The occurrence of *Ixodes ricinus* ticks and important tick-borne pathogens in areas with high tick-borne encephalitis prevalence in different altitudinal levels of the Czech Republic Part I. *Ixodes ricinus* ticks and tick-borne encephalitis virus

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ABSTRACT

Study objective: The aim of the three-year study (2011–2013) was to monitor population density of *Ixodes ricinus* ticks and its infection rate with the tick-borne encephalitis virus in areas with a high incidence of tick-borne encephalitis as reported in the previous decade 2001–2010. Such a comprehensive and long-term study based on existing epidemiological findings has not previously been conducted in Europe.
Material and methods: In the areas of the Ústí nad Labem Region, Olomouc Region, South Bohemian Region, and Highlands Region, 600 m^2 plots were selected in the local optimal *I. ricinus* habitats where tick flagging was performed every year in the spring-summer and autumn seasons of the questing activity. In total, 18,721 *I. ricinus* ticks (1448 females, 1425 males, and 15,848 nymphs) were collected and investigated.
Results and conclusion: The results have shown that the differences in the infection rate of *I. ricinus* observed between regions are driven by variation in the density of the local *I. ricinus* populations which is influenced by the characteristics of the whole local biocenosis. The overall prevalence estimate of TBE virus in *Ixodes ricinus* ticks at the altitudes below 600 m a.s.l. was 0.096 % (95% CI 0.055–0.156) for nymphs, and 0.477 % (95% CI 0.272–0.773) for adults. The dynamics of the seasonal variation in *I. ricinus* populations, depending primarily on the climatic factors, are behind the interyear differences in the infection rate of ticks and, consequently, in the epidemiological situation of tick-borne encephalitis. The nymph to adult ratio was 5.5 on average but showed great interregional variability (from 10.3 in the Ústí nad Labem Region to 1.8 in the Highlands Region). It might be used in the future as one of the indicators of the composition of the local *I. ricinus* population and of the level of the circulation of tick-borne pathogens in zoonotic sphere and also for use in the health risk assessment in a given area. Despite the permanent expansion of ticks and tick-borne pathogens in higher altitudes the high risk limit for human infection with tick-borne encephalitis is 600 m a.s.l. in the Czech Republic.

KEYWORDS:
***Ixodes ricinus* – tick-borne encephalitis virus – occurrence – altitude – region – season**

SOUHRN

Daniel M., Danielová V., Kříž B., Růžek D., Fialová A., Malý M., Materna J., Pejčoch M., Erhart J.: Výskyt klíštěte obecného *Ixodes ricinus* a významných patogenů jím přenášených ve vybraných oblastech se zvýšeným počtem onemocnění klíšťovou encefalitidou v různých nadmořských výškách v České republice
Část I. Klíště obecné *Ixodes ricinus* a virus klíšťové encefalitidy
Cíl práce: Cílem tříletého sledování (2011–2013) výskytu klíštěte obecného *Ixodes ricinus* bylo ověřit v oblastech se zvýšenou incidencí klíšťové encefalitidy (zjištěnou v předchozí dekádě 2001–2010) jednak stavy místních populací tohoto přenašeče, jednak jeho infikovanost virem klíšťové encefalitidy. Takto komplexně zaměřený a dlouhodobý výzkum založený na předchozích epidemiologických zjištěních nebyl dosud v Evropě realizován.
Materiál a metodika: Ve vybraných lokalitách krajů Ústeckého, Olomouckého, Jihočeského a kraje Vysočina byly stanoveny fixní plochy (každá 600 m^2) v optimálních místních habitatech *I. ricinus*, na nichž byl prováděn každoroční kontrolní sběr v jaroletní a podzimní sezoně aktivity klíšťat. Celkem bylo shromážděno a laboratorně vyšetřeno

18.721 klíšťat *I. ricinus* (z toho 1448 samic, 1425 samců a 15.848 nymf).
Výsledky a závěry: Výsledky ukazují, že pozorované regionální rozdíly v infikovanosti *I. ricinus* jsou založeny na stavu místní populace těchto klíšťat ovlivněné charakterem celé lokální biocenózy. Dynamika jejích sezonních změn, podmíněná především klimatickými faktory, určuje meziroční rozdíly infikovanosti *I. ricinus*, a tak i změny epidemiologické situace nákaz klíšťaty přenášených. Číselný poměr aktivních dospělců a nymf klíšťat vykazuje v různých oblastech značnou variabilitu a mohl by být v budoucnu použit jako jeden z indikátorů charakterizujících místní populace *I. ricinus*, úroveň zoonotické cirkulace viru klíšťové encefalitidy, a tak i zdravotní riziko dané oblasti. Dále bylo zjištěno, že populace klíšťat dotčená lokálně jednorázově působícími extrémními meteorologickými jevy, se rychle meziročně obnovuje.
Ačkoliv je stále pozorováno pronikání *I. ricinus* a jimi přenášených patogenů do vyšších nadmořských poloh, vysoké riziko lidských nákaz virem klíšťové encefalitidy je ve sledovaných regionech dosud do úrovně 600 m n. m.

KLÍČOVÁ SLOVA:

klíště obecné *Ixodes ricinus* – virus klíšťové encefalitidy – regionální výskyt – nadmořská výška – sezonnost

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INTRODUCTION

The first attempt to provide a comprehensive review of the occurrence of *Ixodes ricinus* ticks in the Czech lands was made as early as in 1960–1962 by a large group of collaborators from the Regional and District Public Health Centres who monitored the seasonal density of ticks on 46 plots 10 x 10 m in size in five Czech and two Moravian regions. Due to the disunity of the methods used (particularly in terms of the selection of the flagging areas), the results could have been summarized only roughly [2]; however, specific data on the occurrence of *I. ricinus* ticks at altitudes of 200–700 m a.s.l. were obtained. Given the then existing body of knowledge, the results obtained were not considered in context with tick-borne infections. During the following decades, multiple studies were conducted on the ecology of ticks and their role in the transmission of tick-borne encephalitis (TBE). However, the focus was always placed on narrowly specific and regionally targeted issues.
The aim of this three-year study was to monitor the distribution and population density of *I. ricinus* in the selected areas with heightened prevalence of tick-borne encephalitis and the infection rate with the tick-borne encephalitis virus (TBEV).

MATERIAL AND METHODS

1. Selection of areas

In the areas with a high incidence of TBE reported in 2001–2010 [12], superior to the whole-country average, the study plots were selected in northern Bohemia (Ústí nad Labem Region), northern Moravia (Olomouc Region), southern Bohemia (South Bohemian Region), and in the Bohemian-Moravian Highlands (Highlands Region) according to the following key: In each area, two high prevalence municipalities (three municipalities in the South Bohemian Region) were identified. In the cadastres of these municipalities, the monitoring plots representing optimal habitats of *I. ricinus* ticks were selected in the closest vicinity of the built-up area. For this purpose, the Atlas of Tick-Borne Encephalitis [5] served in Bohemia and aerial photographs accessible on the internet were used in Moravia. In addition, in each area, a plot located at a higher altitude than the surrounding landscape and providing a suitable habitat for *I. ricinus* ticks was selected. The middle of each plot was determined by the geographic coordinates. Large urban units (regional cities) were not included among the selected municipalities because the direct contact of TBE cases with the surrounding green spaces is not commonplace.

2. Characteristics of plots

Stožec Mt. 48°52´22˝N, 13°49´41˝E; 910–920 m a.s.l.
A sparse forest, with the predominance of old spruces, adult beeches, and linden trees. Located at the eastern edge of the Natural Monument “Stožec Rock” (since 1989, established to protect the granite rocks with a debris flow and important mixed cover on the rocky outcrops). The forest is a well-preserved residue of a virgin forest on the scree slopes.
Strakonice – Starý Dražejov 49°16´31˝N, 13°52´46˝E; 480–490 m a.s.l.
A sparse oak forest, with a grassy undergrowth, adjacent to the western edge of the Kuřidlo Nature Reserve (1985) established to protect the remains of the original limestone oak forest with the well-preserved original undergrowth vegetation.
Netolice – 49°02´30˝N, 14°10´51˝E; 460–470 m a.s.l.
A sparse mostly deciduous middle-aged forest of oak andlinden tree with additional pines and young spruces, with multiple grassy openings. In the vicinity, „Na Pekle“ holiday cottages and family houses are being built.
Zliv – 49°04´24˝N, 14°22´11˝E; 405–410 m a.s.l.
A transitional area (ecotone) between a mature oak forest (around 80 years of age) with additional pines and spruces, and a young oak, linden tree, and mountain ash forest (around 30–40 years of age). The forest is separated from the edge of the municipality built up with family houses by a sports area (a football ground and tennis courts).

Povrly – 50°40´38˝N, 14°08´31˝E; 280–300 m a.s.l.
An old oak forest with well developed herb, and shrub storeys. The plot is located in the Nature Reserve Kozí vrch (since 1983) established to protect a natural oak-hornbeam forest situated on a geomorphological unit belonging to the range of the Central Bohemian Uplands.
Benešov nad Ploučnicí – 50°43´56˝N, 14°18´58˝E; 280–300 m a.s.l.
An old linden forest scattered with some ash trees and with a well-developed herb storey.
Děčínský Sněžník-Medvědí louka – 50°48´03˝N, 14°05´50˝E; 550–570 m a.s.l.
A loose pine forest with abundant deciduous trees (birch, rowan, and beech), with an undergrowth of the predominating bilberry.
Červenohorské sedlo – 50°07´20˝N, 17°09´08˝E; 1020–1030 m a.s.l.
The loose spruce covers with scattered beeches and a rich herb undergrowth and grassy zones along the path.
Jeseník-Křížový vrch – 50°13´37˝N, 17°13´08˝E; 530–550 m a.s.l.
A mixed forest of ash, maple, and spruce with a well-developed herb storey, where the dominant plants are blackberry bush, touch-me-not, and nettle.
Šumperk-Holubí vrch – 49°58´49˝N, 16°59´08˝E; 410–420 m a.s.l.
A mixed forest of maple and spruce with a well-developed herb storey, where the dominant plants are blackberry bush, touch-me-not, and nettle.
Baliny (Velké Meziříčí) – 49°20´19˝N, 15°58´04˝E; 450–470 m a.s.l.
An adult spruce forest above the valley of the Balinka river with an abundant undergrowth of bush and herbs on the sharp slopes (up to 45°). The monitoring plot is a part of the Balinské údolí Nature Park (425–500 m) which was established to protect the deeply cut valley of the meandering Balinka river (the floodplain is only 10–20 m in width). Sharp slopes on both sides, adjacent to the plains from which the conspicuous mountain ridges rise.
Bystřice nad Pernštejnem – 49°32´41˝N, 16°17´23˝E; 400–450 m a.s.l.
A spruce forest with a rich bush and herb undergrowth on a slope above the valley of the Končinský stream, adjacent to a campsite.
Nedvědice – 49°27´59˝N, 16°20´01˝N; 330–370 m a.s.l.
A spruce forest with an undergrowth on a slope above the valley of the Kolářovský stream.
In July 2012, a mature forest located nearby was severely damaged by a windstorm. After the uprooted trees were removed, the area remained bare soil. During 2013, both the herb and bush storeys were restored.

3. Collection of *Ixodes ricinus ticks*

Ticks were collected by the standard flagging method [20] on the defined plots. The flag (50 x 70 cm) was made of white fabric with a slight nap (flannel). The collection was carried out by flagging for a defined length of time, namely by one person for three hours. To obtain comparable results, collections were performed according to detailed instructions. Based on long-term experience, three hours of work performed by a skilled person (at an average abundance of ticks in the area) correspond to 600 m^2 of the area monitored. This approach makes it possible to focus in detail on the habitat and microrelief of the area where ticks are actually present. The ticks were transported to the laboratory, classified to the species level, and stored at -80 °C until further processed.
For technical reasons, the presence of larvae was only recorded and their abundance was estimated and expressed by crosses.

4. Detection of the tick-borne encephalitis virus in *Ixodes ricinus* ticks

The ticks collected were separated by developmental stage, gender, collection area, and collection date. *I. ricinus* nymphs were pooled into groups of not more than 50 individuals and adult males and females separated into groups of not more than 10 individuals. Nymphs were homogenized in 200 µl of PBS using an automatic homogenizer (Roche) and 14 µl of the homogenate was used for the isolation of RNA using the RNAGEM kit (Zygem). Two microlitres of the isolate were used for the reaction according to the protocol of Růžek [18]. From the positive samples (detected by PCR), the virus was also isolated in PS cell culture. The tick homogenate was added to the semi-confluent culture of PS cells in a 24-well culture plate. After one hour, the culture was washed with sterile PBS and adjusted to a volume of 500 µl with the L-15 medium supplemented with 3% heat inactivated newborn calf serum, 1% glutamine, and 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. A cytopathic effect was evident after four to five days of culture at 37 °C.

5. Statistical analysis

Data are summarized as numbers of ticks and their means. For the statistical testing and analysis of the number of nymphs and adult ticks a generalized linear model with Poisson response and log link was used. Logarithm of the mean number of ticks is modeled as a linear function of observed factors, namely the region, altitude, period and year. Dummy coding of categorical predictor variables was used within the model. The results are presented as prevalence rate ratios with 95% confidence intervals (CI). The statistical significance level was set to 0.05. The data were processed by the R software (R Core Team, 2014, version 3.1.2).
The estimates of pooled prevalence of TBEV and the corresponding exact confidence intervals were calculated using the maximum likelihood method in EpiTool utility [19] with the aid of procedures for perfect test and fixed pool size [1].

RESULTS

1. Occurrence of *Ixodes ricinus* ticks

In total 18,721 *I. ricinus* ticks were collected, of which 15,848 were nymphs (N), 1448 females (F) and 1425 males (M). Larvae were present in tens to thousands of individuals in each cohort of flagged ticks. All ticks collected (except larvae) were screened for the tick-borne encephalitis virus (Table 1).

Table 1. *Ixodes ricinus* ticks found on localities under study and tick-borne encephalitis virus detected in them

Year/Region	Locality	Altitude m a.s.l.	Period					
			Spring-summer			Autumn		
			Nymphs	Females	Males	Nymphs	Females	Males
2011								
Ústí nad Labem	Děčínský Sněžník	550–570	168	7	3	141	4	10
	Povrly	280–300	441 **(2)**	6	9	276	4	12
	Benešov nad Ploučnicí	280–300	255	0	4	143	4	4
Olomouc	Červenohorské sedlo	1020–1030	21	3	5	16	1	1
	Jeseník	530–550	52	12	10	52	5	4
	Šumperk	410–420	335	11	15	204	7	13
South Bohemian	Stožec	910–920	24	3	1	17	0	0
	Strakonice	480–490	72	16	12 **(1)**	87	2	3
	Zliv	405–410	475	16	21	400	51	29
	Netolice	460–470	250	35	28	305	22	29
Highlands	Bystřice n. Pernštejnem	400–450	59	7	10	69	18	14
	Baliny	450–470	105	7	4	61	19 **(1)**	10 **(1)**
	Nedvědice	330–370	160	88 **(1)**	82	25	89 **(1)**	75
Total			**2417 (2)**	**211 (1)**	**204 (1)**	**1796 (0)**	**226 (2)**	**204 (1)**
2012								
Ústí nad Labem	Děčínský Sněžník	550–570	167	18	10	318	1	6
	Povrly	280–300	338	33 **(1)**	43	213	19	15
	Benešov nad Ploučnicí	280–300	323	42	43	333	4	6
Olomouc	Červenohorské sedlo	1020–1030	27	6 **(1)**	6	12	1	1
	Jeseník	530–550	59	19	29	214	4	1
	Šumperk	410–420	219	11	11	541	1	0
South Bohemian	Stožec	910–920	24	3	6	16	3	0
	Strakonice	480–490	200	16	15	450	12	10
	Zliv	405–410	250 **(2)**	78**(3)**	76 **(2)**	220 **(3)**	35	36
	Netolice	460–470	650 **(1)**	35	36	350 **(4)**	22	29
Highlands	Bystřice n. Pernštejnem	400–450	115	72	78	46	28	16
	Baliny	450–470	98	32 **(1)**	25	88	11	12
	Nedvědice	330–370	60	66	63	5	12	3
Total			**2530 (3)**	**431 (6)**	**441 (2)**	**2806 (7)**	**153 (0)**	**135 (0)**
2013								
Ústí nad Labem	Děčínský Sněžník	550–570	423	16	24	254	5	3
	Povrly	280–300	514	38	47	473	26	22
	Benešov nad Ploučnicí	280–300	463	20	23	369 **(2)**	7	8
Olomouc	Červenohorské sedlo	1020–1030	155	13	10	15	2	2
	Jeseník	530–550	190	22	21	125	19	15
	Šumperk	410–420	672	20	29	428	6	9
South Bohemian	Stožec	910–920				50	4	5
	Strakonice	480–490	50	4	5	170	2 **(1)**	2
	Zliv	405–410	260	25	26	240	44	38
	Netolice	460–470	350	47 **(1)**	34	190	23 **(2)**	21
Highlands	Bystřice n. Pernštejnem	400–450	209	20	20	90	12	13
	Baliny	450–470	290 **(1)**	18	23	173 **(1)**	6	4
	Nedvědice	330–370	108	16	26	38	12	11
Total			**3684 (1)**	**259 (1)**	**288 (0)**	**2615 (3)**	**168 (3)**	**153 (0)**
Grand total			**8631 (6)**	**901 (8)**	**933 (3)**	**7217(10)**	**547 (5)**	**492 (1)**

Number in parentheses represents number of TBEV positive pools detected by PCR.

Table 2. Comparison of Ixodes ricinus occurrence on the localities below 600 m a.s.l.

Region	Number of localities	Nymphs		Females		Males	
		Average number		Average number		Average number	
		Per locality	Per 100 m²	Per locality	Per 100 m²	Per locality	Per 100 m²
Ústí nad Labem	3	1871	52.0	85	2.4	98	2.7
Olomouc	2	1546	42.9	69	1.9	79	2.2
South Bohemian	3	1656	46.0	162	4.5	150	4.2
Highlands	3	600	16.7	178	4.9	163	4.5

When evaluating the occurrence of ticks, the main focus is on the nymphs which bite humans most often and thus play an important role in the epidemiology of human infection by TBEV (as well as by other pathogens). The calculations were made with (unless stated otherwise) the results from the areas where the three-year (2011–2013) monitoring was completed without any interference.

The ratio of nymphs to adult ticks was on average 5.5. Clear differences were found between regions. The ratio was 10.3 in the Ústí nad Labem Region and 9.7 in the Olomouc Region, which means a greater proportion of nymphs compared to other regions. In the South Bohemian Region the ratio of 5.3 was close to the overall average, and in the Highlands Region it was only 1.8 which indicates an increased proportion of adult ticks.

The overall female to male ratio was 1.02, i.e. very close to 1 : 1. The regions showed only small variation with females prevailing in Highlands and South Bohemian Regions (ratio 1.09 and 1.08, respectively) and males prevailing in Olomouc and Ústí nad Labem Regions (ratio 0.90 and 0.87, respectively).

a) Altitudinal stratification of the occurrence of *Ixodes ricinus* ticks

The comparison of the results recorded at different altitudes above sea level (a.s.l.) revealed the highest population density of *I. ricinus* ticks at altitudes below 600 m a.s.l. (Table 1). When this zone is divided into two parts, i.e. below 400 m a.s.l. and 401–600 m a.s.l., an insignificant difference is found between them, with the tick populations being more abundant at the lower altitudes, particularly in spring. The highest single absolute number of ticks at altitudes below 400 m (Povrly plot) was 514 N, i.e. the population density of 86 N/100 m², collected along with 38 F (6/100 m²) and 47 M (8/100 m²). At altitudes of 401-600 m a.s.l. the highest single absolute number of ticks collected in the Šumperk plot was 672 N (112/100 m²), along with 20 F (3/100 m²) and 29 M (5/100 m²). A highly similar result was obtained in the Netolice plot, i.e. 650 N, (108 N/100 m²) along with 35 F (6/100 m²) and 36 M (6/100 m²).

Noteworthy are also the results obtained at the highest altitudes. In the Červenohorské sedlo plot, a total of 246 N and an average of 41 N per single control flagging were recorded. At the same time, 26 F (an average of 4 F) and 25 M (an average of 4 M) were collected.

b) Comparison of the population density of *Ixodes ricinus* tick between regions

The selection of distant study plots (within the Czech Republic) makes it possible to compare the population density of *I. ricinus* ticks between different types of landscape with different characteristics of the natural foci of TBEV. Table 2 compares the densities of *I. ricinus* populations, based on the average numbers of nymphs and adult males and females per locality and per 100 m².

The South Bohemian and Olomouc Regions report a highly similar abundance of nymphs (46 and 43/100 m², respectively) which is more than twice as high as that recorded in the Highlands Region (17/100 m²). The situation is different for adult ticks, with the highest numbers per 100 m² found in the Highlands Region (5 F and 5 M/100 m²) and South Bohemian Region (5 F and 4 M/100 m²). On the other hand, these rates are reduced to half in both northern regions (2 F and 3 M/100 m² in the Ústí nad Labem Region and 2 F and 2 M/100 m² in the Olomouc Region). When both nymphs and adult ticks are considered, the leading region is South Bohemia. This finding is relevant to understanding the results of the detection of TBEV in *I. ricinus* ticks collected in this region.

c) Seasonality and interannual differences in the abundance of *Ixodes ricinus* ticks

The study of the population density of *I. ricinus* ticks was conducted over a three-year period (every year in two periods with different seasonal questing activity) which was long enough to see the interannual differences in the seasonal activity of ticks. Nymphs were more abundant in 2013 (particularly in comparison with 2011) and were also more abundant in autumn than in spring-summer in 2012 (Fig 1). This situation was reflected mainly at altitudes of 401–600 m a.s.l. in North Moravia (with the greatest differences of found tick numbers between spring/autumn: 59/214 N and 219/541 N) and in North Bohemia (167/318 N) where the difference was 323/333 N even at the lowest altitudes. A similar balance in the spring/autumn number of nymphs was found in South Bohemia (250/220 N). It suggests a general effect of the long-term trends in weather on the questing activity of *I. ricinus* nymphs.

As for the adult ticks, a hint of the above-mentioned fluctuations appeared in 2011 while in 2012-2013, the curve of their population density clearly peaked in the spring-summer period (Fig. 2).

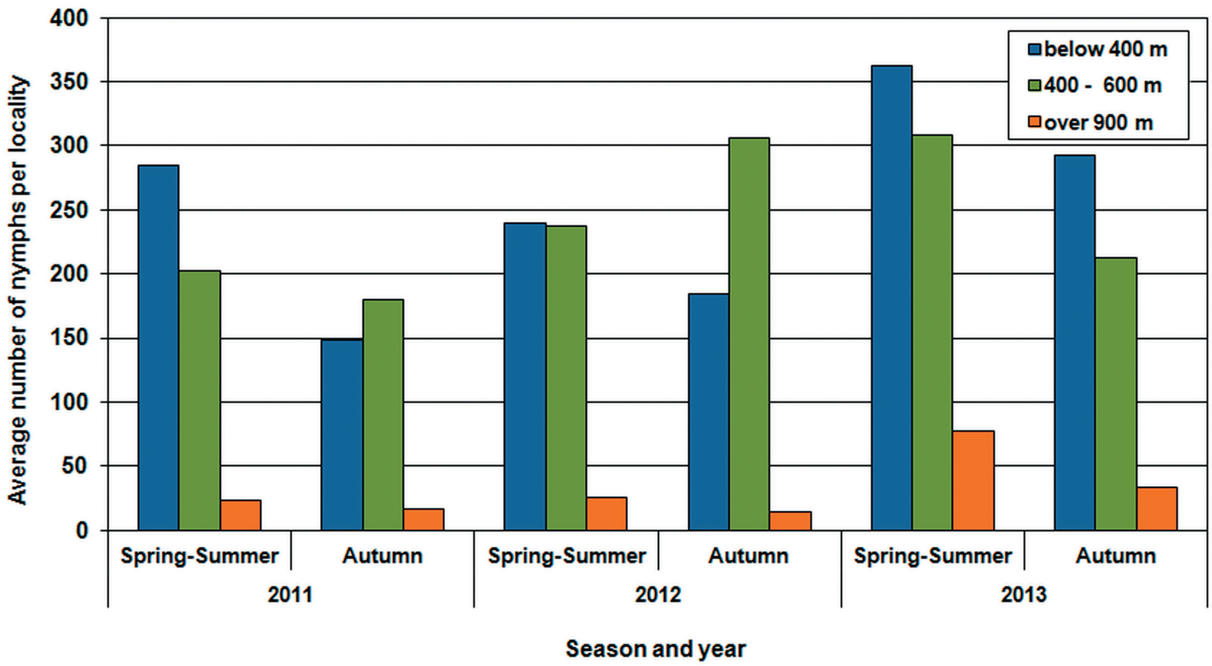

Figure 1. Average number of *Ixodes ricinus* nymphs per locality according to altitudinal level, and season in 2011–2013

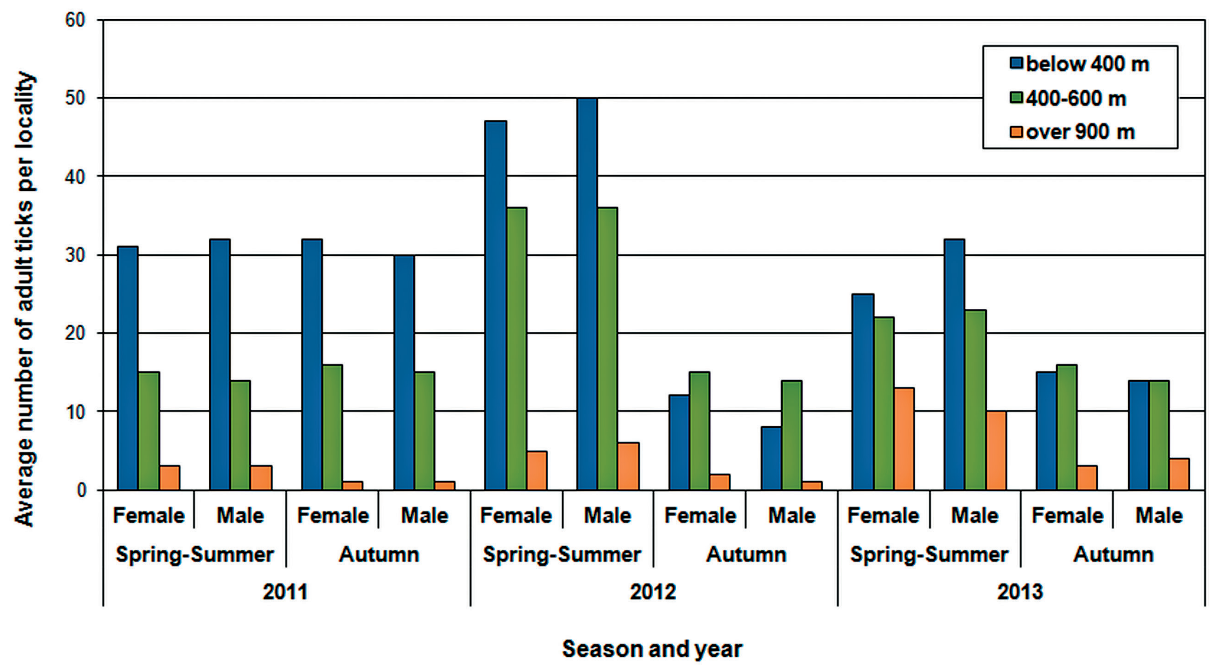

Figure 2. Average number of *Ixodes ricinus* adults per locality according to altitudinal level, season, and gender in 2011–2013

d) Regression model for the number of nymphs and adult ticks

Estimates from the multiple Poisson regression model for the number of nymphs are given in the Table 3. The effect of four explanatory variables (region, altitude, period, and year) is estimated simultaneously. All of them are statistically significant ($p < 0.001$). For detailed comparison, number of nymphs in the South Bohemian Region, below 400 m a.s.l. and in the spring-summer season of the year 2011 is taken as a reference category. Data are presented as coefficient estimates and prevalence rate ratios (exponentiated

Table 3. Poisson multiple regression model for the number of *Ixodes ricinus* nymphs

Category	Regression coefficient estimate	Std. error of the coefficient	Prevalence rate ratio*	95 % CI	p-value
Region					< 0.001
Ústí nad Labem	0.019	0.026	1.019	0.968–1.073	0.467
Highlands	-1.008	0.029	0.365	0.345–0.386	< 0.001
Olomouc	-0.049	0.022	0.952	0.912–0.995	0.029
Altitude					< 0.001
401-600 m a.s.l.	-0.161	0.025	0.851	0.810–0.894	< 0.001
Above 600 m a.s.l.	-2.186	0.059	0.112	0.100–0.126	< 0.001
Period					< 0.001
Autumn	-0.170	0.016	0.844	0.818–0.871	< 0.001
Year					< 0.001
2012	0.256	0.021	1.291	1.240–1.345	< 0.001
2013	0.410	0.020	1.506	1.449–1.566	< 0.001

*Prevalence rate ratio expresses how many times the prevalence rate in the respective category differs from that in the reference one.
Reference categories: South Bohemian Region, altitude below 400 m a.s.l., spring-summer period, year 2011.
CI – confidence interval

coefficients). All comparisons are statistically significant with one exception. Number of nymphs in the Ústí nad Labem Region statistically doesn't differ from South Bohemian Region. From prevalence rate ratio we see that number of nymphs in the Olomouc Region is approximately 5% smaller than South Bohemian Region while in the Highlands Region it is approximately 64% smaller than South Bohemian Region. Number of nymphs decreases with increasing altitude. Compared to the baseline altitude, there is about 85% of nymphs at the altitude of 401–600 m a.s.l. and 11% only at the altitude over 600 m a.s.l. Similarly, average number of nymphs in the autumn season is 16% lower than in the spring-summer season. Finally, at any fixed category, number of nymphs was 29 % and 51 % higher in the year 2012 and 2013 respectively, than in the year 2011.

The regression model for adult ticks was used with the same reference category. The results are in the Table 4. All four variables are statistically significant predictors of tick numbers ($p < 0.001$). The number of adult ticks in the South Bohemian Region is the highest among all the other regions compared and differs significantly from them. Compared to the South Bohemian Region, the number of adult ticks is only 30% of that in the Ústí nad Labem Region, 81% in the Highlands Region, and approximately 52% in the in the Olomouc Region. The effects of altitude and period have the same patterns as for the nymphs' model. The number of adult ticks decreases with increasing altitude and in the autumn period. Finally, compared to the year 2011, the number of adult ticks was 47% higher in 2012 and was not significantly different in 2013.

Table 4. Poisson multiple regression model for the number of *Ixodes ricinus* adult ticks

Category	Regression coefficient estimate	Std. error of the coefficient	Prevalence rate ratio*	95 % CI	p-value
Region					< 0.001
Ústí nad Labem	-1.197	0.071	0.302	0.263–0.347	< 0.001
Highlands	-0.209	0.054	0.811	0.730–0.901	< 0.001
Olomouc	-0.658	0.063	0.518	0.458–0.586	< 0.001
Altitude					< 0.001
401-600 m a.s.l.	-0.903	0.055	0.405	0.364–0.452	< 0.001
Above 600 m a.s.l.	-2.579	0.131	0.076	0.059–0.098	< 0.001
Period					< 0.001
Autumn	-0.499	0.039	0.607	0.562–0.656	< 0.001
Year					< 0.001
2012	0.385	0.046	1.469	1.344–1.606	< 0.001
2013	0.039	0.048	1.040	0.946–1.143	0.421

*Prevalence rate ratio expresses how many times the prevalence rate in the respective category differs from that in the reference one.
Reference categories: South Bohemian Region, altitude below 400 m a.s.l., spring-summer period, year 2011.
CI – confidence interval

Table 5. Estimated prevalence of TBE virus in *Ixodes ricinus* nymphs on the localities below 600 m a.s.l.

Category	Total nymphs	Positive pools	Estimated prevalence (%)	Std. error	95% CI
Region					
South Bohemian	4969	10	0.202	0.064	0.096–0.371
Ústí nad Labem	5612	4	0.067	0.033	0.018–0.171
Highlands	1799	2	0.089	0.063	0.011–0.321
Olomouc	3091	0	0.000		
Altitude					
Below 400 m a.s.l.	4537	4	0.081	0.040	0.022–0.207
401-600 m a.s.l.	10934	12	0.103	0.030	0.053–0.179
Period					
Spring-summer	8380	6	0.066	0.027	0.024–0.144
Autumn	7091	10	0.132	0.042	0.063–0.242
Total	15471	16	0.096	0.024	0.055–0.156

CI – confidence interval

2. Detection of the tick-borne encephalitis virus in *Ixodes ricinus* ticks

All the *I. ricinus* ticks collected (nymphs and adults) were screened for the presence of TBEV. The virus was detected in eight of 13 plots monitored as can be seen in detail from Table 1. In total, TBEV was detected in 16 nymphs (from five areas), 13 females (from seven areas), and in four males (from three areas). Surprisingly, in the Olomouc Region, the TBEV was only detected in an *I. ricinus* female from the Červenohorské sedlo located at the highest altitude while the Šumperk and Jeseník plots yielded no positive result (similarly to the plots at Stožec, Děčínský Sněžník, and Bystřice pod Pernštejnem).

a) Detection of the tick-borne encephalitis virus in *Ixodes ricinus* nymphs

The screening of a total of 15,848 *I. ricinus* nymphs yielded 16 positive results. Table 5 presents the estimates of TBEV prevalence and corresponding 95% confidence intervals derived using maximum-likelihood estimates for pooled samples applied to data collected in the altitudes below 600 m a.s.l. No positive nymph was detected in the areas above 600 m a.s.l.

The overall prevalence estimate at the altitudes below 600 m a.s.l. was 0.096 % (95% CI 0.055–0.156), i.e. one positive detection per 1040 *I. ricinus* nymphs. The prevalences, however, varied depending on the region,

Table 6. Estimated prevalence of TBE virus in *Ixodes ricinus* adult ticks on the localities below 600 m a.s.l.

Category	Total ticks	Positive pools	Estimated prevalence (%)	Std. error	95% CI
Region					
South Bohemian	935	10	0.931	0.293	0.445–1.706
Ústí nad Labem	546	1	0.142	0.142	0.004–0.787
Highlands	1022	5	0.428	0.191	0.139–0.997
Olomouc	294	0	0.000		
Altitude					
Below 400 m a.s.l.	982	3	0.262	0.151	0.054–0.763
401-600 m a.s.l.	1815	13	0.588	0.163	0.313–1.004
Period					
Spring-summer	1778	10	0.494	0.156	0.237–0.907
Autumn	1019	6	0.450	0.183	0.165–0.977
Gender					
Female	1409	12	0.716	0.206	0.370–1.248
Male	1388	4	0.238	0.119	0.065–0.608
Total	2797	16	0.477	0.119	0.272–0.773

CI – confidence interval

altitude and tick collection season. Among regions, the South Bohemian Region clearly showed the highest prevalence of 0.202 %. The prevalence in the Ústí nad Labem and Highlands Regions was less than half of it and was zero in the Olomouc Region. Despite it, no differences between regions can be proven as statistically significant since the prevalence confidence intervals overlap. At the altitudes of 401–600 m a.s.l. the occurrence of infected nymphs was slightly higher than at the altitudes below 400 m a.s.l. Insignificantly higher virus infection rates were found in the autumn periods as compared to the spring-summer period.

b) Detection of the tick-borne encephalitis virus in adult *Ixodes ricinus* ticks

Among 2873 collected adult *I. ricinus* ticks, the TBEV was detected in 17 instances. Similarly to nymphs, the estimates of prevalence for adult ticks were calculated only for the altitudes below 600 m a.s.l., therefore one positive finding in female at higher altitude from the Olomouc Region was not included. The results are summarized in Table 6. The overall prevalence estimate was 0.477 % (95% CI 0.272–0.773). A marked difference in the estimated prevalence was found between females and males with the respective figures of 0.716 % and 0.238 %. Clear differences were also found among regions. The highest prevalence of TBEV in adult *I. ricinus* ticks was detected in the South Bohemian Region, with the absolute number of 10 positive findings (7 females and 3 males) and the resulting prevalence of 0.931 %. Prevalence estimates for other regions were substantially lower, namely 0.142 % in the Ústí nad Labem Region (1 female), 0.428 % in the Highlands Region (4 females and 1 male), and 0.0 % in the Olomouc Region. At the altitudes below 400 m a.s.l. the prevalence estimate of 0.262 % was lower compared to the altitudes of 401–600 m a.s.l. with a respective value of 0.588 %.

DISCUSSION

The present study updates the information on local populations of *I. ricinus* ticks and their involvement in the circulation of the TBEV in the regions of the Czech Republic selected on the basis of high prevalence of TBE infection in humans. The data were obtained from the field research and subsequent laboratory examination of ticks. This comprehensive approach to ticks and TBEV is unique and unprecedented in Europe. The monitoring plots were selected to represent different landscape types of the areas studied and to meet the generally known requirements of *I. ricinus* ticks for a suitable habitat in terms of vegetation. Attention was paid to the size of these plots which had to be adequately large (600 m^2) and to the usage of identical plots during the whole study period. Another important parameter is the length of time during which the monitoring was completed: the three-year period covered the full life cycle of at least one generation of *I. ricinus.*

The first step of such comprehensive research was conducted at the end of the 1990s when a sharp increase in the incidence of TBE in the whole area of distribution of this disease was registered. In the Czech Republic this phenomenon manifested itself mainly in the South Bohemian Region. Therefore, an investigation of density of the local populations of *I. ricinus* was conducted in the year 2000 in this area on cadasters of 8 municipalities with the highest incidence of human cases together with investigation on TBEV prevalence [6]. The present study confirmed the results obtained in the same region in the year 2008 [9].

The expansion of *I. ricinus* ticks at higher altitudes and the increasing abundance of local tick populations have a continuous upward trend, as clearly documented by the data from the Jeseníky Mts. area where the monitoring had already been performed in the same study plots in 2008. In this regard, the results from the Highlands Region are also highly relevant since they extend the previous epidemiological studies dealing with the increase in TBE cases in this region [8, 13]. At present, despite the permanent expansion of ticks and tick-borne pathogens at higher altitudes, the high risk limit for human infection with TBEV is 600 m a.s.l. in the Czech Republic. Nevertheless, the risk of TBE infection at higher altitude exists as confirmed by cases of human infection at 900 m a.s.l. [3]

The comparison of the population density of *I. ricinus* ticks between regions is also important. The nymphs, i.e. the stage which bites humans most often and thus plays a crucial role in the transmission of infections to humans, were comparably abundant at least in three regions. However, differences were found in the nymph to adult ratio (both males and females). The shift towards adult ticks was the most obvious in the study areas in the South Bohemian and Highlands Regions. An opposite result was found particularly in the Olomouc and Ústí nad Labem Regions. This discrepancy can be explained by the differences in the local biocenoses which influence the local animal host range of different developmental stages of ticks. In general, larvae more readily find a host among both small terrestrial mammals such as field mice or bank voles and birds feeding on the ground [15, 16]. Despite the regular considerable losses due to the problem of host finding and due to the microclimatic factors [4], the larvae moult into nymphs usually in sufficient numbers. A problem may arise if in the local habitat there are not enough middle-sized mammals such as squirrels, hedgehogs, hares, etc. which are the main hosts of the nymphs [14]. Based on the investigation of a set of more than 3000 mouse-like rodents for the infestation with *I. ricinus* ticks, Rosický and Černý [16] concluded that among parasiting ticks nymphs represented 1 % only. They doubted whether these hosts would be adequate for the nymphs to have a proper blood meal which would allow their further development. Low numbers of suitable hosts for the nymphs are the reason behind the reduced numbers of adult ticks. This may not endanger the survival of the tick population, but it may endanger the intensity of TBEV circulation in zoonotic sphere due to limited transovarial transmission level. *I. ricinus* females can readily find an appropriate host and lay enough eggs for the new generation to continue. Under the high level of gamekeeping and hunting activities in the Czech Republic, the widespread roe deer populations are maintained in balance with the local landscape conditions. The crucial point in the parasite-host relationship between cloven-hoofed game and ticks is the provision of a successful completion of the final stage of the *I. ricinus* life cycle and the survival of the local tick population. In the last decades, out-of-control wild boar populations create growing concerns and as potential hosts for *I. ricinus* females may have played a role in the increased abundance of ticks [10]. The interference with the tick life cycle in the nymph stage is thus considerably counterbalanced. It should be noted that large mammals themselves do not contribute to the circulation of the TBEV in the biocenosis, particularly with reference to the low and short-term viremia [16] and thus to limited ability to infect the ticks feeding on them.

The limited occurrence of the middle-sized mammals may greatly influence the TBEV circulation in the zoonotic sphere. This permanent negative effect can also have considerable repercussions in the epidemiological situation of human infections. This can be illustrated by the detection rates of TBEV in ticks from the Šumperk and Jeseník plots. They were both selected based on considerable incidence of TBE (2001–2010) as representatives of the Olomouc Region. However, even the considerable number of *I. ricinus* ticks collected was not high enough to allow the detection of TBEV. A low TBEV prevalence in *I. ricinus* ticks was also found in the plots of the Ústí nad Labem Region where heightened incidence of TBE cases was also reported in 2001–2010. A low proportion of infected ticks found in both cases indicates that the risk of human infection also exists in such situation. Under such circumstances, the inter-year weather changes can play an important role which may be reflected in the differences in the incidence of TBE.

The hypothesis that the life cycle of *I. ricinus* ticks is considerably influenced by species composition and abundance of the hosts of the nymphal *I. ricinus* ticks is clearly supported by the results of the detection of the TBEV in the South Bohemian Region.

The nymph to adult ratio might be used in the future on the one hand as one of the indicators of the composition of the local *I. ricinus* population and of the level of the circulation of tick-borne pathogens in zoonotic sphere on the other for use in the health risk assessment in a given area.

Long-term research also confirmed the effect of the climatic conditions on the course of the life cycle of *I. ricinus* ticks. It was evident particularly in 2012 when the abundance of *I. ricinus* nymphal ticks in autumn was superior to that in spring at the altitudes of 401–600 m a.s.l. One possible explanation may be the course of temperatures that in the summer of 2012 showed a significant positive deviation from the 30 year normal temperature (http://portal.chmi.cz/historicka-data/pocasi/uzemni-teploty?l=en).

The effect of a sharp change in the local microclimate was observed in the Nedvědice plot (Highlands Region). In July 2012, a mature forest located nearby was severely damaged by a windstorm. The vegetation in the monitoring plot itself remained untouched, but the climatic conditions changed, as documented by the near-ground temperature and relative humidity measurements made during the collection of *I. ricinus* ticks in autumn. At the same time, a dramatic decline in the number of questing ticks was observed. In the following year (2013), the bush undergrowth was substantially restored in the area damaged previously by the windstorm. Concomitantly, renewal of the questing activity of *I. ricinus* ticks, including the detection of TBEV, was recorded on the adjacent monitoring plot.

The detection of the causative pathogens in the large set of *I. ricinus* ticks collected from distant areas over a three-year period also illustrated the importance of the size of the study set in determining the actual overall vector TBEV infection rate in the study area. The average infection rates determined among the high number of ticks analyzed in this study are relatively low in comparison with the data reported by others [7]. It illustrates the assumption that the size of the study set and the length of time over which the ticks were collected are crucial criteria in considering the general validity of the conclusions.

In the present study, data on the detection of TBEV are shown separately for *I. ricinus* males and females. The fact should be underlined that unlike the *I. ricinus* female, *I. ricinus* male does not feed on blood of animal hosts. The male's role in the life cycle is to fertilize the female (which, at the same time, is a prerequisite for the female to be able to feed to repletion) and to fulfil it, the male has sufficient stored energy reserves accumulated during the nymphal stage. Therefore, the male has no direct relevance in the epidemiology of human infections and also in the zoonotic cycle of the pathogens. Nevertheless, some speculate about the possible transmission of TBEV from an infected male to an uninfected female. While mating, the *I. ricinus* male tick inserts its mouthparts into the female genital pore to deposit its spermatophore to inseminate the female tick. The copulating male tick releases saliva as an effective lubricant [10] which might be a possible route of transmission of the pathogens.

CONCLUSION

Based on the results of the altitudinal distribution of *I. ricinus* populations along with TBEV findings, the high risk limit for human infection is 600 m a.s.l.

The nymph to adult ratio shows great interregional variability and might be used in the future as one of the indicators of the composition of the local *I. ricinus* population and of the level of the circulation of tick-borne pathogens in zoonotic sphere and also for use in the health risk assessment in a given area.

The three-year study justifies the assumption that the regional differences observed in the *I. ricinus* infection rates are driven by the variability of the characteristics of the entire local biocenosis of a given region. The dynamics of its seasonal variation, depending on the climatic factors, determines the interyear differences, including those in the epidemiological situation of tick-borne infections.

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