**University of Veterinary Medicine Hannover**

Institute for Parasitology

# Competence of the vector restricting tick-borne encephalitis virus spread

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by

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# List of abbreviations

| | |
|---|---|
| % | Percent |
| ++ssRNA | positive-sense single-stranded RNA |
| °C | Degree Celsius |
| µl | Microliter |
| *A.* | *Anaplasma* |
| aa | Amino acids |
| *Ae.* | *Aedes* |
| Arbovirus | Arthropode-borne virus |
| *B.* | *Borrelia* |
| BSL3 | Biosafety level three |
| C | Capsid |
| CHIKV | Chikungunya virus |
| CNS | Central nervous system |
| $CO_2$ | Carbon dioxide |
| *D.* | *Dermacentor* |
| DEET | N,N-diethyl-meta-toluamide |
| DENV | Dengue virus |
| E | Envelope |
| *et al*. | *Et ali*; and others |
| FSMEV | Frühsommer-Meningoenzephalitis-Virus |
| *H.* | *Haemaphysalis* |
| *I.* | *Ixodes* |
| IMD | Immune deficiency |
| IOL | Indian Ocean Lineage |
| JAK-STAT | Janus kinase/signal transducers and activators of transcription |
| JEV | Japanese encephalitis virus |
| M | Membrane |
| MAPK | Mitogen-activated protein kinase |
| min. | Minutes |

| | |
|---|---|
| mm | Milimetre |
| NS | Non-structural proteins |
| prM | Precursor of protein M |
| *R.* | *Rickettsia* |
| RdRp | RNA-dependent RNA-polymerase |
| RH | Relative humidity |
| RNA | Ribonucleic acid |
| RNAi | RNA interference |
| s.l. | *Sensu lato;* in the broad sense |
| spp. | *Species pluralis;* multiple species |
| TBE | Tick-borne encephalitis |
| TBEV | Tick-borne encephalitis virus |
| TBV | Tick-borne viruses |
| VEEV | Venezuelan equine encephalitis virus |
| WNV | West Nile virus |
| YFV | Yellow fever virus |
| ZIKV | Zika virus |

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# Summary


## Competence of the vector restricting tick-borne encephalitis virus spread

Katrin Liebig

The tick-borne encephalitis virus (TBEV) is a flavivirus, circulating between ticks and vertebrates. It is considered a major health risk in Germany with 1028 reported human cases in 2018 and 2019. Risk areas of TBEV are mainly located in the south of Germany, primarily in the states of Bavaria and Baden-Wuerttemberg. In contrast to this focal distribution, the main vector of TBEV in Germany, *Ixodes (I.) ricinus*, as well as host species are spread countrywide, indicating that other factors apart from the presence of suitable vectors and hosts are important for enzootic circulation of TBEV. The current state of knowledge concerning the vector competence of geographically separated *I. ricinus* populations for the TBEV is incomplete. Therefore, experimental infection studies using *Ixodes* ticks of different populations, distributed across Germany, and different TBEV isolates were conducted to investigate possible population-based differences in infection susceptibility. To achieve this goal, an artificial feeding system has been used to infect field collected *I. ricinus* nymphs with different TBEV strains. Over three seasons, the susceptibility to TBEV infection have been analyzed involving different impact factors as seasonality, natural co-infection and the correlation of tick population with a respective TBEV strain. *Ixodes ricinus* nymphs were collected in four TBEV endemic foci as well as one non-endemic area. To investigate specific virus isolate/tick population relationships, ticks were exposed to different virus isolates by *in vitro* feeding and compared regarding their feeding behavior as well as their infection susceptibility for the respective TBEV strains. Differences in the intrinsic susceptibility of the *I. ricinus* tick vector to TBEV infection seem to be related to genetic pairings of vector and virus. Furthermore, infection with *Borrelia* spp. may influence the ability of tick populations to spread TBEV. These findings can help to deepen the understanding of TBEV focal transmission cycle as it is critical for predicting and mitigating human disease risk.

# Zusammenfassung


## Untersuchung einer möglichen vektorbedingten Kompetenzbeeinträchtigung bei der Ausbreitung des Erregers der Frühsommer-Meningoenzephalitis

Katrin Liebig

Das Frühsommer-Meningoenzephalitis-Virus (FSMEV) ist ein zwischen Zecken und Vertebraten zirkulierendes Flavivirus. Mit 1028 gemeldeten Humanfällen in den Jahren 2018 und 2019, wird es als erhebliches Gesundheitsrisiko in Deutschland betrachtet. Risikogebiete von FSMEV sind hauptsächlich in Süddeutschland lokalisiert, vorrangig in Bayern und Baden-Württemberg. Im Gegensatz zu dieser fokalen Verbreitung in Deutschland, kommen der Hauptvektor *Ixodes (I.) ricinus* sowie die Wirtsarten gleichmäßig im Land vor. Dies weist darauf hin, dass neben dem Vorkommen von Vektor und Wirt, noch andere Faktoren für den sylvatischen Zyklus von FSMEV wichtig sind. Der derzeitige Wissensstand bezüglich der Vektorkompetenz von geografisch separierten *I. ricinus* Populationen für FSMEV ist unvollständig. Um mögliche Populations-basierte Unterschiede in der Empfänglichkeit für eine Infektion zu untersuchen, wurden experimentelle Infektionsstudien mit *Ixodes* Zecken aus unterschiedlichen Populationen Deutschlands und verschiedenen FSMEV Isolaten durchgeführt. Ein künstliches Fütterungssystem wurde verwendet, um *I. ricinus* Nymphen aus natürlichen Habitaten mit unterschiedlichen FSMEV Stämmen zu infizieren. Die Empfänglichkeit für eine FSMEV Infektion wurde über drei Saisons analysiert. Dabei wurden unterschiedliche Einflussfaktoren wie die Saisonalität, natürliche Koinfektionen und die Korrelation zwischen Zeckenpopulation mit einem bestimmten FSMEV Stamm einbezogen. Die *I. ricinus* Nymphen wurden in vier FSMEV endemischen und einem FSMEV nicht endemischen Gebiet gesammelt. Um spezifische Beziehungen zwischen Virus und Zeckenpopulationen zu untersuchen, wurden die Zecken mit verschiedenen Virusisolaten über die *in vitro* Fütterung infiziert und hinsichtlich ihres Fütterungsverhaltens sowie ihrer Empfänglichkeit für eine Infektion mit einem bestimmten FSMEV Stammes verglichen. Unterschiede in der spezifischen Empfänglichkeit des *I. ricinus* Zeckenvektors für eine FSMEV Infektion scheinen mit der genetischen Paarung von Vektor und Virus in Verbindung zu stehen. Darüber hinaus könnte eine Infektion mit *Borrelia* spp. die Fähigkeit von Zeckenpopulationen FSMEV zu verbreiten beeinflussen. Diese Erkenntnisse können zu einem besseren Verständnis des fokalen FSMEV Übertragungszyklus beitragen, welches für die Vorhersage und Minderung des Erkrankungsrisikos für den Menschen entscheidend ist.

# Introduction

Worldwide over 500 arboviruses are described (Weaver and Reisen 2010; Gubler 2001) of which at least 38 viral species are transmitted by ticks (Labuda and Nuttall 2004). The ongoing geographical expansion of zoonotic diseases like Chikungunya, Dengue, Yellow fever and Zika virus poses a considerable global health threat. Besides mosquitos, ticks are the most important vectors for transmitting arboviruses (de la Fuente *et al*. 2008). Due to globalization and climate change, new arthropod vector species are spread and introduced in formerly non-endemic regions. Consequently, zoonotic diseases can emerge in new geographic regions and the risk of infections with zoonotic pathogens rises. At the same time, the list of tick-borne pathogens which affect humans and animals is constantly growing (de la Fuente *et al*. 2008; Dantas-Torres, Chomel, and Otranto 2012; Rizzoli *et al*. 2014). The tick-borne encephalitis virus (TBEV) is a zoonotic flavivirus which is maintained in a transmission cycle involving small rodents and ticks. The provoked disease is endemic in many countries of Eurasia. With up to 10,000 to 15,000 cases per year in Europe and Asia it poses an important threat to public health (Dobler 2010). In Germany, 1028 human cases were reported in 2018 and 2019, with a higher number of unrecorded cases (RKI 2020).

## Tick-borne encephalitis (TBE)

The tick-borne encephalitis (TBE) is a viral central nervous system (CNS) disease which is transmitted via an arthropod tick vector. In contrast to other important tick-borne diseases, like the Borreliosis, there is no antiviral therapy for TBE. An infection with TBEV can only be treated supportively and symptomatically and prevented by vaccination. In humans, a major proportion of TBEV cases (approximately 70-98%) remain asymptomatic. Following an incubation period of around 4-28 days (Mickienė *et al*. 2002; Kaiser 2008), in around 70% of the cases, unspecific symptoms of a summer flu like fever, headache, catarrhal and gastrointestinal problems occur (Kaiser 1999). The incubation period after infection via the alimentary route is comparably shorter. The disease is described as biphasic. In 74-85% of symptomatic with the European subtype, the patients pass a biphasic course of infection (Haglund and Günther 2003). The first stage of infection occurs in the skin. Virus particles infect the Langerhans cells, replicate and use them as vehicles to the lymph nodes (Labuda *et al*. 1996). Within lymphatic organs, lymphocytes and macrophages get infected resulting in an inhibition of the first immune response. After the first stage, patients enter the asymptomatic phase which last for around 8 days (Kaiser 1999). Between 20-30% of the cases develop the second stage of disease involving the nervous system with symptoms of meningitis (50%), meningoencephalitis (40%) and

myelitis (10%) (Kaiser 2008; Růžek, Dobler, and Mantke 2010). The most severe course of illness is described for the Far-Eastern subtype (lethality around 15-20%, in some cases up to 60%).

In contrast, the European subtype is less severe (1-2% lethality rate). Infection with Siberian subtype results in 6-8% lethality but might lead to chronical disease (Chrdle, Chmelík, and Růžek 2016; Burke and Monath 2001; Barrett 2004). Although higher tick exposure and shorter climbing distance to a suitable biting side pose good conditions for tick infestation, TBEV infections in children seem to be less frequently reported as in adults. Contrary, *Borrelia* infections in young children are five times more common than in older children or adults (Hansson *et al*. 2011). It is assumed that childhood TBE is underdiagnosed. Overall, the clinical course of TBEV is milder in children (Hansson *et al*. 2011; Kaiser 1999; Logar *et al*. 2000; Holmgren and Forsgren 1990). In 70% of reported cases, clinical course proceed biphasic, with a flu-like prodrome, an asymptomatic phase following a varying degree of meningitis to meningoencephalitis (Logar *et al*. 2000; Fritsch *et al*. 2008; Lesnicar *et al*. 2003; Krbková, Štroblová, and Bednářová 2015).

## Tick-borne encephalitis virus (TBEV)

The causative agent of TBE is the tick-borne encephalitis virus. Because of its vectorial transmission via ticks, it is classified as arthropod-borne virus (arbovirus). As a lot of other arboviruses as Zika virus (ZIKV), Dengue virus (DENV), West Nile virus (WNV), and Japanese encephalitis virus (JEV) (Grard *et al*. 2007; Heinze, Gould, and Forrester 2012; Moureau *et al*. 2015; Gaunt *et al*. 2001), TBEV belongs to the family *Flaviridae.* The genus *Flavivirus* comprises over 70 species (Gould and Solomon 2008). Viruses of this genus are small, icosahedral, enveloped particles (~50 nm). The tick-borne encephalitis virus is a positive-sense single-stranded RNA (+ssRNA) virus. The viral genome encodes for one open reading frame which is transcribed to a large polyprotein of around 3400 amino acids (aa).

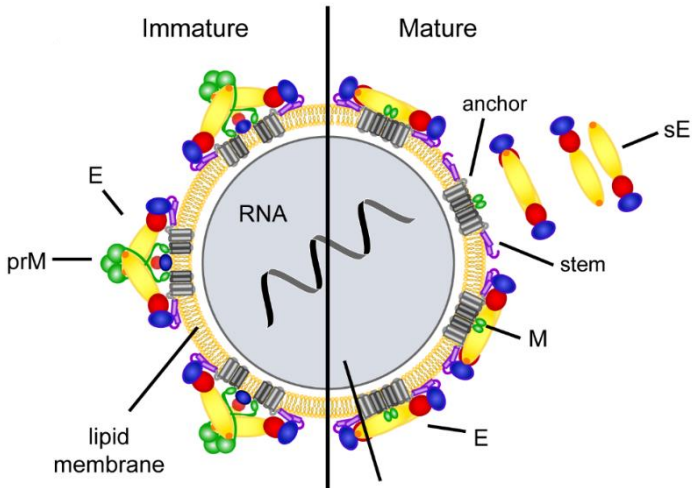


Figure 1 Schematic model of a flavivirus particle (Vratskikh *et al.* 2013).
The left panel show the immature virion, the right panel the mature virion.

The virus has three structure proteins (C=capsid, M=membrane and E=envelope) (Figure1) as well as seven non-structure proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). The RNA is encapsidated by the capsid protein (C) and a lipid bilayer containing the membrane protein (M) and two membrane associated envelope glycoproteins (E). Protein E is responsible for interactions with entry receptors at the plasma membrane of target cells. It mediates viral membrane fusion and is an important determinant for virulence. The host immune response following infection or immunization is targeted against the glycoprotein E (Aberle *et al*. 1999). Protein M plays only a minor role in the mature virus particle. While the pre-membrane protein (prM), its larger precursor protein, acts as chaperone for correct folding of E during its biosynthesis (Heinz and Allison 2001). The role of protein C is poorly understood. As it is rich in basic amino acid residues, it has been proposed that it is involved in the packaging process of viral RNA (Kofler, Heinz, and Mandl 2002). So far, the virus can be distinguished into five genetic subtypes: the European, the Siberian, the Far-Eastern, the Baikalian and the Himalayan (TBEV-Eu, TBEV-Sib, TBEV-FE, TBEV-Bkl, TBEV-Him, respectively) (Figure 2) (Bogovic and Strle 2015; Taba *et al*. 2017; Grard *et al*. 2007; Heinze, Gould, and Forrester 2012; Dai *et al*. 2018; Kovalev and Mukhacheva 2017).

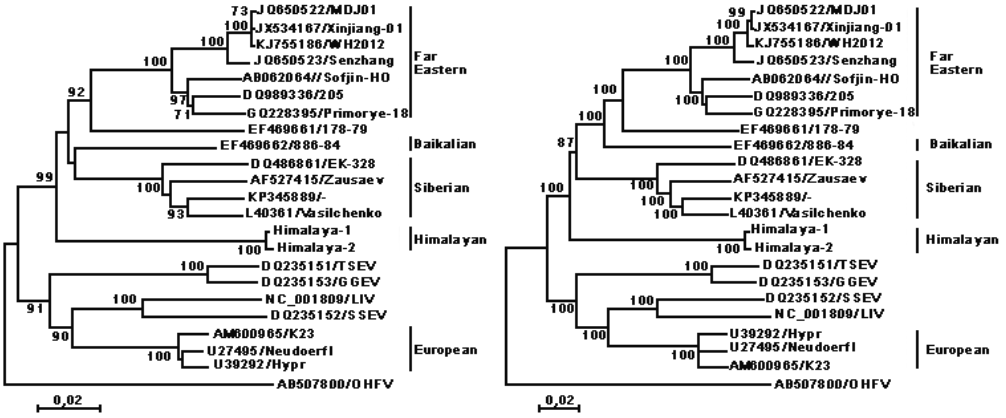


Figure 2 Phylogenetic tree of TBEV (Dai *et al.* 2018), modified. On the left side, the protein E. On the right side, the polyprotein. Both phylogenetic trees were constructed using MEGA 6.0 with neighbor-joining method (1000 bootstrap replications). Bootstrap values (>70%) are shown at the branches. Scale bar below indicates the nucleotide substitutions per site.

In the polyprotein, the subtypes TBEV-Eu, TBEV-Sib and TBEV-FE show difference in the nucleotide sequences (14.6–16.5%) and amino acid sequences (5.0–6.8%) (Dai *et al*. 2018). Within TBEV-Eu observed variation does not exceed 1.8% (Dobler, Erber, and Schmitt 2018). The recently detected subtype TBEV-Him show 82.6–84.6% nucleotide identities and 92.7–95.0% amino acid identities in the E protein and 83.5–85.2% nucleotide identities and 92.6–94.2% amino acids identities for the polyprotein with other three subtypes with other three subtypes (Dai *et al*. 2018). The mortality of TBE varies depending on the viral subtype. TBEV-Eu is associated with a 1-2% mortality rate, TBEV-Sib infections end fatal in 6-8%, whereas TBEV-FE cases have a mortality rate up to 60% (Chrdle, Chmelík, and Růžek 2016; Barrett 2004; Burke and Monath 2001).

Since the recently detected subtype TBEV-Bkl is likely to originate directly from the Far-Eastern subtype, outcome of infection may result in similar mortality rates, but up to now there are no information available.

## Distribution

Worldwide, TBE is endemic in 27 European countries (Amicizia *et al*. 2013). Because of its very severe outcome after infection, 17 countries classified TBE as notifiable disease (Stefanoff *et al*. 2011). During an expedition in Far-East Russia, Lev Zilber discovered the viral disease in 1937. First isolation of the virus could be done in 1948 by Gallia *et al*. (Gritsun, Lashkevich, and Gould 2003). Based on their geographical distribution, five subtypes are known which can be distinguished by their genetic structure. The European subtype (TBEV-Eu) is found in Europe and also western parts of Siberia and the Urals. The Siberian subtype (TBEV-Sib) is found in Siberia, while the Far-Eastern subtype (TBEV-FE) occurs in far-eastern Asia, in the Baltics, as well as in central and eastern Siberia (Dobler *et al*. 2012). Recently two new genetic variances were detected. The Himalyan subtype (TBEV-Him) in Qinghai-Tibet Plateau in China (Dai *et al*. 2018), as well as the Baikailian subtype (TBEV-Bkl) which is considered to be an individual TBEV subtype of TBEV-Sib (Kovalev and Mukhacheva 2017). In the main, distribution of TBEV is linked to the geographical expansion of the vector competent tick species. *Ixodes ricinus* is predominantly in Western Europe, while *I. persulcatus* is the predominant species in East Europe, Russia and Asia (Kaiser 2016). In Russia and Poland, *Dermacentor (D.)* spp. and *Haemaphysalis (H.)* spp. were reported to be competent to transmit TBEV (Gritsun, Lashkevich, and Gould 2003). Virus isolation of field collected *D. reticulatus* demonstrated the role in TBEV transmission in Germany (Chitimia-Dobler, Lemhöfer, *et al*. 2019).

In 2012, TBEV was included in the list of diseases under surveillance by the European Union. The most affected regions in Europe are southern Germany, Austria, the Czech Republic, Slovenia, Baltic States and southern parts of Scandinavia (Chrdle, Chmelík, and Růžek 2016). About half of all reported cases occur in Russia (Chrdle, Chmelík, and Růžek 2016). Starting in 1973, observation of cases showed an increase of 400% (Süss 2008). In addition, TBEV cases have been also reported in countries which were not considered as endemic. In 2019, TBEV was found in the Netherlands as well as in Great Britain (Dekker *et al*. 2019; Kreusch *et al*. 2019). This spread seem to lead back to increased recreational activities in areas inhabited by infected ticks, climate change that affects tick habitats and improvements in the diagnosis and reporting of TBE cases (Kollaritsch *et al*. 2011; Jaenson *et al*. 2012). In Germany, TBEV risk areas are solely based on human case numbers. Concerning that TBEV foci can span a range as small as 500 square meters in size (Dobler *et al*. 2011), district-level risk assessment may not adequately reflect risk of TBE at a smaller scale (RKI 2014).

## Transmission

The transmission of tick-borne pathogens comprises a complex network involving competent vector as well as host species. In the epidemiological cycle, TBEV is maintained involving tick vectors and wild vertebrate like small rodents, birds and larger mammals as hosts (Gritsun, Lashkevich, and Gould 2003; Charrel *et al*. 2004). By transmitting and maintaining the virus within a focus, ticks play a major role as vectors and virus reservoirs. In the natural transmission cycle, infection occurs via blood meal when ticks feed on small rodent species. With onset of tick feeding, TBEV is transmitted via infected tick saliva or blood. Furthermore, transovarial transmission is suggested to be a key factor for preserving TBEV foci (Nuttall *et al*. 1994). For the European subtype, *Apodemus* spp. act as an important host in transmission cycle. Especially for *I. ricinus* larvae and nymphs, *Apodemus flavicollis* and *Apodemus sylvaticus* are meaningful host species (Kožuch *et al*. 1967; Achazi *et al*. 2011; Pintér *et al*. 2014). Moreover, *Microtus spp.* (Achazi *et al*. 2011; Kožuch *et al*. 1967), *Sciurus vulgaris* (Hubálek and Rudolf 2012; Kožuch *et al*. 1967) and *Myodes glareolus* (Kožuch *et al*. 1967; Achazi *et al*. 2011; Burri *et al*. 2012; Pintér *et al*. 2014; Zöldi *et al*. 2015) are rodent species playing an important role as TBEV reservoirs. Between small rodent generations, TBEV can be transmitted vertically (Bakhvalova *et al*. 2009). Most of the bird species which are described to be sentinels for TBEV are members of the order Passeriformes (Imhoff *et al*. 2015; Hubálek and Rudolf 2012). Regarding arthropod vectors, there are a variety of tick species which are known to be vector competent for TBEV. Under experimental settings, it is demonstrated that at least 22 tick species are able to carry the virus (Hayasaka *et al*. 2001; Süss 2003; Chitimia-Dobler, Mackenstedt, *et al*. 2019). The complete vector competence of these species for TBEV is, so far, not proven. For the European subtype, *I. ricinus* act as main vector, whereas the Siberian and the Far-Eastern subtypes are mainly transmitted by *I. persulcatus* (Clarke 1964). Apart from *Ixodes* spp., *D. marginatus* and *D. reticulatus* (Hoogstraal 1966; Kozuch and Nosek 1971; Nosek 1972), *H. concinna* (Kozuch 1980; Khazova and Iastrebov 2001), *H. inermis* (Nosek *et al*. 1972), *H. punctate* (Hubálek 1989) are known vectors for TBEV. Ticks can transmit the virus transstadially (from one tick stage to the next stage) (Jaenson *et al*. 2012), transovarially (via eggs from an infected female to its offspring), viraemically (via feeding on an infectious vertebrate host) and also non-viraemically (between co-feeding ticks) (Havlikova, Lickova, and Klempa 2013) (Figure 3).

In Europe, wild cervids like roe deer (*Capreolus capreolus*) pose an important host for ticks (Hofmeester *et al*. 2017). It could be shown that TBEV infection in ticks is negatively correlated with deer density. The number of co-feeding ticks observed on rodents, was on the contrary positively associated with TBEV infection in ticks and rodents. This phenomenon is described as the 'dilution effect hypothesis'. Deer, which is a dead-end host for TBEV, because of insufficient levels of viremia, diverts ticks from TBEV competent rodent hosts (Cagnacci *et al*. 2012; Bolzoni *et al*. 2012).

However, co-feeding is a key factor in maintenance of TBEV foci and does not require that the host develop a detectable viremia (Labuda *et al*. 1993; Havlikova, Lickova, and Klempa 2013). Nevertheless, deer population density correlates with tick prevalence as deer pose an optimal host for adult tick stage providing sufficient blood meal for questing ticks. Thus, greater deer population leading to a high number of ticks which positively affects TBEV circulation (Jaenson *et al*. 2018). Compared to small rodents, which are in case of TBEV short-living reservoirs with a maximum life span of 18-20 months for *Apodemus sylvaticus* and 18 months for *Myodes glareolus* (Macdonald and Barrett 1993), the multiple life stages of the tick vector favor a long persistence of TBEV within the natural foci up to years (Charrel *et al*. 2004). Regarding behavioral and physiological characteristics of ticks, the virus has adapted well to its vector, especially to blood feeding, blood meal digestion and moulting (Nuttall *et al*. 1994). After blood feeding, it could be shown that viral replication in ticks is enhanced (Belova, Burenkova, and Karganova 2012; Belova *et al*. 2017; Kopáček *et al*. 2018; Slovák *et al*. 2014). Besides the infection via tick vector, TBEV transmission can occur by alimentary route. Consumption of TBEV infected dairy products of cattle, sheep and goats (Holzmann *et al*. 2009; Caini *et al*. 2012) can cause severe symptoms of illness like seen in many cases in the last years. Infections could be traced back to consumption of unpasteurized milk (Balogh *et al*. 2012; Hudopisk *et al*. 2013; Cisak *et al*. 2010; Kerlik *et al*. 2018; Monika Emilia *et al*. 2019; Brockmann *et al*. 2018; Casati Pagani *et al*. 2019). A proven method to inactivate TBEV in milk is the pasteurization technique (Balogh *et al*. 2012).

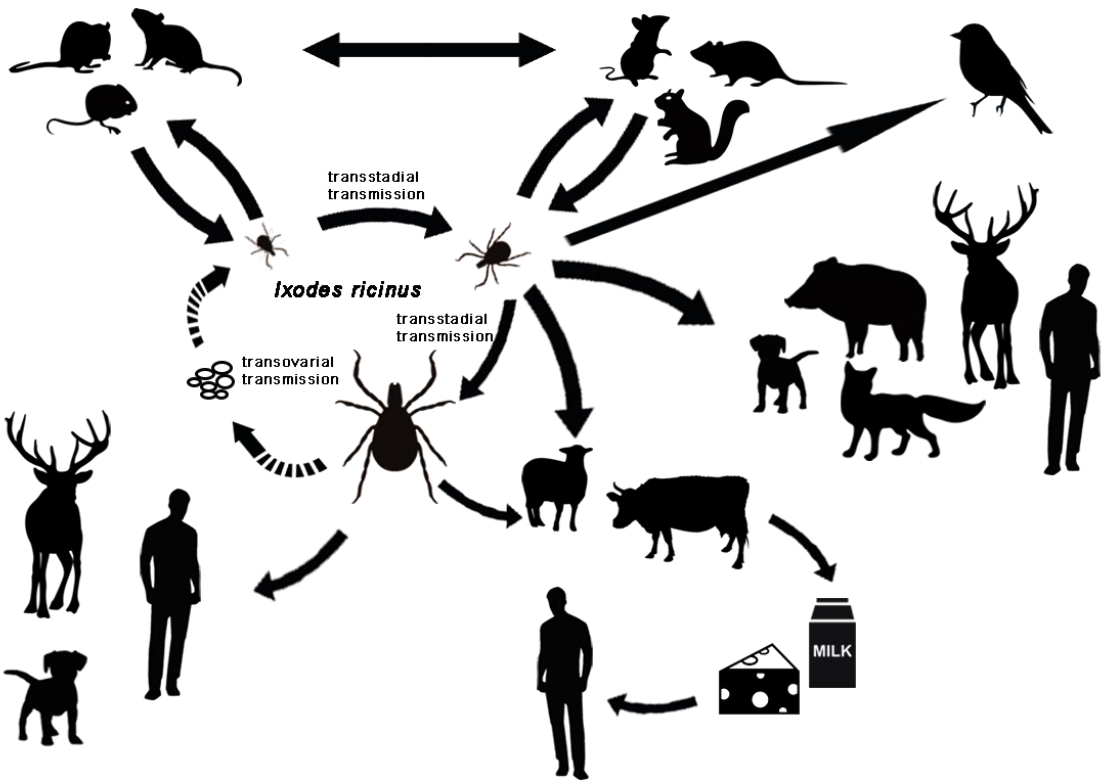


Figure 3 TBEV transmission and *I. ricinus* life cycle. Designed with resources of Freepik.com and vecteezy.com

### *Ixodes ricinus*

The castor bean tick, *I. ricinus* (Linné 1758), is a parasitic member of the class Arachnida belonging to the phylum Arthropoda. Together with mites, they form the subclass Acari. Within the Acari, *I. ricinus* belong to the order Ixodida and the family of Ixodidae (Mehlhorn and Piekarski 1981). Ticks of the genus *Ixodes* transmit a range of zoonotic bacteria, protozoa and viruses that are of concern for veterinary and human public health. *Ixodes ricinus* is known to be a vector for TBEV (TBEV-Eu), *Borrelia* (*B.) burgdorferi* s.l., *Anaplasma (A.) phagocytophilum, Rickettsia (R.) helvetica, R. monacenis*, *Babesia divergens* and *Babesia microti* (Rizzoli *et al*. 2014).

## Life cycle

Due to their hematophagous life cycle, Ixodid ticks require a blood meal to progress through larval, nymphal and adult stage. Depending on factors like climate conditions and the availability of hosts, the completion of the *Ixodes* life cycle requires between two to six years (Liebisch and Liebisch 2003). The three active life stages: larvae, nymph and adult can be discriminated by the number of legs. Larvae have three pairs of legs whereas nymphs and adults have four pairs of legs. Additionally, nymphs and larvae are lacking a genital aperture (Walker *et al*. 2003). During host-seeking, *I. ricinus* actively climbing the stems of grasses. By adopting a 'questing' position in the form of extended front legs, they are ready to grasp a passing host. To recognize the presence of hosts by detecting butanoic acid, carbon dioxide and changes in temperature, ticks use a special sensory organ, the Haller's organ, located on the tarsus of the first pair of legs (Sonenshine and Roe 2013). *Ixodes ricinus* undergoes a three-host life cycle, in which each developmental stage prefers to feed on a different range of hosts (Hillyard 1996) (Figure 3). Larvae favor small rodents, birds or lizards (Dsouli *et al*. 2006), whereas nymphs prefer small to medium sized rodents and also humans. The most important rodent host species for both are the wood mouse (*Apodemus sylvaticus*), the yellow-necked mouse (*Apodemus flavicollis*) and the bank vole (*Myodes glareolus*) (Matuschka *et al*. 1991). Adult ticks usually feed on larger mammals like roe deer, fallow deer, wild boar and ruminants (cattle, sheep, goats). Additionally, humans can serve as host for each life stage (Sonenshine, Lane, and Nicholson 2002). After finding a host, tick may crawl about several hours on the host in search for a suitable attachment site (Waladde and Rice 1982). Feeding areas are often located near the ears, neck or groin area (Lees 1948). At the beginning of attachment, ticks create an entrance wound by inserting the chelicerae into the epidermis. Then the hypostome is pushed into the wound. Detachment during blood meal is prevented through the hypostome's barbs as well as a cement plug, which is created by the tick.

Directly with onset of feeding, ticks inject saliva that contains a mixture of bioactive substances. To facilitate blood meal and suppress host inflammatory response, they produce anticoagulants, histamine binders and cytokine inhibitors.

Injection of local narcotics enable blood feeding for a long period, without disturbing the host, allowing pathogens to be transmitted from ticks to hosts and inversely (Nuttall and Labuda 2008). Copulation of *I. ricinus* takes place on the host. After feeding, larvae and nymphs detach and undergo a molting process on the ground (Oliver Jr 1989) while the female prepares for oviposition. A clutch can have up to 10,000 eggs (Pfister, 2006). Tick activity is dependent on biotic and abiotic factors such as abundance of suitable hosts, temperature and precipitation. Activity peaks for *I. ricinus* are described in spring and autumn (Hillyard 1996). Peak activity of single life stages can overlap which is a crucial factor for the ecology of tick-borne pathogens. Simultaneous presence of different life stages on some rodents is important for the natural maintenance of TBEV (Randolph *et al*. 1999; Matuschka *et al*. 1991; Mihalca *et al*. 2012). To synchronize their life cycle with the seasons, ticks are able to block steps in their development. This phenomenon is termed diapause and results in a delay of embryogenesis, metamorphosis from larvae and nymphs or oogenesis of engorged females (Belozerov 1964). By decreasing the metabolism level, ticks can resist unfavorable conditions (Belozerov 1982).

## Spread

The distribution of *I. ricinus* extends from Europe to Ireland and to the Urals, and from northern Sweden to North Africa (Estrada-Peña 2001; Medlock *et al*. 2013). Their habitats across Europe vary, but typically include rough pasture, heathland, deciduous and coniferous forests, moorland and urban parks (Medlock *et al*. 2013). *Ixodes ricinus* is very vulnerable to climatic changes especially for desiccation. For survival during off-host periods, *I. ricinus* requires specific climatic conditions such as at least 80% humidity. Therefore, this species is restricted to vegetation that holds a high humidity and areas of moderate to high rainfall (Gassner *et al*. 2011; Milne 1949). Higher annual temperature and milder winters are responsible for expansion of the geographical spread of *I. ricinus* northwards and also in higher altitudes (Soleng *et al*. 2018; Smura *et al*. 2019; Jääskeläinen *et al*. 2016; TäLleklint and Jaenson 1998). Additionally, *I. ricinus* has the capacity to adapt to biotopes with high altitudes and low temperature (Jaenson *et al*. 2012).

## Vector competence

The term vector competence comprises traits that are directly associated with the interaction between vector and pathogen. It also involves the infection susceptibility of the arthropod with the pathogen. Furthermore, vector competence describes the ability of the arthropod to transmit the pathogen to a suitable host as well as to its offspring (Tabachnick 1994). Motivated through enhanced importance in zoonotic disease transmission, research on vector competence has attracted increasing interest. So, recognition of the link between intraspecies variability and factors associated with vector competence have involved new questions in terms of vectorial disease transmission. Different factors which might influence the vector competence of ticks have been involved in a variety of studies. Host abundance and host species composition, climate change, anthropogenic factors as land use and enhanced outdoor activities are mentioned in this context (Rizzoli *et al*. 2019). Moreover, biological traits as the co-infection status, colonization with symbionts, the immune response to infection as well as the genetic background of vector and pathogen need to be implemented in vector competence research. Tick-borne viruses (TBV), which are absorbed with blood meal, first infects midgut wall cells. The gut barrier needs to be overcome which means viruses have to withstand the heterophagic bloodmeal digestion of ticks. After this phase, TBV infect the salivary glands to be transmitted with the next blood meal. This requires surviving the molting process by infecting at least one type of cells which do not undergo histolysis. By targeting a high number of tick tissues, TBV developed strategies to endure molting. In this way, viral infection can persist the whole life span of the tick (Nuttall *et al*. 1994). Tick-borne viruses like TBEV have adapted to biological characteristics of their tick vector as shown by nonviremic transmission. Through saliva-activated transmission, the virus can be transmitted from infected to uninfected ticks which are co-feeding on the same host. Thus, the virus do not necessarily need to develop a high viremia in the natural vertebrate host to be transmitted (Nuttall *et al*. 1994). Rapidly and continuously changing environmental conditions require a high plasticity of viruses. Especially arboviruses, which need to adapt to both arthropod vectors and vertebrate hosts, are under high selection pressure. More specific, viruses need to adapt to population-specific differences, especially regarding genetics. Subtypes of TBEV are characterized by their host and vector specificity and the clinical manifestation. Adaptive selection has driven the genetic diversification among the five subtypes (Li, Wang, and Du 2019). *In vitro* experiments with Langat virus revealed an association between genetic determinants with host-specific adaptation and pathogenicity (Mitzel *et al*. 2008). Virus variants showed adaptations to replication in mouse (MNBp20) and tick (ISEp20) cell lines. Amino acid exchanges occurred in adaption to MNBp20 in E, NS4A and NS4B and in M, NS3 and NS4A in adaptation to ISEp20. Results suggest a role for E, M, NS3, NS4A and NS4B in host adaptation of tick-borne flaviviruses. In mosquito vectors, different studies investigated the influence of virus-vector

interactions. The impact of Chikungunya virus (CHIKV) genetics have been shown in the detection of the *Aedes (Ae.) albopictus* adaptive substitution A226V in the E1 envelope glycoprotein which increased virus fitness within the vector (Tsetsarkin *et al*. 2007; Vazeille *et al*. 2007; Schuffenecker *et al*. 2006). Vector adaptive evolution has also been described for DENV (Armstrong and Rico-Hesse 2001; Cologna, Armstrong, and Rico-Hesse 2005) and Venezuelan equine encephalitis virus (VEEV) involving a substitution of the E2 glycoprotein (Brault *et al*. 2004). Enhanced initial infection of *A. albopictus* midguts could be shown the IOL (Indian Ocean Lineage) CHIKV by revealing mutations of E2 and E3 (Tsetsarkin *et al*. 2014; Tsetsarkin and Weaver 2011). Genetic diversity of viruses is characterized by positive selection of mutants which brings phenotypic advantages (Coffey, Failloux, and Weaver 2014). It is assumed that instead of individual mutations alone, multiple mutations act cooperatively to modulate virus replication and pathogenesis (Davis *et al*. 2007).

## *In vitro* feeding

Systems for feeding parasitic arthropods under *in vitro* conditions offer great possibilities to study a wide range of research topics. By using the tick bite as the natural infection route, vector competence studies including infection susceptibility and transmission efficiency on the side of vector, pathogenicity and virulence on the side of pathogens, as well as genetic adaptations in frame of infection can be conducted. Furthermore, artificial feeding allows the reduction of host animals for blood sucking arthropods that goes in line with the concept of 3R principle (replacement, reduction, refinement) of laboratory animals. First artificial feeding experiments with ticks were carried out over hundred years ago. A broad range of experiments based on natural membranes, such as skins of mice (Doube and Kemp 1979), rats (Hindle and Merriman 1912), gerbils (Bonnet *et al*. 2007) and rabbits (Howarth and Hokama 1983) have been conducted. Although these membranes are treatable with antibiotics (Bonnet *et al*. 2007), the incomplete sterilization favoring contamination and rot of the membranes (Howarth and Hokama 1983; Totze 1933). Artificial silicone membranes were designed, which bring further properties such as thermal resistance and an antimicrobial base. Due to the flexible structure, these membranes resemble host skin and can be customized to the hypostome length of different tick species and developmental stages. While feeding, the silicone membrane separates the tick and the blood unit. For infection studies with TBEV, the artificial feeding system needs to fit the requirements of a BSL3 (biosafety level 3) laboratory. Former TBEV infection studies with artificially fed *Ixodes* ticks do not exist. Therefore, adaption of the system of Kröber and Guerin (Kröber and Guerin 2007) have been conducted in form of higher safety precautions (Liebig 2017). Tick and blood containment are both completely sealed up through a screw connection. Additionally, two rubber seals prevent leakage of blood as well as escaping of TBEV infected ticks.

# Aim of the study

As main vector for TBEV in Germany, *I. ricinus* poses a threat for veterinary and public health. Habitats of vector and hosts are evenly distributed in whole Germany. Under these circumstances, the question arises why TBE foci are spread that unevenly countrywide. Despite many years of research in the field of TBE, the current state of knowledge concerning the vector competence of *I. ricinus* for TBEV is incomplete. Therefore, this study aimed to examine population-based differences in infection susceptibility for TBEV among *I. ricinus* nymphs. To explain the spatial distribution of TBE, it is essential to understand the interaction of tick vector and virus. To achieve this goal, ticks of different populations distributed across Germany were infected with different TBEV strains which were isolated of ticks collected in the same areas. For this purpose, a silicone membrane based artificial feeding system has been adapted to the safety requirements of a BSL3 laboratory. This study compared the feeding behavior of different *I. ricinus* populations in addition to susceptibility for infection with TBEV. To understand the influence of different factors on the outcome of TBEV infection susceptibility, the correlation of season, year, tick origin and natural co-infection were included in the study.

# Publications

## Tick populations from endemic and non-endemic areas in Germany show differential susceptibility to TBEV.

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## Abstract

Tick-borne encephalitis virus (TBEV) is endemic in twenty-seven European countries, transmitted via the bite of an infected tick. TBEV is the causative agent of one of the most important viral diseases of the central nervous system (CNS). In Germany, 1028 human cases were registered between the years 2018-2019. The castor bean tick, *Ixodes ricinus*, is the TBEV vector with the highest importance in Central Europe, including Germany. Despite the nationwide distribution of this tick species, risk areas of TBEV are largely located in Southern Germany. To increase our understanding of TBEV-tick interactions, we collected ticks from different areas within Germany (Haselmühl/Bavaria, Hanover/Lower Saxony) and infected them via an *in vitro* feeding system. A TBEV isolate was obtained from an endemic focus in Haselmühl. In two experimental series conducted in 2018 and 2019, ticks sampled in Haselmühl (TBEV focus) showed higher artificial feeding rates, as well as higher TBEV infections rates than ticks from the non-endemic area (Hanover). Other than the tick origin, year and month of the infection experiment as well as co-infection with *Borrelia* spp., had a significant impact on TBEV Haselmühl infection rates. Taken together, these findings suggest that a specific adaptation of the tick populations to their respective TBEV virus isolates or vice versa, leads to higher TBEV infection rates in those ticks. Furthermore, co-infection with other tick-borne pathogens such as *Borrelia* spp. can lower TBEV infection rates in specific populations.

## The stable matching problem in TBEV enzootic circulation: the importance of the perfect tick-virus match.

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## Abstract

Tick-borne encephalitis virus (TBEV), like other arthropod-transmitted viruses, depends on specific vectors to complete its enzootic cycle. It has been long known that *Ixodes ricinus* ticks constitute the main vector for TBEV in Europe. In contrast to the wide distribution of the TBEV vector, the occurrence of TBEV transmission is focal and often restricted to a small parcel of land, whereas surrounding areas with seemingly similar habitat parameters are free of TBEV. Thus, the question arises which factors shape this focal distribution of TBEV in the natural habitat. To shed light on factors driving TBEV-focus formation, we used tick populations from two TBEV-foci in Lower Saxony and two TBEV-foci from Bavaria with their respective virus isolates as a showcase to analyze the impact of specific virus isolate-tick population relationships. We found an association between the virus isolates and the tick population for each TBEV-focus with a 1.85-14.50 fold higher odd of getting infected with the synonymous TBEV isolate as compared to the non-synonymous but genetically closely related TBEV isolate. In addition, median viral RNA copy numbers are 40-112 times higher in the synonymous virus-tick population pairings. These results indicate a co-evolutionary adaptation of virus and tick population and might help to explain the focal distribution of TBEV circulation.

## Introduction

Arthropod-borne viruses (arboviruses) are maintained in nature by cycling between hematophagous arthropod vectors and vertebrate hosts. Most of the arboviruses belong to the *Bunyaviriales*, *Flaviviridae*, *Togaviridae* and *Reoviridae*, which all use RNA to code their genomic information. To succeed in dynamic host environments, especially in the case of arboviruses including two very distinct hosts, viruses need a high genetic plasticity. With an estimated range from $10^{-3}$ to $10^{-5}$ errors / nucleotide / round of replication, the RNA-dependent RNA-polymerase (RdRp) has a high error rate leading to a typical pool of viral sequence variations granting genetic plasticity and fast adaptation of RNA viruses [1,2]. As a member of the *Flaviviridae*, TBEV belongs to the RNA viruses. An infection with TBEV can result in an infection of the central nervous system in humans [3]and animals [4]. TBEV is distributed in many European countries [5,6] and the number of annual reported cases has steadily increased in the past years [7] making TBE one of the most severe arthropod-borne diseases in Germany.

TBEV is transmitted by *Ixodes ricinus* (Linnaeus, 1758) ticks in Central Europe including Germany. Although *I. ricinus* can be found all over the country, TBEV risk areas are mainly found in southern parts of Germany. Furthermore, TBEV risk areas are spatially localized, and fit with the concept of a natural focus. The natural focus is the central, crucial concept of Pavlovsky's theory [8] with a pathogen

circulation in nature independent of human presence and infection with the exception that the human is a dead-end host for the pathogen. TBEV natural foci are usually very small covering only 5000 square meters [9], thus the question arises which parameters define their borders. In addition to the spatial restriction, viral sequences in such TBEV-foci are stable over decades [10]. Considering the high mutation rates of RNA viruses, this is a remarkable characteristic of TBEV indicating a selective pressure for specific genomic sequences of the virus. Almost nothing is known about the interaction of *I. ricinus* with TBEV and the factors shaping TBEV and tick population genetics in a TBEV-focus. However, co-evolution of virus strain and tick population could have driven specific selection of tick and virus genetic markers. Such sequence-based differences are known to affect the outcome of an arbovirus infection and depend on a particular pairing of vector and virus genotypes [11]. In *in vitro* experiments have shown the adaption of virus and vector by demonstrating that growth of TBEV on tick vector cell lines is 100 to 1000-fold higher as in non-vector cell lines [12]. Furthermore, the impact of environmental variations on ticks' vector competence such as the microclimate [13] as well as the coincidence of host and tick population densities [14,15,16] have been demonstrated. Consequently, the outcome of infection seems to be a genotype-genotype-environment complex [17]. To understand this complex, different vector-virus interaction components such as genetic adaption of both, virus and vector, need to be investigated. To do so, we chose two virus isolates and tick population pairings from TBEV-foci in Germany. Two of the selected foci are located in close proximity to each other in Bavaria (Haselmühl/Heselbach) and a similar pairing of foci was recently discovered in Lower Saxony (Barsinghausen/Mooshütte and Rauher Busch [18]). The genetic analysis of selected TBEV isolates from different endemic foci showed exchanges of 10 amino acids (aa) for the TBEV-foci Barsinghausen/Mooshütte and Rauher Busch and 19 aa difference for the TBEV-foci Haselmühl and Heselbach. We tested the susceptibility of the respective *I. ricinus* populations from each TBEV focus for the infection with the synonymous virus isolate or the genetically closely related non-synonymous virus isolate to uncover potential correlations between virus isolate and infection success in different tick populations.

## Materials and Methods

### Tick sampling and maintenance

Questing *I. ricinus* nymphs were collected April-June 2020 by flagging the low vegetation at different TBEV endemic foci in Lower Saxony (Barsinghausen N 52°31`, E 9°39` and Rauher Busch N 52°53`, E 8°87`) as well as in Bavaria (Haselmühl N 49°41`, E 11° 87`and Heselbach N 49°32`E 12°15`). Nymphs of the TBEV endemic foci in Bavaria were sent to the laboratories of the Research Center for Emerging

Infections and Zoonosis (University of Veterinary Medicine Hanover) in falcon tubes with fresh grass to maintain a humid environment. Immediately after receiving, ticks were stored at 4°C for 3-7 days until experiments started. Ticks were retrieved from fridge half a day before starting of *in vitro* feeding to provide time for acclimatization. Ticks were identified by morphological classification and kept in an incubator with a $CO_2$ content of 5%, a relative humidity of about 80% and a temperature of 34°C during the *in vitro* feeding. After *in vitro* feeding, ticks were maintained for 7 days at room temperature (21°C) with 95% relative humidity and a 16/8 light/dark photoperiod.

### Virus cultivation

Four different TBEV isolates of the European subtype were used for this study. Each virus isolate was obtained from ticks sampled in the respective TBEV-focus. Two strains were isolated from *I. ricinus* ticks collected in TBEV-foci in Lower Saxony [18]. The other strains of Bavarian TBEV-foci were kindly provided by the Bundeswehr Institute of Microbiology (Munich, Germany). Regarding virus passage, second passage of TBEV P51 and P19, and first passage of TBEV 303/16 and HB171 was used for *in vitro* infection of ticks. TBEV isolates were cultivated on A549 cells (ATCC® CCL-185™). Cells were grown in MEM (Thermo Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin, Pan Biotech; Aidenbach, Germany; gentamicin/amphotericin, Thermo Fisher, Waltham, MA, USA) and maintained at 37°C under 5% $CO_2$. Cells were inoculated with 100 µL aliquots of TBEV-RNA positive tick homogenate (diluted 1:10 MEM). After 1 h incubation at 37°C and 5% $CO_2$, unabsorbed virus and potential toxic substances from the tick supernatants were removed by rinsing cells three times with sterile PBS. The infected cells were overlaid with 10 mL of MEM supplemented with 2% FBS and antibiotics (penicillin/streptomycin, Pan Biotech; Aidenbach, Germany; gentamicin/amphotericin Thermo Fisher, Waltham, MA, USA). Virus stock titration was performed by serial dilutions and 50 percent endpoint dilution according to Reed & Muench [19] and stored at −150°C.

### *In vitro* feeding

Artificial feeding was done as described in Liebig *et al*. [20]. In brief, an upper tick unit consisting of a glass tube in which one end covered with a silicone membrane is placed into a blood unit consisted of a plastic container. Each blood unit was filled with 5mL of sterile, heparinized bovine blood (Fiebig Nährstofftechnik, Idstein, Germany) supplemented with 4 g/L D-(+)-glucose monohydrate (Sigma-Aldrich, Munich, Germany) and 1 mM adenosine triphosphate and $1x10^6$ PFU/mL of the respective virus strains. During artificial feeding, blood was changed twice a day with a maximum time interval of 14 hours due to the low stability of TBEV in blood to ensure a constant virus titre. Ticks were left in the feeding unit for five days (day -5 to day 0) and at day 0 engorged ticks were removed from the

membrane, cleaned by immersion in 1% hydrogen peroxide and PBS and transferred to fresh glass tubes for further incubation. At time of collection, most ticks were fully engorged. Ticks were then incubated for 7 days prior to PCR analysis, further referred to as day 7 (dpi).

### PCR

Seven days post infection (dpi) ticks were homogenized in 500 µL cell culture medium (Leibowitz L-15 or MEM Eagle, Thermo Scientific, Waltham, MA, USA) using stainless steel beads (3mm) (Isometall, Pleidelsheim ,Germany) and TissueLyser II (Qiagen, Hilden, Germany) at 20 Hz, 2 min. and 3 repetitions. Tick homogenates were clarified by centrifugation, and total RNA was extracted from 140 µL supernatant using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Samples were tested for the presence of TBEV RNA by a quantitative RT-PCR (qRT-PCR) assay and TBEV-specific primers [21]. A standard curve was created using serial dilutions from TBEV RNA of Austrian Neudoerfl strain (U27495.1), RNase-free water served as a negative control. Each sample was run in duplicate, and the data were analysed using AriaMx software version 1.5 (Agilent Technologies, California, USA).

### Statistical methods

TBEV positive rates between ticks from synonymous and non-synonymous areas were compared using Fisher's exact test, virus load between these areas were compares using the Mann-Whitney U test. All comparisons were performed separately for research areas in Lower-Saxony and Bavaria within the statistics software R (version 4.0.2, www.r-project.org). The significance level was set to $\alpha$=0.05 for all tests.

## Results

A total of 1458 *I. ricinus* nymphs were collected by flagging the vegetation in different TBEV endemic foci in Lower Saxony (Barsinghausen/Rauher Busch) as well as two foci in Bavaria (Haselmühl/Heselbach). Nymphs collected in April 2020 (Barsinghausen, n=444; Rauher Busch, n=500), in May 2020 (Haselmühl, n=141; Heselbach, n=158) and in June 2020 (Haselmühl, n=113; Heselbach, n=102) were subjected to *in vitro* feeding with bovine blood spiked with 1 x $10^6$ PFU/mL of the respective TBEV isolate. The ticks from the foci Barsinghausen/Rauher Busch were fed either with blood containing TBEV isolate P51 (Barsinghausen) or P19 (Rauher Busch), and ticks from the TBEV-foci Haselmühl/Heselbach were fed with blood containing either TBEV isolate 303/16 (Haselmühl) or HB171 (Heselbach). The feeding rates (number of engorged ticks divided by the total number of ticks tested) were calculated for April, May and June 2020 (Supplementary Table 1). In April 2020, ticks from

Barsinghausen had higher feeding activity (Barsinghausen 24% (105/444); Rauher Busch 20% (101/500)). In May 2020, higher tick feeding could be observed in nymphs of Haselmühl (Haselmühl 32% (45/141); Heselbach 26% (41/158)).

Analysis of 405 engorged nymphs for TBEV RNA revealed that 90.83% of tested ticks were positive for viral RNA. Maximum infection rates were observed for ticks from TBEV focus Heselbach, infected with the isolate HB171 in May (100%; 27/27) as well as in June (100%; 14/14) and for ticks from Haselmühl with the TBEV isolate 303/16 from Haselmühl in June (100%; 7/7) (Supplementary Table 1). Infected ticks harbor between 9 and $4.4x10^7$ TBEV RNA copy numbers per tick with a median of $2,37x10^3$ TBEV RNA copies per tick over all groups. Highest copy numbers were found in ticks from the sampling area Barsinghausen infected with the synonymous TBEV isolate P51 ($4.6x10^7$ TBEV RNA copies/tick) and the lowest copy number of nine TBEV RNA copies per tick were found in a tick from Haselmühl infected with the non-synonymous TBEV isolate HB171.

To analyze the impact of virus isolate-tick population pairings, we correlated the infection success of TBEV isolates with the respective tick origin; synonymous virus isolate *versus* non-synonymous virus isolate. This analysis was performed separately for the TBEV foci in Lower Saxony and Bavaria. The analysis revealed a higher probability for a tick population to be infected with the synonymous TBEV isolate as compared to a closely related non-synonymous TBEV isolate. The odds of being infected with the synonymous TBEV isolate were 1.85 (p=0.7204) for ticks from Lower Saxony and 14.50 (p=0.0014) for ticks from Bavaria (Table 1).

Table 1 Comparison of TBEV positive rates between ticks with synonymous and non-synonymous pairing, separately for TBEV areas Lower-Saxony and Bavaria. The p-values were calculated by Fisher's exact test.

| **TBEV area** | **Pairing** | **TBEV positive Ticks (%)** | **TBEV negative Ticks (%)** | **Odds ratio** | **95%-CI** | **p-value** |
|---|---|---|---|---|---|---|
| Lower-Saxony | Synonymous | 61 (97%) | 2 (3%) | 1.85 | [0.33, 18.80] | 0.7204 |
| | Non-synonymous | 115 (94%) | 7 (6%) | | | |
| Bavaria | Synonymous | 67 (99%) | 1 (1%) | 14.50 | [2.00, 641.66] | 0.0014 |
| | Non-synonymous | 50 (82%) | 11 (18%) | | | |

Next, we analyzed, if the efficiency of the replication of the TBEV isolates is correlated with the respective tick origin. Mean viral RNA copy numbers of the synonymous and non-synonymous virus-tick population parings were plotted against each other for Lower Saxony and Bavaria (Figure 1)

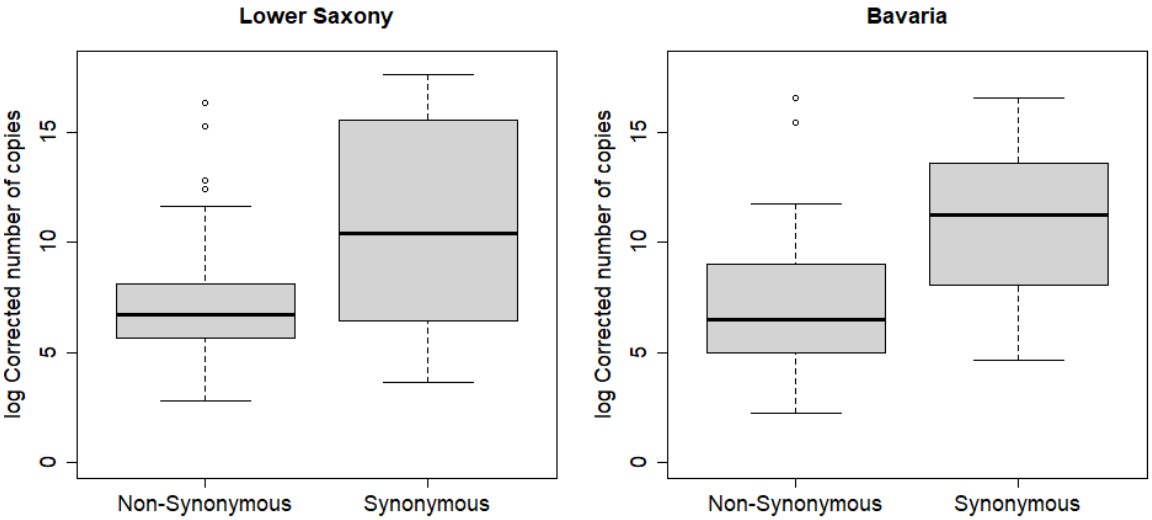


Figure 1: Virus loads measured in synonymous and non-synonymous virus-tick population pairings. TBEV RNA copies were measured by qPCR and compared for each federal state separately. Box plots showing viral RNA copy numbers were created using R and median TBEV RNA copies and Range (Table 2) are illustrated.

Median RNA copy numbers were 842 and 679 TBEV RNA copies/tick for ticks from Lower Saxony and Bavaria, respectively, infected with the non-synonymous TBEV isolate. In contrast, infection with the synonymous TBEV isolate led to significantly higher RNA copy numbers of $3.4x10^4$ ($p< 0.01$) and $7.6x10^4$ ($p<0.01$) TBEV RNA copies/tick in ticks from Lower Saxony and Bavaria, respectively (Table 2).

Table 2: Analysis of synonymous and non-synonymous pairing of TBEV isolate and tick population on TBEV RNA copy numbers per infected tick separately for Lower-Saxony and Bavaria. The p-values were calculated by the Mann-Whitney U test.

| Country | Virus | Median | Minimum, Maximum | p-value |
|---|---|---|---|---|
| Lower Saxony | Non-synonymous<br>synonymous | 842<br>34.200 | 16, 12.400.000<br>38, 46.100.000 | < 0.01 |
| Bavaria | Non-synonymous<br>synonymous | 679<br>76.100 | 9, 15.500.000<br>103, 15.500.000 | < 0.01 |

## Discussion

Besides mosquitoes, ticks are the most important arthropod vectors of human pathogenic diseases. In contrast to their importance, tick-virus interactions are still sparsely understood. To understand the genetic impact of TBEV isolate and tick populations for TBEV enzootic cycles in Germany, we analyzed the relationships between TBEV isolate and tick population for two different TBEV-foci in Bavaria and

Lower Saxony. The TBEV foci were located in close proximity to each other: Barsinghausen *versus* Rauher Busch 35 km beeline and Haselmühl *versus* Heselbach 27 km beeline. However, the virus isolates from Lower-Saxony Rauher Busch P19 and Barsinghausen P51, although being phylogenetically more closely related to each other than to other German isolates, show 10 aa exchanges [18]. A similar relationship is true for the virus isolates from Haselmühl and Heselbach, which exhibit 19 aa exchanges. This degree of diversity is on the lower level of TBEV diversity. For example, Kupča *et al*. [22] describe the relationship of the isolate AS33 and Salem showing 251 nucleotide differences resulting in 26 aa exchanges between those two strains. In general, TBEV sequences are conserved compared to other members of the *Flaviviridae* with only 1.8% variation based on E-gene sequences compared to 6% natural observed variation for Dengue virus, 7% for West Nile Nile-virus and 5% for Yellow fever virus (YFV) [23]. Changes in virus's genetics, as the exchange or deletion of aa can have a significant impact on virus infection, replication and dissemination. For example, the YFV isolates YFV-17D and YFV-DAK differed in their ability to overcome the midgut barrier in *Aedes (Ae.) aegypti* mosquitoes [24] and even more specific, one mutation at the position 226 on the Chikungunya virus E1 glycoprotein (E1-A226V) enhances the transmission in *Ae. albopictus* mosquitoes [25]. Regarding TBEV, Mitzel *et al*. [26] showed that besides the key role in host tropism the E, M, NS3, NS4A and NS4B protein might act as viral determinants for host-specific replication. We found one aa difference between Barsinghausen and Rauher Busch in the E, NS2A, and NS4B sequences, two aa difference in NS3, and five variations in the NS5 sequence, respectively. Regarding the TBEV isolates Heselbach and Haselmühl, we found one aa difference in the C, E, prM, NS2B and NS5 proteins, two differences in the NS4B and four variations in NS1, NS2A and NS3 proteins. None of the variation was identical between the Bavaria and Lower Saxonian strains. However, they might still be located in the same functional domain or affect similar protein functions.

To study the impact of virus sequence differences on infection success in different tick populations, we analyzed the infection rates, TBEV RNA copy numbers for Barsinghausen *versus* Rauher Busch and Haselmühl *versus* Heselbach using an artificial membrane based feeding system, and nymphal stage ticks. We chose nymphs because of two reasons, their high abundance in nature and the important role of infected adult stages for human infections. Studies have shown that infection rates of adult ticks are 5-10 time higher than in nymphs [27,28]. In addition, adult tick stages prefer larger mammalian hosts including humans. Consequently, adult tick stages, infected as nymphs, pose a high risk for TBEV infection in humans. Thus, the infection success of TBEV in nymphal stage can be directly correlated with the risk for TBE. To exclude that pre-existing TBEV infection of ticks from TBEV-foci used for infection experiments influence the results, we had established in a previous study that blood meals do not increase the prevalence of TBEV above the described 0.1-5% [20].

The feeding rates in our study were highly similar between the different study groups, which was to be expected for ticks originating from sampling spots located in close proximity to each other with similar climatic conditions and habitat parameters. However, in this study ticks from Bavaria and Lower Saxony did not show different feeding rates. This observation stands in contrast to our previous study which observed significantly different feeding rates for ticks from different federal states in Germany [20]. This might be due to the reduced sampling scheme, only analyzing ticks from three months as compared to the previous study, which included two consecutive years from April to October. Furthermore, in our first study we compared a TBEV-focus (Haselmühl) with a non-endemic area (Hannover), whereas in this study we only included TBEV-foci from different federal states. Thus, it needs further clarification if ticks from TBEV-foci generally show higher feeding rates irrespective of their geographical origin, or if the relationship between Hannover and Haselmühl specific. Nevertheless, we observed highly similar overall feeding rates in 2020 compared to 2019 (2019=29.38%; 2020=23.5%). Interestingly, feeding rates in 2018 (40.25%) were significantly higher than both following year, indicating that the exceptionally warm weather in 2018 led to enhanced feeding of ticks.

In contrast to the moderate feeding success, the infection rates were exceptionally high in 2020. Of the 405 analyzed ticks, 90.83% were tested positive for TBEV RNA as compared to 38.38% TBEV positive samples in 2018/19 [20]. Analysis of mean viral copy numbers showed also an increase of TBEV RNA loads as compared to our previous study with $1.40x10^6$ in 2020 *versus* $4.81 \times 10^3$ TBEV RNA copies per tick over all experiments in 2018/19. These copy numbers lie well above theoretical values derived from artificial detection of input RNA ($2x10^2$ RNA copies per sample), indicating that the high infection rates are attributed to replication of the virus rather than residual input RNA. The high infection and replication rates shown in this study, if reflected by ticks in natural TBE-foci, might have consequences for the risk of TBEV infection in autumn 2020 and spring 2021 when nymphs from April-June 2020 emerge to adult stage. In this regard, the 17 percent increase in cases for TBE in 2020 (654) over the TBE record year 2018 (558) is especially alarming [29].

Next, we analyzed if the probability of a TBEV infection after artificial feeding is linked to an adaption of TBEV isolate and tick population. Interestingly, we found a correlation of virus-isolate and tick population favoring the synonymous combination. The odds for an infection with the synonymous virus isolate were 1.85 fold higher for the tick populations from Lower Saxony and even 14.50 fold higher for ticks from the Bavarian TBEV-foci (Table 1). Furthermore, the comparison of viral RNA loads in synonymous *versus* non-synonymous virus-tick populations pairing showed significantly higher mean TBEV RNA copy numbers in the synonymous pairing for both, tick populations from Lower Saxony and Bavaria (Figure 1; Table 2). These observations are in line with our hypothesis that co-

evolutionary factors between a TBEV isolate and tick population in a TBE natural focus could be responsible for higher TBEV susceptibility in ticks of the respective TBEV endemic area. Furthermore, the "older" TBEV foci in Bavaria show a stronger correlation of TBEV isolate and tick population (infection rates and viral RNA loads) than the more recent ones in Lower Saxony (only viral RNA loads significantly different), which also fits with the higher genetic divergence of the Bavarian TBEV isolates compared to the isolates from Lower Saxony. These observations are unique for tick-transmitted viruses but are well in line with similar studies conducted in mosquitoes. Intraspecies genetic differences and their influence on vector competence have been explored in *Culex pipiens* complex mosquitoes showing differential susceptibility of *Cx. pipiens* biotype pipiens populations in Germany for West Nile virus [30], and Ae. albopictus populations showing population cluster specific dissemination and transmission efficiencies [31]. Similarly, *Ae. aegypti* susceptibility for dengue-2 virus is linked to yet undetected quantitative trait loci [32] and competence for chikungunya virus transmission is also dependent on mosquito genetics [33].

Factors relevant for those population-specific differences are not clear but it is rational to assume differences in intrinsic infection barriers as one cause for population specific differences in vector competence. Specifically, the midgut and salivary gland barrier may play a crucial role for the development of virus infection in the tick and the transmission of the virus by the tick. Thus far, analysis of the role of the midgut barrier have been conducted in *Amblyomma (A.) variegatum* and *Rhipicephalus (R.) appendiculatus* for Dugbe virus (*Nairovirus*, *Bunyavirales*) [34]. These experiments showed that infection via feeding is possible in vector ticks *(A. variegatum)* and leads to transstadial transmission of the virus, which is not the case in non-vector ticks such as *R. appendiculatus*. This indicates that the midgut barrier may not only determine if an infection is established, but also block transstadial transmission of a virus. It has been shown that TBEV needs to replicate in the lining of the tick midgut where it disseminates to the haemolymph and subsequently infect other tissues reaching the highest titers in the salivary glands and reproductive organs epithelium. The significantly higher viral RNA loads in our synonymous virus-tick population pairings might facilitate the virus escape from the midgut and the spread to other organs and thus influence the probability of the transstadial transmission of the virus, which would influence the probability of this tick to transmit TBEV to the next host.

## Conclusion

In conclusion, our study provides first evidence for a virus isolate-tick population relationship that could be responsible for the focal distribution of TBEV transmission. Which genetic factors in ticks and viruses shape this relationship remains to be further investigated. This information could help to predict novel TBEV-foci and by this improve public health management.

## Supplementary material

**Table 1**. Feeding rates of *I. ricinus* nymphs after *in vitro* feeding and infection with TBEV 2018 and 2019.

| | **2018** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **April** | | **May** | | **June** | | **July** | |
| **Tick origin** | **Feeding rate (%)** | **p value** | **Feeding rate (%)** | **p value** | **Feeding rate (%)** | **p value** | **Feeding rate (%)** | **p value** |
| **Haselmühl** | 5.33 | p< 0.001 | 43.81 | $1.84 \times 10^{-6}$ | 58.24 | 0.9967 | 20.00 | 0.1209 |
| **Hanover** | 28.00 | | 19.44 | | 43.01 | | 38.57 | |
| | **2018** | | | | | | | |
| | **August** | | **October** | | | | | |
| **Tick origin** | **Feeding rate (%)** | **p value** | **Feeding rate (%)** | **p value** | | | | |
| **Haselmühl** | 33.13 | p< 0.0001 | 68.04 | 0.0830 | | | | |
| **Hanover** | 78.08 | | 51.11 | | | | | |
| | **2019** | | | | | | | |
| | **April** | | **May** | | **June** | | **July** | |
| **Tick origin** | **Feeding rate (%)** | **p value** | **Feeding rate (%)** | **p value** | **Feeding rate (%)** | **p value** | **Feeding rate (%)** | **p value** |
| **Haselmühl** | 82.66 | $5.20 \times 10^{-8}$ | 47.33 | $4 \times 10^{-15}$ | 8.00 | 0.1176 | 15.83 | 0.6841 |
| **Hanover** | 53.33 | | 0 | | 2.00 | | 26.00 | |

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# Material and Methods

## *In vitro* feeding system

The *in vitro* feeding system used in this study was modified according to the protocol of Kröber and Guerin (Kröber and Guerin 2007). To meet the safety requirements of an BSL3 laboratory, further components have been included in the design. The upper unit is constructed of a glass tube of which the bottom side is covered with a silicone membrane and the upper side with a plug for drosophila cultivation tubes 28 x 25 mm (Carl Roth, Karlsruhe, Germany). The blood unit consists of a plastic container (wide-mouth straight-sided PPCO jars with closure, Carl Roth, Karlsruhe, Germany). A seal of two rubber rings and a screw connection connects the two units and prevent leakage of infectious blood. The single components of the feeding system are shown in Figure 4.

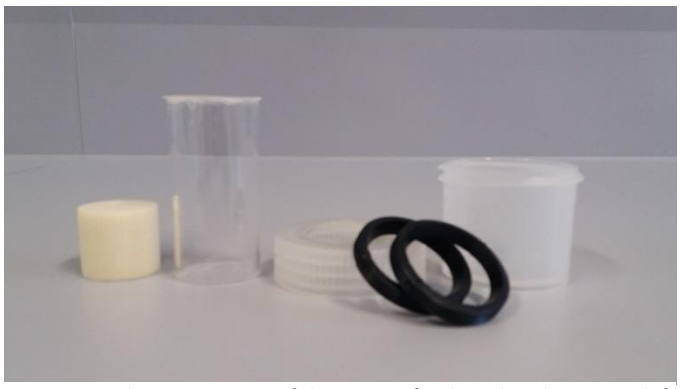

Figure 4 Single components of the *in vitro* feeding chamber. From left to right: plug for drosophila cultivation tubes, glass tube with silicone membrane (inverted, membrane at the top), screw cap, rubber rings and plastic container for blood.

Figure 5 Fully assembled *in vitro* feeding chamber, containing bovine blood.

## Tick dissection

For dissection of *I. ricinus* nymphs, ticks' body were cut with a sterile surgical blade to separate gnathosoma and idiosoma (Figure 6). Using a TissueLyser II (Qiagen, Hilden, Germany) with 20Hz, two min. and three repetitions, body parts were homogenized using stainless steel beads (3mm) (Isometall, Pleidelsheim ,Germany) in 500µl cell culture medium (Leibowitz L-15 or MEM-Eagle, Thermo Scientific, Waltham, MA, USA).

Figure 6 Schematic dissection of *I. ricinus*

# Discussion

The tick-borne encephalitis virus, a human-pathogenic member of the family *Flaviviridae*, causes one of the most dangerous neurodegenerative diseases in humans in Europe and Asia. By its way of transmission via blood-feeding ticks, it is classified in the group of arboviruses and the cause for a significant public health problem in Germany. The risk of infection is rising, due to increased TBEV spread in higher altitudes and to the north of Europe (Soleng *et al*. 2018; Smura *et al*. 2019; Jääskeläinen *et al*. 2016; TäLleklint and Jaenson 1998). Reasons for this ongoing distribution are intensively discussed but key factors shaping TBEV foci are poorly investigated. A wide variety of tick species are known to be competent for carrying TBEV (Hayasaka *et al*. 2001; Süss 2003; Chitimia-Dobler, Mackenstedt, *et al*. 2019). If these species are all able to transmit the virus is unknown. In Germany, the virus subtype TBEV-Eu is transmitted mainly via the tick species *I. ricinus*. Human TBEV cases have increased dramatically in the last years (Süss 2008; Hellenbrand *et al*. 2019). In Germany, reported TBEV cases increased from 839 cases in 2016 and 2017 to 1028 cases in 2018 and 2019 (RKI 2020). In addition, human infection via the alimentary route become more frequent. Enhanced risk for TBEV infection in areas which were, so far, classified as non-endemic regions, requires a well thought out risk management. This includes vaccination as well as education of public in way of behavior recommendations in tick habitats. Risk assessment of TBE in Germany is conducted on base of districts. According to the definition of the Robert-Koch-Institute in Germany, a district is declared as a risk area if the number of recorded TBEV cases within a five year period is significantly higher as the expected number of cases by an incidence of 1 case per 100,000 inhabitants. In Germany, TBEV risk areas are mainly found in Bavaria, Baden-Wuerttemberg, South Hesse, southeast Thuringia and Saxony. In total, currently 164 districts are defined as TBEV risk area. Single TBEV cases in federal states without risk areas are observed more frequently in the past years. As districts vary extremely in size and TBEV foci can be very small (Dobler *et al*. 2011), the TBEV prevalence can vary strong within a TBEV risk area. Observed prevalence of TBEV infected ticks within a focus is 0.1-5% (Süss 2003). Therefore, also districts which are not defined as risk area but exhibit proven TBEV occurrence need to be implied in risk management. Otherwise infection risk for public will be underestimated.

Due to increasing spread of vectors, research on arboviruses are gaining high importance. Despite a variety of studies on tick-borne pathogens and their vectors, there is no deeper knowledge on the vector competence of *I. ricinus* for TBEV. Especially the uneven distribution of the virus in contrast to the even distribution of the vector and reservoirs cannot be explained with current knowledge on TBEV transmission cycles. Understanding which factors are crucial for successful TBEV transmission between host and vector and *vice versa*, is necessary for comprehension of the spatial occurrence of TBEV foci in Germany. A variety of parameters have been implied as critical variable in previous studies.

This includes loss of biodiversity which is leading to changes in host-parasite relationships, climate change as well as anthropogenic factors including land use and enhanced outdoor activities (Rizzoli *et al*. 2019).

The nymphal stage of *I. ricinus* occur in high numbers in nature and have a comparable low host specificity. Therefore, their importance in TBEV transmission is ranked very high (Süss 2003) representing a suitable model for infection studies. We took advantage from the progress in *in vitro* feeding systems for ticks, which have been improved due many studies in the last decades. Artificial feeding systems constitute a helpful tool for research on infectious agents, as the feeding process can be simulated and conducted under constant laboratory settings. The first critical step in pathogen transmission, is the interaction of the tick and the vertebrate host. Pathogens can be transmitted via host blood or tick saliva. As host-seeking and searching for a suitable attachment site pose the first steps in vectorial pathogen transmission, we compared the feeding performance of tick populations collected in different parts of Germany. For infection studies, we examined ticks from the TBEV focus Haselmühl and the TBEV non-endemic area Hanover and analysed potential differences between both sampling spots. For exophilic tick species, such as *I. ricinus,* humidity and temperature play an essential role for host-seeking behaviour (Sonenshine 2018). Concerning this, ticks were hold in an $CO_2$ incubator during feeding. We sought to avoid any variation in temperature, humidity and $CO_2$ level, so we kept parameters constant with respectively 34°C, 90% RH and 5% $CO_2$. Nevertheless, ticks were collected in the field, consequently exposed to environmental conditions beforehand. Although, ticks were sampled at the same time, high variations in feeding rates could be observed between the populations. To include variety in climate conditions of the different tick habitats, we compared temperature and precipitation of the weather stations with the closet proximity to the tick sampling spots. Over the months and the two years, we could observe high variability in feeding rates. Additionally, differences between the groups were significant. Feeding activity of ticks of the TBEV focus Haselmühl were higher in May and June. Contrary, ticks of TBEV non-endemic area Hanover showed higher feeding rates in July and August. Evaluation of measured mean temperature of the two regions, showed no difference. But comparing the mean precipitation over months and years showed large variations between the two sampling spots. In six out of ten months, higher precipitation in the tick habitat resulted in higher feeding activity during *in vitro* infection experiments.

For host-seeking, ticks need to leave their sheltered habitat in the ground litter and climb to the tips of grass (Lees and Milne 1951). Long exposure to low humidity can lead to critical dehydrating of the tick, forcing it to return to the base of the vegetation (Lees 1948). Repeated ascending and descending efforts a high use of energy which consequently results in quick consumption of all available resources (Rosendale *et al*. 2017). Difference in feeding rates between the groups could be detected which might

result of temperature and humidity variations in the two sampling locations as these parameters may affect ticks' fitness and, therefore, the feeding success. Similar results could be observed for the incidence of Lyme disease in the US during nymphal questing and dry weather conditions at the same time (Burtis *et al*. 2016). Nevertheless, also other reasons might be responsible for differences in feeding rates between the groups. In some months, like April and June 2019 for ticks of Haselmühl as well as August 2018 and July 2019 for ticks of Hanover, higher tick feeding results did not match with higher precipitation at this location. Thus, other factors implied in tick biology traits need to be considered.

As ticks, subjected to *in vitro* infection studies, where collected via flagging method, we could ensure that these nymphs were questing. A full life cycle of *I. ricinus* can span several seasons, dependent on external conditions as host abundance and climate (Liebisch and Liebisch 2003). Ticks were collected in natural habitats, defining age and fitness level to unpredictable parameters. A prolonged questing period, which persist over one season can result in low energy level. Even though ticks were questing, the physiological age was unknown. There are a variety of studies on the prediction of ticks physiological age (Uspensky 1995; Balashov Iu, Grigor'eva, and Leonovich 2009; Balashov Iu and Grigor'eva 2010; Pool *et al*. 2017). The tick's lipid content constitutes a measurable parameter and is proposed as an index of the biological age. In case of infection experiments, this method cannot be implemented as ticks need to be sacrificed. As time point of moulting to nymphal stage also remained unknown, it is impossible to assess if nymphs were in the suitable age to feed on a host. Since ticks can synchronize their life cycles with the environment, they are able to overcome unfavourable external conditions. Diapause, as form of blocking metabolism steps, can lead to shifted moulting period. Nymphs collected in April, May and June most likely moulted in autumn of the previous year. In contrast, ticks collected in July, August and October either moulted in the previous year or freshly in the respective year (Gray *et al*. 2016). However, it is hard to prove which tick moulted the previous year and which freshly moulted in the respective year. At least, the uncertainties of physiological age might explain the generally lower feeding activity in July of both years which cannot be traced back to differing precipitation levels. Remarkably is the detection of higher feeding activity of ticks collected in the TBEV focus Haselmühl. Ticks were more active between April and June, which is exactly the period where most of the TBEV transmission events occurred, based on the TBEV case reports (ECDC 2012). Looking at the effects of higher tick feeding activity in nature, tick aggressiveness may end in higher infection risk for humans as it may enhance the chance to locate and infect a passing host. There are less explanations on the feeding behavior of *Ixodes* ticks, but some studies found a correlation between physiological age and tick aggressiveness. *Ixodes persulcatus* adults indicate a higher aggressiveness of physiologically older ticks (Uspensky 1995). With differences in feeding performance, the question

arises if TBEV infection does lead to changes in vector behavior. There are only a few studies which are dealing with tick behavior after pathogen ingestion. Compared to field-collected ticks in TBEV endemic areas, ticks removed from animals or humans showed higher TBEV prevalence. This could be shown in Germany for *I. ricinus* (Süss *et al*. 2004) and in Russia for *I. persulcatus* and *I. pavlovskyi* (Mel'nikova, Botvinkin, and Danchinova 1997; Romanenko and Kondrat'eva 2011). These findings are explained by two hypotheses which are both supported by data provided by Belova *et al*. (Belova, Burenkova, and Karganova 2012). First, feeding is boosting the already present TBEV titer in unfed ticks to a detectable concentration. Second, TBEV infection is assumed to enhance host-seeking activity and tick mobility (Benelli 2020). Infected *I. ricinus* adults, trying to reach a bait, are more active and also tolerant against to exposure with growing concentrations of the repellent N,N-diethyl-meta-toluamide (DEET) than uninfected ticks (Belova, Burenkova, and Karganova 2012). About 6% of the TBEV infected ticks could overcome a DEET concentration of 1% whereas none of the uninfected ticks did. The second hypothesis was demonstrated for TBEV infected *I. persulcatus* reaching higher questing height compared to uninfected conspecifics (Alekseev 1996; Romashchenko *et al*. 2012). Moreover, *I. persulcatus* females showed higher mobility both in terms of moving speed and trajectory length when striving to reach a bait (Alekseev, Burenkova, and Chunikhin 1988). Even if *in vitro* experiments could demonstrate higher activity in TBEV infected ticks, TBEV prevalence of questing ticks, collected in the endemic areas, is on a very low level, which makes that aspect neglectable for infection studies (Boelke *et al*. 2019; Dobler *et al*. 2011; Süss 2003). For deeper understanding of population-based differences in feeding behavior, comparison of the genetic background of the respective populations regarding energy metabolism might be an exciting step forward.

Next to vectors feeding behaviour, vector competence, as described as the ability to acquire an infection and transmit the pathogen to a suitable host, is essential in the transmission cycle of TBEV. Knowledge on factors affecting *I. ricinus* competence for TBEV are on a low level. In former studies, *I. ricinus* was proven to be a competent vector for *Rickettsia (R.) monacensis* (Ye *et al*. 2014), *Powassan virus* (Costero and Grayson 1996) and *B. burgdorferi* s.l. (Eisen 2020). Notable results could be generated in studies concerning the vector competence of *Ixodes* for *Borrelia*, showing the dependency of population and species (Eisen 2020; Couper *et al*. 2020). So far, no studies have examined the vector competence of the tick species *I. ricinus* for TBEV in a natural infection model. Understanding the discrepancy between vector and virus spread requires the detection of key factors which are responsible for ticks' vector competence. Thus, the objective of the first study was a comparison of tick populations from TBEV endemic and non-endemic areas. We aimed to analyze potential differences in TBEV infection susceptibility in ticks of the TBEV focus Haselmühl and the TBEV non-endemic area Hanover. The results of infection experiments between Haselmühl and Hanover of

2018 and 2019 were, except from the months August 2018 and April 2019, rarely significant. Nevertheless, complete analysis showed a trend that TBEV infection susceptibility is higher in ticks of the TBEV focus Haselmühl. Compared to ticks of the non-endemic area Hanover, the odds of getting infected with TBEV via *in vitro* feeding are 2.3 fold higher for a tick from Haselmühl. These results demonstrate that there are variances between tick populations which are, up to now, not understood. Our hypothesis that co-evolution of certain TBEV strains and tick populations is responsible for infection susceptibility is supported by further studies showing that genetic determinants influence vector feeding preferences and vector competence. Gerardi *et al*. compared the ITS2 and 16S rRNA genes among different *Amblyomma sculptum* populations after *R. Rickettsia* infection. Highest infection susceptibility was detected in populations which shared the same 16S rRNA haplotype (Gerardi *et al*. 2019). Adaptation on genetic level between pathogen and vector could be shown in *B. burgdorferi* s.l. infected *I. scapularis* and *I. pacificus*. The sympatric pathogen/vector pairing showed higher infection rates compared to the allopatric pathogen/vector pairing (Couper *et al*. 2020). As major component of vector competence, development of resistance against *Plasmodium falciparum* varied in mosquitoes, dependent on different combinations of parasite isolates and genetically variable vectors, showing that optimal transmission require some specific pairing of the insect's and the parasite's genotypes (Lambrechts *et al*. 2005). Růzek *et al*. could domenstrate adaptation of TBEV and vector via *in vitro* experiment as growth of virus on vector cell-lines was 100-1000 fold higher compared to non-vector cell lines (Růzek *et al*. 2008).

Vector competence is reached, when the arthropod vector acquires the virus from a blood meal, establish an initial infection in the midgut, disseminates the virus to other body tissue and organs, and sub-sequent transmits it to a host by bite (Beaty and Marquardt 1996). To complete our data on *I. ricinus* vector competence for TBEV, we extend our measurement of TBEV RNA copies and looked on the dissemination rate within the ticks. To do so, we measured viral RNA copy numbers in the ticks' gnathosoma and idiosoma to calculate viral dissemination within the tick. The gnathosoma at the anterior contains ticks' mouthparts while the idiosoma comprises the gastrointestinal tract, the neural system, the legs, the genital and anal opening, and an assortment of sensory and tactile structures. As viral replication is stimulated through blood feeding (Belova, Burenkova, and Karganova 2012; Belova *et al*. 2017; Kopáček *et al*. 2018; Slovák *et al*. 2014), we expected an increase in the dissemination rate as incubation time increases. Surprisingly, we could detect only a slight increase of viral RNA in the tick 14 days after compared to 7 days after, suggesting that TBEV infection is completed after 7 days of infection. To disprove the presumption that TBEV infection could be based on natural infection, as ticks derived directly from a TBEV focus, we did some prior feeding experiments. Virus prevalence in ticks of a TBEV focus is described to be very low with 0.1-5% (Süss 2003). Measured TBEV infection rates

reached up to 100% in our experiments and exceeded the natural virus prevalence many times. After *in vitro* feeding of naïve field collected *I. ricinus* nymphs from Haselmühl and Hanover with uninfected blood, TBEV RNA was not detectable in one of the groups (n=40). So, if ticks collected from the TBEV focus would have been positive for TBEV, even low levels of viral RNA should have been detectable after blood meal. Another unpublished study investigated 410 *I. ricinus* nymphs, fed on field collected bank voles in the TBEV focus Haselmühl. Only 1 of 410 pools of nymphs were positive for TBEV, resulting in a prevalence of 0.3% in blood fed ticks within this focus (Gerhard Dobler, personal communication). Additionally, in both, gnathosoma as well as idiosoma, TBEV RNA was detectable which refers to a dissemination of virus within the tick. Consequently, we could also exclude that detected viral RNA was a residue of the infected feeding blood. Viral RNA copy numbers in ticks fed artificially via *in vitro* feeding system reflected RNA copies found in natural TBEV infected ticks detached from humans ($4x10^2$ to $7.7x10^6$ TBEV RNA copies/tick (Lindblom *et al*. 2014). With this we indicate that our *in vitro* feeding system is suitable to simulate the natural route of TBEV infection in ticks.

Co-infections are highly relevant to public health as they cause potential implications in terms of tick-borne diagnosis and treatment (Diuk-Wasser, Vannier, and Krause 2016; Bröker 2012). In addition, simultaneous infections of the tick with more than one pathogen can lead to changes in vector behavior and can also affect vector competence. In comparison to larvae and nymphs, adult ticks, are considered more likely co-infected as they have ingested two blood meals which increase the chance for acquiring pathogens (Otranto *et al*. 2014; Stromdahl *et al*. 2014; Halos *et al*. 2010). In many cases, pathogen infection seems to be a win-win situation for both, tick vector and pathogen. Enhanced pathogen survival on the hand, infection often provides increased vector fitness on the other hand (de la Fuente *et al*. 2016). As seen in *B. burgdorferi*, infection promotes host-seeking and nymphal survival under suboptimal environmental conditions (Herrmann and Gern 2014; Lefcort and Durden 1996; Gassner 2010). Higher survival in nymphs may result from higher fat content which was detected in field collected *I. ricinus* nymphs infected by *B. burgdorferi* s.l. (Herrmann, Voordouw, and Gern 2013). Under experimental settings, *Borrelia* infected *I. ricinus* nymphs were more active when exposed to desiccating conditions for a prolonged time (Perret 2003) and showed longer walking activity and higher velocity compared to uninfected ones (Gassner 2010). Increased phototaxis and attraction to vertical surfaces was also observed in *I. scapularis* nymphs (Lefcort and Durden 1996). As mobility and phototaxis enhance the likelihood of contact between the tick vector and a suitable host it both poses a boost in *Borrelia* transmission. In *I. scapularis*, Lefcort and Durden demonstrated lowered mobility in *B. burgdorferi* infected adults (Lefcort and Durden 1996). Contrary, Romashchenko *et al.* showed that *I. persulcatus* adults reached higher questing height than non-infected ticks (Romashchenko *et al*.

2012). Even though higher activity was observed only in *Ixodes* nymphs, reduced mobility of adult ticks may be considered as adaptive behavior, since reduced movement contribute reducing energy and water loss. Higher tolerance against temperature, could be obtained by induced expression of heat shock proteins (Busby *et al*. 2012) and antifreeze glycoprotein (Neelakanta *et al*. 2010) as result of an *A. phagocytophylum* infection. Expression of *I. scapularis* key genes was manipulated by *A. phagocytophilum* (Cabezas-Cruz, Estrada-Peña, *et al*. 2017) which results in boosted vector fitness and pathogen transmission (Cabezas-Cruz *et al*. 2016; Cabezas-Cruz, Alberdi, *et al*. 2017). Feeding success and survival have been demonstrable improved by *Babesia microti* infection (Randolph 1991). On the other hand, studies showing that infection can lead also to strong disadvantages for the tick. Beginning from decreased proportion of engorged nymphs and reduced subsequent weight (Liu *et al*. 2014) as shown for *Bartonella*, up to lethal effects in *R. rickettsia* infected *D. andersoni* (Niebylski, Peacock, and Schwan 1999). There is an ongoing awareness on the impact of co-infections, so studies focus on this are numerous (May *et al*. 2015; Blazejak *et al*. 2018; Raileanu *et al*. 2017; Moutailler *et al*. 2016; Diuk-Wasser, Vannier, and Krause 2016; Chen *et al*. 2014; Swanson *et al*. 2006; Belongia 2002). To perform correlation studies with TBEV and other tick-borne pathogens, provoked through natural infection, is practically impossible as virus prevalence in foci is on a low level with 0.1-5% (Süss 2003). To overcome the limitations of natural correlation studies, *I. ricinus* nymphs, derived from field-collected populations, contain their natural infection status with other tick-borne pathogens, which provided the opportunity to investigate co-infections. For this reason, we preserved ticks' microbiome in waiving antibiotic treatment of feeding blood. After infection experiments, tick homogenates were tested for DNA of *Borrelia* spp., *Rickettsia* spp., and *A. phagocytophilum*. A TBEV infection rate of 38.38% (n=693) of overall ticks in both years gave the opportunity to measure correlation of co-infections. Of the three tested bacterial pathogens, only *Borrelia* spp. showed correlation with TBEV infection when the origin of *I. ricinus* nymphs was taken into account. *Borrelia* spp. infection decreased the odds ratio for TBEV infection only in ticks of the TBEV focus Haselmühl (OR=0.352; p=0.0188). This was surprisingly as both locations showed similar infections rates (30.0% Haselmühl, 34.5% Hanover; mean values of both years). In a study of 2012, 22.5% of tested ticks of Amberg, a city close to the TBEV focus Haselmühl, were tested positive for *Borrelia* (Vögerl, Zubriková, and Pfister 2012). In the city of Hanover, 24% of tested ticks were infected with *Borrelia* (Blazejak *et al*. 2018). In both locations, *B. afzelii* was the most frequently occurring geno-species. Due to low amounts of sample material, differentiation of *Borrelia* geno-species have not been done in this study but need to be considered in further analyzes. Co-infection of *Borrelia* and TBEV was even detected in altitudes of 1270m in the Krkonoše Mountains, Czech Republic, where Danielová *et al*. (Danielová *et al*. 2010) found *B. burgdorferi* s.l. and TBEV in host-seeking larvae indicating an autochthonic infection. As described

before, landscape structure influences the prevalence of zoonotic pathogens (Millins *et al*. 2018). In case of *B. burgdorferi*, it could be shown that habitat properties have a larger influence on pathogen prevalence, than macroclimate and landscape properties combined (Ehrmann *et al*. 2018). So, food resources and shelter were the most important predictors within habitat patches. Diversity of habitats were shown to have a diluting effect, as it presumably spreads the niches for hosts, resulting in decreased probability of contact between ticks and their hosts and hence the transmission likelihood. Therefore, habitat structure as well as host species composition and abundance pose not only direct impact factors on TBEV transmission but rather can also be indirect influencing the prevalence of pathogen prevalence co-infecting *I. ricinus* nymphs and thereby effecting vector competence. This study give first evidence for a *Borrelia*/TBEV co-infection interference and fits well to the assumption of Alekseev *et al*. that *Borrelia* might suppress viral replication in ticks and in TBE susceptible individuals (Alekseev *et al*. 2001). Although findings need to be proven in further studies, they are supported by the observed increase of TBE cases in Finland (Smura *et al*. 2019), whereas prevalence of *Borrelia* decreased in the last 60 years (Cuellar *et al*. 2020). Further investigation on different *Borrelia* species involved in TBEV infection are needed. For this purpose, *Borrelia* spp./TBEV co-infection models are needed to investigate interaction of both pathogenic infections. This could be implemented by *Borrelia* infected blood meal for larvae following infection with TBEV after moulting. A second possibility would be the combined infection with one pathogen via injection and subsequent infection via *in vitro* feeding with the other pathogen. Infection of both pathogens simultaneously could be conducted via blood meal. In contrast to *Borrelia* spp., *Rickettsia* spp., and *A. phagocytophilum* co-infections did not show any significant influence of the odds of being TBEV infected.

Besides pathogenic bacteria and fungi, also non-pathogenic microbiota colonizing ticks and interfere with tick-borne pathogens. Ticks also carry symbionts which may be important not in a role in tick biology but more in interactions with tick-borne pathogens (Narasimhan and Fikrig 2015). Alteration of microbiota results in modulated immune response which might have an effect on pathogen survival and infection as demonstrated for other arthropod vectors (Cirimotich *et al*. 2011). It could be shown that ticks, bred in a sterile environment, have a reduced microbiota which alters their gut integrity and modifies *B. burgdorferis* ability to colonize this niche (Narasimhan *et al*. 2014). A high prevalence of symbiont species in ticks suggesting that they play a crucial role in tick fitness. In insects, *Wolbachia* is a widespread symbiont which affects reproduction and immunity of hosts (Werren, Baldo, and Clark 2008). Colonizing *Wolbachia* symbionts in ticks (Ahantarig *et al*. 2013) are traced back to the parasitoid wasp *Ixodiphagus hookeri* (Plantard *et al*. 2012). Beside *Wolbachia*, *Midichloria mitochondrii* was detected nearly in all field-collected *I. ricinus* females (Sassera *et al*. 2006). In *Amblyoma* ticks, *Coxiella* related symbionts impairs transmission of *Ehrlichia chaffeensis* (Klyachko *et al*. 2007). The transmission

of the pathogenic *R. Rickettsia* is shown to be altered in *D. andersoni* due to the endosymbiont species *R. peacockii* (Ahantarig *et al*. 2013; Childs and Paddock 2002). Under laboratory conditions, symbionts in tick colonies decline, indicating that advantages coming with symbionts may only be evident under natural conditions (Lo *et al*. 2006). The *in vitro* feeding system, presented in this study, examined field-collected *I. ricinus* nymphs which are naturally colonized by different symbionts. Feeding blood was not supplemented with antibiotics maintaining ticks' microbiota completely. Therefor this approach poses an optimal fundament for investigation of symbiont/pathogen interactions.

Arthropod vectors are exposed to a variety of microorganisms in their habitats and during blood feeding. In the vertebrate host, arboviruses mostly induce morbidity and sometimes mortality, whereas infections in the vector are usually considered non-pathogenic. Different immune mechanisms allow them to maintain pathogens at a level which does not impair their development and fitness. Due to incomplete knowledge on the tick immune response to infection, approximate comparison can be only done with other arthropod vectors. However, even sporadic information indicating defense mechanisms in ticks which protect them against pathogen infection. Within the tick hemolymph, invading pathogens have to face humoral and cellular defense mechanisms. There are a high number of tick specific antimicrobial peptides like lysozymes, defensins and molecules which are not found in other invertebrates (Hajdušek *et al*. 2013). Moreover, most important mosquito immune signaling pathways that have been involved in the antiviral defense, such as the immune deficiency (IMD), Toll and Janus kinase/signal transducers and activators of transcription (JAK-STAT) pathways (Sim, Jupatanakul, and Dimopoulos 2014), also occur in ticks (Gulia-Nuss *et al*. 2016; Oliva Chávez *et al*. 2017; Palmer and Jiggins 2015; Liu *et al*. 2012). Additionally, the RNA interference (RNAi) plays a role in antiviral defense as shown for Langat virus infection in *Ixodes scapularis* (Schnettler *et al*. 2014; Grabowski *et al*. 2017).

Pathogen interactions with the tick immune system have evolved over more than 300 million years (Barker *et al*. 2008) and produce an outcome that probably influence viral genetic diversity. The ability to cope with the tick immune responses are crucial for viral transmission. Arboviruses are confronted with the further complexity of two very distinct hosts. To succeed these conditions, viruses need a high genetic plasticity. The RNA-dependent RNA-polymerase (RdRp) has a high error rate with an estimated range from $10^{-3}$ to $10^{-5}$ errors/nucleotide/round of replication, facilitating fast adaptation and genetic plasticity of RNA viruses. Understanding the genetic influence of TBEV isolate for the infection susceptibility in *I. ricinus* populations is important, so we focused in our second study on the question if there are matching pairings of virus strain and tick population. We investigated the infection success in different tick populations infected with closely related TBEV strains which differed in amino acid sequences. In our study, we used four TBEV isolates which originated from foci located in close

proximity to each other. For testing tick populations of Lower-Saxony we tested the TBEV foci Barsinghausen *versus* Rauher Busch. These foci are 35 km beeline away from each other and exhibit a genetic difference of 10 amino acid exchanges (aa). As second similar pairing of Bavarian TBEV foci, the well explored spots Haselmühl and Heselbach which are 27 km apart in beeline and differentiate in 19 aa, were chosen. These differences are on a very low level, compared to Kupca *et al*. showing 251 nucleotide differences of the isolates AS33 and Salem 251 resulting in 26 aa exchanges (Kupca *et al*. 2010). Differences in E-gene sequences of other members of the *Flaviviridae* are high with 6% natural observed variation for Dengue virus, 7% for West Nile-virus and 5% for Yellow fever virus (YFV). In comparison, TBEV sequences are conserved with only 1.8% variation (Heinz and Stiasny 2019). For TBEV, beside the function of protein E in host tropism, protein M, NS3, NS4A and NSAB might act as viral determinants for host specific replication (Mitzel *et al*. 2008). In the case of Lower-Saxony TBEV isolates, we detected one aa difference in the sequence of E, NS2, and NS4B, two aa differences in NS3 and six aa exchanges in the NS5 protein. In the Bavarian isolates, we found one aa difference in C, prM, NS2B, NS5, two aa differences in the NS4B and four aa differences in NS1, NS2A, NS3. How genetic variations can manifest, is shown in different studies. In the mosquito species *Ae*. *aegypti,* the YFV isolates YFV-17D (live-attenuated vaccine) and YFV-DAK (clinical isolate) differed in the ability to overcome the midgut barrier (Danet *et al*. 2019). A very specific case of genetic mutation, in the position 226 on the Chikungunya Virus E1 glycoprotein, enhances the transmission in *Ae. Albopictus* (Arias-Goeta *et al*. 2013). For TBEV it could be shown that viral adaptation take place in accordance with environmental changes. Viral variants of the strain Hypr, generated through multiple passaging on mammalian porcine kidney stable (PS) and *I. ricinus* tick (IRE/CTVM19) cell lines, exhibited distinct plaque sizes and virulence in a mouse model (Helmová *et al*. 2020). As critical determinant of virus fitness (Martinez *et al*. 1991; Ruiz-Jarabo *et al*. 2000), the diversity of viral quasi species has been shown to be both virus and host dependent (Schneider and Roossinck 2000, 2001). There is growing evidence of field studies (Asghar *et al*. 2014; Asghar *et al*. 2017) and also from laboratory experiments (Romanova *et al*. 2007; Belova *et al*. 2017; Růzek *et al*. 2008) showing the capacity of TBEV to generate quasi species. These findings show that high pressure forces TBEV to adapt quickly to changes in environment and host systems. To investigate the impact of genetic variations of the virus isolate on infection susceptibility, we examined the infection rates of ticks collected in four different TBEV foci. Infection experiments have been conducted with the TBEV isolates Barsinghausen *versus* Rauher Busch (Lower-Saxony) and Haselmühl *versus* Heselbach (Bavaria). Ticks were exposed to infected blood meal with the TBEV isolate of their habitat and a genetic related isolate. Infection rates differed between the groups. In ticks from Lower-Saxony as well as in ticks from Bavaria, infection susceptibility was higher for the virus isolate from the same TBEV focus. We analysed the probability of TBEV infection

using a binomial GLM which revealed that infection results are clearly linked to a possible adaptation of *I. ricinus* population and TBEV isolate. Outcome of binomial testing showed that the odds ratio for infection with the synonymous TBEV isolate is 1.61 fold higher in Lower-Saxony and 16.18 fold higher in Bavaria. Up to now, these results are unique for TBEV infection in ticks and constitute a good fundament for further research. Similar findings in adaptations of virus and vector have been observed in mosquitoes. Ciota *et al*. could demonstrate that Chikungunya virus transmission depends on mosquito genetics (Ciota *et al*. 2018). C*ulex pipiens* biotypes in Germany show different susceptibility to West Nile virus (Leggewie *et al*. 2016). The infection susceptibility of *Ae. aegypti* for Dengue-2 virus is linked to so far undetected trait loci (Bennett *et al*. 2005). Different population cluster of *Ae. albopictus* differ in dissemination and transmission efficiencies (Vega-Rúa *et al*. 2020).

Factors responsible for those population-specific differences are not explored, but it necessarily need to be implied that differences in fundamental infection barriers within the tick play an important role. The pathogens' ability to overcome different barriers within the tick is a crucial parameter determining whether a tick serves as vector. Adaptation to intrinsic physiological and behavioral characteristics of ticks, particularly blood feeding, blood meal digestion, molting and immune responses are essential for persistence of tick-borne pathogens. Different cell types need to be invaded and intestinal, salivary and ovarian barriers need to be passed for successful replication and transmission of the pathogen (Brossard & Wikel, 2004). A crucial role in virus transmission and replication in the tick is attributed to the midgut and salivary gland barrier. As shown for Dugbe virus (*Nairovirus, Bunyavirales*), infection experiments on different tick species can highlight the importance of the midgut barrier. Infection via feeding was possible in vector ticks (*Amblyomma variegatum)* and lead to transstadial transmission of the virus, which was not the case in non-vector ticks such as *Rhipicephalus appendiculatus* (Steele and Nuttall 1989). This indicates that the midgut barrier is also able to block transstadial transmission of viruses in addition for establishment of infection. Studies on TBEV have shown, that the virus need to replicate in the midgut of the tick. From the midgut it disseminates to the haemolymph and then infects other tissues. The highest virus titer is then reached in the salivary glands and reproductive organ epithelium (Dickson and Turell 1992). The knowledge concerning the midgut barrier is on a low level, thus, we can only speculate if similar processes as described for mosquitoes play a role in transmission between virus and ticks. In *Ae. aegypti* it has been shown that RNAi plays an important role in midgut cells to prevent Sindbis virus infection and dissemination (Khoo *et al*. 2010). Key components of the RNAi pathway, Dicer and Argonaute proteins, could be detected in *I. scapularis* and *I. ricinus* cell lines (Gulia-Nuss *et al*. 2016; Weisheit *et al*. 2015). Furthermore, studies on transcriptome level show innate immune pathways including Mitogen-activated protein kinase (MAPK) and JAK/STAT pathways in in *I. scapularis* and *I. ricinus* cell lines (Mansfield *et al*. 2017). Also alteration in form of

weaken vector immune response due advanced age may increases the susceptibility to pathogen infection (Lefevre *et al*. 2013) and need to be implied. Such immunosenescence has been reported in a wide range of insects including crickets, scorpion flies, *Drosophila*, and the mosquito *Ae. aegypti* (Hillyer *et al*. 2005; Stanley 2012).

# Conclusion

In this study, we could demonstrate TBEV/tick vector interactions which possibly can be traced back to genetics of vector and virus. Infection susceptibility was experimentally investigated using an *in vitro* feeding system which was adapted to safety requirements of a BSL3 laboratory. Susceptibility for TBEV infection was higher in *I. ricinus* nymphs collected in a TBEV endemic area. Furthermore, infection outcome differed in accordance with *Borrelia* infection status as well as TBEV strain/tick population pairing, suggesting interdependency in form of co-evolutionary adaptation.

To evaluate all aspects of vector competence of *I. ricinus* for TBEV, data need to be completed in further experiments regarding transmission efficiency to hosts and to the next life stage. In addition, it needs to be investigated if observed infection rates are transferable to the adult life stage. Nevertheless, this study gives first hinds that genetics of vector and virus play a role in the focal distribution of TBEV in Germany. These data provide evidence that more studies on *I. ricinus* population base are needed to predict novel TBEV foci in future.

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