DOI: 10.2478/s11686-007-0015-2

Acta Parasitologica, 2007, 52(2), 165–170; ISSN 1230-2821

VERSITA

Infestation of sand lizards (*Lacerta agilis*) resident in the Northeastern Poland by *Ixodes ricinus* (L.) ticks and their infection with *Borrelia burgdorferi* sensu lato

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Abstract

Sand lizards (*Lacerta agilis*) were trapped and examined for ticks from May to September in 2002 and 2003 in Northeastern Poland. A total of 233 *Ixodes ricinus* (L.) ticks (76 larvae and 157 nymphs) was found on 31 of 235 captured lizards (13.2%). The tick infestation is relatively low compared to that of mammals and passerine birds from the same area (Siński *et al*. 2006, Gryczyńska *et al*. 2002). Tick infestation depended on the month of capture, being the highest in spring. In autumn no ticks were recorded on any of the captured lizards. The oldest lizards carried the highest number of ticks but no differences related to sex of the host were found. All the collected ticks were analysed by PCR for the presence of *Borrelia burgdorferi* sensu lato, the etiological agents of Lyme disease. Spirochetes were detected in 11 out of 233 (4.7%) ticks tested. Genetic analysis confirmed that the spirochetes are members of the *Borrelia afzelii*, *B. garinii* and *B. burgdorferi* sensu stricto genospecies. Mixed infection were not detected. The prevalence of infection was analysed in relation to months of the capture, age and sex of the lizards, but differences were not statistically significant. The obtained results suggest that lizards are probably not *B. burgdorferi* reservoirs, but further studies are required to confirm this.

Key words

Borrelia burgdorferi sensu lato, *Ixodes ricinus*, lizards

Introduction

Ixodes ricinus (L.), as the most common species in Europe (Siuda 1993), may be a vector of numerous pathogens (viruses, rickettsiae, bacteria, protozoans) that cause many serious diseases in humans (Prokopowicz 1995).

In its life cycle, tick *Ixodes ricinus* requires three hosts, on which it engorges as a larva, nymph or adult. The most frequently attacked hosts are mammals, of which rodents and insectivores are the core group of hosts for larvae and nymphs, whereas carnivores and ungulates are the main host chosen by adult ticks (Matuschka 1991, Humair *et al*. 1993). It has also been found that a significant role of the host for immature tick stages is played by passerine birds, gallinaceous birds and seabirds (Olsén *et al*. 1995, Kurtenbach *et al*. 1998a, Gryczyńska *et al*. 2002). It is known from the literature that both birds and mammals constitute a reservoir of tick-borne pathogens and are involved in spreading diseases transmitted by ticks in the environment (Gern *et al*. 1998, Bajer *et al*. 1999, Hulinska *et al*. 2002, Gryczyńska *et al*. 2004, Karbowiak 2004, Stańczak *et al*. 2004).

Reptiles are the third group of vertebrates on which ticks *Ixodes ricinus* can engorge. There are few papers dealing with infestation of various reptile species by ticks. Research has been focused mainly on temporal trends and environmental correlates of prevalence and abundance of ticks infesting western fence lizards (*Sceloporus occidentalis*) (Talleklint-Eisen and Eisen 1999, Eisen *et al*. 2001, Casher *et al*. 2002), five-lined skink (*Eumeces fasciatus*), broad-headed skink (*Eumeces laticeps*), eastern fence lizard (*Sceloporus undulatus*) (Kollars *et al*. 1999) and southern alligator lizard (*Elgaria multicarinata*) (Wright *et al*. 1998) in America, and sand lizard (*Lacerta agilis*) (Matuschka *et al*. 1991) and *Lacerta bilineata* (Scali *et al*. 2001) in Europe. Opinions about the competence of reptiles as a reservoir for tick-borne pathogens differ. Some researchers consider that reptiles have no ability to

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transmit tick-borne pathogens to hosts (Lane and Loye 1989, Lane 1990, Lane and Quistad 1998, Lane *et al*. 2006). The others suggests, that they may serve as an effective source of pathogens in nature (Levin *et al*. 1996). Some investigations demonstrated heterogeneity in complement-dependent bacteriolysis among strains of *B. burgdorferi* s.l. (Breitner-Ruddock *et al*. 1997, Kurtenbach *et al*. 1998b, Kuo *et al*. 2000). In particular, it was suggested that lizards are associated with *B. lusitaniae* (Dsouli *et al*. 2006, Richter and Matuschka 2006).

The primary aim of our study was to quantify the relative importance of the sand lizards, the most common reptile species in Poland, as hosts for the immature stages of ticks. Additionally, we analyzed ticks engorged on lizards for infection with the etiological agents of Lyme disease which is *Borrelia burgdorferi* s.l. (in case of some positive samples identification of genospecies was possible).

Materials and methods

Lizard sampling

The Mazurian Lake region, located in Northeastern Poland, was selected as the study site. This province was chosen on account of the abundance of *Ixodes ricinus* ticks (Siuda 1993). Furthermore, Northeastern Poland is considered an endemic area for many tick-borne diseases (e.g., Lyme disease, tick-borne encephalitis, human anaplasmosis, piroplasmosis) (Prokopowicz 1995).

The catches were performed in 2002 and 2003 during the seasons of the highest tick activity; that is, from May to September. Investigations took place during a subsequent 4 days for 5 consecutive months. Lizards were caught by means of active capturing. They were kept in a terrarium, where their species and sex (on the basis of sexual dimorphism, only in breeding season) were determined. In order to avoid recapturing the same specimens, the lizards were not released until the end of capture fieldwork in a given month. All the lizards were weighed and measured in order to separate three age groups (GW) (Matuschka *et al*. 1992): GW1 (0–2 g), GW2 (3–5 g) and GW3 (above 6 g). Each captured lizard was visually examined for the presence of ticks. Ticks were mainly found on the lizard's neck, under the front limbs and on the sides of the body. All ticks were removed with forceps and preserved in 70% ethanol. Their species and life stages were identified in the laboratory (Siuda 1991). After examination and tick removal lizards were released in the same places where they had been caught.

Borrelia burgdorferi *DNA extraction, amplification and identification*

Extraction of DNA was carried out according to Rijpkema and Bruinink (1996) by lysis of crushed individual ticks in ammonium hydroxide (NH_4OH). Then two primer sets: FL6 and FL7 (in 2002) (Picken 1992), and FLA297 and FLA652 (in 2003) (Levin *et al*. 1999) were used to amplify fragments of conserved regions of the *fla* gene of *Borrelia burgdorferi* s.l. PCR was performed in a reaction volume of 25 µl containing 0.625 U of Ampli*Taq* DNA polymerase, 2.5 µl 10 × PCR Buffer II, 1.5 µl $MgCl_2$ (stock 25 mM) (Perkin Elmer), 2.5 µl dNTPs mixture (stock 2.5 mM) (MBI Fermentas), 0.5 µl primer I (stock 10 mM), 0.5 µl primer II (stock 10 mM), 14 µl double distilled water (DDW) and 2.5 µl of the processed tick sample. In each PCR run, tick lysates from positive reactions obtained in the previous investigations (Stańczak *et al*. 2002, 2004) were used as a positive control. Negative controls used DDW and NH_4OH.

All reactions were carried out in Perkin Elmer Gene Amp PCR System 2400 thermal cyclers. In the case of FL6/FL7 primers, PCR was performed as already described (Stańczak *et al*. 1999) while in the case of FLA297/FLA652 primers, samples were initially denatured for 3 min at 94°C and then thermally cycled 35 times at 94°C for 30 s (denaturation), 55°C for 30 s (annealing), and 72°C for 30 s (extension). Final extension lasted 7 min at 72°C.

The amplification products were visualized in 2% agarose gels stained with ethidium bromide. Resulting bands of 276 bp (FL6/FL7) and 378 bp (FLA297/FLA652) were considered positive results.

Further species identification of *B. burgdorferi* s.l. was done by nested PCR using species specific primers: BB1/BB2, BA1/BA2 and BG1/BG3 for *B. burgdorferi* s.s., *B. afzelii* and *B. garinii*, respectively. The reaction were performed as already described (Stańczak *et al*. 2000). In each PCR run, one negative (NH_4OH) and three positive controls of each species which were originally confirmed by sequencing were added. The primer sets used produced a product of 76 bp for *B. burgdorferi* s.s., 103 bp for *B. afzelii* and 125 bp for *B. garinii*.

The statistical tests used in data analyses were: χ^2, Tukey's test, one-way analysis of variance (UNIANOVA). Due to distribution skewness, data concerning the number of ticks (larvae and nymphs) was transformed logarithmically (function log (n + 1)) before the application of parametric tests.

Results

Tick infestation in sand lizards

The study material was comprised of 235 sand lizards (2002: 136 specimens, 2003: 99). A total of 233 engorging ticks was recorded on the lizards: 47 (17 larvae and 30 nymphs) in 2002 and 186 (59 larvae and 127 nymphs) in 2003. All of them were *Ixodes ricinus*. The number of ticks per lizard ranged from 0 to 38 individuals. The tick infestation intensity, i.e., the average number of ticks engorging a captured lizard over the two years was 0.99 (2002: 0.35; 2003: 1.88). The overall intensity of *I. ricinus* infestation was 0.32 and 0.66 for larvae and nymphs, respectively. The dispersion coefficient (I = variance/mean) shows that the distribution of tick on the captured lizards was not random, but aggregated ($I = 8.96$, $\chi^2 = 268.72$,

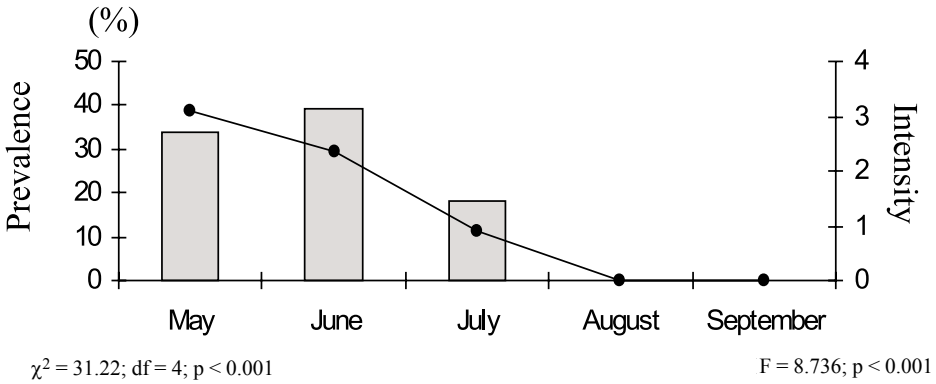

Fig. 1. Changes in prevalence of tick infestation of lizards by month

df = 30, p < 0.001). This data concerns the individuals on which ticks were present. The tick infestation prevalence, that is the percentage of sand lizards with ticks engorging on them, for the two years pooled together, was 13.2% (n = 235) [2002: 7.4% (n = 136) and in 2003: 21.2% (n = 99)]. The overall *I. ricinus* prevalence was 9.8% and 11.5% for larvae and nymphs, respectively.

Infestation prevalence varied depending on the month of capture (2002: χ^2 = 20.69, df = 3, p < 0.001; 2003: χ^2 = 31.22, df = 4, p < 0.001). In 2002, the highest tick prevalence was recorded in May. It decreased in subsequent months and in September no tick infested lizard was caught. In 2003, the tick prevalence reached its highest value in June and, as in the preceding year, no lizard with ticks was captured in autumn. The tick infestation intensity also changed by month (2002: F = 5.573, p < 0.001; 2003: F = 8.736, p < 0.001). The mean number of ticks, during the whole study, was the highest in May and it decreased in the subsequent months (Fig. 1).

In both years, nymphs predominated among the ticks collected from the lizards, constituting 65% in May, 78.2% in June and 53.3% in July. On the basis of the age classes, a relationship between mean tick number and lizard age was found (two-way ANOVA: F = 26.552, p < 0.001) (2002: F = 8.525, p < 0.001; 2003: F = 24.726, p < 0.001). The individuals assigned to age group GW1 and GW2 had on average fewer ticks than those assigned to the oldest age group GW3 (Tukey's test: GW1 vs GW3, p < 0.001; GW2 vs GW3, p < 0.001). The tick prevalence also depended on lizard age (χ^2 = 44.051, df = 2, p < 0.001). The percentage of lizards on which ticks engorged was the highest in the third age class (Fig. 2).

Throughout the two years, the sex of 98 caught lizards was determined (70 females and 28 males), and 20 females and 11 males were found to be infested. This female/male difference was not statistically significant. Tick infestation prevalence in males, for the two years pooled together, was 41% while in females it was 29% (χ^2 = 1.43, df = 1; NS). The mean number of ticks engorging on lizards, for the two years, was 3.7 for males and 1.9 for females (F = 1.771; NS).

Borrelia burgdorferi *infection in ticks parasitized sand lizards*

In total, 233 analyses of ticks engorging on lizards were conducted. The presence of the DNA of these pathogens was

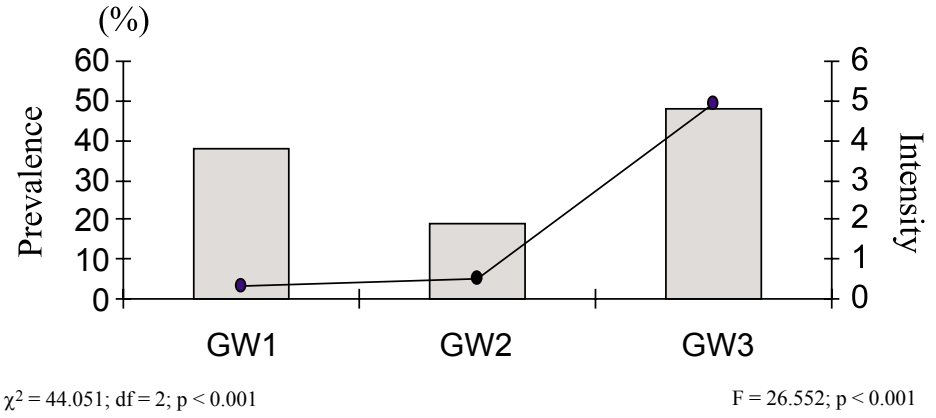

Fig. 2. Changes in prevalence of tick infestation of lizards by lizard age GW1 (0–2 g), GW2 (3–5 g), GW3 (above 6 g)

detected in 11 specimens, which equalled 4.7% of all the ticks studied. The percentage of infected nymphs was higher than in the case of larvae which was 6.4% (n = 157) and 1.3% (n = 76), respectively ($\chi^2 = 2.91$, df = 1, p = 0.09).

The percentage of ticks infected with *B. burgdorferi* s.l. in consecutive months, for both years, amounted to 5.5% in May, 3.6% in June and 0% in July ($\chi^2 = 1.12$; df = 2; NS). No spirochetes were detected in ticks found on lizards from the first and second age group (GW1, GW2). Only ticks engorging on the oldest lizards (GW3) were infected with *B. burgdorferi* s.l. ($\chi^2 = 2.64$, df = 2; NS).

The percentage of infected ticks that engorged on lizards was similar for males and females, amounting to 5% and 4.5%, respectively ($\chi^2 = 0.03$, df = 1; NS).

In case of 7 ticks (nymphs only) out of a total of 11 positive samples identification of genospecies was possible. *Borrelia afzelii* was detected in 5 of them, *B. burgdorferi* s.s. in one sample and *B. garinii* in one sample. Mixed infection were not detected.

Discussion

The highest *Ixodes ricinus* ticks infestation of sand lizards in the study area was recorded in spring. This probably results from high activity of lizards during this period, and is connected with the breeding season (Herczek and Gorczyca 1999). Additionally spring is a season of the highest tick activity. No infestation increase was noted in the lizards in autumn, which may be accounted for by low activity of reptiles at this time of year (Juszczyk 1987).

The general tick infestation prevalence of lizards is relatively low compared to the value observed for rodents and birds inhabiting the same area. Engorging *Ixodes ricinus* ticks were found in 13.2% of captured and examined lizards. However, the prevalence of tick infestation of the forest rodents that were trapped in the same area within the same period varied from 92% for yellow-necked mouse (*Apodemus flavicollis*), and 76% for bank vole (*Clethrionomys glareolus*) to just 37% for common vole (*Microtus arvalis*) (Siński *et al*. 2006). Similarly, the passerine birds' species most frequently captured in the study site during three year period (1996, 1997 and 1998) were more often infested with ticks than lizards. The highest tick infestation prevalence was found for birds' species often visiting undergrowth and shrub plants – dunnock (*Prunella modularis*), tree pipit (*Anthus trivialis*) and hawfinch *(Coccothraustes coccothraustes*), respectively, 67, 56 and 53% individuals were infested. Only species living amongst the canopy or on the tree trunks and branches, for example tree creeper (*Certhia familiaris*) or blue tit (*Parus caeruleus*), were attacked by ticks considerably less frequently 6% (Gryczyńska *et al*. 2002). Therefore, it can be presumed that in the study area, the main tick hosts before reptiles are small forest rodents and passerine birds.

Investigations concerning small rodents and passerine birds infested by ticks have shown that they are attacked principally by immature tick stages, such as larvae and nymphs, and that adult stages are rarely found on them (Gryczyńska *et al*. 2002, Siński *et al*. 2006). In the case of lizards, the results coincide with these findings. The reptiles were infested exclusively by larvae and nymphs, and no adult forms were recorded. Similar results have been obtained in studies of the viviparous lizard (*Lacerta vivipara*), carried out in Germany (Matuschka *et al*. 1992). The high proportion of nymphs engorging on lizards was found in spring. It can be explained by different seasonal activity of each tick stages. In the environment nymphs become active in spring whereas larvae are the most active later – in summer (Kolpy 1961, Nilsson 1988, Siuda 1991).

The differences in tick infestation intensity and prevalence indicate that the host sex may play an important role (Matuschka *et al*. 1992). This is most plausibly connected with the increased mobility of male individuals during the breeding season (Davis and Ford 1983). Published data suggest that, during this period male lizards become more aggressive towards one another and actively defend their territory (Davis and Ford 1983). This behavior exposes them to increased contact with ticks (Matuschka *et al*. 1992, Eisen and Eisen 1999). A similar bias can be noticed in the present study. Although the differences in tick infestation according to the sex are non-significant, it can be noted that higher tick infestation intensity and prevalence in the case of males as compared to females of this species indicate these differences. Similarly, in case of two rodent species, yellow-necked mouse and common vole, the mean abundance values of infestation with immature stages of *I. ricinus* were higher among males (Siński *et al*. 2006).

It has also been shown that the weight of an individual (the heavier, the older) and its territories size may cause heightened infestation with ticks (Matuschka *et al*. 1992). Adult lizards, that explore large area, have greater chances of being infested with ticks than immature ones (Dunlap and Mathies 1993). Studies conducted in Europe revealed that male sand lizards have significantly larger territories than male viviparous lizards. For this reason sand lizards are attacked by ticks at a higher frequency (Bauwens *et al*. 1983). What is more, in studies carried out in Germany it was discovered that lizards weighing less than 3 g had considerably fewer ticks than heavier ones (Matuschka *et al*. 1992). This is confirmed by investigations concerning other tick hosts, for instance the yellow-necked mouse and bank vole. In case of yellow-necked mouse the most frequently infested are males of weight above 35 g, that is, the oldest ones (Siński *et al*. 2006). The results of the present study point to a similar tendency: both the percentage of individuals on which ticks engorged and tick infestation intensity were higher in the case of the oldest (GW3) individuals of this species, as compared to the younger ones (GW1 and GW2).

Due to the growing number of tick-borne diseases diagnosed in humans, researchers have been focusing not only on ascertaining which vertebrate groups are the best tick hosts, but also on determining which ones are the most involved in

cycles maintaining pathogens and parasites in the nature. In Europe increasing Lyme disease morbidity has been a significant epidemiological and veterinary problem in recent years. For this reason, estimation of the infection of *I. ricinus* ticks with *B. burgdorferi* s.l. are more and more frequent.

Published data show that 11.6% of *I. ricinus* larvae and 18.5% of nymphs collected from vegetation in the area of Northeastern Poland (the same study sites as the present study) are infected with *B. burgdorferi* s.l. (n = 336 and n = 270, respectively) (Siński and Pawełczyk 1999). The results obtained for the sand lizards (larvae 1.3%, n = 76; nymphs 6.4%, n = 157) indicate that there are statistically significant differences in the infection relative to ticks that search for hosts in the environment (larvae $\chi^2 = 6.56$, df = 1, $p < 0.01$; nymphs $\chi^2 = 9.43$, df = 1, $p < 0.002$). This suggests that sand lizards inhabiting Northeastern Poland are probably not *B. burgdorferi* s.l. reservoirs (no higher infection values in engorged ticks vs unfed ticks in environment). On the other hand, it can be supposed that sand lizards living in the study site may belong to a group of vertebrates which playing a zooprophylactic role (which is diverting vector ticks from more suitable reservoir competent hosts) with respect to investigated *B. burgdorferi* genospecies (Matuschka *et al*. 2000).

In the similar studies carried out in Germany it was demonstrated that none of the 293 nymphs of *I. ricinus* ticks that engorged as larvae stages on sand lizards were infected by *B. burgdorferi* s.l., but the infection of nymphs engorged on small rodents caught in the same area ranged from 25.2 to 58.2%, depending on the host species (Matuschka *et al*. 1992).

Identification of *B. burgdorferi* s.l. positive samples, revealed that *B. afzelii* was the predominant species among ticks parasitizing lizards. This finding is in agreement with the results of previous studies (Stańczak *et al*. 2000). *B. afzelii* were also the most frequently noted (7.3%) in questing *I. ricinus* collected in different woodlands in Poland, followed by *B. burgdorferi* s.s. (3.3%) and *B. garinii* (0.7%).

It appears that the next essential stage in studies concerning environmental circulation of the pathogens that cause tick-borne diseases should be examining the reptile hosts (blood or internal organs). In case of the southern alligator lizard attempts to isolate spirochetes from lizard blood were unsuccessful (Wright *et al*. 1998). These data suggested that this species is not a competent reservoir for *B. burgdorferi* s.l.

Although, some research regarding the degree of infection with particular *B. burgdorferi* genospecies of lizards living in the natural environment showed the different results. The study in the southeastern United States showed that three *B. burgdorferi* genospecies (*B. andersonii*, *B. bissettii* and *B. burgdorferi* s.s.) are with high prevalence (54%) established in nine lizard species (Clark *et al*. 2005). Another findings carried in central Europe and Africa confirmed that ticks feeding on wild lizards became infected mainly by *B. lusitaniae* (Dsouli *et al*. 2006, Richter and Matuschka 2006). The field work of a similar nature, supported by laboratory studies using xenodiagnostic ticks are also necessary to provide any information on the reservoir status of lizards.

Acknowledgments. Lizards were captured with permission no. BOA-A-5-2939/01/02/RP.

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(Accepted February 5, 2007)