



Prevalence of pathogens in sympatric *Ixodes ricinus* and *Dermacentor reticulatus* ticks in Eastern Poland and their potential impact on oral-anal contacts between ticks

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Abstract

Introduction and Objective. Little is known about interspecific contacts between ticks. Therefore, this study focused on the investigation of factors that may influence interspecific contacts between *Ixodes ricinus* and *Dermacentor reticulatus* ticks.

Materials and method. *Ixodes ricinus* males and *D. reticulatus* females involved in oral-anal contacts (group I) and questing specimens with no such behaviour (group II) collected in eastern Poland were examined using molecular techniques to detect *Borrelia burgdorferi* s.l. (Bb), *Rickettsia* spp. (Rs), *Anaplasma phagocytophilum*, *Babesia microti*, and *Toxoplasma gondii*.

Results. An extremely high infection rate of Bb and Rs was determined in *I. ricinus* males (in groups I: 100% and 46.15% and group II: 90% and 40%, respectively) and *D. reticulatus* females (in group I: 84.61% and 61.53% and in group II: 90% and 20%, respectively). The prevalence of other pathogens in these ticks was substantially lower. Co-infection with pathogens was detected in approximately 53% of ticks.

Conclusions. The study suggests that tick-borne pathogens may have influenced the sexual behaviour of their vectors. The oral-anal contacts between *I. ricinus* and *D. reticulatus* ticks are probably stimulated by Bb and/or Rs. The presence of five pathogens and numerous co-infections in the analysed ticks indicates a risk of various human infectious diseases in the study region. Further studies are required to clarify the implications of oral-anal interspecific tick interactions.

Key words

Ixodes ricinus, *Dermacentor reticulatus*, co-infections, tick behaviour, oral-anal contact between ticks, tick-borne pathogens, vector manipulation

INTRODUCTION

Given the diversity of transmitted pathogens and the wide range of hosts, including domestic and wild animals, *Ixodes ricinus* and *Dermacentor reticulatus* ticks represent arthropods with the greatest epidemiological importance in Europe. *Ixodes ricinus* frequently infests humans [1–3] and can transmit pathogens during human blood ingestion. *Dermacentor reticulatus* is a less frequent human parasite [1, 3, 4]; nevertheless, by transmission of tick-borne pathogens to animal hosts, it contributes to the maintenance of foci of infectious diseases in nature and poses a serious threat to public health. The direct effects of tick parasitism on hosts include skin lesions and systemic reactions caused by the components of tick saliva.

Over the last two decades the distribution range of *I. ricinus* [5] and *D. reticulatus* [6] in Europe has expanded substantially. Currently, these ticks can be found not only in natural habitats but also in parks and other recreation areas

inside or near cities [7–10]. This phenomenon is associated with climate change and environmental changes, which influence the development, survival, and phenology of ticks and the behaviour of their potential animal hosts. The prevalence of *I. ricinus* and *D. reticulatus* in different habitats has increased the risk of infestations of hosts. Hence, investigations of the abiotic and biotic determinants of tick behaviour in the environment arouse great interest and are still gaining in importance.

The impact of climatic parameters (mainly humidity and temperature), environmental conditions [11, 12], and chemical stimuli released by hosts [13–15] on the questing behaviour of ticks has been documented in many studies. In contrast, there are sparse data on the influence of microorganisms on ticks. The results of previous studies indicate that microorganisms can manipulate ticks mainly through their impact on tick questing activity, survival, and energy reserves [16–23]. The question of the potential stimulation of ticks to interact with each other by pathogens has not been addressed to date.

This study was inspired by numerous observations of cases of oral-anal contacts between *I. ricinus* males and *D. reticulatus* females from the same habitat, as described

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in a previous study by the authors [24]. To identify the potential determinants of this atypical behaviour of ticks, the occurrence was examined of pathogens in *I. ricinus* and *D. reticulatus* ticks involved in oral-anal contact, and in questing specimens of these species collected at the same time in their habitat located in eastern Poland. Despite the multi-year experience in investigations of tick biology and behaviour, the authors have never observed a similar phenomenon in ticks collected in other habitats.

MATERIALS AND METHOD

Research procedure. Hungry *I. ricinus* and *D. reticulatus* adults were collected with the flagging method in their habitat near Lubycza Królewska (eastern Poland, 51°27'N 23°10'E), placed in glass containers, and transported to the laboratory. As in the authors' previous study [24], both tick species were kept together in the dark, in a refrigerator at a temperature of approx. 5°C and approx. 80% humidity. The behaviour of the ticks was observed over the next few days. Each pair of ticks of the different species attached to each other on the ventral side was transferred to separate containers and observed every day using an Olympus SZX16 stereoscopic microscope in a room with a monitored temperature of 22°C and approx. 50% humidity. Each time, tweezers were used to assess the degree of attachment of the hypostome of one specimen to the anal aperture of the other tick. The observations were continued until detachment of the ticks from each other. The *I. ricinus* and *D. reticulatus* ticks from each pair (group I) were examined to detect the presence of tick-borne pathogens. The identification of pathogens was also carried out in 20 randomly-selected adult *I. ricinus* (10 females and 10 males) and 20 adult *D. reticulatus* specimens (10 females and 10 males) collected in the same locality during the same period of their activity (group II). No oral-anal contact was exhibited by these ticks during the observations.

Molecular analyses. The *I. ricinus* and *D. reticulatus* specimens from groups I and II were analysed to detect infection with five pathogens: *Borrelia burgdorferi* s.l., *Rickettsia* spp., *Anaplasma phagocytophilum*, *Babesia microti*, and *Toxoplasma gondii*.

DNA was isolated from single ticks by the ammonia method [25]. The concentration was measured spectrophotometrically at the 260/280 nm wavelength in a PEARL nanospectrophotometer (Implen, Munich, Germany). The samples were then frozen at -20°C and stored for further molecular analyses. Pathogens were detected using the PCR and nested PCR methods. Amplification and re-amplification were conducted in a TurboCycler Lite thermocycler (Blue Ray Biotech, Taiwan). Two pairs of primers specific to the flagellin gene were used for the detection of *B. burgdorferi* s.l. in the ticks [26]. In turn, primers specific to the 16S rRNA and 18S rRNA gene fragments were used to detect *A. phagocytophilum* and *B. microti*, respectively [27, 28]. The presence of *Rickettsia* spp. was determined with the use of a pair of primers specific to the *gltA* gene fragment [29]. The presence of *T. gondii* in the studied material was detected with the use of two pairs of primers specific to the B1 gene fragment [30]. The PCR and nested PCR products were separated electrophoretically in ethidium bromide-stained 2% agarose gels and visualised under ultraviolet light in an Omega 10 device (Ultra-Lum,

Claremont, CA, USA). Next, the results were analysed using Total Lab software (TotalLab, Gosforth, UK).

Statistical analysis. To compare the differences between group I of ticks involved in the physical contact and group II of ticks (no physical contact), the Z-test was used for two independent proportions. The results were considered statistically significant at the level of $p \leq 0.05$.

RESULTS

The present study shows that interspecific contact between *I. ricinus* and *D. reticulatus* ticks outside the host is not an incidental phenomenon – 13 cases of oral-anal contacts were observed between the adult *I. ricinus* and *D. reticulatus* ticks collected in the habitat in eastern Poland (Fig. 1). The ticks entered into contact approximately 12–20 hours after transfer to the same container.

All pairs of ticks remained in contact for approximately eight days although they were exposed to strong external stimuli every day. These included thermal shock (a rise in the temperature from 5°C to 22°C), intense illumination, elevated CO₂ concentrations in the air, the smell of the observers, and mechanical irritation caused by checking the attachment of the hypostome of one specimen to the anal aperture of the other. During the observations, the *D. reticulatus* females with the attached *I. ricinus* males moved rapidly in the Petri dishes.

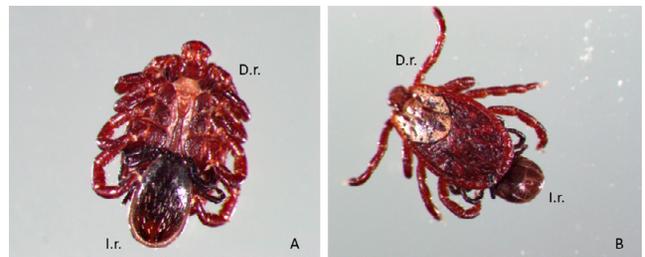


Figure 1. Oral-anal contact between an *Ixodes ricinus* male and a *Dermacentor reticulatus* female; ventral side (A), dorsal side (B), l.r. – *Ixodes ricinus* male, D.r. – *Dermacentor reticulatus* female

B. burgdorferi s.l., *Rickettsia* spp., *A. phagocytophilum*, *B. microti*, and *T. gondii* were detected in the adult *I. ricinus* and *D. reticulatus* ticks (Fig. 2). *B. burgdorferi* s.l. and *Rickettsia* spp. rickettsiae were the most frequently identified bacteria in both groups of ticks collected from vegetation, i.e. specimens involved in oral-anal contact (group I) and those showing no tendency towards interspecies contacts during the observation period.

Borrelia burgdorferi s.l. spirochetes were identified in as many as 92.3% of ticks of both species involved in oral-anal contact (Fig. 2), i.e. in 90% of *I. ricinus* males and 84.61% of *D. reticulatus* females (Fig. 3). Similarly, a high percentage of tick infection with this pathogen (90%) (Fig. 2), i.e. 90% of *I. ricinus* males and 90% of *D. reticulatus* females, was observed in group II, which comprised randomly selected questing ticks from the same habitat (Fig. 3A, 3B). The statistical Z test for two independent proportions did not confirm any statistically significant differences in the prevalence of *B. burgdorferi* between *I. ricinus* males ($Z=0.13$; $p=0.8930$) and *D. reticulatus* females ($Z=-0.24$; $p=0.8070$) from both groups of ticks.

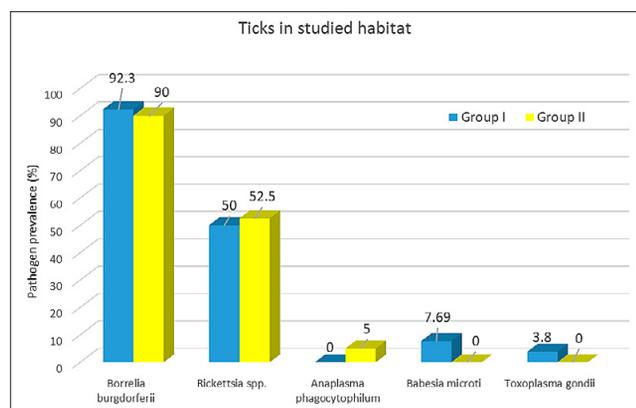


Figure 2. Pathogens identified in adult *Ixodes ricinus* and *Dermacentor reticulatus* ticks involved (group I) and not involved (group II) in oral-anal contacts collected in the habitat of eastern Poland

Rickettsia spp. were detected in 50% of all ticks from group I (Fig. 2), i.e. in 46.15% of *I. ricinus* males and 61.53% of *D. reticulatus* females, and in 52.5% of all ticks from group II, i.e. 40% of *I. ricinus* males and 20% of *D. reticulatus* females (Fig. 3). There were no statistically significant differences in the prevalence of *Rickettsia* spp. between *I. ricinus* males ($Z=-0.13$; $p=0.8973$) and *D. reticulatus* females ($Z=1.22$; $p=0.2233$) in groups I and II.

A high prevalence of *B. burgdorferi* s.l. and *Rickettsia* spp. was also recorded in *I. ricinus* females and *D. reticulatus* males exhibiting no oral-anal contact during the observations (Fig. 3).

The other pathogens were rarely detected in both groups of ticks. The presence of *Babesia microti* DNA was revealed in 15.38% (2/13 analysed specimens) of *I. ricinus* males, and *Toxoplasma gondii* DNA was found in 7.7% (1/13) of *D. reticulatus* females from group I. No *A. phagocytophilum* was identified in either of the species of ticks involved in oral-anal contact (Tab. 1). In group II, only *I. ricinus* males were

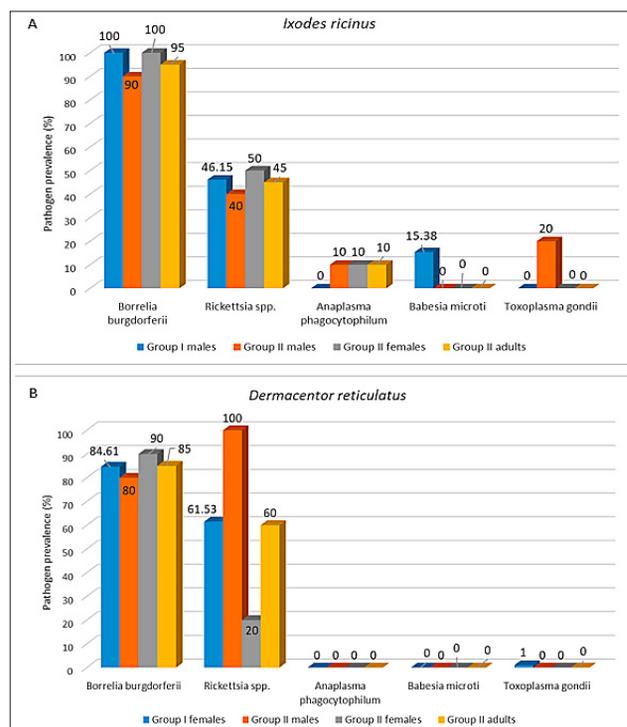


Figure 3. Prevalence of pathogens in *Ixodes ricinus* females and males (A); in *Dermacentor reticulatus* females and males (B) collected in a habitat in eastern Poland

infected by *T. gondii* (20%, 2/10) and *A. phagocytophilum* (10%, 1/10) (Fig. 3).

The statistical Z test did not confirm statistically significant differences in the rates of infection with *A. phagocytophilum* ($Z=0.13$; $p=0.8930$), *B. microti* ($Z=0.55$; $p=0.5812$), and *T. gondii* ($Z=0.94$; $p=0.3467$) between the *I. ricinus* males from groups I and II. Similarly, no significant differences were found in the presence of *T. gondii* between

Table 1. Pathogen co-infections in *Ixodes ricinus* and *Dermacentor reticulatus* ticks involved (group I) and not involved (group II) in interspecific oral-anal interactions (M – males, F – females, A – adult ticks)

| Tick species/life stage | Co-infections | | | |
|--|--|---|---|---|
| | <i>B. burgdorferi</i> + <i>Rickettsia</i> spp. | <i>B. burgdorferi</i> + <i>A. phagocytophilum</i> | <i>B. burgdorferi</i> + <i>B. microti</i> | <i>B. burgdorferi</i> + <i>Rickettsia</i> spp. + <i>Toxoplasma gondii</i> |
| Group I <i>I. ricinus</i> M | 46.15% (6/13) | 0 | 15.38% (2/13) | 0 |
| Group I <i>D. reticulatus</i> F o-a interspecific interactions | 38.46% (5/13) | 0 | 0 | 7.69% (1/13) |
| Group II <i>I. ricinus</i> | | | | |
| F | 50% (5/10) | 10% (1/10) | 0 | 0 |
| M | 40% (4/10) | 10% (1/10) | 0 | 20% (2/10) |
| A | 45% (9/20) | 10% (2/20) | 0 | 10% (2/20) |
| Group II <i>D. reticulatus</i> | | | | |
| F | 20% (2/10) | 0 | 0 | 0 |
| M | 80% (8/10) | 0 | 0 | 0 |
| A | 50% (10/20) | 0 | 0 | 0 |
| All <i>I. ricinus</i> A in studied habitat | 45.45% (15/33) | 6.06% (2/33) | 6.06% (2/33) | 6.06% (2/33) |
| <i>D. reticulatus</i> A in studied habitat | 45.45% (15/33) | 0 | 0 | 3.03% (1/33) |
| Total | 45.45% (30/66) | 3.03% (2/66) | 3.03% (2/66) | 4.54% (3/66) |

the *D. reticulatus* females from groups I and II ($Z=-0.13$; $p=0.8930$).

Pathogen co-infection was detected in 53.8% of ticks involved in the interspecific contact (group I), i.e. in 61.53% of *I. ricinus* males and 38.46% of *D. reticulatus* females, and in 52.5% of specimens from group II, i.e. in 55% of *I. ricinus* and 50% of *D. reticulatus* ticks. The ticks were most frequently infected by two pathogens, mainly *B. burgdorferi* and *Rickettsia* spp. (45.45%), and rarely by three pathogens *B. burgdorferi*+*Rickettsia* spp.+*T. gondii* (4.54%) (Tab. 1).

There were no differences between the *I. ricinus* males from groups I and II in the occurrence of co-infections caused by *B. burgdorferi*+*Rickettsia* spp. ($Z=-0.13$; $p=0.8973$), *B. burgdorferi*+*A. phagocytophilum* ($Z=0.13$; $p=0.8930$), *B. burgdorferi*+*B. microti* ($Z=0.55$; $p=0.5912$), and *B. burgdorferi*+*Rickettsia* spp.+*T. gondii* ($Z=0.94$; 0.3467). No differences in the rates of co-infection by *B. burgdorferi*+*Rickettsia* spp. ($Z= 0.50$; $p=0.6193$) and *B. burgdorferi*+*Rickettsia*+*T. gondii* ($Z=-0.13$; $p=0.8930$) were found between the *D. reticulatus* females from both groups.

DISCUSSION

The knowledge of physical contacts between *I. ricinus* and *D. reticulatus* or other tick species colonising the same habitats is still incomplete. Previous studies have only shown that *I. ricinus* and *D. reticulatus* co-feeding on the host has an impact on the reproductive ability of female ticks and the development of embryonic stages. The egg amount, number of eggs per 1 mg of female engorgement weight, and hatching success were higher in interspecific groups of these ticks than in species-homogenous groups, as evidenced by an earlier study by the authors [31]. The presence of *D. reticulatus* ticks was reported to modify the behaviour on the host and the feeding performance of *D. marginatus* specimens [32]. The authors have not found any literature reports on interspecific interactions of European ticks in the non-parasitic phase of their life cycle.

In all the cases of the oral-anal tick contact described so far by the authors, only *I. ricinus* males were found to insert their hypostomes in the anal aperture of *D. reticulatus* females. The authors have never observed such behaviour of *D. reticulatus* males towards *I. ricinus* females. This may be related to the differences in the morphological structure of the mouthparts and anal apertures of the adult stages of both tick species, mainly their size and shape. *Ixodes ricinus* males have a long lanceolate hypostome and long chelicerae, while females have a small anal aperture. In turn, *D. reticulatus* males have a short, wide, clavate hypostome, while the other mouthparts are short. The anal aperture of *D. reticulatus* females is larger than that of *I. ricinus* females.

No statistically significant differences were found in the pathogen infection rate between the observed ticks from groups I (oral-anal contact) and II (no oral-anal contact). However, it seems that the unusual contacts between *I. ricinus* males and *D. reticulatus* females may be caused by *B. burgdorferi* s.l. and/or *Rickettsia* spp. This hypothesis may be supported by the extremely high prevalence of these pathogens, especially *B. burgdorferi* s.l., in the females and males of both tick species collected in the study area. With the methodology employed in this study, it is not possible to determine precisely which of these pathogens may

stimulate ticks for oral-anal contact. An interaction of both pathogens, i.e. *B. burgdorferi* s.l. and *Rickettsia* spp., in the modification of physiological processes in ticks cannot be ruled out. *Borrelia* spirochetes in double co-infection with *Rickettsia* spp. and in triple co-infection with *Rickettsia* spp. and *T. gondii* were detected in approximately 50% of all the *I. ricinus* and *D. reticulatus* ticks.

The ability of pathogens, especially *Borrelia* spirochetes, to change tick behaviour has been reported by other authors. Alekseev [16] and Alekseev and Dubinina [17] found that *Borrelia*-infected *I. ricinus* larvae, nymphs, and adults as well as *I. persulcatus* adults, covered shorter distances than uninfected specimens. In the case of *I. scapularis*, nymphs infected with *B. burgdorferi* spirochetes covered longer distances and reached higher questing heights, while adults covered shorter distances and reached lower questing heights [18].

It has been reported that *Rickettsia* increased and *Arsenophonus* decreased the motility of larvae of three eastern North American tick species: *Amblyomma americanum*, *Dermacentor variabilis*, and *Ixodes scapularis*. *Rickettsia*- and *Arsenophonus*-infected *D. variabilis* specimens were shown to move faster than *I. scapularis* ticks [33]. The impact of *Rickettsia amblyommatis* on *Amblyomma americanum* questing behaviour was reported by Richardson et al. [34]. In turn, TBE viruses caused adult *I. persulcatus* and *I. ricinus* to walk faster and increased their tolerance to desiccation [16] and repellents [19]. *Ixodes scapularis* ticks infected by *B. microti* protozoa ingested a larger blood meal [35].

Villar et al. [36] suggest that pathogens may influence physiological processes in ticks through modification of tick proteolytic pathways. As indicated by these authors, *A. phagocytophilum* selectively manipulates the levels of vertebrate proteins in *I. scapularis* females in a tissue-specific manner, thereby facilitating pathogen infection, propagation, and transmission with sustenance of tick feeding.

The prevalence of pathogens, especially *B. burgdorferi* s.l. and *Rickettsia* spp., in *I. ricinus* and *D. reticulatus* ticks in the habitat located in eastern Poland is surprisingly high in comparison with the results of similar studies conducted in Poland and in various parts of Europe. Particularly interesting is the detection of *B. burgdorferi* s.l. in many *D. reticulatus* ticks, which are not competent vectors of this pathogen, as suggested by some researchers [12, 37, 38]. Rudolf and Hubalek [39] showed that the salivary gland and midgut extracts in this species inhibited the development of one of the *B. burgdorferi* s.l. genospecies, i.e. *B. afzelii*, *in vitro*. In contrast, extracts from this gland and the midgut of *I. ricinus*, which is the main vector of borreliosis, were reported to stimulate the growth of these spirochetes.

The high *Borrelia* infection rate in the *D. reticulatus* ticks collected in the analysed habitat, which has not been reported in the literature to date, is probably associated with the favourable conditions for the development of ticks and for the zoonotic reservoirs of this pathogen. However, this aspect was not investigated in the present study.

In other localities in the Lublin Province of eastern Poland, the *B. burgdorferi* s.l. infection rate in *I. ricinus* and *D. reticulatus* collected from vegetation over the last 11 years typically ranged from 4.94% – 15.3% [40, 41] and from 0% – 2.5% [42], respectively. In the northern part of Lublin Province, *B. burgdorferi* spirochetes were detected in 0.7% of *D. reticulatus* specimens removed from dogs

[43]. The percentage of *D. reticulatus* ticks infected with this bacterium and collected from vegetation and dogs was higher, and reached 22.8% only in urban settlements in this region [44]. Podlaskie Province, bordering the north of Lublin Province, is characterised by the highest incidence of tick-borne diseases recorded in Poland, and the prevalence of *B. burgdorferi* s.l. in this region, on average, is 25.2% in questing *I. ricinus* ticks and only 0.7% in *D. reticulatus* [12].

In an area of co-occurrence of both tick species in Mazovia Province (east-central Poland), *B. burgdorferi* s.l. spirochetes were identified in 12.2%–22.2% of questing *I. ricinus* ticks [45] and in 0.09%–3.6% of *D. reticulatus* specimens [46, 47]. In the Masuria region (north-eastern Poland), these bacteria were detected in 27% of questing *I. ricinus* ticks [48] and in 1.2% of *D. reticulatus* removed from wild ungulates [49]. Pawełczyk et al. [3] confirmed the presence of *B. burgdorferi* in 12.7% of *D. reticulatus* specimens removed from human skin.

In south-western Poland, the prevalence of *B. burgdorferi* s.l. in *I. ricinus* and *D. reticulatus* collected from vegetation or animals was reported to be in the range of 0.9%–24.8% [50–53] and 2% [51], respectively, depending on the habitat.

No infections by *Borrelia burgdorferi* s.l. spirochetes or a low infection rate ranging from 0.25% – 7% can be observed in *D. reticulatus* in almost the entire occurrence range of this species [8, 37, 38, 54–56]. Besides the present study, the largest numbers of questing *D. reticulatus* ticks infected by this bacterium (31.9%) have been detected in Western Ukraine [57].

The prevalence of *Borrelia burgdorferi* s.l. in questing *I. ricinus* adults in other regions of Europe most often ranges from 6.8% – 25% [10]. In some areas, however, the percentage of infected *I. ricinus* adult ticks is substantially higher; for example, *Borrelia* spirochetes were identified in $48.9 \pm 8.4\%$ of adult *I. ricinus* ticks [58] in Finland, and 49.1% of adult stages were infected by the bacteria in northern Germany (Western Pomerania) [56].

The present results showed a similar prevalence of *Rickettsia* spp. in *I. ricinus* and *D. reticulatus* ticks co-occurring in the same habitat in Lublin Province (Tab. 1). In other habitats in the region, these rickettsiae were detected in 43.8% of questing adult *D. reticulatus* ticks [42]. The *Rickettsia* spp. infection rate in questing *I. ricinus* and *D. reticulatus* from their co-occurrence areas located in various parts of Poland ranges from approx. 4% – 15.6% [59, 60] and from 35% – 57% [61, 62], respectively. The *Rickettsia* infection rate in adult *I. ricinus* and *D. reticulatus* ticks in some regions of Europe may be as high as $6.4 \pm 3.4\%$ (*R. helvetica*) [58] and 82.0% (*Rickettsia* spp.) [56], respectively.

A. phagocytophilum, *Babesia microti*, and *T. gondii* pathogens in *I. ricinus* and *D. reticulatus* ticks exhibiting atypical sexual behaviour and in the other questing ticks collected in the same habitat were detected less frequently than spirochetes and rickettsiae, which is consistent with most studies conducted in various regions of Poland [12, 40, 46, 52, 59] and other European countries [8, 37, 54, 56].

The frequency of co-infection in the *I. ricinus* and *D. reticulatus* specimens examined in the present study is higher than that in specimens of these species collected in other habitats of Poland [40, 42, 46, 47, 52] and European countries [2, 9, 55, 63], where it ranges from 0.7% – 45%. As reported by other authors [9, 40, 42, 55, 63], the ticks were most often co-infected by two or, less often, three pathogens.

Given the current state of knowledge, it is impossible to determine whether the atypical sexual behaviour of ticks contributes to transmission of pathogens, or whether it has epidemiological significance. Possibly, some pathogens contained in tick excrements are transferred from one tick specimen to another during the interspecific oral-anal contact. Analyses have revealed the presence of *Francisella tularensis* in the faeces of *I. ricinus* nymphs and adults [64], viable *Coxiella burnetii* in faeces of *I. ricinus* and *D. marginatus* [65], and *Bartonella henselae* bacteria in the faeces of *Rhipicephalus sanguineus* larvae [66]. As shown by these authors, these bacteria are not able to survive in the external environment.

Without comprehensive ecological studies, it is difficult to identify environmental factors that contribute to the surprisingly high *B. burgdorferi* s.l. and *Rickettsia* spp. infection rate in *I. ricinus* and *D. reticulatus* ticks from the habitat in eastern Poland. The detection of *Borrelia* spirochetes in the high number of *D. reticulatus* adults suggests the necessity for further research to clarify the role of this tick species in the circulation of bacteria in nature.

CONCLUSIONS

Due to the increasing threat to human and animal health posed by tick attacks and tick-borne diseases around the world, it is extremely important to elucidate the interactions occurring in the biological systems created by these arthropods. It is essential to explore the impact of pathogens on the physiology of various tick species, including their behaviour in the habitat.

Although previous studies have confirmed that pathogens can manipulate tick behaviour, their ability to initiate interspecific tick contacts has not been reported to date. Research by the authors of the current study indicates that the sympatric *I. ricinus* and *D. reticulatus* tick species enter into contact in the non-parasitic phase of the life cycle. The oral-anal contacts between *I. ricinus* males and *D. reticulatus* females, described for the first time by the authors, are probably associated with the extremely high prevalence of *Borrelia burgdorferi* s.l. and/or *Rickettsia* spp. in these tick species from the habitats in eastern Poland. However, the contribution of the interspecific contacts to transmission of some pathogens in nature and their epidemiological importance are still unknown.

The presence of five pathogens: *Borrelia burgdorferi* s.l., *Rickettsia* spp., *Anaplasma phagocytophilum*, *Babesia microti*, and *Toxoplasma gondii*, was detected in the questing *I. ricinus* and *D. reticulatus* adult ticks collected in eastern Poland. This indicates a potential threat to humans and animals of various zoonotic diseases. Pathogen co-infections in ticks may result in polymicrobial tick-borne infections in humans and animals.

The high *B. burgdorferi* s.l. infection rate in the *D. reticulatus* ticks collected in eastern Poland suggests the need for further research on the involvement of this species in the maintenance and circulation of this pathogen in nature. It would also be interesting to identify biological and abiotic factors that may influence the wide spread of such pathogens as *B. burgdorferi* s.l. and *Rickettsia* spp. in *I. ricinus* and *D. reticulatus* ticks occurring in the same habitat.

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