



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 Metastricata 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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