticks present and their relative importance in the community of hosts and pathogens.

Adequate identification methods based on DNA or proteome analysis of ticks such as barcoding and mass spectrometry, are under development (Lv et al., 2013; Yssouf et al., 2013), but much work is still necessary to verify their significance. The complete genome has been sequenced for only *I. scapularis* (Pagel Van Zee et al., 2007), and the reliability of the sequences of other species deposited in GenBank is uncertain, as has proved to be the case for other taxa (Bridge et al., 2003; Wägele et al., 2011). Knowledge about the intra- and interspecific variation of the few molecular sequences so far examined is limited and therefore the significance of sequence variation is difficult to interpret. At present, identification of ticks based only on molecular data should be discouraged, and while a potential source for future research, sequences available in GenBank should not be considered as the "gold standard" for tick identification.

IDENTIFICATION OF TICK HOSTS BY DETERMINING THE SOURCE OF TICK BLOOD-MEALS

Another biotic component of these systems are the hosts. Several studies have addressed the importance of examining the complete community of hosts in a territory to estimate their relative contribution in supporting the various tick species present and the pathogens the ticks transmit (Levin and Fish, 1998; Ostfeld and Keesing, 2000; Logiudice et al., 2003; Tyre et al., 2003). In sylvatic habitats the accessibility of the hosts is an obvious problem and determination of their role in the maintenance of ticks and pathogens has traditionally required expensive and exhaustive trapping or shooting. However, several variations on a method to detect host DNA in the remnants of blood within questing ticks are now available. These consist of amplifying fragments of conserved genes such as *cytochrome b* (Kirstein and Gray, 1996); 18SrRNA (Pichon et al., 2003) and 12SrRNA (Humair et al., 2007), followed by reverse line blot (RLB) or sequence analysis. Some success has been achieved in determining the role different host species play in maintaining populations of *Ixodes ricinus* and in identifying possible reservoir hosts of *Borrelia burgdorferi* sensu lato (Estrada-Peña et al., 2005; Pichon et al., 2005; Moran Cadenas et al., 2007), but the method has

proved highly susceptible to contamination and sensitivity problems. While there is impetus for such an approach, at present it cannot be considered sufficiently robust for general deployment in field studies. Recently developed methods to identify the blood meal source in questing ticks using proteome profiling techniques (Wickramasekara et al., 2008; Onder et al., 2013) or natural isotope analysis (Schmidt et al., 2011) may be interesting alternatives.

EVALUATION AND STANDARD DENOMINATIONS OF TICKS-HOSTS-PATHOGENS RELATIONSHIPS

The interactions between the elements of any tick-host-pathogen system and interactions with other transmission cycles are key features to understand pathogen prevalence in questing ticks and in vertebrates. The evaluation of such interactions is a necessary preliminary (though complex) step in risk assessment. There are several reviews addressing the survival strategies of tick-transmitted pathogens, and how the ecology of ticks and their hosts may shape such prevalence (Mannelli et al., 2012), but these aspects are beyond the scope of this review. It is necessary to summarize a framework of relevant ecological terminology proposed by Kahl et al. (2002) that was largely based on terms introduced by Pavlovsky (1966), and continued by Balashov (1972), Ginsberg (1993); Nuttall and Labuda (1994); Sonenshine and Mather (1994), and Randolph and Craine (1995). Such a framework has been constructed to address major pitfalls in ecological research, identifying common errors and faulty interpretations occurring in the literature.

The eco-epidemiological status of ticks and their hosts cannot be determined by field studies alone. Complementary laboratory studies should be designed to capture the essentials of the role played by each element of the system. Some hypotheses can only be confirmed after tests have been conducted in the laboratory to remove the inherent variability of the field data. To designate a tick as a vector of a given pathogen, the three following criteria must be confirmed:

(1) Ability of ticks to become infected by feeding on the infected hosts. Whenever possible these studies should be carried out using uninfected ticks and natural, presumed reservoir hosts.
(2) The ability of fed ticks to trans-stadially pass the pathogen through the following molt to the next stage of the life cycle, or the F1 generation.
(3) The ability of molted stages to transmit the pathogen to naive hosts during blood feeding.

In summary, to qualify as a vector, the tick must (1) feed on infectious vertebrates (2) acquire the pathogen during the blood meal (3) maintain the pathogen through one or more trans-stadial molts and (4) transmit the pathogen to previously unexposed hosts while feeding again (Kahl et al., 2002). Assigning vector or reservoir status to a tick or a host species based solely on the detection of the DNA of the pathogen or evaluation of hosts by serology is not acceptable. Detection of pathogen DNA in a tick or host indicates that it possesses *carrier* status (see below for the definitions), and detection of antibodies in host sera merely indicates that an animal has been exposed to the pathogen. Whether or not transmission from tick to host or from host to tick is involved must be addressed experimentally in the laboratory.

Figure 2 outlines the steps in the chain of host-tick-pathogen relationships. Tick hosts (**Figure 2A**) include all vertebrates on

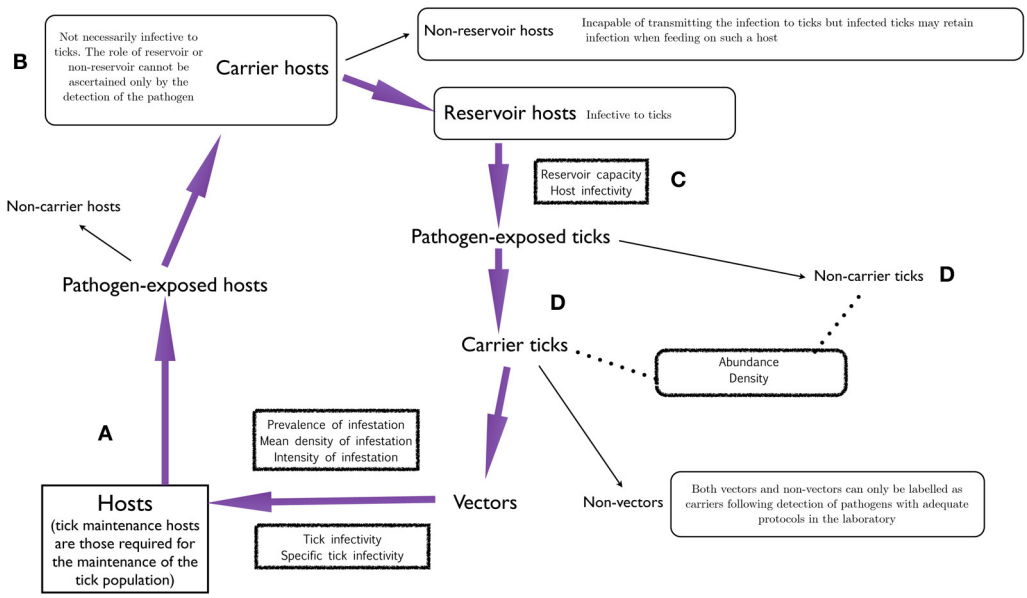

FIGURE 2 | A chart describing the chain of events in the interface of ticks, hosts, and pathogens, aimed to delineate a set of standard denominations following a common framework (Kahl et al., 2002). Hosts **(A)** may be exposed to pathogens and then be carrier hosts **(B)**. These may be reservoir hosts **(C)** that feed ticks exposed to pathogens. The ticks feeding on these reservoir hosts may be the carrier ticks **(D)** that are vectors of pathogens to new hosts.

which ticks feed in nature. Local and regional tick abundance largely depends on the abundance of *tick maintenance hosts* (sometimes called *amplifying hosts*, a term also applied occasionally to vertebrates that transmit pathogens and in which the pathogens replicate). The lack of sufficient numbers of hosts or even the absence of a key host in a particular area may prevent the occurrence of a species of tick even if weather and other abiotic features are permissive. When hosts are fed on by *infected vector ticks* they may be *pathogen-exposed* and these hosts may be *carriers* or *non-carriers*. The latter cannot infect naive ticks. A carrier host (**Figure 2B**) is not necessarily infective to ticks and therefore the terms *non-reservoir* and *reservoir* must be used to define infectivity status. The former are incapable of transmitting the infection to uninfected ticks. There are two important terms that apply to reservoir hosts and are relevant as to how ticks feeding on them are exposed to pathogens (**Figure 2C**). *Reservoir capacity* describes the absolute contribution made by a particular reservoir host species to the natural prevalence of infection by a given pathogen within a certain site. *Host infectivity* denotes the efficiency with which the host transmits the infection to ticks feeding on it. Reservoir capacity is dependent upon its infectivity for feeding ticks and the duration of the infective period (Mather et al., 1989; Mannelli et al., 1993; Randolph and Craine, 1995; Kahl et al., 2002).

By feeding on reservoir hosts, ticks are exposed to pathogens and they may become *non-carrier* or *carrier* ticks (**Figure 2D**). Carrier ticks may be *vectors* or *non-vectors*, as already discussed. The terms *tick infectivity* and *specific tick infectivity* refer to the efficiency with which the infection is transmitted from ticks to hosts and the efficiency with which the infection is transmitted from a given tick species to a particular species of host, respectively, and these features must be addressed in the laboratory.

MOLECULAR DIAGNOSTICS OF TICK-BORNE PATHOGENS

For all eco-epidemiological studies on tick-borne diseases, correct identification of the tick-borne pathogen(s) in question is pivotal. Examples of conventional methods for the identification of pathogens include cell culture, immunofluorescence and microscopic techniques (Kahl et al., 2002), but the detection of pathogens was revolutionized following the advent of the polymerase chain reaction (PCR) in the mid-1980s. The PCR made it possible to amplify minute amounts of (pathogen) DNA from biological samples such as ticks or blood for subsequent analysis by means of gel electrophoresis, restriction fragment length polymorphism (RFLP), sequencing, or hybridization probing techniques such as the RLB (Persing et al., 1990; Rijpkema et al., 1995; Richards, 2012). Quantification of the copy number of pathogen DNA initially present in tick or host samples became reality following the development of real-time PCR, in which the amount of product formed during the PCR reaction can be monitored (Pusterla et al., 1999).

In general, a well-designed PCR assay has a superior sensitivity and specificity vs. direct-detection methods, such as visual inspection of blood or tick hemolymph smears. It also offers the advantage over serological methods in that it can document current infection in ticks and animals and not simply past exposure to a pathogen, and does not require adaptation to different animal species, which is particularly useful in the examination of samples collected from wildlife. Unfortunately, the widespread use of PCR has led to a tendency to incriminate tick species as vectors based solely on the detection of pathogen DNA. Although the screening of ticks for pathogen DNA by PCR may be useful to identify potential tick vectors or reservoir vectors and to determine the tick-borne pathogens which circulate in a defined area, the findings must always be interpreted with great caution, as positive results may be caused by remnants of imbibed blood meals containing pathogen DNA, which does not necessarily implicate the tick as a vector (Kahl et al., 2002). This is further illustrated by experimental studies in which pathogen DNA was detected by PCR in non-vector species that had fed on infected animals as larvae, but were not capable of transmitting the pathogen *in vivo* (Des Vignes et al., 1999; Soares et al., 2006). To circumvent this problem, an alternative approach is to detect pathogens or pathogen DNA in the salivary glands of ticks. It should, however, be noted that certain pathogens such as *B. burgdorferi* s.l. are restricted primarily to the midgut of infected ticks and migrate to the salivary glands only during feeding (De Silva and Fikrig, 1995); pathogen DNA of *B. burgdorferi* s.l. is therefore unlikely to be found in the salivary glands of unfed ticks. For the tick-borne bacterium *Anaplasma centrale*, it has been demonstrated that colonization of the salivary glands does not prove that a tick can transmit the pathogen to a host; the salivary glands may act as a distinct barrier for the efficient pathogen transmission (Ueti et al., 2007). Hence it is necessary to perform transmission experiments to confirm the vector capacity of a given tick species.

Following collection and the correct identification of ticks, the first step in the laboratory is the extraction of nucleic acids. Collected ticks can be used directly for DNA extraction, stored in 70% ethanol or cryopreserved (Mtambo et al., 2006). Since PCR inhibitors can be present in engorged as well as in unfed ticks (Schwartz et al., 1997), DNA extraction methods which are proven to eliminate inhibitors from the purified DNA template should be used. Other methods to overcome PCR inhibition are reviewed elsewhere (Rådström et al., 2004). Ticks should be clean and free of debris such as plugs of cement prior to homogenization or dissection. Live or freshly killed ticks can be dissected to isolate tissues such as salivary glands for subsequent DNA extraction. Pictorial guides on commonly used methods to collect tick tissues, saliva or hemolymph were published recently (Edwards et al., 2009; Patton et al., 2012). When DNA is extracted from tick tissues, adequate cleaning of materials between processing individual samples and washing of tissues is required to rule out carry-over contamination between different ticks and tissues.

When designing PCR experiments, it is important to prevent the unspecific amplification of DNA. Since the nucleotide sequences of primers presented in scientific papers are not always accurate or may be outdated when novel nucleotide sequence information for related species has become available (Baker et al., 2003), it is advisable to check the specificity of the primers which are to be used. This can for instance be done using BLAST (http://blast.ncbi.nlm.nih.gov/). Similarly, the design of oligonucleotide

probes used for hybridization assays such as the RLB also requires critical appraisal and validation, to ensure their specificity and prevent cross-reactions that may lead to false-positive results (Nagore et al., 2004; Bhoora et al., 2009).

The high sensitivity of PCR and other PCR-based technologies such as loop-mediated isothermal amplification (LAMP) renders it prone to false-positive results due to exogenous contamination. Guidelines to avoid contamination by following the principles of GLP have been published and also include a strict spatial separation of pre-PCR and post-PCR activities, a unidirectional workflow, decontamination measures, and sufficient and adequate negative controls (Lo and Chan, 2006). An additional measure that can be taken to prevent carry-over contamination of PCR amplicons formed during previous PCRs is the use of dUTPs with Uracil DNA Glycosylase (UDG) which renders uracil-containing DNA from previous PCR reactions incapable of being copied by Taq DNA polymerases (Longo et al., 1990). The dUTP/UDG protocol is not compatible with proof-reading DNA polymerases. Read-out of results from certain LAMP tests can be done without opening the reaction tubes, which also reduces the chances of amplicon contamination (Francois et al., 2011).

Whatever method is used for post-PCR analysis, it remains important to use common sense when analyzing the data. Unconventional results in particular have to be verified, and visual inspection of blood smears is often essential, even in the post-genomic era.

MATHEMATICAL MODELS APPLIED TO CAPTURE THE NICHES OF TICKS FROM SURVEYS

Climate change and climatic variations within seasons are likely to influence the epidemiology of vector-borne diseases (Patz et al., 2003). There are reports on field observations of the spread of ticks in several areas of the world (Tälleklint and Jaenson, 1998; Daniel et al., 2003; Materna et al., 2005; Ogden et al., 2008; Jaenson and Lindgren, 2011; Madder et al., 2012). This has kindled interest in capturing the basic patterns of climate and other environmental features regulating the geographical ranges of ticks and their associated pathogens. Since this is a growing discipline in the field, we only outline some misconceptions that may affect the performance and conclusions drawn from modeling exercises.

The capture of data to estimate the direct and indirect effects of weather on the distribution, phenology or spread of ticks is commonly based on the idea of "climate" or "environmental" niche (Soberón and Nakamura, 2009). This is defined as the "intersection" of values of the climatic variables at which optimal development and mortality occur, resulting in the best performance of the population. This concept assumes that the most important factors driving the performance of ticks are related to weather and that niches can be reconstructed by relating data on the occurrence of the tick with datasets summarizing climate, topographic, edaphic, and other "abiotic" or "ecological" variables. It is also assumed that (1) there is a *niche conservatism* and the organism tracks the sites where adequate conditions exist (not taking into account adaptations of local populations to local weather) and (2) the complete distribution range of the organism has been surveyed. The tick's niche is evaluated to *infer* its associations with environmental variables. Inference is later *projected* into a target area to obtain a map that explains "how similar" the conditions in space are, compared with the ones where the tick has been collected. Such a measure of similarity is not an estimate of abundance or of distribution, although it is incorrectly assumed to be a "risk map," in which "climate similarity" is interpreted as a direct estimator of abundance. Many other variables, including the fine scale distribution of hosts, human factors altering the habitat, geographical barriers to host movements and the uncertain effects of the vegetation, influence the abundance of ticks (Estrada-Peña et al., 2012) and therefore the crude map is not a direct projection of the spatial risk.

Other procedural gaps in the evaluation of the environmental niche of a tick involve the inadequate utilization of interpolated climate datasets, which inflate the models because of autocorrelation (Legendre, 1993) and colinearity (Storch et al., 2003). Both factors strongly modify the apparent influence of variables that affect the distribution of an organism, resulting in the false perception of a well-fitted model. Determination of climate niche is also affected by the partial and subjective use of a limited number of collections of the tick to be modeled (see Porretta et al., 2013), by the inclusion of variables that are correlated with others to build the model, such as altitude and temperature (i.e., Diuk-Wasser et al., 2006), or by the selection of variables based on the predictive performance of the best model, but lacking a biological significance [as mentioned by Braunisch et al. (2013)]. The utilization of physical altitude or elevation in attempts to relate tick occurrence patterns to environmental variables is misconceived. It is obvious that, at a local scale, elevation greatly affects the patterns of climate, decreasing the temperature over the altitudinal gradients and influencing the rainfall. In every case the performance of the model will be inflated giving the false perception of a robust model, biasing the conclusions and the projections. Because of the structure of the algorithms explaining the distribution of an organism (Elith et al., 2010), the use of covariates such as rainfall will produce a false estimate of a good fit, apparently covering the known distribution of the tick. It will, however, be a fortuitous correlation, and the response of the distribution of the tick will not parallel changes in rainfall.

CONCLUSIONS

Increasing interest in tick-borne zoonotic agents, accompanied by the development of accurate and accessible molecular tools, has resulted in a marked upsurge of published papers on the topic over the past three decades. Although this has resulted in distinct progress in knowledge, some of the hard-learned lessons of the past have inevitably been overlooked during this period of intense activity. Typical problems include errors in tick identification (especially of immature instars), premature or erroneous reporting of ticks as new vectors and hosts as reservoirs, superficial data on the abundance and seasonal activity of ticks, inappropriate use of statistical methodology, and uncontrolled laboratory diagnostic procedures. The very ready accessibility of research publications online, at least in abstract form, tends to exacerbate the situation in that, as a result of superficial reading and subsequent citing, an erroneous conclusion can quickly become

embedded in the literature. In this review we have described what consensus opinion views as the correct methodologies for obtaining and interpreting data in research on ticks and tick-transmitted pathogens and have highlighted and illustrated some of the common procedural missteps, with suggested solutions where possible. It is hoped that this review will contribute to a more considered and thoughtful approach to this complex topic in the future.

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The role of rodents in the ecology of *Ixodes ricinus* and associated pathogens in Central and Eastern Europe

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Rodents comprise more species than any other mammal order. Most rodents are considered keystone species in their ecological communities, hence the survival of many other species in the ecosystem depend on them. From medical point of view, this is particularly important for rodent-dependent pathogens. In the particular case of tick-borne diseases, rodents are important as hosts for vector ticks and as reservoir hosts (Lyme borreliosis, human granulocytic anaplasmosis, Crimean-Congo hemorrhagic fever, Tick-borne relapsing fevers, tick-borne rickettsioses, babesiosis). Community and population ecology of rodents was shown to be correlated with disease ecology in the case of many tick-borne diseases. In Eastern Europe, several adult hard-tick species use rodents as their principal hosts: *Ixodes apronophorus*, *I. crenulatus*, *I. laguri*, *I. redikorzevi*, *I. trianguliceps*. However, the majority of ticks feeding on rodents are immature stages of ticks which as adults are parasitic on larger mammals. Larvae and nymphs of *Ixodes ricinus*, the most abundant and medically important tick from Europe, are commonly found on rodents. This is particularly important, as many rodents are synanthropic and, together with other micromammals and birds are often the only available natural hosts for ticks in urban environments. This work reviews the correlated ecology of rodents and *I. ricinus*.

Keywords: rodents, *Ixodes ricinus*, Europe, ecology, synchronous activity

BACKGROUND

Vector-borne diseases generally have a very complex ecology, and various factors are influencing their distribution, natural prevalence, seasonal, and multiannual dynamics. As compared to other hematophagous arthropods, hard-ticks have a particular biology, as they take only one, very large meal per life stage. As most hard-ticks follow a three-host life cycle this means that during a complete generation, each individual tick will feed on three individual hosts. Hence, the contact between a potential vector tick and a susceptible/reservoir host is limited to maximum three per generation. As a consequence, each acquired pathogen is transmitted to a subsequent host by the following developmental stage of the tick. Therefore, the prerequisite for vectorial competence in ixodid ticks is the transstadial maintenance and/or transovarial transmission of infection (Randolph, 2004).

From medical and social points of view, tick-borne pathogens are particularly important if they are able to infect humans. However, not all known tick-borne pathogens are recognized as human pathogens. This does not necessarily mean that they are not theoretically infectious to humans, but certain ecological factors are rather limiting the possibility of transmission to humans. Although in some areas of the world the number of tick species recorded on humans is relatively high (Estrada-Peña and Jongejan, 1999; Dantas-Torres et al., 2012), usually the vast majority of them belong to very few species. In South America, the most important human ticks are certain species of the genus *Amblyomma* (Dantas-Torres et al., 2012), in Turkey it is the genus *Hyalomma* (Bursali et al., 2010), while in Central and Eastern Europe the majority of ticks collected from humans are *Ixodes ricinus* (Briciu et al., 2011). In Central and Eastern Europe, *I. ricinus* is the dominant tick on humans, birds and rodents in forest environment (Briciu et al., 2011; Mihalca et al., 2012a,b,c). *I. ricinus* is the most generalist tick of Europe, with more than 300 host species reported (Anderson, 1991).

However, the importance of these hosts in the ecology of tick-borne diseases is variable and depends on certain factors: (i) the abundance of hosts during the activity peaks of ticks; (ii) the availability of the host for questing ticks; (iii) the possibility of an individual host to harbor simultaneously multiple developmental stages (i.e., larvae and nymphs); (iv) the capacity of the hosts to maintain and transmit the pathogens to ticks; (v) the capacity of the host to allow bridging of pathogens between ticks, both intraspecifically and interspecifically.

The density of questing *I. ricinus* is influenced by various factors, among which the local abundance of hosts is among the most important (Randolph et al., 2002). Additionally, the prevalence of infection with certain pathogens in ticks depends on the ecological pattern of tick infestation in reservoir hosts. Because of their abundance, proven role as reservoir hosts for important human pathogens and host for ticks, rodents represent a good epidemiological model for the study of disease ecology. Hence, our review will focus on the role of rodents in the ecology of *I. ricinus*. Population ecology and distribution of certain rodent species seems to be connected to the epidemiological situation

in humans, mainly in the case of certain tick-borne pathogens as *Borrelia burgdorferi* sensu lato or tick-borne encephalitis virus (TBE).

LARGE AND UNCOMMON OR SMALL AND ABUNDANT?

There are various direct and experimental evidences which show that larger hosts have higher level of infestation with macroparasites. These size-dependent differences in parasitism intensity can be found both between hosts from different species as well as between individuals form the same species (Randolph, 2004). The factors incriminated are physical, behavioral or immunological and act individually or synergistically (Randolph, 2004). Larger hosts (e.g., deer, fox), which are usually carrying many ticks, are generally found in much lower densities than rodents. On the other hand, even if intensity of tick parasitism on small rodents is much smaller, their abundance certainly compensates (Randolph, 2004). Hence, overall small and abundant rodents might account for hosting the majority of local tick population in any given time. Rodents in temperate areas show a cyclic population fluctuation, which has usually yearly cycles (in contrast to the multiyear boreal rodent cycles), with most species in Central European region having the population maxima in late summer/early autumn (Krebs, 2013). This peak in abundance overlaps with the density peak observed in many areas for subadult stages (especially nymphs) in Europe, of questing *I. ricinus* (Randolph et al., 2002). Moreover, this is a high mobility period of most rodent species, thus the hosts transport the resistant, but abundant developmental stage of the tick life-cycle. Population minima in most rodents occurs in early spring, a period overlapping with the highest abundance of adult stages, which do not require rodent host, but generally parasitize larger mammals (species lacking such yearly population fluctuations). In this way there is a synchronization in *I. ricinus* development and rodent population densities in temperate European forest rodents, making these later optimal hosts for this tick species.

Various rodents were reported as hosts for *I. ricinus* in Central and Eastern Europe. A comprehensive list of hosts include: *A. agrarius, A. flavicollis, A. sylvaticus, A. uralensis, Arvicola amphibius, Chionomys nivalis, Cricetus cricetus, Dryomys nitedula, Eliomys quercinus, Glis glis, Micromys minutus, Microtus agrestis, M. arvalis, M. subterraneus, M. tatricus, Mus musculus, Muscardinus avellanarius, Myodes glareolus, Rattus norvegicus, Sciurus vulgaris* (Nosek and Sixl, 1972; Matuschka et al., 1991; Mihalca et al., 2012b; Pérez et al., 2012).

However, several studies showed that certain rodent species are more commonly used as hosts by *I. ricinus*. In Germany, among the most important hosts for larvae and nymphs are *A. flavicollis*, *A. agrarius,* and *M. glareolus* (Matuschka et al., 1991). In France (L'Hostis et al., 1996) and Romania (Mihalca et al., 2012b) the main host for immature stages was *M. arvalis*. *A. uralensis* and *A. sylvaticus* were also reported to be important hosts for larvae and nymphs of *I. ricinus* in Romania (Mihalca et al., 2012b). On the other hand, although certain species of rodents are generally or locally abundant (*M. musculus*, *M. spicilegus*, *R. norvegicus*), they rarely harbor *I. ricinus*, and if they do so, the intensity is usually low (Paulauskas et al., 2009; Mihalca et al., 2012b). This pattern may be related to the differences in home ranges and activity patterns of these species, with *Mus* species tending to hold small home ranges in comparison to most *Apodemus* species (Krebs, 2013), while most *Microtus* voles have their daily activity peaks overlapping with the most active periods of questing ticks (Randolph et al., 2002).

While this correlation of the peak tick activity with the synchronous abundance of rodent populations is common, there are species-specific differences observed, which may be caused by differences in host ecology. Matuschka et al. (1991) showed that certain hosts (i.e., *A. agrarius*) tend to be more heavily parasitized by nymphal *I. ricinus* than others (i.e., *M. glareolus* or *A. flavicollis*) because of the synchronous host-tick abundance. Additionally, they also suggested that during the summer, when specific rodent abundance is locally low and the questing ticks are still active, they prefer to feed on lizards which might impair the transmission cycle of *B. burgdorferi* s.l. Pérez et al. (2012) noted a discrepancy between the seasonal activity patterns of questing ticks (measured by flagging) and the tick abundance on sympatric rodents.

Another important factor in the ecology of tick-borne pathogens is the correlation of peak activities of larvae and nymphs. Rodents are particularly important as some species are able to host both stages, synchronously. This is crucial for the natural maintenance of pathogens like TBEV, which produce non-systemic infections and it is maintained in the rodent host only for short time. Field studies have shown this in rodents which are able to host together larvae and nymphs of *I. ricinus* (Matuschka et al., 1991; Randolph et al., 1999; Mihalca et al., 2012b). This is uncommon in other host categories, like mesocarnivores (Széll et al., 2006) or large herbivores (Ruiz-Fons and Gilbert, 2010).

WHAT HAPPENS AFTER THE TICK DETACHES FROM A RODENT?

After feeding, each developmental stage of the tick detaches. Nevertheless, the duration of feeding and survival rates of detached ticks is dependent on various factors, and the host species is among the most important. Post larval bite acquired resistance to *I. ricinus* has been described in *M. glareolus* (Dizij and Kurtenbach, 1995). This results in reduced engorged weight and reduced survival in nymphs. The same study showed that post-bite immunity for *I. ricinus* was not present in *A. flavicollis*. Similarly, *A. sylvaticus* was shown to develop significantly lower acquired immunity to *I. trianguliceps* than laboratory mice (Randolph, 1979, 1994). Immunity of rodents to ticks is also dependent on the level of sexual hormones. In *M. glareolus* and *A. sylvaticus*, the individuals with high testosterone levels showed reduced innate and acquired resistance to feeding *I. ricinus* (Hughes and Randolph, 2001). However, a recent study found no difference between the tick burdens of different sexes of two commonly parasite rodent species, *A. sylvaticus* and *M. glareolus* (Kiffner et al., 2011). Moreover, several experimental studies showed that certain rodent species are more infective to ticks than others. Pérez et al. found that in the case of *B. burgdorferi* s.l., *M. glareolus* was more infective to ticks than *A. sylvaticus* (Pérez et al., 2012). When infectivity to *I. ricinus* was assessed for *B. afzelii*, *M. glareolus* was still more infective than *A. sylvaticus* and *A. flaviocollis* (Humair et al., 1999). When two species

of genus *Apodemus* (*A. flavicollis* and *A. sylvaticus*) were compared for their infectivity with *B. burgdorferi* s.l. to *I. ricinus*, no significant differences were found (Gern et al., 1994).

CONCLUDING REMARK

Rodents are one of the most important hosts for the maintenance of a number of *Ixodes* species in Central and Eastern Europe and crucial in shaping the population dynamics of *I. ricinus*. The spatial and temporal synchrony of temperate forest rodents and the parasitizing *I. ricinus* developmental stages, create optimal conditions not only for high abundance of the ticks, but for pathogen transmission for important diseases. This makes rodent species the key players in the yearly cycle of a number of important diseases, like Lyme borreliosis or TBE.

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