



Copulation of *Ixodes ricinus* males and females on the host and its potential impact on pathogen transmission

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Abstract

Introduction and Objective. The common tick *Ixodes ricinus* is one of Europe's most important vectors of tick-borne diseases. The increased risk of attacks by this tick suggests the need for identification of factors contributing to the transmission of tick-borne pathogens, and the routes of pathogen circulation in nature.

Materials and method. Polymerase chain reaction was used to investigate the prevalence of four pathogens, i.e. *Borrelia burgdorferi* s.l. (Bb), *Anaplasma phagocytophilum* (Ap), *Babesia* spp. (Bs), and *Bartonella* spp. (Ba) in *I. ricinus* females and males mating on dogs in south-central Poland.

Results. The study revealed the presence of three pathogens: Bb, Ap, and Bs in 9.4%, 5.4%, and 5.4% of all *I. ricinus* adults in copula, respectively. Co-infection with two pathogens was detected in one tick specimen. *Borrelia burgdorferi* spirochetes were isolated in two females and two males in copula, but the sexual transfer of the spirochetes between these specimens could not be clearly confirmed.

Conclusions. By increasing the feeding dynamics in females, the copulation of *I. ricinus* males with females attached to the host's skin may stimulate pathogen replication in tick tissues and migration from the gut to the salivary glands. Further investigations of the *I. ricinus* copulation on the host on female feeding and pathogen transmission may contribute to the elucidation of the eco-epidemiology of tick-borne diseases transmitted by this tick species.

Key words

Borrelia burgdorferi, *Anaplasma phagocytophilum*, *Ixodes ricinus*, pathogen transmission, tick-borne pathogens, tick copulation

INTRODUCTION AND OBJECTIVE

Ticks are haematophagous arthropods and one of the most important vectors of pathogens of great importance for public health. In Europe, the common tick *Ixodes ricinus* is the most widely distributed species [1]. Compared with other tick species present in this area, *I. ricinus* ticks are characterized by an extraordinary ability to colonize various environments, a wide range of hosts, and a variety of transmitted pathogens. This species transmits e.g. *Borrelia burgdorferi* s.l. spirochetes, European tick-borne encephalitis viruses, *Anaplasma phagocytophilum*, and *Babesia* spp., and infects its hosts during feeding [e.g. 2–8]. Human diseases caused by these pathogens, in particular Lyme disease and tick-borne encephalitis, are a serious social problem and generate large economic losses [9–11]. In the case of Lyme disease, approximately 850,000 human cases are reported each year in Europe [11]. Tick-borne diseases are also increasingly being diagnosed in companion animals, including dogs [e.g. 12–14].

Similar to other tick species, *I. ricinus* ticks inject bioactive substances with a wide range of activity contained in their saliva into the host. They may cause local skin lesions and systemic reactions [e.g. 15–18] and induce meat allergies (alpha-gal syndrome) [19–21].

The dramatic increase in the number of human cases of tick-borne diseases transmitted by *I. ricinus*, mainly Lyme disease, indicates the necessity to monitor the spread of this tick species and associated pathogens. Special importance for identification of the causes of the persistence of tick-borne disease outbreaks and the circulation routes of tick-borne pathogens (TBPs) in nature is ascribed to studies on the biology of this tick species, including the mechanisms of its feeding on the host and reproduction.

In a large area of Europe, *Ixodes ricinus* is characterized by the highest rates of infestation of humans [e.g. 15, 16, 18, 22, 23] and companion animals [e.g. 24–30]. This is associated with the presence and increase in the population size of these ticks in urban and suburban areas that are often visited by humans and their companion animals [e.g. 31–36].

As shown by research, the intensity of *I. ricinus* infestation on the host does not result in statistically significant changes in the duration of the attachment and feeding of females, but has a considerable effect on their body weight [37]. The increase in the amount of blood ingested by females is accompanied by an increase in the number of oviposited eggs [38]. Preoviposition, oviposition, and the development and number of eggs laid by engorged *I. ricinus* females are also influenced by other factors, e.g. host immunity [39, 40], temperature and humidity conditions prevailing in the environment [41]. Changes in the development of non-parasitic stages, i.e. egg maturation and oviposition or larval hatching, were noted during observations of *I. ricinus* and

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Dermacentor reticulatus co-feeding in the same area. The values of such parameters as the egg amount, number of eggs per 1 mg of female engorgement weight, female oviposition-related weight loss, and hatching success were higher in inter-specific groups than in mono-specific groups of these tick species [42]. Some bacteria and viruses may be transmitted from infected to non-infected specimens during co-feeding [43–47].

As in most ticks, excluding parthenogenetic species of ixodid ticks and some autogenetic ticks from the family Argasidae, the insemination of *I. ricinus* females by males is a prerequisite for the completion of the entire gonotrophic cycle. The mating process has an impact on the course and efficiency of feeding of female ticks, egg development, oviposition [48–50], and, consequently, the size and structure of the tick population. *I. ricinus* females cannot fully engorge without mating [51] (authors' observations), likewise in other species of ixodid ticks, such as *Ixodes persulcatus* [52]. Virgin females of ixodid ticks do not lay eggs [50]. In contrast to Metastriata ticks, whose males and females copulate only on the host, representatives of the Prostriata group, e.g. *I. ricinus*, mate both in the environment and on the host [51, 53–56].

In this study, the prevalence of pathogens in *I. ricinus* females and males mating on the host was determined, given the importance of this phenomenon in the circulation of pathogens. The analyses were also focused on the increased risk of host infection with pathogens transmitted by ticks *in copula* and on the possibility of sexual transmission of pathogens in the tick-tick system.

MATERIALS AND METHOD

Collection of ticks and morphological studies. *In copula* specimens were found in the collection of ticks harvested from 37 dogs during tick seasonal activity in south-central Poland in 2010–2019. The specimens were subjected to morphological analyses performed with the use of a Stemi/DV4 stereoscopic microscope (Carl Zeiss, Germany). The species affiliation of these specimens was confirmed using the tick identification key compiled by Nowak-Chmura [57]. The *in copula* female feeding phase was determined based on morphometric features described by Bartosik and Buczek [58].

The ticks were kept in 70% ethanol prior to the molecular tests. The presence of four pathogens, i.e. *Borrelia burgdorferi* s.l., *Anaplasma phagocytophilum*, *Bartonella* sp., and *Babesia* spp., was examined in each tick of the mating pair. The polymerase chain reaction (PCR) method was used to identify TBPs.

Molecular studies. The DNA was isolated from single *I. ricinus* ticks with the ammonium hydroxide method [59]. Next, its concentration was measured in a NanoPhotometer PEARL (Implen, Germany) at the 260/280 nm wavelength. In the next step, the samples were frozen and stored at -20°C for further analysis. *B. burgdorferi* s.l. spirochetes were detected in the ticks by *real time* PCR, which was conducted with the use of a ready *Borrelia* qPCR detection Kit (EURx, Poland) in accordance with the manufacturer's protocol. In turn, *A. phagocytophilum* was detected in *I. ricinus* by nested PCR with the use of two pairs of primers specific to the 16S rRNA gene [60]. The presence of the other pathogens

in ticks, i.e. *Babesia* spp. and *Bartonella* spp., was detected with the use of the single PCR method. The primers used in these assays were specific to the 18S rRNA gene and the *rpoB* gene, respectively [61, 62]. The amplification products were separated electrophoretically in 2% ethidium bromide-stained agarose gels and visualized in a device for agarose gels analysis (Vilber Lourmat, France). The presence of products with a size of 932 base pairs (bp) and 546 bp for *A. phagocytophilum*, 620 bp for *Babesia* spp., and 825 bp for *Bartonella* sp., was considered positive.

RESULTS AND DISCUSSION

In the collection of ticks removed from the skin of dogs, 37 pairs of copulating *I. ricinus* females and males were found. Although specimens of this species are most frequently found on wild [63, 64, 65] and companion animals [26, 28–30, 66, 67] in most habitats in northern and central Europe, research on this species mating on hosts and the biological and epidemiological implications of the phenomenon has rarely been undertaken.

Most *I. ricinus* females mate in the field before climbing on the host, which ensures a proper course of feeding [51, 54]. Virgin females attach to and remain on a host's skin for some time (usually 14 days) without ingestion of large amounts of blood. In the presence of male specimens, copulation starts immediately and then the females cease feeding within 3–5 days [51]. In our previous study, inseminated females fed on rabbits for 8–10 days at a temperature of 18.5±1 °C and ca. 38±1% humidity [37].

The methodology employed in the present study did not allow determination of the percentage of all *I. ricinus* adults *in copula* collected from dogs in south-central Poland. In southern Poland, Kocoń et al. [29] recorded 7.9% and 7.7% of females and males *in copula* among 1,849 specimens collected from dogs, and 794 specimens removed from cats, but the researchers did not specify the feeding stage of these females.

In our collection, three (8.2%) of the 37 females *in copula* with males were at the very early stage of feeding process (1st phase of feeding), 17 (45.9%) partially blood-engorged (2nd phase of feeding) and 17 (45.9%) were in the final feeding phase (3rd feeding phase) (Fig. 1). We did not find any literature reports on the feeding period of *I. ricinus* females mating with males on the host in natural conditions. Researchers have usually only reported that most females collected from dogs are partially or fully engorged [e.g. 24] (authors' observations).

The present observations are consistent with laboratory studies conducted by Zemek et al. [68], who confirmed that

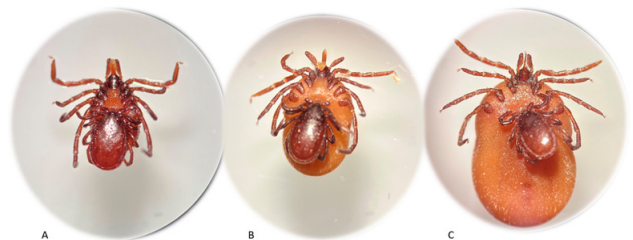


Figure 1. *Ixodes ricinus* adults *in copula* collected from dogs in south-central Poland. Pair with a female at the beginning of the feeding process: 1st phase of feeding (A), pair with a female partially blood-engorged: 2nd phase of feeding (B) and pair with a female in the final feeding phase: 3rd feeding phase (C). Original magnification 16x

the feeding status of *I. ricinus* females had an impact on their sexual attractiveness. The highest attractiveness was observed in engorged females, whereas unengorged females in a field population of this species were found to be the least attractive. The secretion of increased amounts of pheromones by partially and almost fully engorged female ticks, which is also noted in representatives of Metastratiata ticks, enhances the chance to attract a male and to be inseminated [69, 70].

Among the 74 adult *I. ricinus* ticks in copula, 14 (18.9%) of the total specimens were infected with TBPs, namely 6 (8.1%) with *Borrelia burgdorferi* s.l. spirochetes alone, 3 with *Anaplasma phagocytophilum* alone (4.0%), 1 with *B. burgdorferi* s.l. together with *A. phagocytophilum* (1.4%) and 4 with *Babesia* spp. (5.4%). Co-infection with *B. burgdorferi* s.l. and *A. phagocytophilum* was recorded in one *I. ricinus* male. No genetic material of *Bartonella* spp. was identified in the analyzed ticks (Tab. 1). The presence of pathogens in the ticks collected from dogs and the results of previous studies on their prevalence in host-seeking ticks in southern and south-central Poland [71–74], indicate a high risk of tick-borne diseases posed to humans and animals in this area. The incidence of *Borrelia burgdorferi* s.l. infection in *I. ricinus* tick ranges from 4% in Upper Silesia to up to 62% in Beskid Żywiecki [71, 72]. In these regions, *B. microti* was detected in 23.3–50.87% of *I. ricinus* ticks [73, 74], and *Toxoplasma gondii* was found in 64.91% of specimens [73].

Table 1. Prevalence of 3 pathogens in *Ixodes ricinus* adults collected from dogs in south-central Poland

<i>Ixodes ricinus</i>	<i>Borrelia burgdorferi</i> s.l. N (%)	<i>Anaplasma phagocytophilum</i> N (%)	<i>B. burgdorferi</i> + <i>A. phagocytophilum</i>	<i>Babesia</i> spp. N (%)
Females (n=37)	5 (13.5)	2 (5.4)	0	4 (10.8)
Males (n=37)	1 (2.7)	1 (2.7)	1 (2.7)	0
Total Females + Males	6 (8.1)	3 (4.0)	1 (1.4)	4 (5.4)
Pairs (n=37)	2 (5.4)	0	0	0

Interestingly, *B. burgdorferi* s.l. genetic material was detected in 2 pairs of ticks (a female and a male) in copula. Sexual transmission of spirochetes in these ticks cannot be clearly confirmed by the present results, as the 2 mating specimens may have been infected earlier. The potential trans-spermal transmission of pathogens, e.g. *Borrelia burgdorferi* s.l. spirochetes, has rarely been studied in *Ixodes* ticks, and the frequency and importance of the phenomenon for pathogen circulation within tick populations have not been fully elucidated. Experimental studies have demonstrated transmission of *B. burgdorferi* s.l. or tick-borne encephalitis virus between infected males and non-infected females of the taiga tick *I. persulcatus* during mating [75, 76]. The *Borrelia burgdorferi* infection rate in the sexual pairs of ticks was 1.75–2.00 times higher than in individuals of both genders kept separately. *Borrelia burgdorferi* s.l. spirochetes were detected in 22.9% of copulating ticks and in 17.4% of females and 16.5% of males without sexual contact [76]. As suggested by the authors, the spirochetes were transmitted in a venereal or cannibalistic mode. Infection with *Borrelia* spirochetes in tick couples was observed more frequently in specimens harvested in the environment in a hot spring and summer period than in those collected in seasons with moderate temperatures [77].

Alekseev and Dubinina [77] confirmed the ability of *I. persulcatus* males infected with a single spirochete species (*B. afzelii* or *B. garinii*) and co-infected with both species, to transmit the pathogens into females during copulation. In their study, the sexual transmission of *B. garinii* occurred only from an infected male to a non-infected female. In turn, no transmission of this bacterium from an infected *I. persulcatus* female to a non-infected male was observed in the case of infection with a single spirochete species.

Transspermal transmission of relapsing fever *Borrelia* was previously described in soft ticks [78, 79]. Via this route, *Borrelia crociduræ* spirochetes were transmitted during copulation of an infected *Ornithodoros* (*Pavlovskyella*) *erraticus* male with females. The spirochetes were observed in 23% and 37% of females after the first and second gonotrophic cycles, respectively.

Sexual transmission of tick-borne encephalitis virus from infected ixodid males to non-infected females was detected in *Ixodes persulcatus* (in 50% of cases) and *Hyalomma anatolicum* (6.2%) [75]. Females infected with this virus during insemination may infect their host while feeding. Additionally, tick-borne encephalitis virus can be transferred from an infected female to the developing egg (transovarial transmission). The possibility of *Rickettsia rickettsii* transmission from infected male ticks to non-infected females during copulation has also been confirmed [80], but the scale of this phenomenon in nature is still unknown. It is also unknown whether the transspermal transmission leads to generalized infection in female ticks. Researchers have detected rickettsiae in spermatogonia, spermatocytes, and maturing spermatids of male *I. ricinus* males.

Through changes in the physiology of feeding specimens and increasing the amount of blood ingested by females, copulation and insemination of females by males on the host may have an impact on the dynamics of pathogen transmission between the infected tick and the host. Tick feeding involves alternating cycles of injection of saliva portions into host's skin damaged by the chelicera and hypostome and ingestion of portions of host's blood. A large amount of ingested blood increases the possibility of effective transmission of spirochetes to the host. An important role in the colonization of the tick gut by *B. burgdorferi* s.l. spirochetes is played by bacterial surface proteins, e.g. OspA, which bind to the TROSPA receptor (tick receptor for OspA) present on the gut surface. Tick feeding is accompanied by a decline in the OspA expression and an increase in the expression of OspC, which disrupts the connection between OspA and TROSPA. *Borrelia* spirochetes that have multiplied in the midgut cross the midgut barrier (between cells) to reach the haemolymph and salivary glands [81–84].

The insemination of females attached to host's skin by males stimulates the females to ingest blood and may initiate the replication and/or expansion of bacteria in tick's tissues. In addition to *Borrelia* spp., the tick gut harbors *Bartonella* spp., which migrate to tick's salivary glands during ingestion of a blood meal from the host. Bacteria of the genera *Anaplasma* and *Ehrlichia* can multiply in the midgut and migrate to salivary glands of non-feeding ticks [85].

The number of spirochetes in the midgut of *Ixodes scapularis* nymphs exhibited a 6-fold increase at 48 h after attachment of infected specimens to host's skin (from 998 per tick before attachment to 5,884 after 48 h). The number of spirochetes in salivary glands increased over 17 times

from 1.2 per salivary gland pair before feeding to 20.8 at 72 h after tick attachment. The greatest increase in the number of spirochetes in salivary glands of *Ixodes scapularis* nymphs was recorded between 48 and 60 h after the beginning of feeding [86].

The transmission of pathogens by infected ticks to the host depends on, e.g. the duration of feeding, pathogen type and dose, location of the pathogen in tick tissues during blood ingestion, and physiological characteristics of the host [e.g. 87–90]. Tick-borne pathogens, e.g. viruses, rapidly replicating and migrating in tick tissues, can be injected into the host with the first portions of tick saliva. In turn, spirochetes, whose replication and/or migration in tick tissues are slower, can usually infect the host only after some time of tick attachment and feeding. Therefore, instantaneous removal of the tick from the host's skin reduces the risk of infection with TBPs.

Pathogens can influence the host feeding behaviour in ticks [91–95] and even initiate their interspecies contacts [96]. Enhancement of the activity of tick vectors and their atypical behaviour stimulated by *Borrelia* spp., *Rickettsia* spp., and tick-borne encephalitis virus increase the risk of attacks of potential hosts and transmission of these pathogenic factors during blood ingestion.

An interesting study conducted by Kempf et al. [97] has revealed differences in the mating patterns between *I. ricinus* populations, probably caused by biological differences between ticks. As highlighted by these authors, assortative mating in *I. ricinus* may contribute to changes in the circulation of TBPs in the environment through its influence on the population dynamics. These results may inspire further research on the biology of *I. ricinus* from various populations, including their reproduction and development, which may contribute to the elucidation of the mechanisms of pathogen circulation in various environments.

CONCLUSIONS

The copulation of *I. ricinus* adults on the host by influencing the feeding dynamics in females may propagate multiplication of TBPs in tick tissues and promote pathogen transmission to the host. Further research on the reproductive processes of *I. ricinus* during the parasitic phase of the life cycle and their relationship to feeding dynamics, may contribute to elucidating the ecoepidemiology of tick-borne diseases transmitted by this tick species.

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REFERENCES

1. ECDC. European Centre for Disease Prevention and Control and European Food Safety Authority. *Ixodes ricinus* – current known distribution: May 2020. Stockholm. 2020. <https://www.ecdc.europa.eu/en/publications-data/ixodes-ricinus-current-known-distribution-may-2020>
2. Jongejans F, Uilenberg, G. The global importance of ticks. *Parasitology*. 2004;129(Suppl.):S3–S14. <https://doi.org/10.1017/S0031182004005967>
3. Wójcik-Fatla A, Szymańska J, Wdowiak L, Buczek A, Dutkiewicz J. Coincidence of three pathogens (*Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum* and *Babesia microti*) in *Ixodes ricinus* ticks in the Lublin macroregion. *Ann Agric Environ Med*. 2009;16(1):151–158.
4. Pettersson JHO, Golovljova I, Vene S, et al. Prevalence of tick-borne encephalitis virus in *Ixodes ricinus* ticks in northern Europe with particular reference to Southern Sweden. *Parasit Vectors*. 2014;7:102. <https://doi.org/10.1186/1756-3305-7-102>
5. Bertola M, Montarsi F, Obber F, Da Rold G, Carlin S, Toniolo F, Porcellato E, Falcaro C, Mondardini V, Ormelli S, Ravagnan S. Occurrence and identification of *Ixodes ricinus* borne pathogens in Northeastern Italy. *Pathogens*. 2021;10(9):1181. <https://doi.org/10.3390/pathogens10091181>
6. Markowicz M, Schötta AM, Höss D, et al. Infections with tickborne pathogens after tick bite, Austria, 2015–2018. *Emerg Infect Dis*. 2021;27(4):1048–1056. <https://doi.org/10.3201/eid2704.203366>
7. Olsthoorn F, Sprong H, Fonville M, et al. Occurrence of tick-borne pathogens in questing *Ixodes ricinus* ticks from Wester Ross, Northwest Scotland. *Parasit Vectors*. 2021;14:430. <https://doi.org/10.1186/s13071-021-04946-5>
8. Kubiak K, Szymańska H, Dmitryjuk M, Dzika E. Abundance of *Ixodes ricinus* Ticks (Acari: Ixodidae) and the diversity of *Borrelia* species in Northeastern Poland. *Int J Environ Res Public Health*. 2022;19(12):7378. <https://doi.org/10.3390/ijerph19127378>
9. Kunze U. The International Scientific Working Group on Tick-Borne Encephalitis (ISW TBE): review of 17 years of activity and commitment. *Ticks Tick Borne Dis*. 2016;7(3):399–404.
10. van den Wijngaard CC, Hofhuis A, Simões M, et al. Surveillance perspective on Lyme borreliosis across the European Union and European Economic Area. *Eurosurveillance*. 2017;22. <https://doi.org/10.2807/1560-7917.ES.2017.22.27.30569>
11. European Parliament. European Parliament resolution on Lyme disease (Borreliosis) (2018/2774(RSP)). 2018.
12. Adaszek Ł, Winiarczyk S, Kutrzeba J, Puchalski A, Dębiak P. Przypadki boreliozy u psów na Lubelszczyźnie. *Życie Wet*. 2008b;83:311–313.
13. Beugnet F, Marie JL. Emerging arthropod borne diseases of companion animals in Europe. *Vet Parasitol*. 2009;163:298–305.
14. Bajer A, Beck A, Beck R, et al. Babesiosis in Southeastern, Central and Northeastern Europe: an emerging and re-emerging tick-borne disease of humans and animals. *Microorganisms*. 2022;10:945. <https://doi.org/10.3390/microorganisms10050945>
15. Bartosik K, Sitarz M, Szymańska J, Buczek A. Tick bites on humans in the agricultural and recreational areas in south-eastern Poland. *Ann Agric Environ Med*. 2011;18(1):151–157.
16. Wilhelmsson P, Lindblom P, Fryland L, et al. *Ixodes ricinus* ticks removed from humans in Northern Europe: seasonal pattern of infestation, attachment sites and duration of feeding. *Parasit Vectors*. 2013;6:362. <https://doi.org/10.1186/1756-3305-6-362>
17. Buczek W, Buczek AM, Bartosik K, Buczek A. Comparison of Skin Lesions Caused by *Ixodes ricinus* Ticks and *Lipoptena cervi* Deer Keds Infesting Humans in the Natural Environment. *Int J Environ Res Public Health*. 2020;17(9):3316. <https://doi.org/10.3390/ijerph17093316>
18. Pawełczyk A, Bednarska M, Hamera A, et al. Long-term study of *Borrelia* and *Babesia* prevalence and co-infection in *Ixodes ricinus* and *Dermacentor reticulatus* ticks removed from humans in Poland, 2016–2019. *Parasit Vectors*. 2021;14(1):348. <https://doi.org/10.1186/s13071-021-04849-5>
19. Hamsten C, Starkhammar M, Tran TA, et al. Identification of galactose- α -1,3-galactose in the gastrointestinal tract of the tick *Ixodes ricinus*; possible relationship with red meat allergy. *Allergy*. 2013;68(4):549–552. doi:10.1111/all.12128
20. Fischer J, Lupberger E, Hebsaker J, et al. Prevalence of type I sensitization to alpha-gal in forest service employees and hunters. *Allergy*. 2017;72(10):1540–1547. <https://doi.org/10.1111/all.13156>
21. Rutkowski K, Sowa P, Mroczko B, et al. Sensitisation and allergic reactions to alpha-1,3-galactose in Podlasie, Poland, an area endemic for tick-borne infections. *Infect Dis (Lond)*. 2022;54(8):572–579. <https://doi.org/10.1080/23744235.2022.2057583>
22. Banović P, Díaz-Sánchez AA, Galon C, et al. Humans infested with *Ixodes ricinus* are exposed to a diverse array of tick-borne pathogens in Serbia. *Ticks Tick Borne Dis*. 2021;12(2):101609. <https://doi.org/10.1016/j.ttbdis.2020.101609>
23. Jumpertz M, Sevestre J, Luciani L, Houhamdi L, Fournier PE, Parola P. Bacterial agents detected in 418 ticks removed from humans during 2014–2021, France. *Emerg Infect Dis*. 2023;29(4):701–710. doi:10.3201/eid2904.221572
24. Földvári G, Máriaigetű M, Solymosi N, et al. Hard ticks infesting dogs in Hungary and their infection with *Babesia* and *Borrelia* species. *Parasitol Res*. 2007;101:25–34. doi:10.1007/s00436-007-0608-6

25. Claerebout E, Losson B, Cochez C, et al. Ticks and associated pathogens collected from dogs and cats in Belgium. *Parasit Vectors*. 2013;6:183. <https://doi.org/10.1186/1756-3305-6-183>
26. Król N, Obiegala A, Pfeffer M, et al. Detection of selected pathogens in ticks collected from cats and dogs in the Wrocław agglomeration, South-West Poland. *Parasit Vectors*. 2016;9:351. <https://doi.org/10.1186/s13071-016-1632-0>
27. Abdullah S, Helps C, Tasker S, et al. Ticks infesting domestic dogs in the UK: a large-scale surveillance programme. *Parasit Vectors*. 2016;9:391. <https://doi.org/10.1186/s13071-016-1673-4>
28. Geurden T, Becskei C, Six RH, et al. Detection of tick-borne pathogens in ticks from dogs and cats in different European countries. *Ticks Tick Borne Dis*. 2018;9(6):1431–1436. <https://doi.org/10.1016/j.ttbdis.2018.06.013>
29. Kocóń A, Nowak-Chmura M, Asman M. Assessment of the species composition of ticks attacking dogs and domestic cats in the Małopolska and Silesian provinces. *Syst Appl Acarol*. 2022;27(8):1509–1517. <https://doi.org/10.11158/saa.27.8.3>
30. Probst J, Springer A, Strube C. Year-round tick exposure of dogs and cats in Germany and Austria: results from a tick collection study. *Parasit Vectors*. 2023;16(1):70. <https://doi.org/10.1186/s13071-023-05693-5>
31. Lejal E, Marsot M, Chalvet-Monfray K, et al. A three-years assessment of *Ixodes ricinus*-borne pathogens in a French peri-urban forest. *Parasit Vectors*. 2019;12(1):551. <https://doi.org/10.1186/s13071-019-3799-7>
32. Kubiak K, Dziekońska-Rynko J, Szymańska H, Kubiak D, Dmitryjuk M, Dzika E. Questing *Ixodes ricinus* ticks (Acari, Ixodidae) as a vector of *Borrelia burgdorferi* sensu lato and *Borrelia miyamotoi* in an urban area of north-eastern Poland. *Exp Appl Acarol*. 2019;78(1):113–126. <https://doi.org/10.1007/s10493-019-00379-z>
33. Grochowska A, Milewski R, Pancewicz S, et al. Comparison of tick-borne pathogen prevalence in *Ixodes ricinus* ticks collected in urban areas of Europe. *Sci Rep*. 2020;10(1):1–9. <https://doi.org/10.1038/s41598-020-63883-y>
34. Hauck D, Springer A, Chitimia-Dobler L, Strube C. Two-year monitoring of tick abundance and influencing factors in an urban area (city of Hanover, Germany). *Ticks Tick Borne Dis*. 2020;11(5):101464. <https://doi.org/10.1016/j.ttbdis.2020.101464>
35. Vogelgesang JR, Walter M, Kahl O, Rubel F, Brugger K. Long-term monitoring of the seasonal density of questing ixodid ticks in Vienna (Austria): Setup and first results. *Exp Appl Acarol*. 2020;81(3):409–420. <https://doi.org/10.1007/s10493-3-020-00511-4>
36. Hansford KM, Wheeler BW, Tschirren B, Medlock JM. Questing *Ixodes ricinus* ticks and *Borrelia* spp. in urban green space across Europe: A review. *Zoonoses Public Health*. 2022;69:153–166.
37. Bartosik K, Buczek A. The impact of intensity of invasion of *Ixodes ricinus* and *Dermacentor reticulatus* on the course of the parasitic phase. *Ann Agric Environ Med*. 2012;19(4):651–655.
38. Honzáková E, Olejníček J, Černý V, Daniel M, Dusbábek F. Relationship between number of eggs deposited and body weight of engorged *Ixodes ricinus* female. *Folia Parasitol (Praha)*. 1975;22:37–43.
39. Bowessidjaou J, Brossard M, Aeschlimann A. Effects and duration of resistance acquired by rabbits on feeding and egg laying in *Ixodes ricinus* L. *Cell Mol Life Sci*. 1977;33(4):528–530.
40. Schorderet S, Brossard M. Changes in immunity to *Ixodes ricinus* by rabbits infested at different levels. *Med Vet Entomol*. 1993;7(2):186–192. [doi:10.1111/j.1365-2915.1993.tb00673.x](https://doi.org/10.1111/j.1365-2915.1993.tb00673.x)
41. Buczek A, Barańska E, Buczek W, Bartosik K, Ciura D, Buczek AM. Preoviposition and oviposition of female *Ixodes ricinus* (L., 1758) (Ixodida: Ixodidae) at different levels of humidity under laboratory conditions. *Ann. Parasitol*. 2022;(68):s247.
42. Bartosik K, Buczek A, Borzęcki A, Kulina D. Study of the non-parasitic stage in *Ixodes ricinus* after co-feeding with *Dermacentor reticulatus* in three infestations. *Ann Agric Environ Med*. 2017;24(1):90–95. [doi:10.5604/12321966.1234005](https://doi.org/10.5604/12321966.1234005)
43. Alekseev AN, Chunikhin SP. The exchange of the tick-borne encephalitis virus between ixodid ticks feeding jointly on animals with a subthreshold level of viremia. *Med Parazitol*. 1990;2:48–50.
44. Gern L, Rais O. Efficient transmission of *Borrelia burgdorferi* between cofeeding *Ixodes ricinus* ticks (Acari: Ixodidae). *J Med Entomol*. 1996;33(1):189–192.
45. Labuda M, Alves MJ, Eleckova E, Kozuch O, Filipe AR. Transmission of tick-borne bunyaviruses by cofeeding ixodid ticks. *Acta Virol*. 1997;41(6):325–328.
46. Randolph SE. Transmission of tick-borne pathogens between cofeeding ticks: Milan Labuda's enduring paradigm. *Ticks Tick Borne Dis*. 2011;2:179–182.
47. Voordouw MJ. Co-feeding transmission in Lyme disease pathogens. *Parasitology*. 2015;142(2):290–302. [doi:10.1017/S0031182014001486](https://doi.org/10.1017/S0031182014001486)
48. Diehl PA, Aeschlimann A, Obenchain FD. Tick reproduction: oogenesis and oviposition. In: Obenchain FD, Galun R, editors. *The physiology of ticks*. Oxford: Pergamon Press; 1982. p. 277–350.
49. Gillott C. Male accessory gland secretions: modulators of female reproductive physiology and behavior. *Ann Rev Entomol*. 2003;48:163–184.
50. Donohue KV, Khalil SMS, Ross E, Mitchell RD, Roe RM, Sonenshine DE. Male engorgement factor: Role in stimulating engorgement to repletion in the ixodid tick, *Dermacentor variabilis*. *J Insect Physiol*. 2009;55:909–918.
51. Graf J-F. Écologie et éthologie d'*Ixodes ricinus* L. en Suisse (Ixodoidea: Ixodidae). 3e note: Copulation, nutrition et ponte. *Acarologia*. 1974;16(4):636–641.
52. Ioffe-Uspensky I, Uspensky I. The influence of preprandial insemination on feeding and oviposition of *Ixodes persulcatus* females (Acari: Ixodidae) and some thoughts concerning mating strategies in ticks of the genus *Ixodes*. *Ticks Tick Borne Dis*. 2017;8(6):866–871. [doi:10.1016/j.ttbdis.2017.07.003](https://doi.org/10.1016/j.ttbdis.2017.07.003)
53. Graf J-F. Copulation, nutrition et ponte chez *Ixodes ricinus* L. (Ixodoidea: Ixodidae) – 2e partie. *Bull Soc Ent Suisse*. 1978;51:241–253.
54. Balashov YS. Bloodsucking ticks (Ixodoidea) – vectors of diseases of man and animals. *Misc Publ Entomol Soc Am*. 1972;8:161–376.
55. Gray JS. Mating and behavioral diapause in *Ixodes ricinus* L. *Exp Appl Acarol*. 1987;3(1):61–71. [doi:10.1007/BF01200414](https://doi.org/10.1007/BF01200414)
56. Kiszewski AE, Matuschka FR, Spielman A. Mating strategies and spermiogenesis in ixodid ticks. *Ann Rev Entomol*. 2001;46:167–182.
57. Nowak-Chmura, M. Fauna kleszczy (Ixodida) Europy Środkowej. Kraków: Wydawnictwo Naukowe Uniwersytetu Pedagogicznego; 2013. pp. 300.
58. Bartosik K, Buczek A. Determination of the parameters of the parasitic stage in *Ixodes ricinus* females. *Ann Agric Environ Med*. 2013;20(3):441–446.
59. Rijpkema S, Golubić D, Molkenboer M, Verbeek-De Kruif N, Schellekens J. Identification of four genomic groups of *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks collected in a Lyme borreliosis endemic region of northern Croatia. *Exp Appl Acarol*. 1996;20(1):23–30. [doi:10.1007/BF00051474](https://doi.org/10.1007/BF00051474)
60. Massung RF, Slater K, Owens JH, et al. Nested PCR assay for detection of granulocytic ehrlichiae. *J Clin Microbiol*. 1998;36(4):1090–1095. [doi:10.1128/JCM.36.4.1090-1095.1998](https://doi.org/10.1128/JCM.36.4.1090-1095.1998)
61. Blaschitz M, Narodslavsky-Gföller M, Kanzler M, Stanek G, Walochnik J. *Babesia* species occurring in Austrian *Ixodes ricinus* ticks. *Appl Environ Microbiol*. 2008;74(15):4841–4846. [doi:10.1128/AEM.00035-08](https://doi.org/10.1128/AEM.00035-08)
62. Renesto P, Gouvenet J, Drancourt M, Roux V, Raoult D. Use of rpoB gene analysis for detection and identification of *Bartonella* species. *J Clin Microbiol*. 2001;39(2):430–437. [doi:10.1128/JCM.39.2.430-437.2001](https://doi.org/10.1128/JCM.39.2.430-437.2001)
63. Mysterud A, Hatlegjerde IE, Sørensen OJ. Attachment site selection of life stages of *Ixodes ricinus* ticks on a main large host in Europe, the red deer (*Cervus elaphus*). *Parasit Vectors*. 2014;13(7):510. [doi:10.1186/s13071-014-0510-x](https://doi.org/10.1186/s13071-014-0510-x)
64. Ciebiera O, Łopińska A, Gabryś G. Ticks on game animals in the fragmented agricultural landscape of western Poland. *Parasitol Res*. 2021;120(5):1781–1788. [doi:10.1007/s00436-021-07132-9](https://doi.org/10.1007/s00436-021-07132-9)
65. Mysterud A, Hügli C, Viljugrein H. Tick infestation on medium–large-sized mammalian hosts: are all equally suitable to *Ixodes ricinus* adults? *Parasit Vectors*. 2021;14:254. <https://doi.org/10.1186/s13071-021-04775-6>
66. Pavlović I, Jovčevski S, Rogožarski D, Csordás F, Motrović N, Mijatović I, Marčić D, Ilić Ž, Čirković D, Šekler M, Jovčevski S, Ristić M. Biodiversity of ticks and fleas of dogs in the Western Balkans – Preliminary Examinations. *Bull UASVM Vet Med*. 2016;73(2):1–4. [doi:10.15835/buasvmcn-vm:11344](https://doi.org/10.15835/buasvmcn-vm:11344)
67. Cull B, Pietzsch ME, Hansford KM, Gillingham EL, Medlock JM. Surveillance of British ticks: An overview of species records, host associations, and new records of *Ixodes ricinus* distribution. *Ticks Tick Borne Dis*. 2018;9(3):605–614. [doi:10.1016/j.ttbdis.2018.01.011](https://doi.org/10.1016/j.ttbdis.2018.01.011)
68. Zemek R, Bouman E, Socha R, et al. The effect of feeding status on sexual attractiveness of *Ixodes ricinus* (Acari: Ixodidae) females. *Exp Appl Acarol*. 2002;27:137–149. <https://doi.org/10.1023/A:1021511505415>
69. Feldman-Muhsam B, Borut S. Copulation in Ixodid Ticks. *J Parasitol*. 1971;57(3):630–634.
70. Feldman-Muhsam B. Observations on the mating behaviour of ticks. In: Sauer JR, Hair JA, editors. *Morphology, physiology, and behavioral biology of ticks*. Chichester, UK: Ellis Horwood; 1986. p. 217–232.

71. Pet'ko B. Lyme borreliosis in carpathian region of central Europe – ecological aspect of diagnostics. In: Buczek A, Błaszak C, editors. Arthropods. Arthropods in medicine. Lublin: Koliber; 2002, p. 93–104.
72. Asman M, Gąsior T, Pająk C, Cuber P, Szilman P, Szilman E, Solarz K. Occupational risk of infections with *Borrelia burgdorferi* sensu lato, *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii* in agricultural workers on the territory of Beskid Żywiecki. In: Buczek A, Błaszak C, editors. Arthropods. The medical and economic importance. Lublin: Akapit; 2012. p. 163–170.
73. Asman M, Solarz K, Cuber P, et al. Detection of protozoans *Babesia microti* and *Toxoplasma gondii* and their co-existence in ticks (Acari: Ixodida) collected in Tarnogórski district (Upper Silesia, Poland). Ann Agric Environ Med. 2015;22(1):80–83.
74. Asman M, Solarz K, Szilman E, Szilman P, Sikora B, Jakubas-Zawalska J. The occurrence of three tick-borne pathogens in *Ixodes ricinus* ticks collected from the area of the Kraków-Częstochowa Upland (Southern Poland). Acarologia. 2018;58(4):967–975.
75. Chunikhin SP, Stefuktina LF, Korolev MB, Reshetnikov IA, Khozinskaia GA. Sexual transmission of the tick-borne encephalitis virus in ixodid ticks (Ixodidae). Parazitologiya. 1983;17(3):214–217.
76. Alekseev AN, Dubinina HV. Exchange of *Borrelia burgdorferi* between *Ixodes persulcatus* (Ixodidae: Acarina) sexual partners. J Med Entomol. 1996a;33(3):351–354.
77. Alekseev AN, Dubinina HV. Venereal and cannibalistic ways of *Borrelia burgdorferi* sensu lato exchange between males and females of *Ixodes persulcatus* (Ixodidae, Acarina). Roczniki Akademii Medycznej w Białymstoku (1995). 1996b;41(1):103–110.
78. Gaber MS, Khalil GM, Hoogstraal H. *Borrelia crocidurae*: Venereal transfer in Egyptian *Ornithodoros erraticus* ticks. Exp Parasitol. 1982;54:182–184.
79. Gaber MS, Khalil GM, Hoogstraal H, Aboul-Nasr AE. *Borrelia crocidurae* localization and transmission in *Ornithodoros erraticus* and *O. savignyi*. Parasitology. 1984;88:403–413.
80. Hayes SF, Burgdorfer W, Aeschlimann A. Sexual transmission of spotted fever group Rickettsiae by infected male ticks: detection of Rickettsiae in immature spermatozoa of *Ixodes ricinus*. Infect Immun. 1980;27:638–642.
81. De Silva AM, Fikrig E. Growth and migration of *Borrelia burgdorferi* in *Ixodes* ticks during blood feeding. Am J Trop Med Hyg. 1995;53(4):397–404. doi:10.4269/ajtmh.1995.53.397
82. Hojgaard A, Eisen RJ, Piesman J. Transmission dynamics of *Borrelia burgdorferi* s.s. during the key third day of feeding by nymphal *Ixodes scapularis* (Acari: Ixodidae). J Med Entomol. 2008;45(4):732–736. doi: 10.1603/0022-2585(2008)45[732:TDOBBS]2.0.CO;2. PMID: 18714875.
83. Dunham-Ems SM, Caimano MJ, Pal U, et al. Live imaging reveals a biphasic mode of dissemination of *Borrelia burgdorferi* within ticks. J Clin Invest. 2009;119(12):3652–3665. doi:10.1172/JCI39401
84. Kurokawa C, Lynn GE, Pedra JHF, Pal U, Narasimhan S, Fikrig E. Interactions between *Borrelia burgdorferi* and ticks. Nat Rev Microbiol. 2020;18:587–600. doi:10.1038/s41579-020-0400-5
85. Lejal E, Moutailler S, Šimo L, Vayssier-Taussat M, Pollet T. Tick-borne pathogen detection in midgut and salivary glands of adult *Ixodes ricinus*. Parasit Vectors. 2019;12(1):152. doi:10.1186/s3071-019-3418-7
86. Piesman J, Schneider BS, Zeidner NS. Use of quantitative PCR to measure density of *Borrelia burgdorferi* in the midgut and salivary glands of feeding tick vectors. J Clin Microbiol. 2001;39(11):4145–8. doi:10.1128/JCM.39.11.4145-4148.2001
87. Ebel G, Kramer L. Short report: Duration of tick attachment required for transmission of Powassan virus by deer ticks. Am J Trop Med Hyg. 2004;71:268–271.
88. Richards SL, Langley R, Apperson CS, Watson E. Do tick attachment times vary between different tick-pathogen systems? Environments. 2017;4(2):37. https://doi.org/10.3390/environments4020037
89. Eisen L. Pathogen transmission in relation to duration of attachment by *Ixodes scapularis* ticks. Ticks Tick Borne Dis. 2018;9(3):535–542. doi:10.1016/j.ttbdis.2018.01.002
90. Rocha SC, Velásquez CV, Aquib A, Al-Nazal A, Parveen N. Transmission cycle of tick-borne infections and co-infections, Animal models and diseases. Pathogens. 2022;11(11):1309. https://doi.org/10.3390/pathogens11111309
91. Alekseev AN. Tick pathogen interactions: behavior of infected and uninfected ticks (Ixodidae). In: Mitchell R, Horn DJ, Needham GR, Welbourn W, editors. Acarology. Columbus: Biological Survey; 1996. p. 113–15.
92. Alekseev AN, Dubinina HV. Abiotic parameters and diel and seasonal activity of *Borrelia*-infected and uninfected *Ixodes persulcatus* (Acarina: Ixodidae). J Med Entomol. 2000;37(1):9–15. doi:10.1603/0022-2585-37.1.9
93. Belova OA, Burenkova LA, Karganova GG. Different tick-borne encephalitis virus (TBEV) prevalences in unfed versus partially engorged ixodid ticks--evidence of virus replication and changes in tick behavior. Ticks Tick Borne Dis. 2012;3(4):240–246. doi:10.1016/j.ttbdis.2012.05.005
94. Herrmann C, Gern L. Do the level of energy reserves, hydration status and *Borrelia* infection influence walking by *Ixodes ricinus* (Acari: Ixodidae) ticks? Parasitology. 2012;139(3):330–337. doi:10.1017/S0031182011002095
95. Pipová N, Peňazziová K, Baňas M, Majláth I, Majláthová V. The Behavior of Rickettsia-positive *Dermacentor reticulatus* ticks under laboratory conditions. Life (Basel). 2023;13(3):612. doi:10.3390/life13030612
96. Buczek W, Buczek A, Witecka J, Asman M. Prevalence of pathogens in sympatric *Ixodes ricinus* and *Dermacentor reticulatus* ticks in Eastern Poland and their potential impact on oral-anal contacts between ticks. Ann Agric Environ Med. 2023;30(2):259–265. doi:10.26444/aaem/167427
97. Kempf F, de Meeûs T, Arnathau C, Degeilh B, McCoy KD. Assortative pairing in *Ixodes ricinus* (Acari: Ixodidae), the European vector of Lyme Borreliosis. J Med Entomol. 2009;46(3):471–474.