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Dermacentor reticulatus in Berlin/Brandenburg (Germany): Activity patterns and associated pathogens

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ABSTRACT

Dermacentor reticulatus is one of the most important European tick species. However, its spatial distribution, seasonality and regional vector role are not well known. This study aimed to gather information about abundance patterns of questing ticks and associated pathogens in unfed female adult *D. reticulatus* in the Berlin/Brandenburg area. Using the flagging method, questing ticks were collected at four sites in 2010–2012 and 2000 *D. reticulatus* were analysed regarding infection with *Rickettsia*, *Babesia*, *Borrelia* and Anaplasmataceae by conventional or real-time PCR. *Dermacentor reticulatus* showed a bimodal activity pattern: highest numbers of adult ticks were recorded between March and end of May (mean 50 ticks/h) and from mid-August until end of November (mean 102 ticks/h). During summer, almost complete inactivity was observed (mean 0.4 ticks/h). Sporadic samplings from December to February revealed tick activity also during winter (mean 47 ticks/h), which was characterised by large fluctuations. Using negative binomial regression analysis, significant influences of the variables sampling site, season and temperature on the abundance of questing *D. reticulatus* were determined. The parameters relative humidity and year were not of significant importance. PCR analyses showed an average prevalence of 64% for *Rickettsia* sp. Large differences in pathogen frequencies were observed between sampling sites (31.4–78.3%). Regression analysis demonstrated a significant influence of the sampling site but not of season and year. Examinations regarding other pathogen groups indicated prevalences of 0.25% (*Borrelia* sp.) and 0.05% (Anaplasmataceae) but absence of *Babesia* sp. Sequencing of positive samples revealed infections with *Rickettsia raoultii*, *Borrelia miyamotoi*, *Borrelia afzelii* and *Anaplasma phagocytophilum*. The study shows stable populations of *D. reticulatus* in Berlin/Brandenburg. People should be aware of ticks throughout the year since *Ixodes ricinus* is co-endemic and active in spring, summer and autumn while adult *D. reticulatus* are active throughout the year and even in winter during periods of frost as long as it is warming up during the day. Prevalence of *R. raoultii* in the present study is among the highest described for *D. reticulatus*. *Borrelia miyamotoi* was detected for the first time in *D. reticulatus*, illustrating the importance of screening studies to evaluate the pathogen structure in *D. reticulatus* populations.

1. Introduction

Ticks and tick-borne diseases are gaining increasing importance in the field of human and veterinary medicine worldwide (de la Fuente and Estrada-Peña, 2012). In Europe, the second most important tick species is the hard tick *Dermacentor reticulatus* (Karbowiak, 2014). Studies in many different European countries indicate an expansion of the geographic range of this tick species (Dautel et al., 2006; Mierzejewska et al., 2016; Paulauskas et al., 2015). The geographic range currently extends from northern parts of Portugal in the West to the region of Yenisei River (Siberia) in the East. Regarding the north-to-south-expansion in Europe, *D. reticulatus* has been observed between the latitudes 41 °N and 57 °N (Karbowiak, 2014; Rubel et al., 2016). However, in Germany the distribution of the tick is apparently still quite patchy (Dautel et al., 2006; Liebisch and Liebisch, 2007; Rubel et al., 2016; Zahler et al., 2000). In the Berlin/Brandenburg area, *D. reticulatus* populations have already been described (Dautel et al., 2006; Heile et al., 2006; Richter et al., 2012; Robert-Koch-Institut, 2009) and

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recent data have shown that the tick has a similar abundance on local dogs as *Ixodes ricinus*, the most abundant tick in Central Europe (Beck et al., 2014).

Dermacentor reticulatus has been found in moist areas such as river basins, alluvial forests and meadows, but also in clearings in mixed forests, coastal dune systems and heaths with sufficiently moist soil (Földvári et al., 2016). However, it can also occur in high numbers in quite dry areas (Hornok and Farkas, 2009; Pfäffle et al., 2015) indicating that *D. reticulatus* is able to adapt to very different habitats. Tick density was shown to be higher on undisturbed fallow land than in cultured areas (Mierzejewska et al., 2015a). On the other hand findings of *D. reticulatus* have also been described in parks and fallow land in urban areas (Hornok et al., 2014; Zahler et al., 2000).

Dermacentor reticulatus is a 3-host tick with each developmental stage showing a characteristic pattern of activity throughout the year. Adult ticks show a biphasic activity pattern and are present on the vegetation in spring and autumn (Bartosik et al., 2011; Duscher et al., 2013; Immler, 1973). Nevertheless, active adults sometimes can be observed also during winter (Buczek et al., 2014; Dautel et al., 2008). Juvenile stages primarily feed on small mammals. Therefore they are assumed to be endophilic, predominantly living in the burrows of their hosts (Immler, 1973). Highest activities of larvae and nymphs on rodents and mustelids are recorded in July and August, respectively (Paziewska et al., 2010; Pfäffle et al., 2015). In contrast, adult ticks particularly feed on larger mammals such as dogs but also horses and cervids. Moreover, infestations of cattle, wild boar, humans and other mammals have been described (Dautel et al., 2006; García-Pérez et al., 2016; Jongejan et al., 2015).

In some of these host species, serious diseases are caused by *D. reticulatus* transmitted pathogens. In Europe, vector competence has been proven for e.g. *Rickettsia raoultii*, *Rickettsia slovaca*, *Babesia canis*, *Babesia caballi*, *Theileria equi* and *Anaplasma marginale* (Földvári et al., 2016; Rubel et al., 2016).

The obligate intracellular bacteria *R. raoultii* and *R. slovaca* are important members of the spotted fever group rickettsia belonging to the order Rickettsiales (Sekeyova et al., 2013). They are distributed throughout Europe and parts of Asia and North Africa, causing SENLAT (scalp eschar and neck lymph adenopathy after tick bite) in humans (Parola et al., 2013). In south-western parts of Germany, autochthonous cases of the disease have already been described (Rieg et al., 2011). Average infection rates of up to 70.5% of host seeking *D. reticulatus* with *R. raoultii* illustrate the major importance of this species in Germany (Obiegala et al., 2017; Pluta et al., 2010; Silaghi et al., 2011). In contrast, no infections of *D. reticulatus* with *R. slovaca* have been described so far in Germany. However, in other European countries prevalences up to 9.3% confirm the presence of this pathogen in *D. reticulatus* (Hodžić et al., 2016; Spitalská et al., 2012).

Babesia and *Theileria* species are piroplasms infecting red blood cells (Irwin, 2009). *Dermacentor reticulatus* is considered as the main vector of *B. canis*, one of the most important agents of canine babesiosis. In Europe, severe cases of disease are associated with mortality rates of 1.520% (Matijatko et al., 2012). In Germany, autochthonous cases of babesiosis in dogs have been reported from almost all parts of the country, particularly from the South-West (Saarland, Bavaria, North Rhine-Westphalia) (Barutzki et al., 2007; Beelitz et al., 2012; Halos et al., 2014). In contrast, positive host seeking ticks were detected only in the Saarland, which is in the far most western part of Germany at the border to France where *B. canis* is endemic (Beelitz et al., 2012). Concerning the geographical occurrence in Europe, higher *B. canis* prevalence in *D. reticulatus* questing ticks was seen in south-eastern and eastern countries with prevalences up to 21.6% (Hornok et al., 2016; Karbowiak et al., 2014; Mihaljica et al., 2012; Wójcik-Fatla et al., 2012). In contrast, *B. canis* was rarely found in the north-west of Europe (Cochez et al., 2012; Jongejan et al., 2015; Silaghi et al., 2012a).

In addition to various other tick species (*Rhipicephalus* spp., *Hyalomma* spp.), *D. reticulatus* is also discussed as a vector of *B. caballi* and *T. equi*, the aetiological agents of equine piroplasmosis. The pathogens are distributed in (sub)tropical and temperate climate zones all over the world (Rothschild, 2013; Scoles and Ueti, 2015). In Europe, reports of seropositive horses predominantly originate from southern and eastern parts. Until now, only one case of autochthonous equine piroplasmosis has been described in Germany (Scheidemann et al., 2003). Presence of these pathogens in host seeking *D. reticulatus* has been reported in Spain, Belgium and the Netherlands with prevalences of 2.1% (*T. equi*) and 0.2–1.0% (*B. caballi*) (García-Sanmartín et al., 2008; Jongejan et al., 2015).

In addition, a variety of different pathogens (e.g. *Anaplasma* spp., *Borrelia* spp., *Rickettsia helvetica*, *Babesia microti*, *Babesia* cf. *microti*, *Babesia bigemina*, *Babesia divergens*, *Coxiella burnetii,* Bartonella spp., *Francisella tularensis* and *Francisella philomiragia*) were detected in *D. reticulatus* during studies conducted in Europe and Asia. However, vector competence for most of these pathogens has not been proven (Földvári et al., 2016; Hodžić et al., 2017; Rubel et al., 2016).

Borrelia sp. are gram-negative bacteria of the order Spirochaetales. Further taxonomy is still discussed at the moment (Adeolu and Gupta, 2014; Barbour et al., 2017; Margos et al., 2017; Casjens et al., 2010). Regardless of the point of view, both, the Lyme disease *Borrelia* (*Borrelia burgdorferi* sensu lato complex) and the relapsing fever *Borrelia* species are important groups within this order. Species of the *B. burgdorferi* sensu lato complex are the aetiological agents of Lyme disease, the most common infectious tick-borne disease in humans and animals in the northern hemisphere and are primarily transmitted by *Ixodes* spp. (Margos et al., 2011). Worldwide-distributed relapsing fever group Borrelia are of great medical importance in Africa, while in Europe only sporadic clinical cases have been described. Depending on the genospecies, lice and ticks (*Ornithodoros* and *Ixodes* spp.) are considered as vectors (Cutler, 2010; Siński et al., 2016). Genospecies found in *D. reticulatus* so far exclusively belong to the *B. burgdorferi* sensu lato complex (Földvári et al., 2016; Rubel et al., 2016). Studies, which carried out sequencing of positive samples, predominantly detected *Borrelia afzelii* and *Borrelia garinii* (Mierzejewska et al., 2015b; Nijhof et al., 2007; Rar et al., 2005). Even though infection rates between 0.009 and up to 11% have been recorded in *D. reticulatus* in Germany and other European countries, vector competence of this tick species has not been finally clarified yet (Bonnet et al., 2013; Kahl et al., 1992; Mierzejewska et al., 2015b; Nijhof et al., 2007; Robert-Koch-Institut, 2009).

The family Anaplasmataceae also belongs to the order Rickettsiales and contains important genera of pathogens such as *Anaplasma*, *Ehrlichia* and *Candidatus* Neoehrlichia (Dumler et al., 2001; Silaghi et al., 2016). Considering infections in questing *D. reticulatus*, *Anaplasma phagocytophilum* and *A. marginale* have sporadically been described in Europe (Bonnet et al., 2013; Paulauskas et al., 2012; Zając et al., 2017). *Anaplasma phagocytophilum* is of particular medical importance as the causative agent of granulocytic anaplasmosis in humans, dogs and horses and of tick-borne fever in ruminants (Dugat et al., 2015). Clinical infections caused by *A. marginale* are restricted to ruminants (bovine anaplasmosis) leading to great economic losses in cattle production (Aubry and Geale, 2011). In Germany, no case of Anaplasma infection in *D. reticulatus* has been detected so far. *Anaplasma phagocytophilum* is usually transmitted by *Ixodes* spp. In Europe, *I. ricinus* is the main vector (Atif, 2015). Due to partially high prevalence (25%) in *D. reticulatus* in Europe, further experiments are required to understand the epidemiological significance of the tick in the transmission cycle of *A. phagocytophilum* (Bonnet et al., 2013; Karbowiak et al., 2014). In contrast to that, Zivkovic et al. (2007) have experimentally proven the transmission of *A. marginale* by *D. reticulatus*, considering this tick species as a further potential vector in addition to different *Hyalomma*, *Haemaphysalis* and *Rhipicephalus* species.

Regarding the genera *Ehrlichia* and *Ca*. Neoehrlichia, *Ehrlichia canis* and *Ca*. Neoehrlichia mikurensis are members of major importance in Europe (Rar and Golovljova, 2011). Cases of disease induced by the recently discovered species *Ca*. Neoehrlichia mikurensis have already

been observed in humans and dogs in Germany, Sweden, Switzerland and the Czech Republic (Andréasson et al., 2015; Diniz et al., 2011; Fehr et al., 2010; Grankvist et al., 2014; Maurer et al., 2013; Pekova et al., 2011; Richter and Matuschka, 2012; von Loewenich et al., 2010; Welinder-Olsson et al., 2010). However, infections in questing *D. reticulatus* seem to be rare compared to the suspected European vector *I. ricinus* (Krücken et al., 2013; Silaghi et al., 2012b). Only a few studies on prevalence regarding *E. canis* infections in *D. reticulatus* have been reported and no cases of *E. canis* have been described in host seeking *D. reticulatus* in the Netherlands, Austria and in the Asian part of Russia (Duscher et al., 2016; Nijhof et al., 2007; Shpynov et al., 2006).

To assess the risk of vector-borne diseases in humans and animals, extensive investigations considering the epidemiology of pathogens and their vectors are needed. In Germany, only a few studies analysing tick transmitted pathogens in *D. reticulatus* have been conducted (Pluta et al., 2010; Richter et al., 2012; Silaghi et al., 2011, 2012a). Since *D. reticulatus* has vastly expanded its geographic range in Germany (Dautel et al., 2006) and is in the Berlin area apparently as abundant on dogs as *I. ricinus* (Beck et al., 2014), its importance for veterinary public health has presumably increased a lot in recent years. The objective of this study was to extend information about the seasonal activity pattern of *D. reticulatus* throughout the year. Furthermore, PCR analyses for the detection of a variety of tick-borne pathogens (*Rickettsia* sp., *Babesia* sp., *Borrelia* sp., *Anaplasma* sp., *Ehrlichia* sp., *Neoehrlichia* sp.) were carried out to identify prevalences in *D. reticulatus* in the Berlin/Brandenburg area in Germany. Furthermore, multivariate regression analyses were conducted to identify potential factors influencing first the number of collected questing ticks to estimate tick activity patterns and second pathogen prevalence.

2. Material and methods

2.1. Tick sampling

Four sub-urban sampling sites (Güterfelde N 52° 21.019′, E 13° 11.621′, Gatow N 52° 28.685′, E 13° 8.119′, Falkenberg N 52° 34.716′, E 13° 33.421′, Königs Wusterhausen (K. Wusterhausen) N 52° 17.767′, E 13° 33.878′) were selected around the city of Berlin (Fig. S1). Due to the uncultivated land and almost complete absence of trees, all sampling sites are preferred habitats of *D. reticulatus* and preliminary visits before the study period had confirmed this. Three of the sites were former wastewater farms with a grass/herbage vegetation that was mowed once per year in Güterfelde and K. Wusterhausen but located in a conservation area and thus not cut in Falkenberg. In contrast, the site in Gatow consisted of an area of fallow land (no mowing) previously used for military purposes with heath as the predominant vegetation. All study sites were sampled biweekly from March to November 2010 and January to November 2011 to determine the presence of the ornate/meadow tick *D. reticulatus*. To detect any potential residual tick activity during winter months, one of the study sites (Güterfelde) was additionally examined between December 2011 and April 2012 when the vegetation was not completely covered with snow and air temperatures were not below 0 °C. Questing ticks were captured by dragging a 1.20 m × 0.50 m cotton cloth through the vegetation (flagging method). Every 10–20 steps, attached ticks of any species were collected from the cloth and transferred to a 50 ml falcon tube. All tick samplings were conducted by the same person. In order to avoid depletion of ticks by repeatedly sampling of large, continuous areas, it was decided to sample along narrow tracks. This made it difficult to define exact areas of the same size for all study sites and therefore a time-based approach was chosen. Tick samplings were usually performed for 1 h. Despite the fact that samplings were conducted time-based, it was aimed to always sample the same track and keep walking speed and frequency of tick collection from the cloth as constant as possible. Samplings were conducted either during early morning and late evening hours (summer) or in the early afternoon (winter, autumn and spring). At each sampling date, the temperature und relative humidity were recorded once at a height of approximately 20 cm above the ground by a portable weather meter (PCE-222, PCE Deutschland GmbH, Meschede, Germany).

Using taxonomic keys (Babos, 1964), all collected ticks were identified according to their species, sex and developmental stage under a stereomicroscope. Ticks were transferred into individual 1.5 ml reaction tubes and stored at −80 °C.

2.2. DNA extraction

A subset of 1000 adult female *D. reticulatus* per year, collected in 2010 and 2011, was screened for the presence of vector-borne pathogens. It was aimed to investigate 125 *D. reticulatus* per sampling site and per half of a year. For each sampling day, a number of ticks proportional to the number of ticks collected on that day in comparison to the same site over the whole half a year was randomly chosen: Number analysed at day_x,$site_y$ = 125 × (ticks collected at day_x,$site_y$/ticks collected in the half year at $site_y$). For DNA extraction, ticks were initially homogenised with sterile pestles. DNA of ticks collected in the year 2010 was isolated with the NucleoSpin® 8 Blood Kit according to the manufacturer's instructions using the vacuum system (NucleoVac 96 Vacuum Manifold, Macherey & Nagel, Düren, Germany). DNA was eluted with 70 µl buffer BE. DNA extraction of ticks captured in 2011 was performed with the Maxwell® 16 LEV Blood DNA Kit using the Maxwell® 16 Research Instrument System (Promega, Madison, USA) and eluted in 50 µl elution buffer. DNA samples were stored at −20 °C until further use.

2.3. PCR amplification

Specific conventional or real-time PCR assays were applied for the detection of DNA of the different pathogens. Depending on the pathogen group, *D. reticulatus* were analysed individually (*Borrelia* sp., *Rickettsia* sp.) or in pooled samples, obtained by mixing equal amounts of DNA from five ticks (*Babesia* sp., *Anaplasma* sp., *Ehrlichia* sp.). DNA from pools tested positive were then reanalysed individually. In each PCR run, a negative control (nuclease-free water) and a positive control (plasmid DNA containing the PCR target as insert) were run in parallel. All PCR reactions were carried out in thermocyclers (ICyclerThermal Cycler, CFX96™Real-Time System, Biorad, Munich, Germany). Products of conventional PCR were analysed on agarose gels stained with GRGreen (Labgene, Switzerland) (*Borrelia* sp. 2.5% agarose; all other pathogens 1.5%), purified and sequenced.

For the detection of *Babesia* sp., primers amplifying the ITS-1 region of the gene encoding rRNAs, were designed using the Software Clone Manager Professional 9. They show a complete sequence identity to the following *Babesia* species: *B. canis* (GenBank accession no. AF394533.1), *Babesia vogeli* (AF394534.1), *B.* cf. *microti* (AF510203.1), *Babesia rossi* (AF394535.1), *B. caballi* (AF394536.1), *B. divergens* (EU185801.1), *Babesia major* (HM538228.1), *B. bigemina* (HM538227.1), *Babesia venatorum* (=*B.* sp. EU1) (HM113372.1), *B. microti* (AB190435.1) and *Babesia gibsoni* (FJ769389.1). Depending on the species, the amplified fragment is predicted to have a size of 333-499-bp. Due to the high sequence variability of the ITS-1 region, species differentiation could be performed. PCR reactions contained 0.3 mM dNTPs, 0.4 µM of the primer pair BabFOR (5'-TAGGTGAACCTGCGGAAGGATCAT-3′) and BabREV2 (5'-AGCCRAGACATCCAYCGCTGAAA-3′), 2 mM $MgCl_2$ and 1.25 U Maxima Hot Start Taq DNA polymerase (Thermo Fisher Scientific, Inc., Waltham, USA) in 25 µl 1× Maxima Hot Start Taq Buffer and genomic DNA (60–120 ng) of pooled samples. Ten copies of a plasmid containing the *B. microti* PCR product served as positive control. After initial denaturation at 95 °C for 4 min, 40 cycles of 95 °C for 30 s, 67 °C for 30 s and 72 °C for 30 s were conducted. Finally, reactions were incubated at 72 °C for 7 min.

To detect *Anaplasma* and *Ehrlichia* species described in domestic

animals in Europe so far, a PCR published by Tabar et al. (2009) was slightly modified, including primer sequences as reported previously (Krücken et al., 2013). The new primers show complete sequence identity to *A. phagocytophilum* (AY055469.3), *Anaplasma platys* (EF139459.1), *A. marginale* (AF311303.1), *Anaplasma bovis* (AB211163.1), *Anaplasma centrale* (AF318944.1), *Anaplasma ovis* (AY262124.1) and *E. canis* (AF162860). Moreover, the alignment showed complete compatibility to the species *Ehrlichia ruminantium* (NR_074513.1), *Ehrlichia chaffeensis* (M73222.1), *Ehrlichia muris* (AB196302.1) and *Ehrlichia ewingii* (NR_044747.1), which have not been described in Europe or in domestic animals. Pathogens were identified amplifying and sequencing a 257-bp sequence of the 16S rRNA gene. The reaction mixture of 25 µl contained 0.3 mM dNTPs, 0.3 µM of the primer pair E + Afor (5'-GGGGATGATGTCAARTCAGCAY-3′) and E/Arev (5'-CACCAGCTTCGAGTTAAGCCAAT-3′), 1.5 mM $MgCl_2$, 1.25 U Maxima Hot Start *Taq* DNA polymerase (Thermo Fisher Scientific), 1 × Maxima Hot Start *Taq* Buffer and DNA (60–120 ng) of pooled samples. After initial denaturation at 95 °C for 5 min, 40 cycles of 94 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s were performed. A final extension was carried out at 72 °C for 7 min. Plasmid DNA (20 copies) with the *A. phagocytophilum* target region served as positive control. Furthermore, analyses of ticks in other studies using this PCR described an amplification of Ca. Neoehrlichia mikurensis (Krücken et al., 2013). The alignment showed three mismatches in the forward or reverse primer. To determine the bacterial species, samples tested positive in gel electrophoresis were investigated by high resolution melt analyses according to Krücken et al. (2013). Moreover, purified samples were sequenced by GATC Biotech (Constance, Germany).

According to Schreiber et al. (2014), *Rickettsia* species were detected by two different PCR assays amplifying 203-bp and 676-bp sequences of the citrate synthase gene (*gltA*). Using the primers Rmasglta863up (5'-GCTAAAGCTAAGGATAAAAATGAT-3′) and Rmasglta1065lo (5'-TCAA TAAAATATTCATCTTTAAGAGC-3′), 2000 female *D. reticulatus* were initially screened for the 203-bp fragment to determine the prevalence of rickettsial infections. After initial denaturation at 98 °C for 30 s, 50 cycles with 98 °C for 10 s, 52 °C for 30 s and 72 °C for 15 s were performed, followed by a final elongation at 72 °C for 5 min. A subset of tick samples tested positive was subjected to amplification of the 676-bp fragment to identify the species. Positive samples were sent in for sequencing by LGC Genomics (Berlin, Germany). The primer pair CS409d (5'-CCTATGGCTATTATGCTTGC-3′) and Rmasglta1065lo was used. Reaction mixtures were first heated for 4 min at 95 °C, followed by 50 cycles denaturation at 95 °C for 15 s, annealing at 53 °C for 30 s and extension at 72 °C for 40 s. Finally, samples were incubated at 72 °C for 10 min. Positive controls of both assays contained 50 copies of a plasmid carrying the amplicon from *R. helvetica*.

Ticks were analysed for the presence of a 67-bp sequence from the 5S-23S intergenic spacer of the rRNA gene of *Borrelia* species using the primers IGS-MGB Borrelia for (5'-TCCTAGGCATTCACCATAGACT-3′) and IGS-MGB Borrelia rev (5'-TGGCAAAATAGAGATGGAAGAT-3′) described by Strube et al. (2010). To investigate all 2000 female *D. reticulatus*, primers were initially used in a conventional PCR. Ticks collected in the year 2010 were analysed using 1.4 mM dNTPs, 0.2 µM primer, 3.5 mM $MgCl_2$, 1 U Maxima Hot Start Taq DNA Polymerase (Thermo Fisher Scientific) in 25 µl of 1 × Maxima Hot Start Taq Buffer and genomic DNA (60–120 ng). After initial denaturation at 94 °C for 2 min, 40 cycles of 94 °C for 15 s, 56 °C for 60 s and 72 °C for 45 s were conducted. To increase efficiency, ticks collected in the year 2011, were investigated using the following PCR protocol: 0.2 mM dNTPs, 0.25 µM primers, 0.4 U Phusion® Hot Start ll Polymerase (2 U/µl; Thermo Fisher Scientific) in 20 µl of 1 × Phusion® HF Buffer and genomic DNA (60–120 ng). Reaction mixtures were first heated to 98 °C for 30 s. Then, 50 cycles of 98 °C for 10 s, 56 °C for 30 s and 72 °C for 30 s were performed. The final extension was at 72 °C for 10 min. Positive samples of the conventional PCR assays were reanalysed using the more specific quantitative real-time PCR. The reaction mixture (20 µl) contained 12.5 µl Maxima Probe qPCR Mastermix, 0.2 µM of the primer pair, 0.25 µl IGS-MGB Borrelia probe (6-FAM-ATTACTTTGACCATATTT-MGBNFQ) (Strube et al., 2010) and genomic DNA. After initial denaturation at 95 °C for 5 min, 40 cycles of 95 °C for 20 s, 56 °C for 60 s and 72 °C for 45 s were conducted. Ten copies of a plasmid containing the *Borrelia* spp. PCR product (identical for all *B. burgdorferi* sensu lato) served as positive control, both in the conventional and in the quantitative real-time PCR. For species identification, a further PCR detecting a 153-bp fragment of the histone-like protein gene (*hbb*) was carried out according to Schreiber et al. (2014), which is based on a real-time PCR conducted by Portnoï et al. (2006). As positive control, reactions containing a plasmid with an insert derived from *B. afzelii* were run in parallel. Amplicons were sent in for sequencing by LGC Genomics (Berlin, Germany).

2.4. Statistical analysis

Frequency and total time of sampling varied between the years and sampling sites. Reasons for this included differences in the beginning of sampling periods in 2010 and 2011 due to the study design. Furthermore, occasional sampling dates prematurely ended (8/149) or had to be postponed, because of limiting weather conditions, in particular starting rainfall during the sampling action. Thus, for comparisons of tick numbers between sampling sites, years and seasons the average number of ticks/h was calculated for each sampling action.

Statistical analysis was performed using the R software version 3.3.2 and the graphical user interface RStudio version 0.99.903. Prevalence of pathogens and frequencies of collected tick species and sex were calculated with their 95% confidence intervals (Wilson score) using the command “propCI” in the R-package “prevalence”. To identify if proportions were significantly different from 50%, a binomial test was performed (“chisq.test” function). Analyses to determine pairwise differences in sex ratios between different seasons and years have been applied using the “tab2by2.test” (Mid-P Exact test) in the package “epitools”. To identify potential effects of the recorded parameters location, year of sampling, season, temperature and relative humidity on the abundance of questing *D. reticulatus*, a negative binomial regression using the “glm.nb” function in the package “mass” was conducted. Calculations were carried out on the basis of the standardized data (1 h-values) documented in the whole sampling periods of 2010 and 2011. Since integer numbers are required for negative binomial regression analyses, decimal numbers, which emerged in a few cases through extrapolating tick numbers when sampling times were shorter than 1 h, were rounded with the command “round”. The initial model, containing all variables mentioned above, was improved by removing categorical variables with no impact, which were identified by the Wald-test (command “wald.test”, package “aod”) and by determining the lowest AIC (Akaike information criterion) with the help of the “drop1” command. Significant influences ($p < 0.05$) between individual levels of a factorial variable were shown by t-tests as integrated in the “glm.nb” function. Using the “confint” function, 95% CIs were calculated for each level. Additionally, the exponential function (“exp”) of the estimates and the 95% CIs was computed to determine the rate ratios and to enable a better comparability of different levels of the specific variables. The goodness of the final model was verified by comparing it to a null model, the fully parameterised model and a corresponding Poisson-Modell by means of the AIC and likelihood ratio tests (LRTs). Likelihood ratio tests were conducted using the “lrtest” function in the package “lmtest”. To determine how much of the variance is explained by the analysed variables, pseudo r-squared measures (McFadden, Nagelkerke) were calculated with the “pR2” function in the “pscl” package.

Prevalence of pathogens and pairwise differences in their prevalences between individual sampling sites, seasons and years were determined using again the functions “propCl” with Wilson score intervals and “tab2by2.test” for Mid-P exact tests. Applying the Holm

correction implemented within the "p.adjust" function, p-values were corrected for multiple testing. Multivariate analysis has also been applied to identify influencing factors to the infection rate of *D. reticulatus* with *Rickettsia* sp. using a linearized logistic regression ("glm" function). For this purpose, the potential effect of the variables sampling year, season and location were tested. To create the final model, selection of variables with no impact was carried out as described before (see model abundance of questing *D. reticulatus*). The 95% CIs of the estimates and odds ratios were obtained using "confint" function. Odds ratios were calculated by exponentiation of the estimators. In accordance with tick activity calculations, pseudo r-squared measures (McFadden, Nagelkerke) were determined. Assessing again the AIC and LRTs, the goodness of the final model was proven by comparing it both to a null model and the fully parameterised model.

3. Results

3.1. Collection of ticks

Altogether, 9186 questing ticks belonging to the species *D. reticulatus* (n = 8166) and *I. ricinus* (n = 1020) were collected from the vegetation starting March 2010 until April 2012 at four different sampling sites. For all further analyses, only the *D. reticulatus* ticks were considered. Between March and November 2010, 3176 *D. reticulatus* were collected (total sampling time 3410 min). In the year 2011 (5000 min) a number of 4693 *D. reticulatus* was documented between January and November. From December 2011 until April 2012 only one sampling site (Güterfelde) was analysed for ticks. In this period, 297 *D. reticulatus* were collected during a total sampling time of 390 min. Regarding the study years as well as the sampling sites, total sampling time differed due to the study design on the one hand and limiting weather conditions (in particular rainfall and wet vegetation, which were reasons to stop or postpone sampling) on the other hand (Table 1). All collected *D. reticulatus* were identified as adult stages. The dominance of *D. reticulatus* over *I. ricinus* illustrates the characteristic of the sampling sites as habitats for the analysed tick species. In comparison to *I. ricinus*, significantly more *D. reticulatus* were found in all three sampling years (relative *D. reticulatus* prevalence: 85.1–98.3%; respectively $p < 0.001$) and sampling sites (2010, 2011, 2012 relative *D. reticulatus* prevalence: 56.1–96.4%; $p < 0.001$–0.005). Except during summer (all sites) and spring period (Falkenberg) significantly higher numbers of *D. reticulatus* than *I. ricinus* were collected in autumn and winter (all sites) as well as in spring (Güterfelde, Gatow and K. Wusterhausen) (89.4–99.8%; respectively $p < 0.001$). The sex ratio of *D. reticulatus* showed moderately but significantly higher numbers of female than male ticks at all sampling sites and years (female: 55.1–60.6%, $p < 0.005$).

3.2. Seasonal abundance of questing Dermacentor reticulatus ticks

In the course of the study years 2010 and 2011, a characteristic seasonal questing behaviour of adult *D. reticulatus* could be observed at all four sampling sites (Fig. 1). Showing a bimodal pattern of abundance of questing *D. reticulatus*, ticks were found especially during spring and autumn. Spring activity covered the period from March until the end of May. Depending on the sampling site, 11.3–46.7 (2010) or 31.3–89.0 (2011) *D. reticulatus* were obtained on average per collection time of 1 h (Table 2). Within this period of time, the maximum number of questing ticks was noticed in the last decade of March. From June until the mid of August, almost complete inactivity of adult *D. reticulatus* was recorded (0–0.8 ticks/h). The following period of autumn activity approximately lasted until the end of November. Per 1 h collection 70-178.3 (2010) or 41-142.4 (2011) *D. reticulatus* were collected on average. During this time, the highest number of questing *D. reticulatus* was recorded between the last ten days of September and the middle of October. Samplings performed from December to February, showed an irregular occurrence of ticks also during winter months. In January 2010, an average number of 88.8 questing *D. reticulatus*/h was found after melting of a long-lasting snow cover at temperatures between 3.4–6 °C. In contrast, samplings at temperatures of 11.5 °C in the second half of February showed a decrease of tick numbers to ≤3 *D. reticulatus*/h.

3.3. Investigation of the abundance of questing Dermacentor reticulatus in relation to abiotic factors

Within this study, the abundance of questing adult *D. reticulatus* was analysed with regard to the sampling site, year, season and different climate factors (temperature, relative humidity). Both in 2010 and 2011, the highest number of *D. reticulatus* per collection (1 h) was found in Gatow (mean 88.5), followed by Güterfelde (mean 61), K. Wusterhausen (mean 41) and Falkenberg (mean 31) (for detailed information see Table 2).

Between seasons and years, a pronounced variability in numbers of questing ticks was observed. For instance, 23.3 (2010) or 65.3 (2011) *D. reticulatus*/h (average of all sampling sites) were collected in spring. With 121.4 (2010) or 85.3 (2011) *D. reticulatus*/h, highest numbers of questing ticks were obtained in autumn. In winter 2010/2011, an average of 60 questing ticks/h was collected. In contrast to that, a much lower number of questing ticks/h (2010: 0.42; 2011: 0.3) was recorded during summer months. Comparing the periods of main activity throughout the year (spring and autumn), the number of questing *D. reticulatus* was on average 5.21-fold (2010) or 1.31-fold (2011) higher during autumn time. In this context, the late beginning of the study in spring 2010 has to be considered. Generally, the increase of questing adult *D. reticulatus* in autumn compared to spring was noted at all sampling sites, except in K. Wusterhausen in the year 2011.

Comparison of the main sampling years 2010 and 2011 was difficult since the beginning of the spring peak was missed in 2010. Nevertheless, the mean number of collected *D. reticulatus*/h was almost identical between 2010 and 2011. Average tick numbers of 55.6/h (2010) and 55.4/h (2011) were obtained when all data were included while 55.6/h (2010) and 54.8/h (2011) were noticed including only March to November data available for both years.

Numbers of questing adult *D. reticulatus* were considered also in relation to the temperature and relative humidity, which were determined locally at every sampling site and day (2010–2012, Fig. 1). As expected, the data of measured temperatures showed a bell-shaped curve with maximum values in July. In contrast to that, measurements of the relative humidity demonstrated enormous variations between individual sampling days within the same season and sampling site. During spring and autumn, average monthly temperatures of 11.3 °C (March) to 18.0 °C (May) or 22.1 °C (mid-August) to 6.7 °C (November) were documented during the collections. The range for spring and autumn were 5.0–25.0 °C and-0.5–29 °C, respectively. Data of average monthly relative humidity during collections showed relatively

Table 1
Overview of the absolute number of collected *Dermacentor reticulatus*, the sampling site, number of samplings and total sampling time at each location from 2010 to 2012.

	Güterfelde	Gatow	Falkenberg	K. Wusterhausen
	Total number of collected *D. reticulatus*			
	Number of samplings/total sampling time			
2010	906	1,214	400	656
(Mch–Nov)	16/975 min	14/840 min	14/755 min	14/840 min
2011	1,359	1,928	629	777
(Jan–Nov)	21/1,230 min	21/1,270 min	21/1,250 min	21/1,250 min
2011/12	297	–	–	–
(Dec–Apr)	7/390 min			

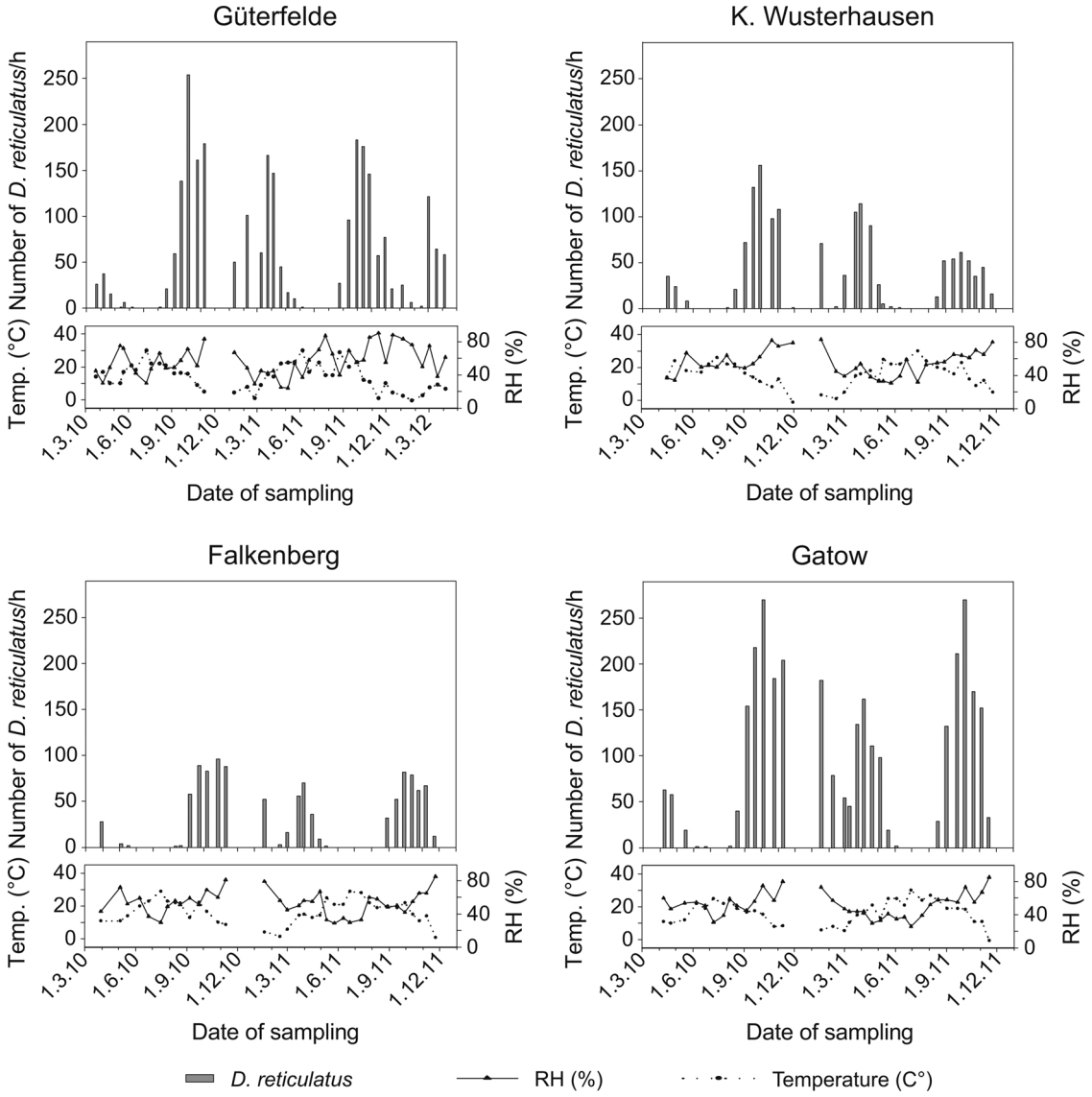


Fig. 1. The numbers of adult *Dermacentor reticulatus* obtained per individual collection (1 h) from March 2010 until November 2011 are shown in form of bar charts for each sampling site. Seasonal activity of ticks in Güterfelde was recorded until April 2012. From December 2010 until February 2011 no sampling was performed (gaps in graphs). Climate graphs, belonging to the respective tick activity bar chart, reflect the locally measured temperatures and relative humidities.

constant values during springtime (44.4–47.7%). In autumn months, the relative humidity gradually increased from 54.9% to 75.9%. During these periods of time the number of questing *D. reticulatus* initially increased with rising (spring) or falling (autumn) temperatures. After reaching a maximum, numbers of questing ticks subsequently declined with further increase (spring) or drop (autumn) of temperature. In summer, when almost no adult *D. reticulatus* were found, highest average monthly temperatures for the year (21.9–24.5 °C) were measured. Relative humidity varied between 44.3% and 60%. In contrast, tick activities were even noticed in winter months. The number of ticks as well as measured climatic factors were subject to large fluctuations. Temperatures and relative humidities of −0.5 to 7.8 °C and 29–88% were documented at individual sampling days. All collected data illustrate the activity of adult *D. reticulatus* in a wide range of temperatures. However, within similar temperature ranges differences in tick activity were noticed. For example, tick numbers varied from 0 (July) to 166 (end of March) individuals/h at temperatures around 15 °C at Güterfelde in 2011. At days with maximum temperatures (average of all sampling sites) in spring (24.5 °C) or autumn (22.5 °C) an average number of 8.75 *D. reticulatus*/h or 29.6 *D. reticulatus*/h were collected.

3.4. Calculation of potential impacts of abiotic factors using multivariate analysis

To determine potential impacts of the factors sampling site, season, year (2010, 2011), temperature and relative humidity on the abundance of questing *D. reticulatus*, all variables were investigated by multivariate analysis using a negative binomial regression model. By identification and elimination of variables with no impact, the best model with the lowest AIC (1,110.9) was obtained. Calculations of pseudo r-squared measures (Nagelkerke 0.805, McFadden 0.176) illustrated a good adaptation of the regression model to the collected data on the basis of the analysed variables. The final model consisted of the factors sampling site, season, temperature and year (Table 3). Except for the year, all other remaining variables were of significant importance for abundance of questing ticks. Nevertheless, to create the final regression model the factor year was essential as a confounding factor. For the purpose of graphical illustration of the final model, Fig. 2 compares actual numbers of captured *D. reticulatus* per 1 h and modelled values calculated by negative binomial regression analysis. Selected graphs illustrate differences in abundance of questing ticks

Table 2
Mean numbers and range of adult *Dermacentor reticulatus* collected per 1 h at each location with regard to sampling year and season. Data are based on standardized values.

	Güterfelde		Gatow		Falkenberg		K. Wusterhausen	
	Mean number of ticks per hour (Number of samplings)							
	Range							
2010								
Spring	17.1	(5)	46.7	(3)	11.3	(3)	22.3	(3)
	1–37		19–63		2–28		8–35	
Summer	0.4	(5)	0.8	(5)	0.2	(5)	0.25	(4)
	0–1		0–2		0–1		0–1	
Autumn	135.3	(6)	178.3	(6)	70	(6)	84	(7)
	21–254		40–270		6–96		1–156	
Total 2010	56.2	(16)	86.7	(14)	32.5	(14)	46.9	(14)
	0–254		0–270		0–96		0–156	
2011								
Winter	50.3	(3)	130.5	(2)	27.5	(2)	36.5	(2)
	0–101		79–182		3–52		2–71	
Spring	74.2	(6)	89	(7)	31.3	(6)	62.7	(6)
	10–166		19–162		1–70		5–114	
Summer	0.2	(5)	0.4	(5)	0	(6)	0.6	(5)
	0–1		0–2		0–0		0–2	
Autumn	108.9	(7)	142.4	(7)	55.5	(7)	41	(8)
	27–183		29–270		24–82		13–61	
Total 2011	64.7	(21)	89.7	(21)	30.1	(21)	37.1	(21)
	0–183		0–270		0–82		0–114	
2012								
Winter	18.8	(4)	n.d.[a]	n.d.	n.d.	n.d.	n.d.	n.d.
	2–42							
Spring	81	(3)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	58–121							
Total 2012	45.4	(7)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

[a] no data.

depending on the variables sampling site, season and temperature. With the exception of the graph in Fig. 2B showing data for K. Wusterhausen in autumn 2011, calculated numbers of ticks completely reflect actual collection data, indicating a good adaption of the model. In Fig. 2B "K. Wusterhausen", the actual number of collected ticks was lower compared to the modelled activity curves. Due to reforestation of individual sampled areas within the sampling site K. Wusterhausen in the beginning of the year 2011, collection of ticks partly had to be switched to new areas, showing a lower activity of *D. reticulatus*, which presumably explains this discrepancy.

According to the model, 2.95 times or 2.06 times more *D. reticulatus* were collected at the sampling location Gatow than in Falkenberg or K. Wusterhausen ($p < 0.001$). In contrast, no significant differences were recognised between Gatow and Güterfelde as well as between the remaining sampling sites. Regarding the impact of the factor season, the number of questing *D. reticulatus* was significantly lower in summer compared to spring, autumn and winter ($p < 0.001$). In accordance with the previously described observations, a 2.58-fold higher

Table 3
Results of the final model, calculated by negative binomial regression analysis to identify potential significant impacts of the different variables on activity of adult *Dermacentor reticulatus*. The factor relative humidity (RH/RH^2) has been eliminated, because no relevant influence of this variable was determined in the initial model. Data are based on standardized values of ticks collected per 1 h.

	Estimate	Standard error	*P* value	Wald test *P* value	Rate ratio	95% CI
Intercept	2.345	0.377	< 0.001		10.438	4.817–23.568
Climate factors						
Temperature	0.284	0.043	< 0.001	< 0.001	1.328	1.212–1.448
$Temperature^2$	−0.010	0.001	< 0.001		0.989	0.987–0.993
RH	–					
RH^2	–					
Year: 2010 vs.						
2011	0.288	0.155	0.063	0.063	1.334	0.962–1.845
Sampling site: Gatow vs.						
Falkenberg	−1.083	0.206	< 0.001	< 0.001	0.339	0.226–0.508
Güterfelde	−0.355	0.197	0.072		0.701	0.474–1.034
K. Wusterhausen	−0.724	0.202	< 0.001		0.485	0.325–0.723
Season: spring vs.						
Summer	−4.264	0.332	< 0.001	< 0.001	0.014	0.007–0.027
Autumn	0.948	0.159	< 0.001		2.582	1.838–3.619
Winter	0.674	0.329	0.040		1.962	1.066–3.742

AIC: 1110.9.
R^2: McFadden: 0.176.
R^2: Nagelkerke: 0.805.

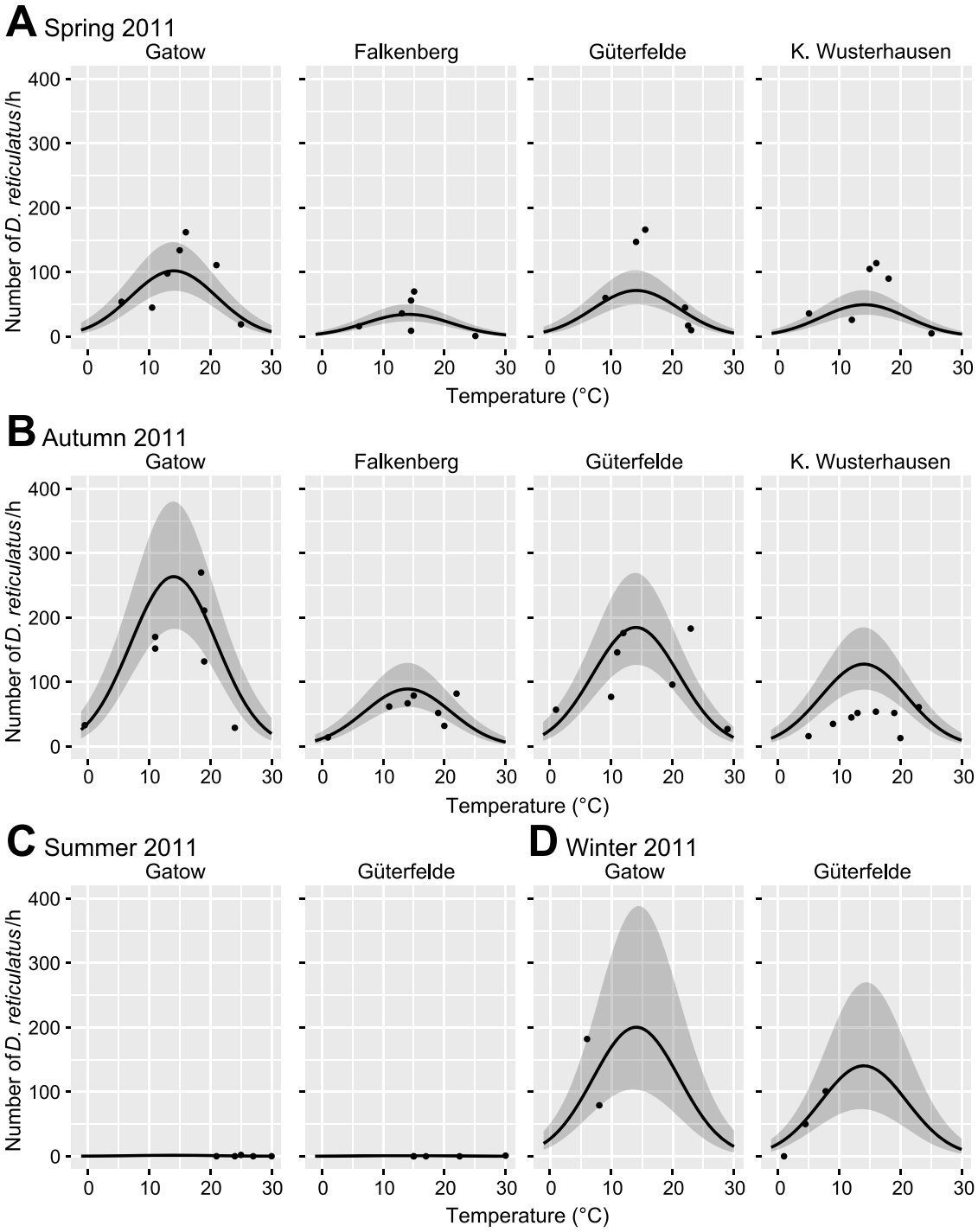


Fig. 2. Graphic representation of the final regression model showing the activity of Dermacentor reticulatus/h at different sampling sites and seasons depending on the temperature. Both, actual collection data (points) as well as data calculated by negative binomial regression analyses (curves) with 95% confidence bands (dark grey area) are illustrated (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

abundance of questing ticks was confirmed during autumn time compared to springtime ($p < 0.001$). Under the given circumstances, a comparison of abundance of questing ticks in spring and winter showed significantly more active adult *D. reticulatus* (1.96-fold increase) even during the colder season ($p < 0.05$). However, it must be kept in mind that no sampling actions were conducted in winter when the temperature was below freezing or the vegetation was fully covered with snow.

On the basis of the collected data, a quadratic instead of a linear relationship was expected for the climate factors temperature and relative humidity in connection with tick activity which is able to model an optimum curve. Therefore, variables were reflected by the parameters "temperature + temperature^{2}" and "relative humidity + relative humidity^{2}". In contrast to the factor relative humidity, multivariate analysis showed a significant impact of the factor temperature ($p < 0.001$). In accordance with field observations, the number of questing ticks initially increased with rising temperatures up to an optimum value of 14.03 °C, whereas further increase of temperature entailed a decline of abundance of questing ticks (Fig. 2). According to the negative binomial regression analysis, 80% of the questing *D.*

Table 4
Prevalence and 95% confidence intervals (95% CI) of tick-borne pathogens in *Dermacentor reticulatus* collected at four different sampling sites in the area of Berlin/Brandenburg in 2010 and 2011.

Sampling year and site	Ticks analysed	*Babesia* spp. (95% CI)	*Borrelia* spp. (95% CI)	*Rickettsia* spp. (95% CI)	Anaplasmataceae (95% CI)
2010					
Güterfelde	256	0 (0–1.05)	0.39 (0.07–2.18) [d]	75.4 (69.8–80.3)[a]	0 (0–1.05)
Gatow	256	0 (0–1.05)	0 (0-1.05)	28.5 (23.3–34.3)[b]	0 (0–1.05)
Falkenberg	231	0 (0–1.16)	0.43 (0.08–2.41)	71.9 (65.7–77.3)[a]	0.43 (0.08–2.41)
K. Wusterhausen	257	0 (0–1.04)	0 (0–1.04)	77.0 (71.5–81.8)[a]	0 (0–1.04)
Total 2010	1000	0 (0–0.27)	0.2 (0.05–0.73)	63.0 (60.0–65.9)	0.1 (0.02–0.56)
2011					
Güterfelde	250	0 (0–1.07)	0 (0–1.07)	81.2 (75.9–85.6)[a]	0 (0–1.07)
Gatow	250	0 (0–1.07)	0.4 (0.07–2.23) [e]	34.4 (28.8–40.5)[c]	0 (0–1.07)
Falkenberg	250	0 (0–1.07)	0 (0–1.07)	71.2 (65.3–76.5)[b]	0 (0-1.07)
K. Wusterhausen	250	0 (0–1.07)	0.8 (0.22–2.87)	73.2 (67.4–78.3)[ab]	0 (0–1.07)
Total 2011	1000	0 (0–0.27)	0.3 (0.1–0.88)	65.0 (62.0–67.9)	0 (0–0.27)
Total 2010/11	2000	0 (0–0.14)	0.25 (0.11–0.58)	64.0 (61.9–66.1) [g]	0.05 (0.01–0.28) [f]

[a,b,c] Prevalences with different indices are significantly different with $p < 0.05$.
[d]Borrelia afzelii, [e]Borrelia miyamotoi, [f]Anaplasma phagocytophilum, [g] Rickettsia raoultii.

reticulatus were collected between 5.0-23.0 °C (calculation of the area under the curve).

3.5. Prevalence of Rickettsia sp. in Dermacentor reticulatus

Among the 2000 ticks initially examined by amplifying the 203-bp fragment of the *Rickettsia gltA* gene, 1280 (64.0%; 95% CI 61.9–66.1%) samples were detected as positive (Table 4). Between ticks of the sampling years 2010 and 2011, infection frequencies were almost identical (63.0% and 65.0%; Mid-P Exact test $p = 0.35$). Regarding the different sampling sites, *Rickettsia* were most frequently found in ticks in Güterfelde, Falkenberg and K. Wusterhausen with prevalences of 71.9–77.0% (2010) and 71.2–81.2% (2011). In contrast to that, significantly fewer ticks were infected in Gatow in 2010 (28.5%) and 2011 (34.4%) ($p < 0.001$; Mid-P Exact test followed by Holm correction). No significant differences were found between the number of infected ticks in the different seasons ($p = 0.15–1.0$; Mid-P Exact test followed by Holm correction). Thus, infection rates (average of all sampling sites) of 57.8% (n = 244; 2010) and 61.6% (n = 383; 2011) in spring, 64.8% (n = 755; 2010) and 67.2% (n = 500; 2011) in autumn and 66.4% (n = 113) in the winter 2010/2011 were documented. Using a general linearized logistic regression model (model A, Fig. 3), potential influences of the parameters year, sampling site and season on prevalence of *Rickettsia* in *D. reticulatus* were calculated. Differences in the odds of *D. reticulatus* ticks being positive for *Rickettsia* were observed only for the factor sampling site ($p < 0.001$). Thus, the odds that *D. reticulatus* were positive for *Rickettsia* sp. were 5.48-fold (Falkenberg), 6.6-fold (K. Wusterhausen) and 7.86-fold (Güterfelde) higher than in Gatow (model B, Fig. 3).

To determine the *Rickettsia* species, samples were reanalysed detecting a 676-bp fragment of the same gene region. In total PCR products from 18 ticks, representing all four study sites, were sent in for sequencing. Results showed a 100% identity to several *R. raoultii* entries (e.g. GenBank accession no. KT261764.1).

3.6. Prevalences of selected Anaplasmataceae as well as Borrelia- and Babesia sp

Only a few ticks of the analysed subset contained DNA of *Anaplasma* sp. or *Borrelia* sp. *Borrelia* specific DNA was detected in five ticks (0.25%; 95% CI 0.110.58%) of the 2000 female *D. reticulatus* analysed. Positive samples came from all four sampling sites. For species identification, two positive samples were reanalysed by a PCR detecting a 167-bp fragment of the *hbb* gene. One sequenced product was 100% identical to *Borrelia miyamotoi* isolated from *I. ricinus* and *Ixodes hexagonus* in Germany (GenBank accession no. HE993870.1). The second positive sample showed 100% identity to different isolates of *B. afzelii* (e.g. GenBank accession no. CP009212.1).

Analysing a 257-bp fragment of the 16S rDNA of Anaplasmataceae revealed a negligible small rate of only one positive *D. reticulatus* (0.05%; 95% CI 0.01–0.28%). Sequencing of the amplicon showed a 99% identity to *A. phagocytophilum* (GenBank accession no. AY055469.3) and *A. platys* (GenBank accession no. EF139459.1). Since *A. platys* has not been described in Germany yet, the positive sample most likely harboured *A. phagocytophilum* DNA. Moreover, DNA of *Ehrlichia* sp. and Ca. *Neoehrlichia* sp. was not detected (0%; 95% CI 0–0.14%).

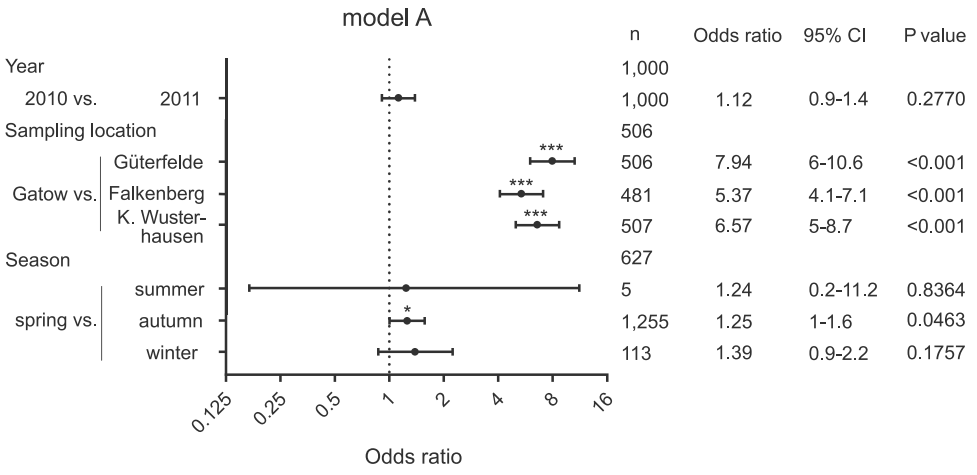
Investigation of the 333-499-bp amplicon of the ITS-1 region of different *Babesia* spp. showed that no tick harboured DNA of these pathogens (0%; 95% CI 0–0.14%).

Detailed information regarding prevalence of all pathogens at the different sampling sites and sampling years is summarised in Table 4.

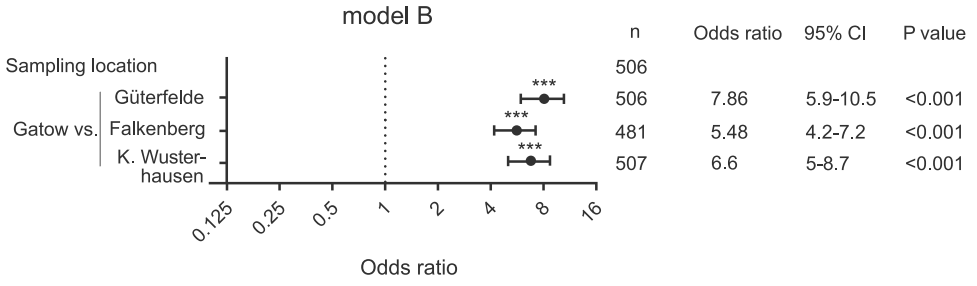
4. Discussion

Dermacentor reticulatus is a vector of a variety of pathogens of medical importance (Földvári et al., 2016; Rubel et al., 2016) and is considered the second most important tick species in Europe (Karbowiak, 2014). However, major differences in tick distribution are noticed depending on the region. To establish a basis for risk assessment in the area of Berlin/Brandenburg, comprehensive investigations concerning the activity of *D. reticulatus* and its influencing factors as well as the prevalence of different tick-borne pathogens were carried out in the course of the present study. Only questing ticks were analysed to reflect actual pathogen prevalence in tick populations.

In the beginning of the study, it was decided to achieve comparable sampling areas at the different study sites by sampling always for one hour. This option was chosen to avoid marking a continuous area at each study site since it was not possible to introduce permanent marks and more importantly to avoid dragging the cloth every two weeks over a continuous area. The latter would probably lead to a reduction of tick density due to sampling. By sampling only narrow tracks bordered by much larger untouched areas it was assumed that sampling would have only minimal effects on tick abundance. However, this approach introduces an important bias: With increasing number of collected ticks, the time spent to remove the ticks from the cloth also increases and the length of the track is expected to decrease. However, this effect is only small since the vast majority of the ticks were adult *D. reticulatus*, which are large and thus easy to see and grasp. We determined that the person performing the sampling required 16 s to collect 10 ticks from the cloth. With a maximum of 270 ticks collected per hour, this would lead to



Wald-test (P values): year (0.28), season (0.19), sampling location (<0.001)
AIC: 2,313.5
R^2: Nagelkerke 0.200/ Mc Fadden 0.121



AIC: 2,311.1
R^2: Nagelkerke 0.197/ Mc Fadden 0.119

Fig. 3. Influence of variables on presence of *Rickettsia* sp. in *Dermacentor reticulatus* analysed by logistic regression analysis. Odds ratios with 95% CIs for variables with and without influence are shown as forest plots of the initial model (model A) and the final model (model B). In the initial model A, the reference variables year 2010, Gatow and spring were compared to the other levels of the factors year, sampling site and season. The final model B contained exclusively the parameter sampling site, which was the only variable with an impact on presence of Rickettsia. The lower AIC value illustrates the final model B as the best model. Pseudo r^2 values (Nagelkerke, McFadden) are almost the same in both models. Low values indicate that not all variables, explaining the variance of the prevalences, are known and were included.

7 min time (11.6% of total sampling time) spent with collecting ticks from the cloth and not with dragging the cloth. Since there is also time required to search the cloth for the presence of ticks at days when no ticks were found, we assume that the difference in time spent for dragging the cloth was less than 10% between days with the highest and lowest number of ticks collected. Nevertheless, it must be clearly stated that there is a systematic bias in sampling time, which leads to an underestimation of the abundance of questing ticks on days with high tick activity. Therefore, differences between the high activity seasons spring and autumn on one and the low activity season summer on the other hand are most likely even more pronounced than the recorded data suggest.

The number of ticks collected by flagging was used as a proxy for abundance of questing ticks. The latter is influenced by two different effects: First, short term effects of variables such as temperature on activity of the tick population, i.e. the percentage of ticks that are questing. Second, long term effects of parameters such as the sampling sites, reforesting or tick survival during winter on the total tick abundance at the study sites. Variables with medium term effects are likely to affect both, tick abundance and tick activity. In the context of the present study, the season is most likely such a medium term factor with strong effects on tick activity (depression of questing behaviour in summer) but presumably also moderate effects on tick abundance (higher numbers of questing ticks in autumn compared to spring). For the present study, it was aimed to identify variables with effects on tick activity but due to the nature of the data it was not always possible to discriminate effects on tick abundance and effects on tick activity.

In accordance with many other studies (Bartosik et al., 2011; Duscher et al., 2013; Hornok, 2009; Immler, 1973; Széll et al., 2006), collected data show a characteristic bimodal pattern of abundance of questing adult *D. reticulatus* during a year. This is in marked contrast to a recent publication describing seasonal activity from a region in South Europe (northern Italy) where questing adult *D. reticulatus* were only observed between February and June and there was no evidence for a second activity peak in autumn (Olivieri et al., 2017). Potential impacts of primarily abiotic parameters (e.g. temperature, humidity, day length, precipitation, wind speed) on the activity of this tick species have already been published in several studies (Bartosik et al., 2011, 2012; Buczek et al., 2013; Hubálek et al., 2003; Zając et al., 2016). In contrast to most of the studies, using univariate tests for statistical analyses, negative binomial regression analysis was conducted here. Multivariate analyses better reflect the natural conditions, because the influences of all parameters are considered together in context.

The factor sampling site was of significant importance and in turn, comprises various biotic and abiotic variables influencing overall tick abundance. Therefore, the kind of habitat obviously plays an important role. In the present study completely uncultivated areas (fallow land: Gatow, Falkenberg) as well as meadows (Güterfelde, K. Wusterhausen), which were mowed once a year, were included. Results of tick samplings showed that the lowest as well as the highest number of ticks was recorded on fallow land. Thus no differences in tick abundance of questing ticks were noticed between both habitats, which confirms the results of Biaduń (2011). In contrast, a 3.5-time higher number of collected *D. reticulatus* on fallow land compared to meadows was reported by Mierzejewska et al. (2015a) and recently an even 6-fold difference was recorded after mowing three times per year over three

years (Bajer et al., 2017). However, abundance of questing ticks clearly differed between these two kinds of habitats and intensively managed areas such as pastures (Hornok and Farkas, 2009; Mierzejewska et al., 2015a). This is in line with observations in the present study according to which tick numbers sharply decreased on a section of the sampling site K. Wusterhausen in the beginning of 2011. Cultivation in the form of planting dense lines of trees and regular conducted weed control possibly destroyed the previously existing habitat of *D. reticulatus* in this section. Variations in tick abundance depending on the habitat could be explained by differences in the vegetation structure and the availability of hosts (Hornok et al., 2014; Mierzejewska et al., 2015a).

The significant effect of the parameter temperature on tick activity corresponds to the results of various previous studies (Bartosik et al., 2011, 2012; Hubálek et al., 2003; Zając et al., 2016) where univariate statistical analyses showed a negative correlation between adult *D. reticulatus* activity and rising temperatures. However, this apparently oversimplifies the temperature effect since decreased tick activity also occurs at temperatures around the freezing point. Raw data and the multivariate model show an initial increase of tick numbers with rising temperatures, followed by a decrease after reaching a temperature optimum that results in a quadratic equation to estimate temperature effects. According to the regression model, 80% of active *D. reticulatus* can be found in a range of 523 °C, which is in line with the observations of Bartosik et al. (2011), who collected most of the ticks between 421 °C. The tolerance of this tick species to a variety of temperatures was also reported by Zahler and Gothe (1995) and Zając et al. (2016). In the present study the activity of adult D. reticulatus was strongly reduced above temperatures of 23 °C (total tick number: 110) and below temperatures of 5 °C (total tick number: 512). Below the limit of 5 °C, tick numbers were subject to large fluctuations (0–182 ticks/h) showing a large or total inactivity of *D. reticulatus* on colder days.

In contrast to the temperature, no significant impact has been found for the parameter relative humidity in various studies (Bartosik et al., 2011, 2012; Buczek et al., 2013; Zając et al., 2016), which is in line with the results presented here. The relative humidity is the critical parameter governing the water balance of ticks and uptake of water can only take place above the “critical equilibrium activity“-limit (CEA). Investigations defined a *D. reticulatus*-specific CEA at a relative humidity of 84% (Knülle and Rudolph, 1982; Meyer-König et al., 2001a). In the present study, this limit was not achieved at 96.6% of the sampling days, which was primarily caused by sampling conditions (bi-weekly intervals, dry vegetation). However, it must also be considered that the humidity data determined here were not always recorded within the vegetation but constantly 20 cm above the ground, which was occasionally outside of the vegetation in the early vegetation period or after mowing. Within the vegetation and between individual sampling actions (e.g. at night), relative humidity most likely was at least temporarily higher, improving the water balance of the ticks. Relative humidity within the vegetation cannot be assumed to be constant for a study site at a particular time point but will depend on vegetation density and height, exposure to sun light and humidity of the soil, which might e.g. be higher in local sinks. Measuring the relative humidity within the vegetation at multiple positions at each study site and allocation of collected ticks to small scale local grids would promise to collect very interesting data. However, this would require much more complicated collection and measurements protocols as well as complex spatio-temporal microclimate models to analyse the data which was far beyond the scope of the present study. Moreover, humidity data are systematically biased by the fact that no samplings were performed when it rained. Thus, the impact of the factor relative humidity at a particular sampling point has to be considered with caution. In addition, *D. reticulatus* is regarded as a drought-tolerant tick, attributed to an integument characterised by a low transpiration rate. Furthermore, only a loss of 40% of the total body water mass was shown to result in decreased motility of adult *D. reticulatus* (Meyer-König et al., 2001a; Meyer-König et al. (2001b). Observations of the locomotion pattern on vegetation demonstrated a low motility compared to *I. ricinus*, resulting in a reduced loss of water via respiration (Immler, 1973; Knülle and Rudolph, 1982).

According to the negative binomial regression analysis, the variable year was of importance to improve the fit of the model to the data but had no significant effect on the abundance of questing *D. reticulatus*. Differences between years can be caused e.g. by changes in weather conditions, host density, vegetation structure or simply by successful parasite reproduction.

Negative binomial regression analysis confirmed a significant impact of the parameter season. The seasons reflect distinct periods within the characteristic activity pattern of *D. reticulatus* throughout the year and almost completely conform to the meteorological seasons. In accordance with the results presented here, highest activity of adult *D. reticulatus* was noticed during spring and autumn, while tick numbers strongly fluctuated or completely decreased in the period of winter or summer (Bartosik et al., 2011; Hornok, 2009; Immler, 1973; Nosek, 1972). Varying seasonal biotic and abiotic factors such as vegetation structure, host abundance and climatic conditions as well as the seasonality of the *D. reticulatus* live cycle with moulting of nymphs to adults in summer (Paziewska et al., 2010; Pfäffle et al., 2015) have to be considered to explain the differences in abundance of questing ticks between the seasons. In the present study, no information was gathered concerning the diversity and density of the vegetation and hosts. Highest recorded abundance of questing ticks in spring and autumn suggest the best environmental conditions in these seasons. Regarding climatic factors, average temperatures of 14.5 °C (spring) or 14.3 °C (autumn) were documented. These data correspond well to the optimum temperature (14.03 °C) for abundance of questing ticks, calculated by regression analysis. In contrast, the nearly complete absence of collected *D. reticulatus* during summer months indicates adverse conditions for tick survival. Consequently, adult stages undergo a diapause and remain inactive even if weather conditions would be temporarily suitable for activity in summer. Indeed, no ticks were found here on colder sampling days in summer, although temperatures were nearly identical to recorded temperatures on days with high tick activity in spring or autumn. The diapause is a pre-adaptive, genetically determined behavior to avoid poor environmental conditions triggered by an increase of day length (photoperiod) that classifies *D. reticulatus* as a short day species (Belozerov, 1982; Tokhov et al., 2014). In winter months, highly fluctuating activity of adult stages was noticed as described previously (Buczek et al., 2014; Hornok, 2009; Kiewra et al., 2016; Széll et al., 2006). Due to the sometimes very quick reappearance of active *D. reticulatus*, ticks probably undergo only a quiescence when temperatures drop in winter to levels preventing activity (Zahler and Gothe, 1995) and not a diapause as supposed by Buczek et al. (2013), Földvári et al. (2016), Hornok (2009) and Kiewra et al. (2016). Buczek et al. (2014) and Hornok (2009) showed that variation of the tick activity in winter is correlated with temperature changes. This is in line with the results of the present study but sampling during winter was carried out relatively irregular and only on snow-free vegetation. It is striking to note the sudden high activity of ticks after the snowmelt in January 2011. Hence further studies are needed to identify additional factors (e.g. closed snow cover) influencing the activity of *D. reticulatus* during winter.

The previous explanations illustrate a significant impact of the parameters temperature and season. However, significantly higher tick activity in autumn than spring indicates a potential impact of additional factors and in particular the rapid moulting of engorged nymphs to adults in summer might explain this phenomenon (Immler, 1973; Paziewska et al., 2010; Pfäffle et al., 2015). Higher numbers of active ticks in autumn were also recorded in Poland and Hungary (Bartosik et al., 2011, 2012; Buczek et al., 2013; Hornok, 2009; Széll et al., 2006). In contrast, higher activities of questing *D. reticulatus* in spring than in autumn were noticed in France, Poland and Belgium (Cochez et al., 2012; Martinod and Gilot, 1991; Mierzejewska et al., 2015a). The

reasons for these contrasting observations are not known so far.

The high abundance of adult *D. reticulatus* in the area of Berlin/Brandenburg substantiates the importance to identify pathogens in this tick species and to determine their prevalences. *Dermacentor reticulatus* positive for *Rickettsia* have been found in nearly every European country. The average prevalence of 64% presented here, is one of the highest described infection rates at all. Previous German studies recorded prevalences of 33.1%, 50%, 51.3% and 70.5% in questing *D. reticulatus* in the area of Berlin and in the mid and south of the country (Obiegala et al., 2017; Pluta et al., 2010; Robert-Koch-Institut, 2009; Rumer et al., 2011; Silaghi et al., 2011). Pathogen frequencies, published in other European countries, strongly vary ranging from 4.953% (Barandika et al., 2008; Dobec et al., 2009; Hornok et al., 2017; Nijhof et al., 2007; Radzijevskaja et al., 2015; Spitalská et al., 2012; Stańczak et al., 2018; Svehlová et al., 2014; Tijsse-Klasen et al., 2011; Wójcik-Fatla et al., 2013; Zając et al., 2017). Only three studies in Austria, Ukraine (Chernobyl) and Denmark reported prevalences (6586%) being at least as high as described here (Duscher et al., 2016; Karbowiak et al., 2016; Klitgaard et al., 2017). However, it should be noted that the very high frequency of 86% reported from Denmark was calculated after analysing only 21 male ticks collected from a single migrating golden jackal, a species which is not endemic to the region (Klitgaard et al., 2017). In German studies including the present, *R. raoultii* was the only species found in *D. reticulatus*. The pathogen was also detected in the majority of other European studies. However, DNA of *R. slovaca* and *R. helvetica* was also detected in some cases (Dobec et al., 2009; Hodžić et al., 2016, 2017; Radzijevskaja et al., 2015; Rudolf et al., 2016; Spitalská et al., 2012; Svehlová et al., 2014; Tijsse-Klasen et al., 2013). Although *R. raoultii* is known to rarely cause SENLAT in humans (Földvári et al., 2013), quantitative information about the risk caused by *R. raoultii* infected *D. reticulatus* for humans is still insufficient. Despite the high prevalences of *R. raoultii* in *D. reticulatus*, clinical cases were reported only sporadically in Germany and have been exclusively described in southern parts of the country (Pluta et al., 2009; Rieg et al., 2011). Serological studies, reflecting the contact of humans with this pathogen, are also rare. In Berlin/Brandenburg (Germany), a seroprevalence of 5% was detected in the risk group of forestry workers (Wölfel et al., 2017). The low infection rate can possibly be traced back to the comparatively low host preference of *D. reticulatus* for humans and underdiagnosis due to low pathogenicity. Clinical cases in animals induced by *R. raoultii* have not been described so far. In asymptomatic dogs, a seroprevalence of 2.8% in Germany (Wächter et al., 2015) and a prevalence of 0.68% as determined by PCR in Brandenburg (Liesner et al., 2016) were reported. In the area of Berlin/Brandenburg *D. reticulatus* is almost as abundant on dogs as *I. ricinus* (Beck et al., 2014). High frequency of *R. raoultii*-positive *D. reticulatus* collected from dogs (39%) in this region (Schreiber et al., 2014) but also from the vegetation (present study), stand in contrast to the low prevalence detected by PCR in canine blood samples (Liesner et al., 2016). Further evaluation of the role of *D. reticulatus* as an important vector of *R. raoultii* to humans and animals is required but awareness of tick-borne disease caused by *R. raoultii* needs to be increased in human and veterinary medicine.

The probability to find *D. reticulatus* being positive for *Rickettsia* is significantly influenced by the sampling site as shown by logistic regression analysis. Silaghi et al. (2011), Stańczak (2006) and Stańczak et al. (2016) also noticed large differences in prevalences between various locations. *Dermacentor reticulatus* is a reservoir of *R. raoultii* due to successful transstadial survival (98%) and transovarial (90%) transmission (Alberdi et al., 2012; Samoylenko et al., 2009). Nevertheless, there is a progressive loss of bacteria over many generations. This is probably compensated by amplification in mammalian hosts, which differ in number, competence (ability to transmit the infection to another host) and capacity (ability to sustain transmission of a pathogen) depending on the sampling site. Possibly certain hosts act as additional reservoirs. Therefore, a low number or capacity of competent hosts would reduce the probability of rickettsial transmission. A high diversity of hosts, which is probably connected with an increasing proportion of non-competent hosts, is discussed as a reason for lower pathogen prevalence as shown for *Borrelia* spp. (Ostfeld, 2009; Turney et al., 2014; Wood and Lafferty, 2013). However, until now the role of hosts as potential reservoirs for *R. raoultii* is insufficiently investigated (Karbowiak et al., 2016a). Differences in prevalence can also arise from co-feeding. Transmission of *Rickettsia conorii* and *Rickettsia massiliae* via co-feeding has already been proven (Matsumoto et al., 2005; Zemtsova et al., 2010). The chance of successful co-feeding increases with the number of ticks attaching to the same host and hence depends on host and tick density. In contrast, occurrence of zooprophylactic hosts would reduce the transmission of tick-borne pathogens as previously reported for *B. burgdorferi* sensu lato (Richter and Matuschka, 2010).

In contrast to the high frequency of detected *Rickettsia*, no *Babesia* sp. were found in the analysed subset of ticks. This is in line with most of the studies conducted in questing *D. reticulatus* in Germany (Silaghi et al., 2012a; Weis, 2014), West Europe and the western parts of Poland and Slovakia (Bonnet et al., 2013; Cochez et al., 2012; Hofmeester et al., 2016; Król et al., 2016; Kubelová et al., 2011; Nijhof et al., 2007). In Germany (Saarland) the only proven detection of *Babesia* sp. (2.5%) in questing *D. reticulatus* was published by Beelitz et al. (2012). Species identification revealed an infection with *B. canis*. In contrast, reported prevalences are much higher (2.721.6%) in most parts of East and South-East Europe (eastern Poland, south eastern Slovakia, Serbia, Ukraine), predominantly showing DNA sequences identical to *B. canis* (Karbowiak, 2014; Kubelová et al., 2011; Mierzejewska et al., 2015b; Mihaljica et al., 2012; Tomanović et al., 2013; Wójcik-Fatla et al., 2012, 2015). In comparison to these frequencies, *D. reticulatus* currently plays no apparent role as a carrier of *Babesia* spp. in north western Europe. Nevertheless, autochthonous cases of clinical babesiosis in dogs caused by *B. canis* are more and more frequently reported in previously non-endemic areas including Berlin/Brandenburg (Barutzki et al., 2007; Berzina et al., 2013; Halos et al., 2014; Heile et al., 2006; Krücken et al., 2016; Matjila et al., 2005; Øines et al., 2010; Sánchez-Vizcaíno et al., 2016; Schaarschmidt et al., 2013). This suggests that the risk of infection arising from German *D. reticulatus* populations is not sufficiently investigated. Further studies are needed, especially against the background of increasing importance of pet travel and importation of dogs from southern and eastern Europe (Hamel et al., 2011; Naucke, 2011; Pantchev, 2012). Pathogens can possibly establish in local tick populations due to infected animals brought into the country (Földvári et al., 2016).

Analyses of *Anaplasma* sp., *Ehrlichia* sp. and *Ca.* Neoehrlichia mikurensis were conducted by a single PCR-assay revealing an overall prevalence of 0.05%. Sequencing of the positive sample showed only the presence of *A. phagocytophilum* DNA. This is the first detection of *Anaplasma* sp. in questing *D. reticulatus* in Germany so far. Members of the Anaplasmataceae were investigated in *D. reticulatus* samples in a variety of European and Asian studies, predominantly showing no infection of ticks (Barandika et al., 2008; Hornok et al., 2016; Jahfari et al., 2012; Opalińska et al., 2016; Reye et al., 2013; Shpynov et al., 2006; Sixl et al., 2003; Tijsse-Klasen et al., 2013). In a few studies, single *D. reticulatus* ticks were diagnosed as positive for Anaplasma spp. in Latvia, France, Serbia, Hungary and Bosnia-Herzegovina, demonstrating prevalences of 1.35–8% (n = 53–87) (Bonnet et al., 2013; Hodžić et al., 2016; Paulauskas et al., 2012; Szekeres et al., 2015; Tomanović et al., 2013). Ca. Neoehrlichia mikurensis has been described in questing *D. reticulatus* only once in the course of a study in the Havelland (Brandenburg) conducted in parallel to the present investigation (Krücken et al., 2013). In conclusion, *D. reticulatus* does not seem to play an important role in connection with these medically important agents due to the very low prevalences. Furthermore, vector competence has not been proven for any of the species of the family Anaplasmataceae, except for *A. marginale*.

The prevalence of *Borrelia* spp. (0.25%) in the area of Berlin/

Brandenburg was similar to the results (0–0.47%) published by many other German and European studies analysing questing *D. reticulatus* (Barandika et al., 2008; Dobec et al., 2009; Hodžić et al., 2016; Mierzejewska et al., 2015b; Nijhof et al., 2007; Obiegala et al., 2017; Opalińska et al., 2016; Richter et al., 2012). On the other hand comparatively high frequencies of DNA from this pathogen group in the range of 1.5–4.3% (France, Belarus, Poland, western Siberia) or even up to 11.3% (Germany) were occasionally described in certain areas (Bonnet et al., 2013; Kahl et al., 1992; Król et al., 2015; Rar et al., 2005; Reye et al., 2013; Robert-Koch-Institut, 2009; Zając et al., 2017). A few studies carried out analyses to determine the genospecies, only showing infections with agents of the *B. burgdorferi* sensu lato complex. Thus *B. afzelii* was primarily detected (Mierzejewska et al., 2015b; Nijhof et al., 2007; Rar et al., 2005; Reye et al., 2013). In individual cases, *B. garinii* (Rar et al., 2005), *Borrelia valaisiana* und *B. burgdorferi* sensu stricto (Reye et al., 2013) were documented as well. Sequencing of a subset of positive samples analysed here, identified *B. afzelii* (*B. burgdorferi* sensu lato complex) and even *B. miyamotoi* (relapsing fever Borrelia). To the best of the authors' knowledge, this is the first description of *B. miyamotoi* in *D. reticulatus*. Both genospecies are pathogenic to humans. Single cases of human infections caused by *B. miyamotoi* were described in Russia, Japan, the USA, the Netherlands and Germany (Boden et al., 2016; Siński et al., 2016). In comparison, *B. afzelii* is one of the most important tick-borne infectious agents in humans in Europe (Stanek et al., 2012). The pathogenicity of *B. afzelii* and *B. miyamotoi* to animals has not sufficiently been investigated so far (Pantchev et al., 2015). However, *D. reticulatus* is usually not considered to be a competent vector of *Borrelia* sp. but experimental information is only available for a *B. garinii* genospecies. After experimental inoculation of *B. garinii* into *D. reticulatus* motility of the spirochetes rapidly decreased within a few days (Mátlová et al., 1996; Rudolf and Hubálek, 2003). A missing vector competence for various isolates of *B. burgdorferi* sensu lato has also been demonstrated for other *Dermacentor* spp., such as *Dermacentor andersoni*, *Dermacentor silvarum* and *Dermacentor occidentalis* (Dolan et al., 1997; Lane et al., 1994; Sun and Xu, 2003).

5. Conclusion

In contrast to most other important tick species in Germany (excluding *Dermacentor marginatus* in the south west), adult *D. reticulatus* are comparatively active during winter months albeit with high fluctuations in activity. Field collection data suggest an impact of the variable temperature, although further analyses are needed to detect additional potential parameters influencing the tick activity in this time. On the basis of these observations, tick prophylaxis should be recommended in areas with high activity of *D. reticulatus* throughout the year. By analysing both the activity pattern of this tick species and its role as pathogen carrier, a basis to evaluate the risk caused by *D. reticulatus* for transmission of tick-borne diseases was established. The detected prevalences suggest a negligible role of the tick for transmission of the members of Anaplasmataceae, absence or very low prevalence of *B. canis* or *Borrelia* spp. in the investigated populations, and a significant role of *D. reticulatus* as a reservoir of *R. raoultii*. Nevertheless, prevalences have to be considered together with other factors such as tick activity pattern, host preference, vector competence and incidence of clinical cases. No cases of clinical rickettsial infections were described in humans and animals in the study area although one of the highest prevalences that has ever been described in *D. reticulatus* so far was noticed here. In contrast, several cases of autochthonous canine babesiosis were reported in the area Berlin/Brandenburg, despite the fact that no Babesia were found in the present study (Barutzki et al., 2007; Heile et al., 2006; Krücken et al., 2016). Due to the much higher pathogenicity of *B. canis*, rare cases of canine babesiosis are usually recognised due to the clinical implications while many infections with *R. raoultii* are usually inapparent and barely detectable which explains the fact that more cases of babesiosis than of rickettsiosis were reported although prevalences in the vector are much higher for *R. raoultii* than for *B. canis*. Especially with regard to *B. canis* cross-sectional studies are needed to detect potential tick populations being positive for this pathogen. To identify changes in pathogen prevalence, but also in the types of pathogens, which is of particular importance in times of increasing pet travel and import of animals, regular screening of tick populations should be conducted in general. Infected animals such as dogs (Fitzi-Rathgen, 2016; Hamel et al., 2011; Menn et al., 2010; Naucke, 2011) can introduce pathogens into local tick populations and regular surveillance offers a chance to detect such pathogens before larger epidemics are caused by *D. reticulatus*-borne pathogens. This can e.g. be achieved by increasing awareness of local veterinary practitioners and dog owners for recommended tick prophylaxis. For mosquitoborne diseases, such screening programs have been established in recent years (Czajka et al., 2014; Engler et al., 2013; Jöst et al., 2010; Kampen et al., 2017; Scheuch et al., 2018) but tick-borne diseases are still largely neglected in this regard in Germany.

Conflict of interest

Stefan Pachnicke and Klemens Krieger are employees of Bayer Vital GmbH and Bayer Animal Health GmbH, which produce and sell drugs to prevent and treat infestation of animals with ticks.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2018.10.003.

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