**MEDICAL UNIVERSITY - PLEVEN**

**FACULTY OF MEDICINE**

**DEPARTMENT OF ANATOMY, HISTOLOGY, CYTOLOGY AND BIOLOGY**

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# Investigation of *Borrelia burgdorferi* Sensu Lato Infection in Ixodid Ticks in the Pleven Region

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The dissertation includes 173 pages, 29 tables, 39 figures and 13epizootological maps. The bibliography comprises 266 publications, 17 of them are written in Cyrillic

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List of Abbreviations

| | |
|---|---|
| FQI | Female questing index |
| GIS | Geographic information system |
| igs | Intergenic spacer |
| LB | Lyme borreliosis |
| MQI | Male questing index |
| nPCR | nested Polymerase Chain Reaction |
| NQI | Nymph qesting index |
| PCR | Polymerase Chain Reaction |
| RH | Relative humidity |
| s.l. | Sensu lato |
| SD | Standard deviation |
| TF | Ticks frequency |
| TQI | Ticks questing index |
| U | Urbanised; maintained grass area |
| W | Wild; natural areas not maintained |
| $\bar{x}$ | Arithmetic mean |

## Contents

# I. INTRODUCTION

Ixodid ticks (family Ixodidae within the Parasitiformes) are a relatively small, taxonomically isolated group of acari, obligate hematophagous parasites of terrestrial vertebrates (mainly mammals, birds and reptiles). In Bulgarian fauna 5 genera with 39 species have been described. Ixodid ticks are vectors and often hosts of many pathogens of natural infectious diseases and are involved in the spread of bacteria, rickettsiae, viruses and protozoa among wild and domestic animals.

Lyme borreliosis (LB) is the most common naturally occurring tick-borne zoonotic infection in temperate countries, which includes Bulgaria. The causative agents of LB are spirochetes belonging to the *Borrelia burgdorferi* sensu lato (s.l.) complex, spread by ticks of the genus *Ixodes*.

The World Health Organization (WHO) is developing strategies and control approaches for this at times underestimated infection. Investments are being made to introduce vaccines, both routinely in livestock and for pets, to improve conditions in ecosystems. Lyme borreliosis is also a socio-economic problem, so controlling, monitoring and limiting tick populations must also be considered. All this will provide a boost in the campaign against this health problem.

Laboratory diagnosis of the disease requires rapid and accurate methods that allow detection of the pathogen directly in its vectors. At this stage, there is still no effective method to eradicate the pathogen and its tick vector. Moreover, no vaccines are currently available to protect against *Borrelia*. Research on pathogen transmission via the main vectors (ticks of the genus *Ixodes*) will contribute to the faster eradication or containment of the causative agents already in their natural foci. The use of rapid and easily implemented techniques for the detection of *Borrelia* in ticks will contribute to early diagnosis and adequate treatment, especially in cases with fever of unknown origin.

More thorough studies of ixodid tick populations and their associated pathogens will contribute to optimizing epidemiological investigation of the disease. This will help to clarify the overall epidemic situation in the country and contribute to a more effective prevention of Lyme borreliosis.

# II. AIMS AND OBJECTIVES

The aim of the present study is to identify and analyse the infectivity of *Ixodes ricinus* with *Borrelia burgdorferi* s.l. in the Pleven region.

To identify the outbreaks of tick populations and their population density and to assess the risk of infection of the population in the region based on the established data. To accomplish this goal, we set the following tasks:

1. To perform a retrospective epidemiological study of vector-borne infections in the Pleven region.
2. To investigate the ixodofauna in the study area by taxonomic identification by species, sex and developmental stage of the ticks and to form biogeocenotic groups on this basis.
3. To investigate *Borrelia burgdorferi* s.l. infestation in specimens of the ixodofauna of the Pleven region by identifying “hot spots” with high population density of ixodid ticks that represent an increased epidemiological risk for the public.
4. To identify the presence of the *Borrelia burgdorferi* s.l. complex in the collected *Ixodes* ticks by dark-field microscopy.
5. To isolate DNA from the vectors and perform PCR analysis for positivity for the *Borrelia burgdorferi* s.l. complex.
6. To develop a map of established outbreaks of tick populations and their infectivity with *Borrelia burgdorferi* s.l. in the region of Pleven.
7. To compare and analyse the density of tick populations and their infectivity with *Borrelia burgdorferi* s.l. in maintained green areas treated with acaricides and in natural unmaintained and untreated areas.
8. To assess the risk of infection of the population in Pleven and its district based on the infectivity of the ticks examined.

# III. MATERIALS AND METHODS

## 1. A RETROSPECTIVE EPIDEMIOLOGICAL STUDY ON THE PREVALENCE OF LB IN THE PLEVEN REGION AND BULGARIA

### 1.1. Morbidity study

The object of a population-based morbidity study is the sum of the incidence of diseases among people. The unit of observation represent the registered cases or sick people who sought medical care. The technical unit and source of information is the health facility where the cases were registered.

### 1.2. Sources of information

The main sources of information for the annual surveys are the annual forms submitted by all health care facilities in the country to the National Statistical Institute and the National Center for Public Health and Analysis, Directorate for National Health Data and eHealth https://nsi.bg/bg.

The data on the prevalence and incidence of LB in the Pleven region for the period 2001 - 2016 were provided by the Regional Health Inspectorate (RHI) - Pleven with permission for access to public information granted under the Public Information Act No. 16-02942 dated 05.04.2016. From 2016 to 2021, data from the published bulletins on the RHI-Pleven website, as well as from the annual publications "Health" on the NSI website https://www.nsi.bg/bg were used. Detailed information regarding the sources of information is presented in Table 1.

*Table 1. Data used for a retrospective epidemiological study on registered cases of Lyme borreliosis in Bulgaria and the Pleven region.*

| Indicators | Source of information | Data available for the period: |
|---|---|---|
| **Age** | NSI – "Health" | Data not available |
| | RHI - Pleven | 2011 - 2016 г. |
| **Gender** | NSI – "Health" | 2011 - 2016 |
| | RHI - Pleven | 2002 - 2015 г.[1] |
| **Registered Morbidity** | NSI – "Health" | 2015 - 2020 г. |
| | RHI - Pleven | 2001 - 2020 г. |
| **Registered number of cases per month** | NSI – "Health" | 2016 - 2020 г. [2] |
| | RHI - Pleven | 2001 - 2020 г. |
| **Registered cases by district** | NSI "Health" | 2016 - 2020 г. |
| | RHI - Pleven | Data not available |

1 For 2008, the data provided did not include information on the sex of those registered with LB.

2 The data are total for Bulgaria.

## 2. COLLECTION AND IDENTIFICATION OF TICKS

Tick collection campaigns were conducted in March, April, May, and June, over a 5-year period from 2016 to 2021, using a flagging method with a white cotton cloth (approximately 100 × 100 cm in size) (Mays et al., 2016) mounted on a handle. The distance to be flagged for each sampling site on every visit depended on the available time and weather conditions, as well as local tick abundance.

Sampling days were selected based on the following criteria: no rain and heavy dew, wind speed less than 3 on the Beaufort scale, air temperature above 10°C. For this purpose, a weather station (Auriol IAN 94604 Owim GmbH & Co. KG, Neckarsulm, Germany) was placed on the ground in each sampling area to monitor the local temperature and hygrometry. The total sampling time and surface area were recorded for each visit. The time spent sampling (including that required to extract the ticks from the flag with tweezers and place them separately in 1.5 ml Eppendorf-type tubes (Eppendorf AG, Hamburg, Germany) ranged from 5 to 60 min. The collected ticks were transferred to the laboratory, where they were washing with sterile water in an ultrasonic cleaner (Silvercrest, HOYER Handel GmbH, Hamburg, Germany), identification of the tick species, developmental stage and sex according to morphological keys provided in Georgieva, Filippova and Estrada-Peña et al., using a stereomicroscope (Olympus SZ4045, Olympus American Inc., Melville, NY) were carried out.

## 3. DENSITY OF THE QUESTING TICKS

Tick densities are represented as the number of ticks captured per 100 $m^2$ of flagging area. The flagging area was calculated using the Google Maps application (https://www.google.com/maps/@43.4107218,24.6295521,15z) and Google Earth (http://earth.google.com).

Because flagging was performed unevenly across sampling sites (different times, duration of flagging, and number of samples), several indexes were used to compare and determine the abundance of actively searching ticks in different study areas. Tick abundance was evaluated using a so-called questing index (QI), where the number of ticks collected from vegetation was divided by the flagged area. The QI was calculated for *I. ricinus* only (separately for adults, nymphs) as follows: males (MQI); females (FQI); nymphs (NQI); and total number of *I. ricinus* ticks in the field (TQI). The conversion was performed using the following formula: QI = n /S, where n is the number of ticks collected and S is the area from which specimens were collected in square meters. The area and corresponding indices were calculated immediately after each tick collection campaign. The resulting indices were used for statistical analysis.

### 3.1. Frequency of the questing ticks (Ticks Frequency TF)

TF represents the total number of ticks collected from each location per minute regardless of stage and sex. The total number of ticks collected was divided by the collection time at each event.

When determining the collection time, the time from the first pass of the flag over the vegetation to the harvest of the last captured tick was recorded.

## 4. Study areas

The survey included areas that have not been studied previously in Bulgaria. They are as follows:

### 4.1. “Kaylaka” Protected area (PA), Pleven

“Kaylaka” Protected Area (PA) is located in the karst valley of the Tuchenitsa River in the outskirts of the town of Pleven, the village of Tuchenitsa, the village of Bohot, and the village of Radishevo. It was declared a protected area with Order No. 3700/29.12.1972 of the Ministry of Environmental Protection. It covers the canyon of the Tuchenitsa River and the Bohot Forest (Map 2).

Over a period of seven years (2016-2022), eight sampling sites divided into two categories - Urbanised (U) and Wild (W) were surveyed in the PA “Kaylaka”.

#### 4.1.1. Urbanised Areas in PA “Kaylaka”

Sampling sites U1 to U4 (Central Park Lane 2.8 km). There are various deciduous and coniferous trees and glades on both sides of the lane. The urban sections are under strong anthropogenic influence, regularly visited by citizens, maintained by mowing and pruning the shrubs and trees. Intensively used by visitors for a variety of outdoor activities, especially sports, walking children, dog walking, cycling etc (Map 4 Map 7).

All urban sites are inhabited by rodents and birds as well as synanthropic predators such as dogs (*Canis lupus familiaris*), ferrets (*Mustela putorius*) and hedgehogs (*Erinaceus europaeus*). Larger mammals such as roe deer (*Capreolus capreolus*) and European badger (*Meles meles*) are also found (although rarely) (according to personal observation during the study period).

#### 4.1.2. Wild Areas in PA “Kaylaka”

The unmaintained, wild areas (W1 - W4) are known to be inhabited by numerous large mammals, especially a free-living population of roe deer (*Capreolus capreolus*) or wild boar (*Sus scrofa*). Jackals (*Canis aureus*), foxes (*Vulpes vulpes*), European badger (*Meles meles*) are permanent residents of the forest, which is also the habitat of many rodents, insectivores, many bird species and reptiles (personal observation) (Map 8 Map 12). Wild sampling sites have a much lower level of daily human activity and are mainly visited by forest workers, hikers, herb collectors and mushroom pickers, during certain times of the year.

### 4.2 “Karst Gorge on the river Chernelka” Protected Area (“Chernelka” PA) stretching from the village of Gortalovo to the village of Kartozhabene

The gorge is located in the middle part of the Danube plain. It is a karst canyon of Upper Cretaceous limestones and sandy clay rocks. It is located between the two villages of Gortalovo and Kartozhabene, about 12 km away from the town of Pleven. Its forest fund has a total area of

300 hectares. Along its 6.5 km length, the small river Chernelka has formed a canyon with a width of 60 to 200 metres and a cliff height of 10 to 35 - 40 metres (Map 1and Map 13). It is characterised by low anthropogenic interference with dense vegetation. Among the predominant plant species are black elder (*Sambucus nigra*), Jerusalem thorn (*Paliurus spina-christi*), sloe (*Prunus spinosa*), spindle tree (*Euonymus europaeus*), hawthorn (*Crataegus* sp.).

### 4.3. Village of Sadovets, area "Garvan dol"

The area is situated about 3 km southeast of Sadovets, between the Sadovsko fortress and the Kozhuharovo fortress. There are plenty of walnuts, lindens, poplars, elms and other centuries-old trees in the area. Cattle and small livestock grazing is daily. It is characterised by the presence of open meadows with tall herbaceous vegetation (Map 1).

### 4.4. Village of Grivitsa, locality "Tachova Cheshma"

The area is located next to the so-called "Alleys" near two swampy (until 2019) areas. Specimens of the Ixodofauna were collected until 2019 due to draining of the swamps and turning the area into an illegal dumpsite. The vegetation consist mostly of low trees and shrubs. The predominant species are Jerusalem thorn (*Paliurus spina-christi*), wild pear (*Pyrus pyraster*), sloe (*Prunus spinosa*), hawthorn (*Crataegus* sp.), and black elder (*Sambucus nigra*). There are artificial forests of honey locust (*Robinia pseudoacacia*). The area is rich in wildlife (Map 1).

## 5. DARK-FIELD MICROSCOPY FOR THE DETECTION OF *BORRELIA BURGDORFERI* S.L. IN *IXODES RICINUS* INTESTINAL CONTENTS

A dark-field condenser microscope was used for the detection of *Borrelia burgdorferi* s.l. Only ticks that were collected from vegetation were subjected to microscopic analysis. Ticks were examined immediately after collection or after being in a refrigerator at 4°C within a week. In the process, the tick was fixed on a slide using entomological forceps. Using the tip of a sterile needle, an incision was made between 2 – 3 limbs, aiming to rupture the midgut. A small drop (20-30 µl) of PBS or saline solution was added to the haemolymph containing some of the intestinal contents. It was observed at 400x magnification. Those ticks in which at least one spirochaete was observed were considered positive. Negative ticks were those in which microscopic examination of at least 50 fields did not reveal the presence of *Borrelia*.

## 6. TICKS DNA EXTRACTION

### 6.1 Isolation with Phenol-Chloroform Isoamyl Alcohol as Described by Barker et al (1998).

MATERIALS:

- Proteinase K
- Phenol-chloroform isoamyl alcohol (25:24:1)
- Chloroform isoamyl alcohol (24:1)
- Ice-cold 100% ethanol
- 70% ethanol
- TRIS - EDTA (TE) buffer

LABORATORY EQUIPMENT:

- Thermostat
- Laminar flow hood
- Vortex
- 1,5 ml sterile microcentrifuge tubes
- Freezer
- Microcentrifuge
- Thermo Shaker

**TISSUE LYSIS:**

1. Ticks were dissected using a sterile needle and/or a pipette tip in 1.5 ml ependorf-type tubes;
2. A 400 µl lysis buffer [50 mM Tris-HCl (pH 8.25), 25 mM EDTA, 25 mM NaCl, 1% SDS, Proteinase K (500 mg/ml)] were added;
3. Samples were incubated for 3-4 hours at 56°C on a thermal shaker.

## DNA extraction and precipitation:

1. An equal volume (400 µl) of phenol-chloroform isoamyl alcohol (25:24:1) was added to each tube and mixed for 5 min at room temperature;
2. Samples were centrifuged at 10 000 g for 10 min at room temperature;
3. The upper (aqueous) phase containing DNA was carefully removed;
4. The DNA fraction was transferred to a new tube. If precipitate had formed between the two phases, steps 2-4 were repeated again;
5. An equal volume of chloroform-isoamyl alcohol was added (24:1), stirred gently for 2 min and centrifuged for 1 min at 10 000 g at 4°C;
6. The upper (aqueous) phase containing DNA was gently pipetted off and transferred to a new tube;

volume of 3M sodium acetate (pH = 5) and 2-2.5 volumes of ice-cold 100% ethanol were added and incubated at -70°C overnight;

8. The samples were centrifuged at 4°C for 20 min at 10 000 g and the supernatant was removed;
9. One ml of 70% ethanol was added to each sample after which they were centrifuged at 4°C for 20 min per 10 000 g.
10. The supernatant was removed; the tubes were dried at room temperature for 1-2 hours;
11. The resulting isolate was dissolved in 50 µl TE buffer (pH 8);
12. The resulting DNA was stored at -70°C.

### 6.2 DNA extraction using commercially available kits

Genomic DNA was extracted from individual ticks using NucleoSpin® Tissue kit for DNA from cells and tissues (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). DNA extraction was performed according to the manufacturer's instructions.

## 7. NESTED PCR TOR THE DETECTION OF *BORRELIA BURGDORFERI* S.L. IN *IXODES RICINUS*.

### 7.1. NESTED POLYMERASE CHAIN REACTION nPCR

Nested polymerase chain reaction is a two-step variation of PCR used to reduce the amount of nonspecific reaction products.

The detection of *B. burgdorferi* s.l. was carried out at the Department of Molecular Biology, Immunology and Medical Genetics of the Faculty of Medicine at Trakia University, Stara Zagora, Bulgaria. GeneAmp PCR System 9700 (Applied Biosystems, USA) was used.

In order to increase the sensitivity of PCR analysis to the presence of Borrelia DNA in ticks, we performed two nPCRs targeting (1) the non-coding region, the intergenic spacer of *5S-23S* rRNA (PCR5S-23Sigs) and (2) the gene encoding flagellin B (*FlaB*). The sequences of the primers used are given in Table 2.

### 7.2. Nested PCR

The steps by which nPCR for detection of *Borrelia* was carried out are as follows:

1. The laminar box was pre-sterilized with UV light and 70% ethanol;
2. After thawing, the samples were gently mixed on a vortex mixer and briefly centrifuged;
3. A reaction mixture (master mix) was prepared at room temperature by adding the components presented in Table 3 and
4. 
5. 

6. *Table 4* into a test tube;
7. The master mix thus prepared was mixed thoroughly on a vortex mixer and centrifuged briefly;
8. 17µl of the reaction mixture was placed into each well of the PCR plate;
9. 3µl of the isolated DNA was added;
10. In parallel, positive control series were prepared consisting of verified *Borrelia* DNA, as well as negative control series involving reactions without the presence of DNA matrix (nuclease-free water instead of DNA) to detect contamination from reagents and aerosols;

11. The PCR apparatus was programmed according to the scheme in
12. Table *5* The plate was placed in the apparatus and amplification was started.

*Table 2. Internal and external primers for nPCR of B. burgdorferi s.l. 5S-23S intergenic spacer and FlaB.*

| **Primer name** | **Gene** | **Sequence** (5′-3′) | **Amplicon size** | Tm | GC% | **Annealing temperature** | **References** |
|---|---|---|---|---|---|---|---|
| **23S3 Out Fw** | *rrf (5S)-rrl (23S) intergenic spacer* | CGACCTTCTTCGCCTTAAAGC | 411 bp | 59.61 | 52.38 | 55°C | Chu et al., 2008 Schwartz et al., 1992 |
| **23Sa Out Rv** | | TAAGCTGACTAATACTAATTACCC | | 53.43 | 33.33 | | |
| **Primer 1 In Fw** | *rrf (5S)-rrl (23S) intergenic spacer* | CTG CGA GTT CGC GGG AGA | 254 bp | 67.1 | 66.67 | 59°C | Postic et al., 1994 |
| **Primer 2 In Rv** | | TCC TAG GCA TTC ACC ATA | | 57.7 | 44.44 | | |
| **FlaB Out Fw** | *FlaB* | GCATCACTTTCAGGGTCTCA | 503 bp | 62.8 | 50 | 55°C | Wills et al. |
| **FlaB Out Rv** | | TGGGGAACTTGATTAGCCTG | | 62.7 | 50 | | |
| **FlaB In Fw** | | CTTTAAGAGTTCATGTTGGAG | 447 bp | 57.4 | 38.10 | 58°C | |
| **FlaB In Rv** | | TCATTGCCATTGCAGATTGT | | 61.7 | 40 | | |

13. After the first amplification, the reaction mixture for the second (nested) PCR was prepared as shown in Table 3 and
14.
15.

16. *Table 4*. The difference with the first PCR was:
- The use of the appropriate internal primers (Table 2);
- 2 µl of DNA matrix obtained from the first PCR and a hybridisation (annealing) temperature matching that of the internal primers presented in Table 2;

17. A negative control (nuclease-free water) and a positive control were included in each amplification. For the positive control, purified genomic DNA (isolated from *Borrelia burgdorferi*; strain B31, [ATCC®35210D5™] supplied by LGC, Germany, Hanover) diluted to a concentration of 10 ng/µl was used.

*Table 3. Required starting quantities for one nPCR5S-23Sigs reaction (20 µl).*

| Ingredients | $PCR_{5S\text{-}23Sigs}$ №1 | $PCR_{5S\text{-}23Sigs}$ № 2 |
|---|---|---|
| **Taq Polymerase Master Mix 2X** | 12.5 µl | 12.5 µl |
| **Nuclease-free water** | 3.7 µl | 4.7 µl |
| **10 µM forward and reverse primers** | 0.8 µl external primers:<br>0.4 µl Forward Primer<br>0.4 µl Reverse Primer | 0.8 µl internal primers:<br>0.4 µl Forward Primer<br>0.4 µl Reverse Primer |
| **DNA template** | 3.0 µl, sample extract | 2.0 µl, from PCR product No 1 |

*Table 4. Required starting amounts to run one nPCRflaB per sample (20 µl).*

| **Ingredients** | PCR №1 | PCR № 2 |
|---|---|---|
| **Taq Polymerase Master Mix 2X** | 12.5 µl | 12.5 µl |
| **Nuclease-free water** | 3.5 µl | 4.5 µl |

| | | |
|---|---|---|
| **10 µM forward and reverse primers** | 1.0 µl external primers:<br>0.5µl Forward Primer<br>0.5µl Reverse Primer | 1.0 µl internal primers:<br>0.5µl Forward Primer<br>0.5µl Reverse Primer |
| **DNA** | 3.0 µl, sample extract | 2.0 µl, from product No. 1 |

*Table 5. Temperature modes of nPCR performed for the detection of 5S-23Sigs and flaB.*

| | | | |
|---|---|---|---|
| **nPCR$_{5S\text{-}23Sigs}$ №1** | | | |
| 95°C | Primary denaturation | 5 min | |
| 95°C | Denaturation | 30 s | 35 repetitive amplification cycles |
| 55°C | Hybridization of primers | 30 s | |
| 72°C | Elongation | 40 s | |
| 72°C | Final elongation | 5 min | |
| nPCR$_{5S\text{-}23Sigs}$ №2 | | | |
| 95°C | Primary denaturation | 5 min | |
| 95°C | Denaturation | 15 s | 35 repetitive amplification cycles |
| 59°C | Hybridization of primers | 30 s | |
| 72°C | Elongation | 45 s | |
| 72°C | Final elongation | 5 min | |
| PCR$_{flaB}$ №1 | | | |
| 95°C | Primary denaturation | 5 min | |
| 95°C | Denaturation | 15 s | 35 repetitive amplification cycles |
| 55°C | Hybridization of primers | 30 s | |
| 72°C | Elongation | 45 s | |
| 72°C | Final elongation | 5 min | |
| PCR$_{flaB}$ №2 | | | |
| 95°C | Primary denaturation | 5 min | |
| 95°C | Denaturation | 15 s | 35 repetitive amplification cycles |
| 58°C | Hybridization of primers | 30 s | |
| 72°C | Elongation | 45 s | |
| 72°C | Final elongation | 5 min | |

# 8. ELECTROPHORESIS AND VISUALIZATION:

The PCR products were analysed by electrophoresis on a 1.5% agarose gel in TAE buffer [40 mM Tris-acetate, 2 mM EDTA (pH 8.5)] for 1 h at 107 V (5 V/cm) stained with ethidium bromide. GenLadder 50bp (Genaxxon bioscience) was used to estimate the length of the resulting product. PCR amplification results were visualized using a UST-20M-8E BIO View Transilluminator photo-documentation system, Biostep, Germany.

## 9. MAPPING OF THE AREAS

For map generation, the free and open source desktop information system QGIS 3.18.0-Zürich was used. QGIS functions as geographic information system software, allowing users to analyse and edit spatial information. The geographic coordinates of each tick were extracted from Google Maps and exported to Microsoft Excel 2016 immediately after collection.

Data processing was performed using the QGIS 3.18.0-Zürich platform. Layers from Open Street Map (OSM Standard) freely available and ESRI World Topo also freely available were used for terrain visualization. The geographical system used was EPSG:3857 - WGS 84 / Pseudo-Mercator. All flagging areas were represented as polygons which were manually drawn. Separate layers were created from the *csv files for all *Ixodes ricinus* ticks captured by flagging, for the positive with *Borrelia burgdorferi* s.l. and for the negative ones.

## 10. STATISTICAL ANALYSIS

The data were entered and processed with the statistical packages IBM SPSS Statistics 23.0, GraphPad Prism v.8 and Excel of MS Office 2021. A significance level rejecting the null hypothesis was taken as $p < 0.05$.

The following methods were applied:

- Descriptive analysis.
- Graphical analysis.
- Shapiro-Wilk test.
- Student's t-criterion.
- Non-parametric Mann-Whitney U test.
- Pearson's chi-square ($\chi 2$) test.
- One Way ANOVA and Post-hoc test (LSD)
- To test the extent to which the results of the TPM and the two PCR methods agreed, an analysis of the reliability of the tests was performed by calculating Cohen's kappa coefficient (κ), Data are presented as kappa coefficient (κ) and 95% confidence interval (95% CI). Table 6 presents the Kappa interpretation scale.

*Table 6. Cohen's Kappa coefficient interpretation scale (κ) by Landis and Koch.*

| Value of Kappa | STRENGTH OF AGREEMENT |
|---|---|
| < 0 | Poor |
| 0.01 - 0.20 | Slight |
| 0.21-0.40 | Fair |
| 0.41-0.60 | Moderate |
| 0.61-0.80 | Substantial |
| 0.81 - 1.00 | Almost perfect |

# VI. RESULTS

## 1. A Retrospective Epidemiological Study Of Tick-Borne Infections In Bulgaria And Pleven Region

In the comparative analysis of tick-borne infections for the period from 2000 to 2016, data from the Communicable Diseases Surveillance Directorate, RHI - Pleven, and the Epidemiology and Surveillance of Communicable Diseases Section, NCIPD - Sofia were used.

During the study period, 9827 cases of Lyme Borreliosis (LB) were registered in Bulgaria, 352 (3.58%) of them in the Pleven district. The registered cases of Mediterranean spotted fever (MST) were 11732, of which 25 were in the Pleven district. There were 195 cases of tularaemia in the country, and only one of them was registered in Pleven region. With Q fever, 1115 cases have been registered in Bulgaria, of which one in Pleven district.

The data show that the country leads in the number of registered cases of MST, followed by LB. Pleven district has the highest number of registered cases of LB. Analysing the incidence of LB in Pleven district and the country (Figure 1), it was found that: cases were registered annually in Pleven District, with the lowest incidence recorded in 2001 - 0.31%$_{000}$ and the highest in 2009 - 14.61%$_{000}$. The average annual incidence was 7.22%$_{000}$. Across the country for the same period, the lowest incidence was recorded in 2000 at 3.61%$_{000}$ and the highest in 2005 at 12.61%$_{000}$. The average annual incidence across the country is 7.58%$_{000}$. It is noteworthy that in some of the years the incidence in the Pleven region exceeded the national incidence (2009, 2012, 2013 and 2016).

From the MST data, Pleven district single cases were recorded from 2006 to 2016, the incidence was low (0.33%$_{000}$ to 3.04%$_{000}$). The average annual incidence is 0.897%$_{000}$. In Bulgaria, cases of MSF are registered annually and an average annual incidence of 8.90%$_{000}$ has been found (from 2.96%$_{000}$ in 2016 to 19.35%$_{000}$ in 2001).

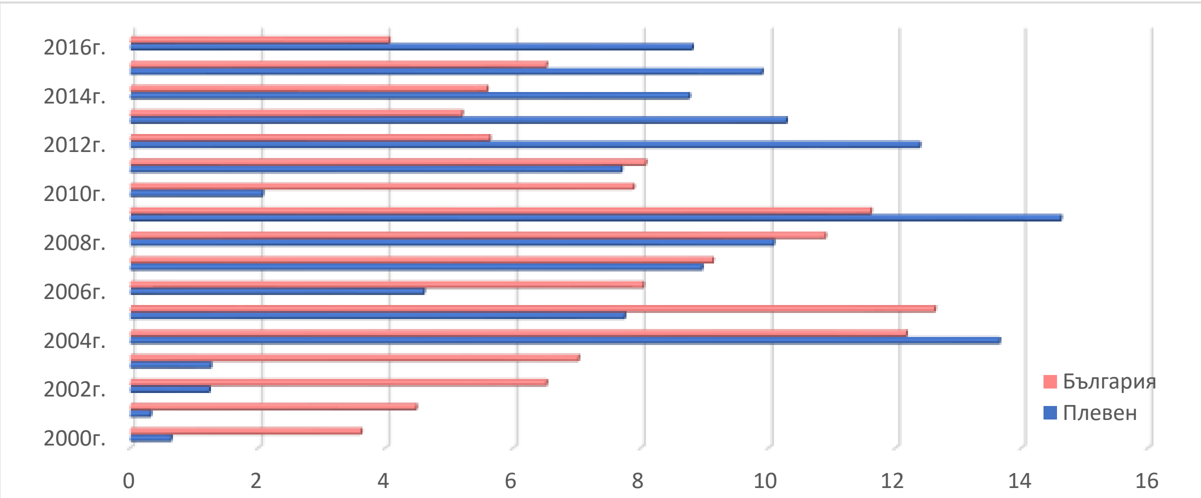



*Figure 1. Lyme borreliosis morbidity (%$_{000}$) in Pleven distrinct and Bulgaria for the period 2000 - 2016.*

In the Pleven district, one case of tularaemia (2014), which was the only case in the country for the same year, and one case of Q fever (2006), which was fatal, were registered during the study period. The morbidity rate for both infections in the district is low, at 0.38%000 for tularaemia and 0.33%000 for Q-fever. Nationwide, 195 cases of tularaemia, with an incidence of 0.01%000 to 0.21%000, and 1115 cases of Q fever, with an incidence of 0.22%000 to 3.48%000, were recorded for the period 2000-2017.

From the data published in the available NSI Health publications, there were 4678 cases of LB in Bulgaria from 2010 to 2020. On average, 425 ± 135.6 cases are registered annually in the country (5.9 ± 1.8 per 100,000 people). For the Pleven region in the same period, 232 cases were reported, with an annual average of 21 ± 9.2 (8.6 ± 3.6 per 100 000 people), which is statistically higher than the national average ($p = 0.037$) (*Figure 2*).

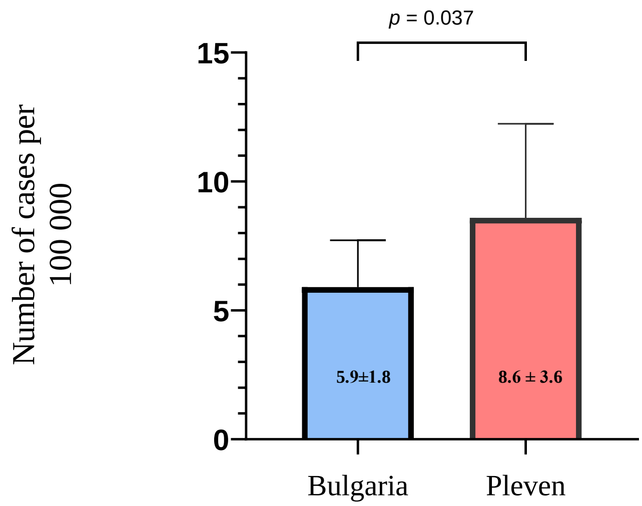



*Figure 2. Number of LB cases in Bulgaria and Pleven for the period 2010 - 2020 according to NSI data. Box-plot is represented* $\overline{x}$ *and SD.*

Analysis of the data from RHI - Pleven (2001-2020) and NSI (2016-2020) shows that from 2001 to 2020, a total of 422 (21.1 ± 12.5 annually) cases of LB were registered in the Pleven region. The highest number of registered LB cases (n = 49) was found in 2004 and 2009.

Based on publicly available data from the National Statistical Institute (2015-2020), the Pleven district ranks 9$^{th}$ in Bulgaria in the number of registered cases of LB (Figure 3).

The sex ratio from the data of the Regional Health Inspectorate - Pleven for 2002-2015 (n = 296) was 62% infected women compared to 38% men (14.08 ± 8.71 compared to 8.7 ± 5.22). The difference was statistically non-significant ($p = 0.068$).

From the monthly reports of the RHI - Pleven and the NSI it is found that for both Bulgaria and Pleven the highest number of cases of LB is observed in the summer months with a peak in July. For the general Bulgarian population, there is a small second peak in September-October, which is absent in the Pleven region (*Figure 4*). Regarding the age distribution, it is observed that the majority of cases are in persons over 30 years of age, with the highest rate in persons over 60 years of age.

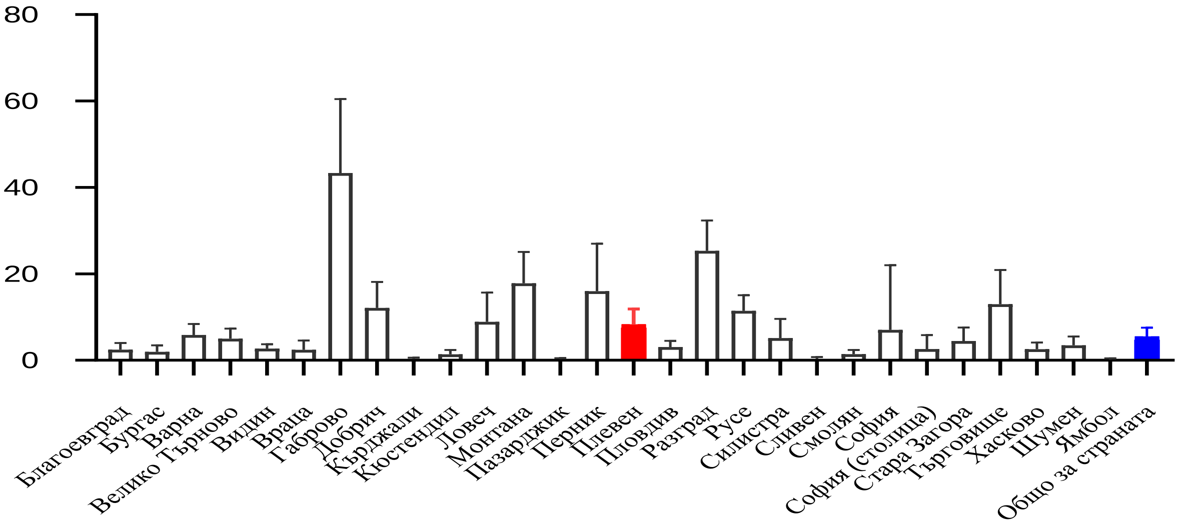
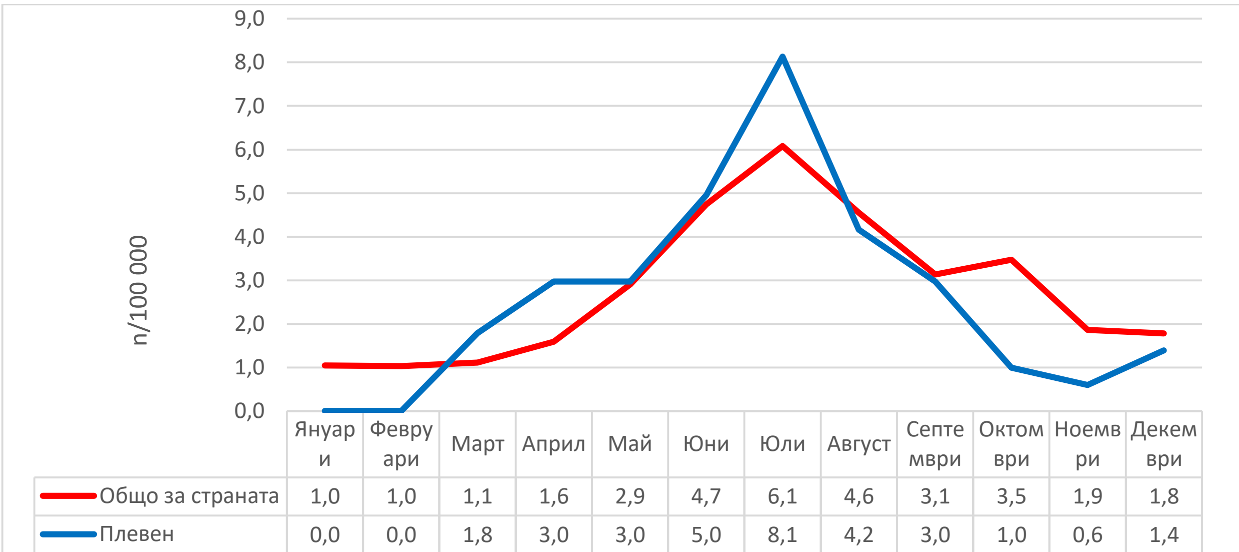



*Figure 3. Number of registered cases of LB in Bulgaria by districts for the period 2015 - 2020 according to the National Statistics Institute.*




| | Януари | Февруари | Март | Април | Май | Юни | Юли | Август | Септември | Октомври | Ноември | Декември |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Общо за страната | 1,0 | 1,0 | 1,1 | 1,6 | 2,9 | 4,7 | 6,1 | 4,6 | 3,1 | 3,5 | 1,9 | 1,8 |
| Плевен | 0,0 | 0,0 | 1,8 | 3,0 | 3,0 | 5,0 | 8,1 | 4,2 | 3,0 | 1,0 | 0,6 | 1,4 |

*Figure 4. Registered morbidity per 100 000 population (%$_{000}$) from LB by month in Bulgaria and Pleven for the period 2015-2020.*

## 2. IXODID TICK COLLECTION CAMPAIGNS

Collection of ixodid ticks was conducted over a 7-year period (2016-2022) during the spring-summer season from March to late June, according to meteorological conditions. A total of 75 collection campaigns were performed during which 1459 ticks (1376 of which by flagging) were collected from the Pleven region. Of these, 1298 (88.91%) were representatives of the genus *Ixodes*; 52 (3.56%) - of the genus *Dermacentor*; 108 (7.4%) - of the genus *Rhipicephalus*; 2 (0.1%) - of the genus *Haemaphysalis*. No members of the genus *Hyalomma* were captured. The sex and developmental stage distribution of *Ixodes ricinus* collected by flagging was 42.5% (n = 551) females, 40.2% (n = 521) males, 17.2% (n =223) nymphs, and 0.15% (n =2) larvae. The results of the campaigns in the four main zones are presented in Table 7.

*Table 7. Distribution by sex and stage of development of collected ticks by region.*

| **Study area** | **Development stage/gender** | | | | Total |
|---|---|---|---|---|---|
| | Female | Male | Nymph | Larvae | |
| **PA "Kaylaka"** | 387 | 364 | 108 | 1 | 860 |
| **PA "Chernelka"** | 114 | 103 | 48 | 1 | 266 |
| **Grivica "Tachova Cheshma"** | 26 | 38 | 61 | 0 | 125 |
| **Sadovets "Garvan dol"** | 16 | 12 | 4 | 0 | 32 |
| **Other** | 8 | 4 | 2 | 0 | 14 |
| **Total** | 551 | 521 | 223 | 2 | 1297 |

The mean temperature at which *Ixodes ricinus* collections were made during the seven years of the study was 19.27 ± 4.48°C. The lowest temperature at which samples were collected was 9°C and the highest temperature at which samples were collected was 29°C. The relative humidity (RH) at which the flagged ticks were successfully captured ranged from 32% to 96%, with a mean of 63.12 ± 15.53% RH. Maximum activity expressed by TQI and TF of *Ixodes ricinus* was at 20°C and 60% RH (Table 8).

*Table 8. Summary statistics for TF, TQI, RH and temperature as determined for the entire study period.*

| | **N** | **Minimum** | **Maximum** | $\bar{x}$ | **SD** |
|---|---|---|---|---|---|
| **TF[1]** | 73 | 0.02 | 3.67 | 0.56 | 0.43 |
| **TQI[2]** | 73 | 0.07 | 44.00 | 4.84 | 7.52 |
| **FQI[3]** | 67 | 0.5 | 24 | 2.33 | 3.64 |
| **MQI[4]** | 66 | 0.2 | 20 | 2.26 | 3.54 |
| **NQI[5]** | 45 | 0.6 | 8.57 | 1.28 | 1.92 |
| **Temperature (ºC)** | 73 | 9.00 | 29.00 | 19.27 | 4.48 |
| **Relative humidity (RH%)** | 73 | 32.00 | 96.00 | 63.12 | 15.53 |

1. TF –- Ticks Frequency. Frequency of active questing *Ixodes ricinus* (n/min).
2. TQI – Total Ticks Questing Index of *I. ricinus* (n/100 $m^2$).
3. FQI – Female Ticks Questing Index of *I. ricinus* (n/100 $m^2$).
4 MQI – Male Ticks Questing of *I. ricinus* (n/100 $m^2$).
5. NQI – Numph Ticks Questing Index of *I. ricinus* (n/100 $m^2$).

For the Pleven region, the mean density of *Ixodes ricinus* over the study period, expressed by TQI, was 4.84 ± 7.52 individuals per 100 $m^2$ with a maximum of 44 individuals per 100 $m^2$. The mean Ticks Frequency (TF) was 0.56 ± 0.43 ticks per minute and the maximum frequency was 3.67 ticks per minute (Table 8).

*Ixodes ricinus* host-seeking activity has been found to begin once the environmental temperature passes 10°C, usually in early March. Peak questing activity occurs at the end of April and lasts until the end of May, after which in the month of June it gradually declines with increasing temperature and drought.

In order to establish the maximum presence of questing *Ixodes ricinus*, the collection period was divided into intervals of 10 days and the mean TQI and TF indexes were recorded (Figure 5 and Figure 6). In both comparisons, the maximum of questing ticks was in the period from late April to early June.

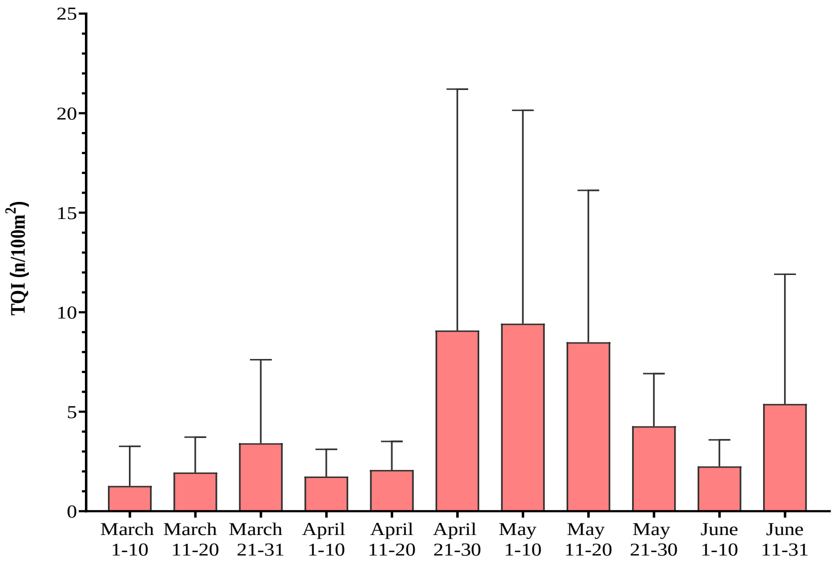



*Figure 5. Dynamics of questing ticks expressed by their TQI (density) at 10-day intervals. Mean frequencies ± standard deviation for the study period (2016-2022) are presented.*

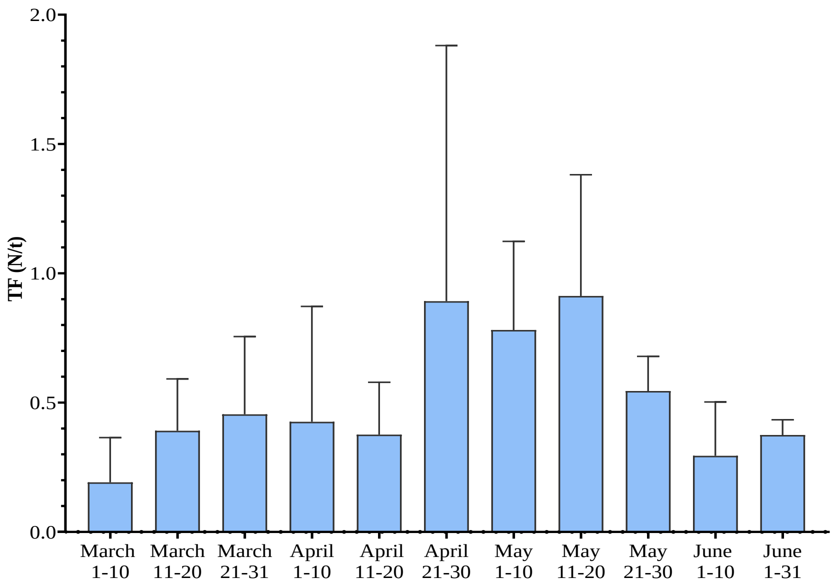



*Figure 6. Dynamics of questing ticks expressed by their frequency (TF) at 10-day intervals. Mean frequencies ± standard deviation for the study period (2016-2022) are presented.*

The effect of temperature on questing ticks was evaluated by calculating the mean values of TQI and TF for the corresponding temperature intervals to the nearest 1ºC. The obtained results are presented graphically in Figure 8 and Figure 9. It was found that at 20ºC the density of *Ixodes ricinus* was the highest ($\overline{x}$ = 12.54) and the maximum was 31.19 per 100 $m^2$. At the same temperature, the maximum frequency was $\overline{x}$ = 1.17, with a maximum of 2.65 individuals per minute.

The effect of relative humidity on the questing activity of Ixodes ricinus was studied similarly to temperature. The TQI and TF indexes were plotted by averaging the values recorded at 10% RH intervals and presented graphically in

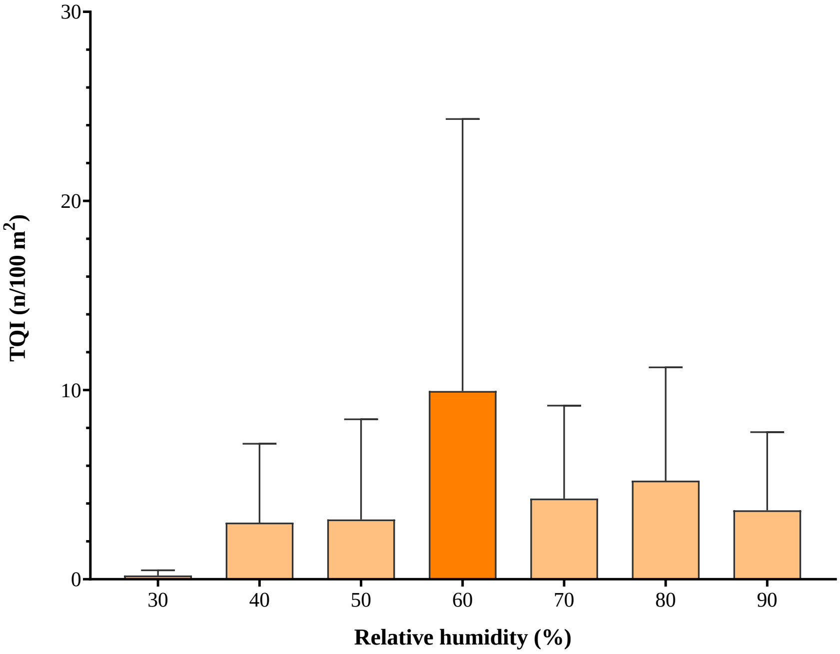



Figure *7* and Figure 8. The highest density and frequency were found at 60% RH. As for TQI, $\overline{x}$ = 9.96, with a maximum of 24.33 individuals per 100 $m^2$. For TF, $\overline{x}$ = 0.8, with a maximum of 1.76 mites captured per 1 min.

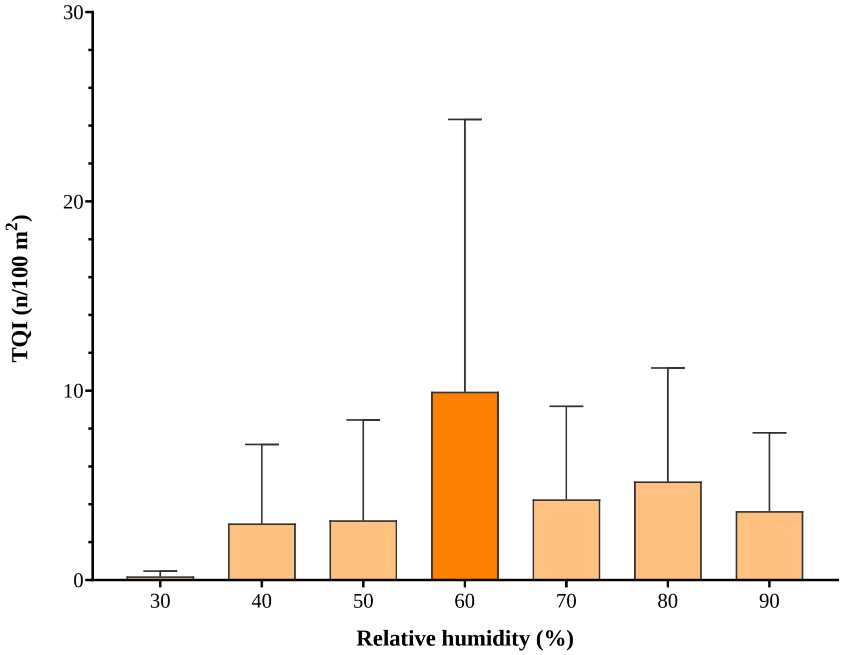



*Figure 7. Effect of relative humidity on the density of questing ticks per 100 $m^2$. Mean values for the study period 2016-2022 ± SD are presented.*

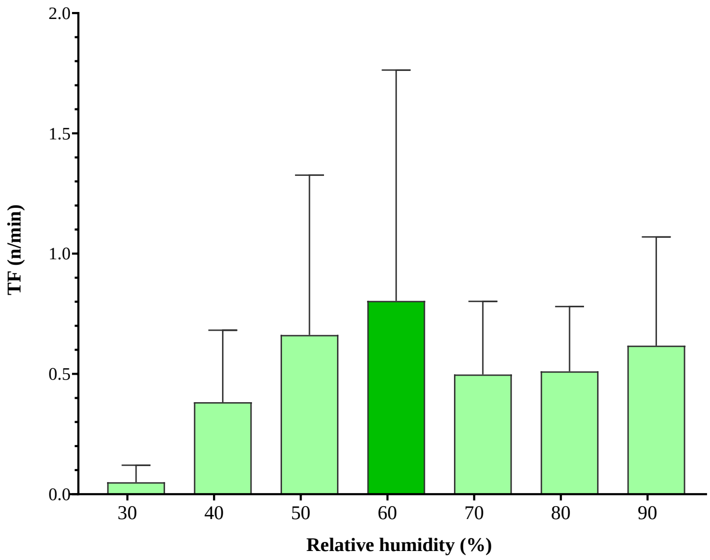



*Figure 8. The effect of relative humidity on the frequency of questing I. ricinus. Mean TF values for the study period 2016-2022 ± SD are presented.*

## 2.1. Protected area PA "Kaylaka"

The dominant tick species collected by flagging in the area of the PA "Kaylaka" was *Ixodes ricinus*. In the area of Hotel Spartak, for the first time in Bulgaria, a questing female tick of the species *Ixodes frontalis* was caught. A male *Haemaphysalis punctata* was captured from the grass areas of the zoo, during flagging. During the study period, the largest number of *Ixodes ricinus* (n = 860) was collected and surveyed from the areas of the PA “Kaylaka”. In table 9 the information about sex distribution is presented, as well as stage of development and years of collection. The total estimated density (TQI) in the PA “Kaylaka” was 5.89 ± 8.60 and the frequency (TF) was 0.61 ± 0.46. The highest tick density was found in 2017 (n = 49; x̄ = 20.48 ± 19.53), the highest capture frequency was measured in 2018 (n = 142; x̄ = 0.93 ± 0.57).

*Table 9. Distribution of collected Ixodes ricinus ticks from the PA “Kaylaka” by zone, according to use and maintenance of green areas.*

| Zones in PA | Female | Male | Nymph | Larvae | Total | TQI (n/100 m²) x̄ ± SD | TF (n/min) x̄ ± SD |
|---|---|---|---|---|---|---|---|
| …ed | **142** | **112** | **34** | **1** | **289** | **5.56 ± 9.08** | **0.53 ± 0.43** |
| | 23 | 14 | 9 | 0 | 46 | 2.08 ± 1.68 | 0.22 ± 0.19 |
| | 39 | 27 | 6 | 0 | 72 | 4.55 ± 3.89 | 0.5 ± 0.28 |
| | 28 | 30 | 12 | 0 | 70 | 7.43 ± 9.72 | 0.58 ± 0.26 |
| | 52 | 41 | 7 | 1 | 101 | 9.93 ± 19.07 | 0.84 ± 0.76 |
| | **245** | **252** | **74** | **0** | **571** | **6.29 ± 8.14** | **0.73 ± 0.49** |
| | 120 | 127 | 36 | 0 | 283 | 5.48 ± 5.31 | 0.70 ± 0.48 |
| | 46 | 41 | 21 | 0 | 108 | 11.14 ± 12.79 | 0.76 ± 0.50 |
| | 20 | 20 | 7 | 0 | 47 | 1.57 ± 1.73 | 0.61 ± 0.73 |
| | 59 | 64 | 10 | 0 | 133 | 5.59 ± 8.37 | 0.92 ± 0.21 |
| **Total** | **387** | **364** | **108** | **1** | **860** | **5.89 ± 8.60** | **0.61 ± 0.46** |

*Table 10. Distribution by year and developmental stage of questing Ixodes ricinus collected from the area of river Chernelka.*

| Year | Sex/stage of development: Female | Male | Nymph | Larvae | Total | TQI | TF |
|---|---|---|---|---|---|---|---|
| **2016** | 67 | 44 | 10 | 0 | 121 | 2.13 ± 1.63 | 0.45 ± 0.32 |
| **2017** | 28 | 14 | 7 | 0 | 49 | 20.48 ± 19.53 | 0.62 ± 0.5 |
| **2018** | 54 | 76 | 11 | 1 | 142 | 7.68 ± 13.83 | 1.15 ± 1.01 |
| **2019** | 102 | 108 | 30 | 0 | 240 | 5.82 ± 7.20 | 0.55 ± 0.42 |
| **2020** | 54 | 42 | 9 | 0 | 105 | 5.14 ± 4.71 | 0.66 ± 0.47 |
| **2021** | 66 | 59 | 26 | 0 | 151 | 5.36 ± 3.92 | 0.59 ± 0.39 |
| **2022** | 16 | 21 | 15 | 0 | 52 | 1.2 ± 0.8 | 0.27 ± 0.16 |
| **Total** | 387 | 364 | 108 | 1 | 860 | 5.89 ± 8.60 | 0.64 ± 0.58 |

In the PA “Kaylaka”, a total of 289 *Ixodes ricinus* were collected from urbanised, maintained grasslands during the study period with TQI = 5.56 ± 9.08 and TF = 0.53 ± 0.43, respectively, and 571 from wild (unmaintained) areas with TQI = 6.29 ± 8.14 and TF = 0.73 ± 0.49. In the wild areas, the highest frequency was found in the Bohot Forest area (W4) TF = 0.92 ± 0.21 and in the urbanised parts of the park in U4 (TF = 0.84 ± 0.76). The highest density of ticks in the maintained natural areas was again found in the U4 area (TQI = 9.93 ± 19.07), and of the unmaintained areas in the area around the W2 “Srebrostrui” Hut (TQI = 11.14 ± 12.79) (Table 9).

## 2.2. Protected area “Karst Gorge of the River Chernelka”

From the region of the PA “Chernelka” a total of 266 ticks were collected during 12 collection campaigns. One hundred and fourteen of them were females, 103 males, 48 nymphs and 1 larva. The distribution of collected ticks by year and developmental stage is presented in *Table 11*. All ticks were of the species *Ixodes ricinus*. Overall, TQI=1.61 ± 3.16 and TF=0.012 ± 0.16 were found for the whole period. The highest density and frequency of ticks was recorded in the area of the former "Kapchuka" Hut TQI =3.25 ± 0.49; TF = 0.58 ± 0.06.

*Table 11. Distribution by year and developmental stage of actively searching Ixodes ricinus collected from the Gorge area of the PA "Chernelka".*

| | | Stage of development/gender | | | | Total |
|---|---|---|---|---|---|---|
| | | Female | Male | Nymph | Larvae | |
| **Year** | 2016 | 25 | 15 | 1 | 1 | 42 |
| | 2017 | 22 | 22 | 8 | 0 | 52 |
| | 2018 | 8 | 14 | 4 | 0 | 26 |
| | 2019 | 1 | 1 | 0 | 0 | 2 |
| | 2021 | 5 | 15 | 10 | 0 | 30 |
| | 2022 | 53 | 36 | 25 | 0 | 114 |
| | **Total** | 114 | 103 | 48 | 1 | 266 |

### 2.3. Village of Grivitsa – "Tachova Cheshma"

Tick collections in this area took place during the period 2016 to 2019. In total 125 *Ixodes ricinus* ticks were collected, 26 females, 38 males and 61 nymphs. Individuals of *Dermacentor marginatus* and *Haemaphysalis punctata* were also captured in the area. The density of *I. ricinus* in "Tachova Cheshma" was TQI = 6.82 ± 6.5 and the frequency TF = 0.75 ± 0.25.

### 2.4. Village of Sadovets, - "Garvan dol"

In this area, 56 *Ixodes* ticks were collected - 32 members of *Ixodes ricinus* and 24 representatives of *Dermacentor marginatus* from a total of 4 campaigns (one without collected material). The sex and developmental stage distribution of *I. ricinus* are as follows: 16 females, 12 males and 4 nymphs - TQI = 0.69 ± 0.08, TF = 0.21 ± 0.54.

## 3. COMPARISON OF *I. RICINUS* POPULATIONS BETWEEN DIFFERENT STUDY AREAS.

The distribution of the data for the indexes TF, TQI, MQI, NQI, t, RH was verified with the Shapiro-Wilk test. At test significance ($p < 0.05$), the null hypothesis of normal distribution was rejected, i.e. the data distribution was different from normal. The distributions for the quantitative variables studied showed significant deviation from normality for TF, TQI, FQI, MQI, NQI and relative humidity. Only the indicator temperature had a normal distribution (*Table 12*).

*Table 12. Shapiro-Wilk test for data distribution of the studied indexes.*

| Indicator | Shapiro-Wilk | | |
|---|---|---|---|
| | W | Df (degrees of freedom) | Sig.(p) |
| **TF**[1] | 0.865 | 75 | 0.001 |
| **TQI**[2] | 0.606 | 75 | 0.001 |
| **FQI**[3] | 0.566 | 67 | 0.001 |
| **MQI**[4] | 0.601 | 66 | 0.001 |
| **NQI**[5] | 0.611 | 45 | 0.001 |

| | | | |
|---|---|---|---|
| **Temperature** | 0.979 | 75 | 0.240 |
| **Relative humidity** | 0.961 | 75 | 0.021 |

[1] TF – Ticks Frequence of *Ixodes ricinus* (n/min).
[2]. TQI – Total Ticks Questing of *Ixodes ricinus* (n/100 $m^2$).
[3]. FQI – Female Ticks Questing Index of *Ixodes ricinus* (n/100 $m^2$).
[4] MQI – Female Ticks Questing Index of *Ixodes ricinus* (n/100 $m^2$).
[5] NQI – Numph Ticks Questing Index of *Ixodes ricinus* (n/100 $m^2$).

The abundance of I. *ricinus* estimated by the TF and TQI indices showed that in "Tachova Cheshma" is was the highest. Calculated mean frequency - 0.75 ± 0.25 n/min; mean density 6.82 ± 6.5 n/100 $m^2$. In the PA "Kaylaka" TF = 0.61 ± 0.46 n/min; TQI = 5.89 ± 8.60 n/100 $m^2$. In the PA "Chernelka" TF = 0.28 ± 0.16 n/min; TQI = 1.48 ± 1.2 n/100 $m^2$. The lowest values were calculated for ticks collected from "Garvan Dol" TF = 0.13 ± 0.18 n/min; TQI = 1.18 ± 1.83 n/100 $m^2$ (*Figure 9* and *Figure 10*).

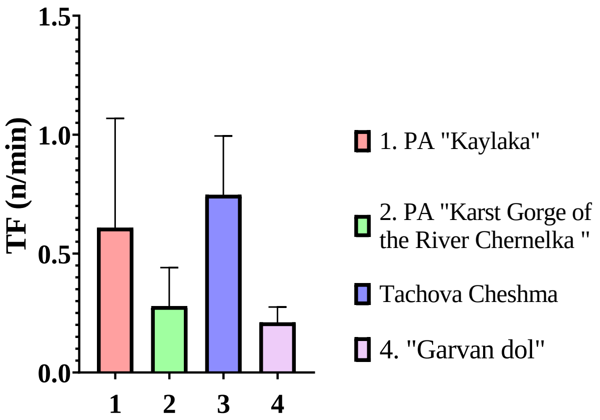



*Figure 9. Frequency of questing I. ricinus in the different flagging areas for the period 2016-2022. The box-plot is represented by the mean TF and standard deviation.*

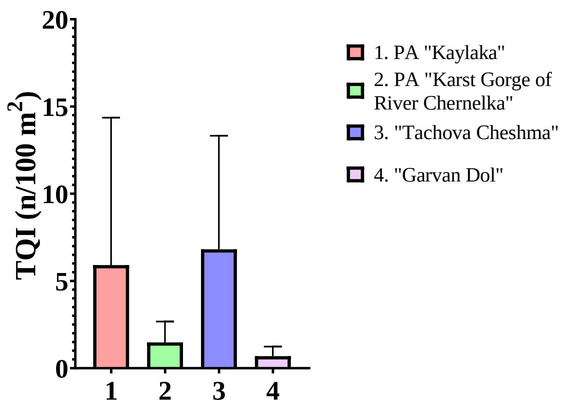



*Figure 10. Density of questing I. ricinus in the flagging areas for the period 2016-2022. Boxplots are represented by the mean TQI and standard deviation.*

When the analysis of variance (Kruskal-Wallis) was performed between major collection areas (PA "Kaylaka", PA Chernelka", "Tachova Cheshma", and "Garvan Dol") with different density and frequency indexes, differences were found with the TF H (3) = 11.77; p = 0.008; TQI H(3) = 9.7, p = 0.021; MQI H(3) = 10.84, p = 0.013; NQI H(3) = 8.51, p = 0.037.

To determine if there were differences in the indices between the different areas from which the ticks were collected, the TF and TQI indices between any two of the areas were compared using the Mann-Whitney U test. Statistically significant differences were found as follows:

### 3.1. PA "Kaylaka"– PA "Chernelka"

- TQI U($N_{Kaylaka}$ = 54, $N_{\text{Chernelka}}$ = 12) = 179.5, $z$ = -2.402, $p$ = 0.016 (Figure 11 A);
- TF U($N_{Kaylaka}$ = 54, $N_{\text{Chernelka}}$ = 12) = 166.0, $z$ = -2.628, $p$ = 0.009 (Figure 11 B);
- FQI U ($N_{Kaylaka}$ = 50, $N_{\text{Chernelka}}$ = 10) = 150, $z$ = -1.984, $p$ = 0.047 (Figure 12 A);
- MQI U ($N_{Kaylaka}$ = 49, $N_{\text{Chernelka}}$ = 11) = 131.5, $z$ = -2.636, $p$ = 0.008 (Figure 12 B);
- NQI U ($N_{Kaylaka}$ = 30, $N_{\text{Chernelka}}$ = 8) = 62, $z$ = -2.077, $p$ = 0.038 (Figure 12 B).

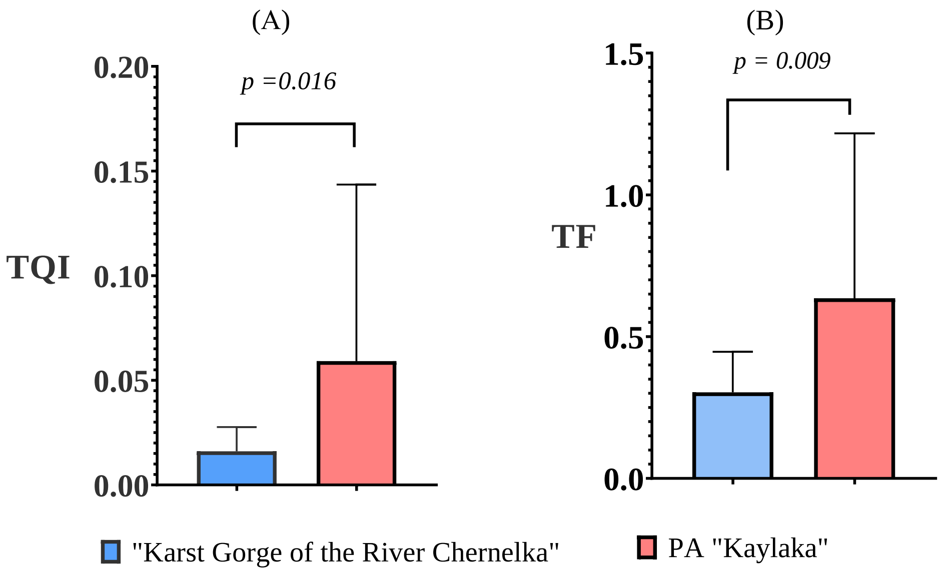



*Figure 11. Statistically significant differences comparing TQI (A) and TF (B) in the PA "Chernelka" and PA "Kaylaka". The boxplots show the mean and standard deviation.*

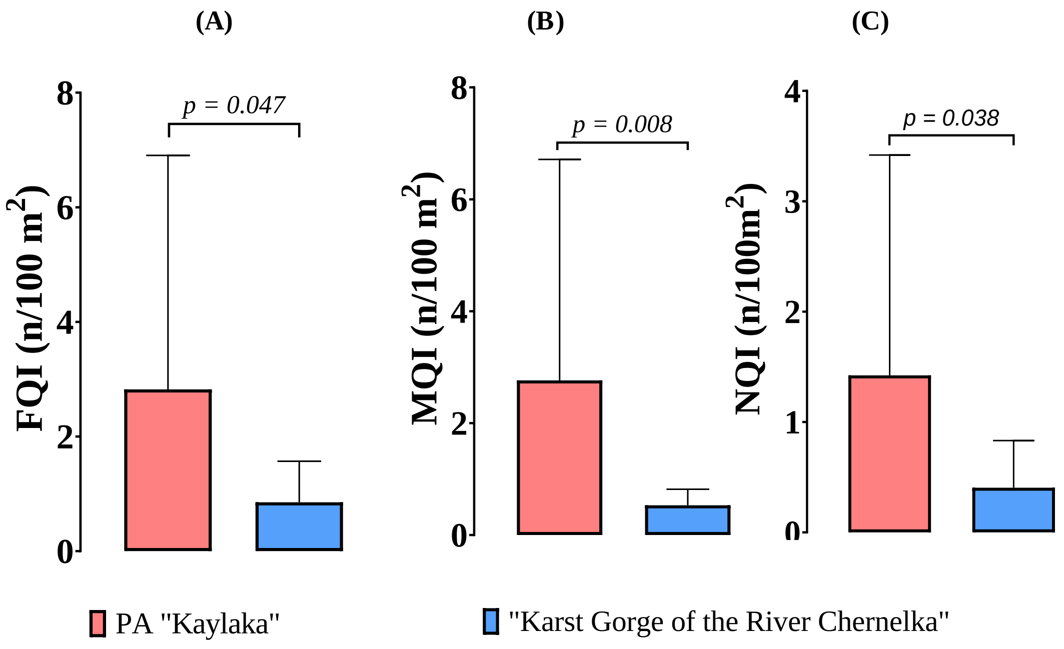



*Figure 12. Statistically significant differences comparing the FQI (A), MQI (B) and NQI (C) between the PA "Chernelka" and PA "Kaylaka". Mean values and standard deviation are presented on the box plot.*

## 3.2 PA "Chernelka" – "Tachova Cheshma"

- TQI U ($N_{Chernelka}$ = 12, $N_{T. Cheshma}$ = 3) = 4, $z$ = -2.021, $p$ = 0.048 (Figure 13 A);
- TF U ($N_{Chernelka}$ = 12, $N_{T. Cheshma}$ = 3) = 0, $z$ = -2.598, $p$ = 0.004 (Figure 13 B);
- MQI U ($N_{Chernelka}$ = 11, $N_{T. Cheshma}$ = 1) = 2, $z$ = -2.258, $p$ = 0.022 (Figure 14 A);
- NQI U ($N_{Chernelka}$ = 8, $N_{T. Cheshma}$ = 3) = 0, $z$ = -2.449, $p$ = 0.012 (Figure 14 B).

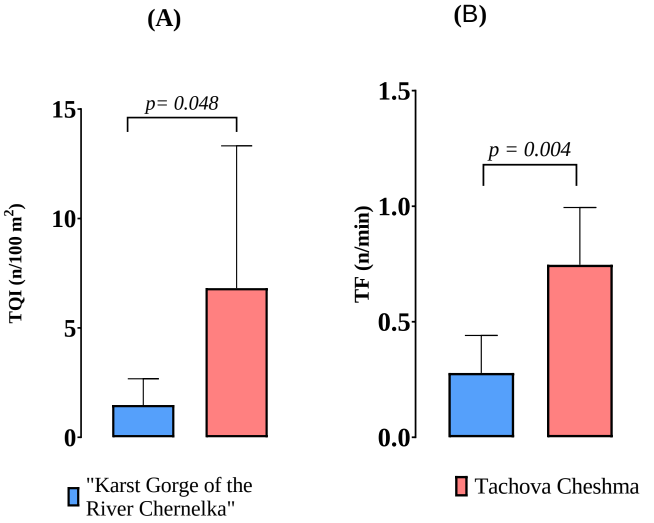



*Figure 13. Statistically significant differences comparing the TQI (A) and TF (B) and NQI (C) between the PA "Chernelka" and "Tachova Cheshma" near the village of Grivitsa.*

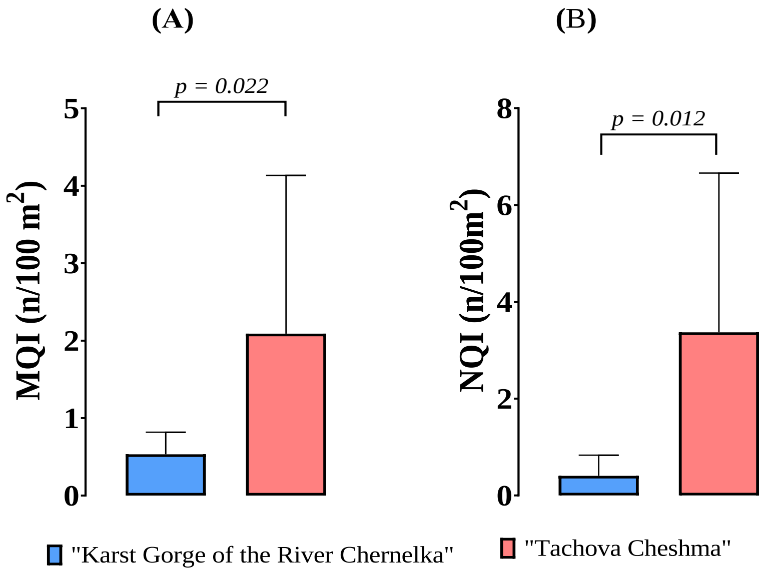



*Figure 14. Statistically significant differences comparing the MQI (A) and NQI (B) in the PA "Chernelka" and Tachova Cheshma".*

When comparing the areas of the PA "Kaylaka"- s. Grivitsa m. "Tachova Cheshma", PA "Kaylaka"- m. "Garvan dol", PA "Chernelka" - "Garvan dol" and "Tachova Cheshma" - "Garvan dol", no statistically significant differences were found.

# 4. RESULTS OF THE IXODES RICINUS COLLECTION CAMPAIGNS IN PA "KAYLAKA"

In an attempt to determine whether the presence of questing *Ixodes ricinus* ticks was influenced by human activity, we compared the estimated indexes from different areas in the PA "Kaylaka". The Mann-Whitney test was used to compare maintained (U) and (W) unmaintained areas in the PA "Kaylaka". There were no statistically significant differences between the maintained (urbanized) and unmaintained (natural) areas in the PA "Kaylaka"( *Table 13* and *Figure 15*).

*Table 13. Mann-Whitney test to compare indices of questing Ixodes ricinus presence in urbanized (U) and (W) unmaintained areas in the PA "Kaylaka".*

| PA "Kaylaka" | | No. of flagging event (N) | Mean Rank | Sum of Ranks | Mann-Whitney U | Wilcoxon W | Z | Asymp. Sig. (2-tailed) |
|---|---|---|---|---|---|---|---|---|
| TF | U | 29 | 23.09 | 669.50 | 234.5 | 669.5 | -1.83 | 0.068 |
| | W | 23 | 30.80 | 708.50 | | | | |
| | Total | 52 | | | | | | |
| TQI | U | 29 | 25.21 | 731.00 | 296 | 731 | -0.69 | 0.49 |
| | W | 23 | 28.13 | 647.00 | | | | |
| | Total | 52 | | | | | | |
| FQI | U | 25 | 25.32 | 633.00 | 267 | 543 | -0.42 | 0.672 |
| | W | 23 | 23.61 | 543.00 | | | | |
| | Total | 48 | | | | | | |
| MQI | U | 24 | 23.83 | 572.00 | 272 | 572 | -0.09 | 0.932 |
| | W | 23 | 24.17 | 556.00 | | | | |
| | Total | 47 | | | | | | |
| NQI | U | 12 | 16.25 | 195.00 | 75 | 211 | -0.98 | 0.330 |
| | W | 16 | 13.19 | 211.00 | | | | |
| | Total | 28 | | | | | | |

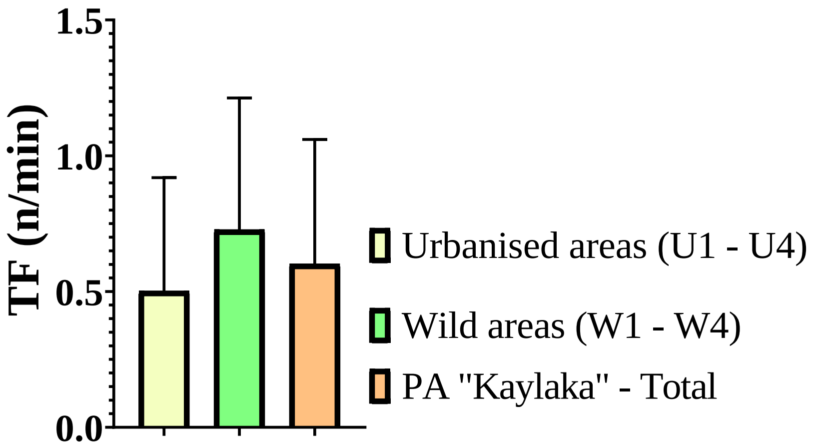



*Figure 15. Frequency of questing I. ricinus collected from the PA "Kaylaka" in urbanized and wild areas. The box-plot is represented by the mean TF and standard deviation.*

The Mann-Whitney U test was used to determine differences in the distribution of *Ixodes ricinus* between different pairs of zones. The results are presented in *Table 14* and *Figures* 16-20.

*Table 14. Mann-Whitney U test for comparison of urbanized (U) and wild (W) areas in the PA "Kaylaka".*

| Areas | Statistically significant difference between | Mean rank) | Median | $\bar{X}$ | SD | U | N | Z | p |
|---|---|---|---|---|---|---|---|---|---|
| U1 | TF[1] | 3.83 | 0.14 | 0.23 | 0.19 | 2 | 6 | -2.58 | 0.009 |
| U3 | | 9.17 | 0.64 | 0.58 | 0.26 | | 6 | | |
| U1 | TF | 4.33 | 0.14 | 0.23 | 0.19 | 5 | 6 | -2.72 | 0.005 |
| W1 | | 11 | 0.55 | 0.70 | 0.48 | | 10 | | |
| U1 | TF | 4.17 | 0.14 | 0.23 | 0.19 | 4 | 6 | -2.26 | 0.026 |
| W2 | | 8.83 | 0.77 | 0.76 | 0.49 | | 6 | | |
| U1 | TF | 3.5 | 0.14 | 0.23 | 0.19 | 0 | 6 | -2.36 | 0.024 |
| W4 | | 8 | 1.00 | 0.91 | 0.21 | | 3 | | |
| U2 | FQI[2] | 9.45 | 0.50 | 0.49 | 0.28 | 6 | 11 | -2.09 | 0.04 |
| W3 | | 4 | 0.43 | 0.69 | 0.76 | | 4 | | |
| U2 | TF | 6.71 | 2.36 | 2.32 | 1.50 | 2.5 | 12 | -2.24 | 0.018 |
| W4 | | 13.17 | 1.00 | 0.91 | 0.21 | | 3 | | |
| W1 | TQI[3] | 8.9 | 3.16 | 5.48 | 5.31 | 6 | 10 | -1.98 | **0.048** |
| W3 | | 4 | 0.86 | 1.57 | 1.73 | | 4 | | |

1. TF – Ticks Frequence of *Ixodes ricinus* (n/min).
2. FQI – Female Ticks Questing Index of *Ixodes ricinus* (n/100 $m^2$).
3. TQI – Total Ticks Questing of *Ixodes ricinus* (n/100 $m^2$).

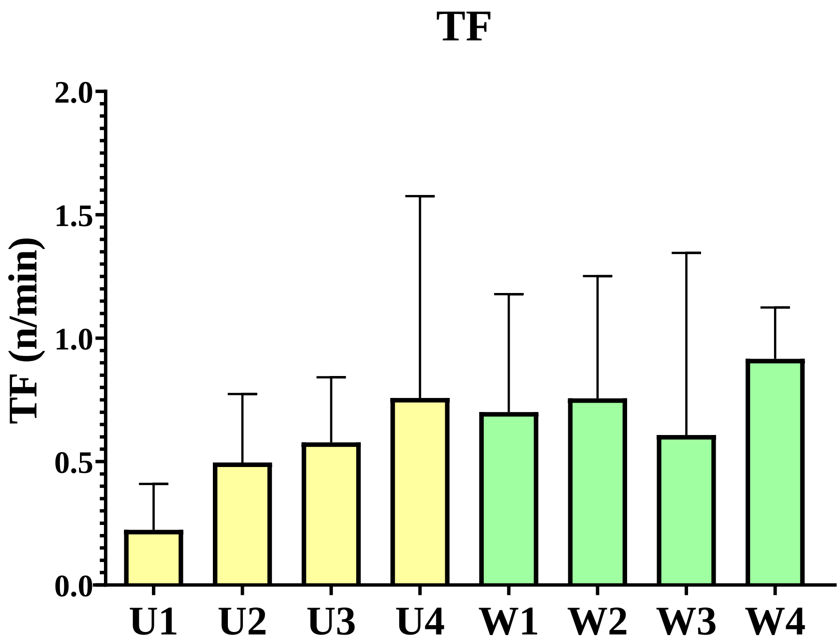



*Figure 16. Frequency of questing Ixodes ricinus (TF) in eight flagging zones in the PA "Kaylaka". The box-plot is represented by TF means and standard deviation.*

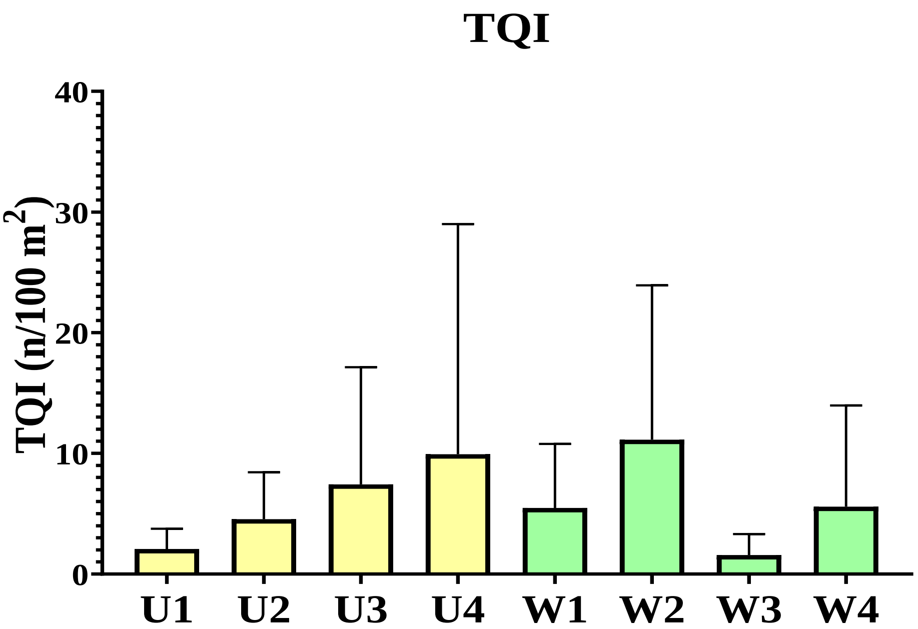



*Figure 17. Ticks questing index of Ixodes ricinus (TQI) in eight flagging zones in the PA "Kaylaka". The box-plot is represented by TQI means and standard deviation.*

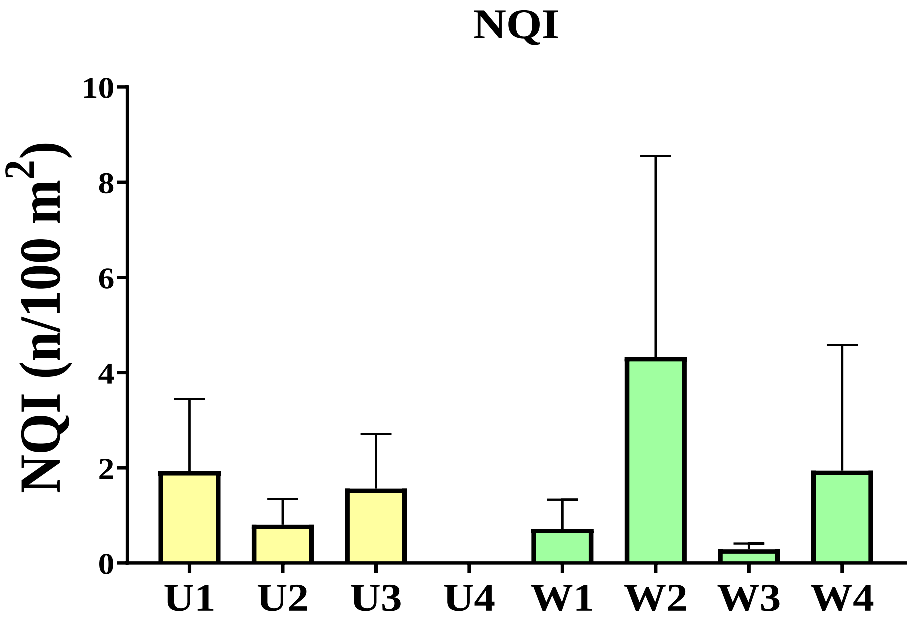



*Figure 18. Nymphs questing index of Ixodes ricinus (NQI) in eight flagging zones in the PA "Kaylaka". The box-plot is represented by NQI means and standard deviation.*

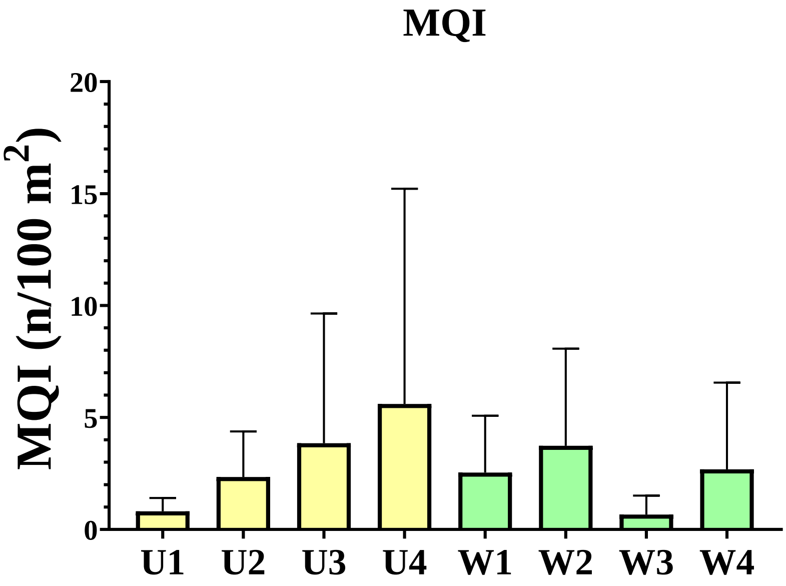



*Figure 19. Male questing index of Ixodes ricinus (MQI) in eight flagging zones in the PA "Kaylaka". The box-plot is represented by MQI means and standard deviation.*

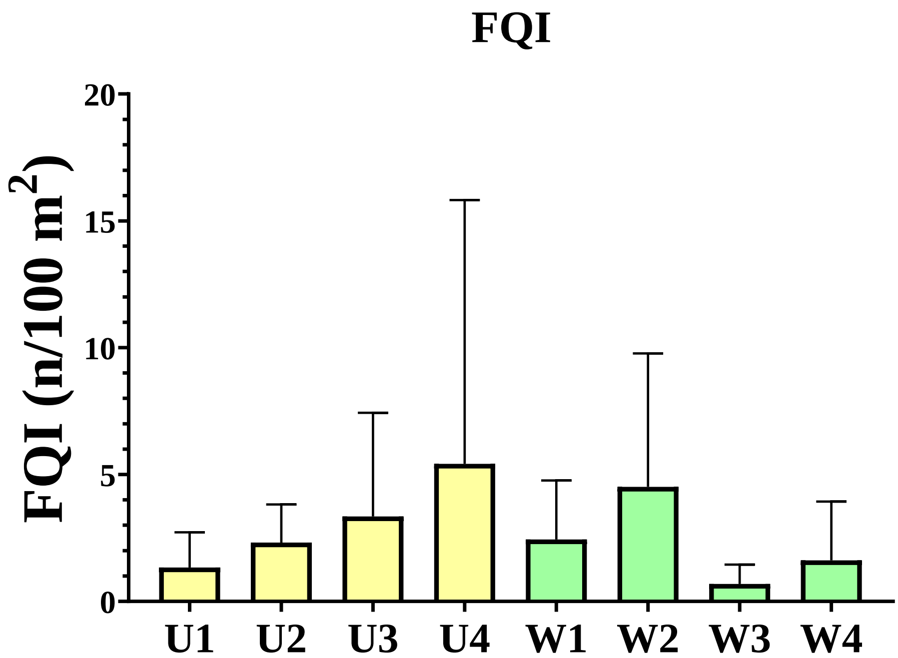



*Figure 20. Female questing index of Ixodes ricinus (FQI) in eight flagging zones in the PA "Kaylaka". The box-plot is represented by FQI means and standard deviation.*

## 5. DARK-FIELD MICROSCOPY FOR THE DETECTION OF *BORRELIA BURGDORFERI* S.L.

During the study period, a total of 394 ticks were examined for the presence of *Borrelia* sp. Spirochaetes were observed in 129 (32.94%) of them (68 females; 41 males and 20 nymphs). The results of DFM from the different study areas are presented in Table 17. During the years in which DFM was performed, the highest number of infected ticks was observed in 2017 with 49.25% of 67 surveyed individuals (*Table 15*). There was an association between years and infectivity with *Borrelia* $\chi 2$ (3, N = 394) = 14.94, p = 0.002. When comparing the adult forms, a difference was found in the infection rate with *B. burgdorferi* s.l. between females and males $\chi 2$ (2, N = 330) = 5.72, p = 0.019.

*Table 15. Dark-field microscopy data for the presence of Borrelia burgdorferi s.l. in areas of flagging, grouped by developmental stage and sex.*

| Gender/stages | DFM | PA "Kaylaka" | PA "Chernelka" | " Garvan Dol" | "Tachova Chashma" | Pleven |
|---|---|---|---|---|---|---|
| **Female** | Negative | 74 (62.18%) | 18 (47.37%) | 6 (100%) | 8 (72.73%) | 1 (100%) |
| | Positive | 45 (37.82%) | 20 (52.63%) | 0 (0%) | 3 (27.27%) | 0 (0%) |
| **Male** | Negative | 71 (73.2%) | 21 (61.76%) | 3 (100%) | 19 (90.48%) | 0 (0%) |
| | Positive | 26 (26.8%) | 13 (38.24%) | 0 (0%) | 2 (9.52%) | 0 (0%) |
| **Nymph** | Negative | 13 (68.42%) | 3 (42.86%) | 0 (0%) | 27 (72.97%) | 1 (100%) |
| | Positive | 6 (31.58%) | 4 (57.14%) | 0 (0%) | 10 (27.03%) | 0 (0%) |
| **Total** | Negative | 158 (67.23%) | 42 (53.16%) | 9 (100%) | 54 (78.26%) | 2 (100%) |
| | Positive | 77 (32.77%) | 37 (46.84%) | 0 (0%) | 15 (21.74%) | 0 (0%) |

## 6. SURVEY OF THE "KAYLAKA" PA FOR THE PRESENCE OF *BORRELIA BURGDORFERI* S.L. IN *IXODES RICINUS* TICKS

Of a total of 860 *Ixodes ricinus* ticks collected, 405 were tested for the presence of *Borrelia* by at least one of the three methods. The number of positive samples was 183 (45.2%). By dark-field microscopy, 235 individuals were examined and 77 (32.5%) were found to have the presence of spirochaetes. By PCR, 202 ticks were tested for the presence of spirochaetes of the *Borrelia burgdorferi* s.l. complex, and 97 (48%) of them tested positive for the amplification of the *23S/5S igs* gene and 104 (51.5%) for the *FlaB* gene. The results of the tests conducted for the urbanized and wild areas in the PA "Kaylaka" are presented in Table 16. Figure 21 presents the percentages of positive and negative *Ixodes ricinus* in the different areas of the PA “Kaylaka”. The wild zone W4 was found to have the highest percentage of positive ticks (63.9%), followed by those collected from urbanized zone U2 (57.6%).

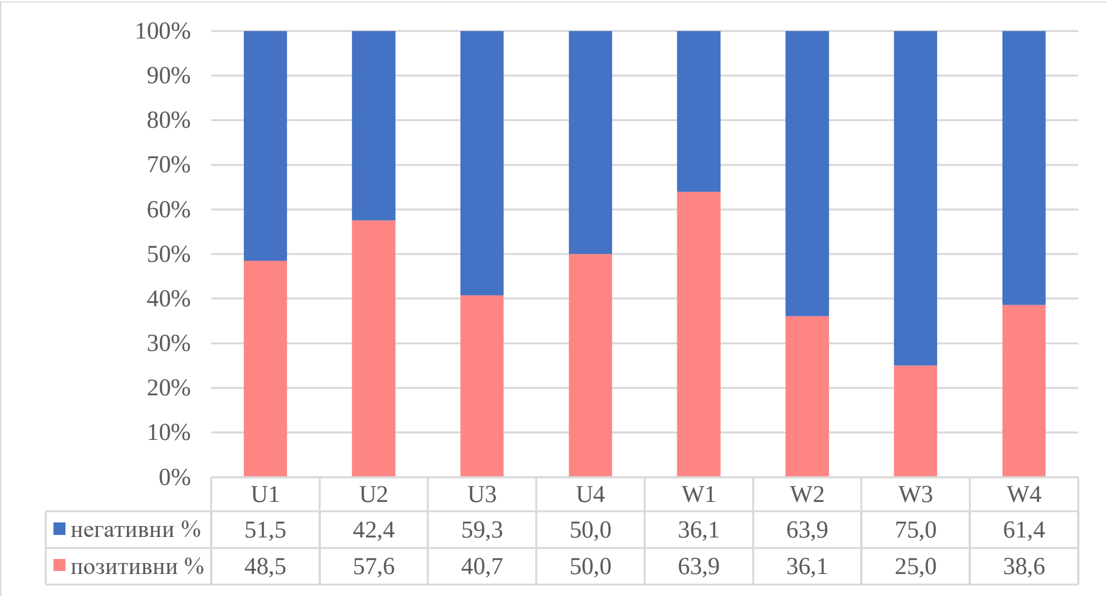



*Figure 21. The rate of DMF and/or PCRs positive ticks in which Borrelia burgdorferi sl was detected regardless of the method of testing in urban (U1-U3) and wild (W1-W4) areas within PA ‘‘Kaylaka’’. Data are presented as percentages.*

A higher percentage of infected ticks, regardless of the method of testing, was observed in those from urban maintained areas, but there was no statistically significant difference - 49.66% versus 42.69% ($\chi2$ = 1.822 n = 405; df = 1 p = 0.177).

*Table 16. Results of Ixodes ricinus testing for the presence of Borrelia burgdorferi sl within the PA "Kaylaka" study areas.*

| Areas | **Number of collected ticks** | **Type of test** | | | Detection of *Borrelia* sp. in I. *ricinus* by any test |
|---|---|---|---|---|---|
| | | **nPCR** $_{23S/5S}$ | **nPCR** $_{FlaB}$ | **ТПМ** | |
| | | Infected ticks (%) and number of positive versus tested samples (n) | Infected ticks (%) and number of positive versus tested samples (n) | Infected ticks (%) and number of positive versus tested samples (n) | Percentage of ticks infected (%) and number of positive samples (n) |
| Urban areas | **289** | **46.25 (*n* = 37/80)** | **51.251 (*n* = 41/80)** | **40.54 (*n* = 30/74)** | **49.66 (*n* = 72/145)** |
| **U1** | 46 | 55.56 (*n* = 10/18) | 61.11 (*n* = 11/18) | 43.48 (*n* = 10/23) | 51.52 (*n* = 16/33) |
| **U2** | 72 | 38.10 (*n* = 8/21) | 52.38 (*n* = 11/21) | 40.00 (*n* = 6/12) | 57.6 (*n* = 19/33) |
| **U3** | 70 | 47.06 (*n* = 8/17) | 47.06 (*n* = 8/17) | 22.22 (*n* = 2/9) | 40.7 (*n* = 11/27) |
| **U4** | 101 | 45.83 (*n* = 11/24) | 45.83 (*n* = 11/24) | 44.44 (*n* = 12/27) | 50 (*n* = 26/52) |
| Wild areas | **571** | **49.18 (*n* = 60/122)** | **51.64 (*n* =63/122)** | **29.19 (*n* = 47/161)** | **42.69 (*n* = 111/260)** |
| **W1** | 283 | 54.10 (*n* = 33/61) | 54.10 (*n* = 33/61) | 25.00 (*n* = 1/4) | 63.93 (*n* = 39/61) |
| **W2** | 108 | 58.82 (*n* = 10/17) | 64.71 (*n* = 11/17) | 23.40 (*n* = 11/47) | 36.07 (*n* = 22/61) |
| **W3** | 47 | 0.00 (*n* = 0/3) | 0.00 (*n* = 0/3) | 27.27 (*n* = 6/22) | 25 (*n* = 6/24) |
| **W4** | 133 | 41.46 (*n* = 17/41) | 46.34 (*n* = 19/41) | 32.95 (*n* = 29/88) | 38.6 (*n* = 44/114) |
| Total | **860** | **48.02 (*n* = 97/202)** | **51.49 (*n* = 104/202)** | **32.77 (*n* = 77/235)** | **45.19 (*n* = 183/405)** |

When comparing ($\chi 2$ method) the positive samples by any of the methods between the different areas in the PA “Kaylaka”, statistically significant differences were found between some of them (*Table 17*). In the U2 maintained zone, ticks positive for *B. burgdorferi* s.l. were significantly higher than those in the unmaintained wild areas W2, W3 and W4. In zone U4, the number of positive ticks was significantly higher than in zone W3. Wild zone W1 positives were significantly greater than those in zone U3 and the remaining wild zones (W2, W3 and W4).

*Table 17. Statistically significant differences between the different zones in the PA “Kaylaka”. The results of a $\chi 2$ test are presented ($p<0.05$). The number of positives is based on the number of ticks positive by any method of testing.*

| | Compared Areas | Number of positive (%) | Number negative (%) | Pearson $\chi 2$ (Df = 1) | Total (n) | *p* |
|---|---|---|---|---|---|---|
| **Detection of *Borrelia* sp. in I. *ricinus* by any test** | U2 | 19 (57.58) | 14 (42.42) | 4.029 | 33 | 0.045 |
| | W2 | 22 (36.07) | 39 (63.93) | | 61 | |
| | U2 | 19 (57.58) | 14 (42.42) | 5.988 | 33 | 0.014 |
| | W3 | 6 (25.00) | 18 (75.00) | | 24 | |
| | U2 | 19 (57.58) | 14 (42.42) | 3.764 | 33 | 0.05 |
| | W4 | 44 (38.60) | 70 (61.40) | | 114 | |
| | U3 | 11 (40.74) | 16 (59.26) | 4.104 | 27 | 0.043 |
| | W1 | 39 (63.93) | 22 (36.07) | | 61 | |
| | U4 | 26 (50.00) | 26 (50.00) | 4.21 | 52 | 0.04 |
| | W3 | 6 (25.00) | 18 (75.00) | | 24 | |
| | W1 | 39 (63.93) | 22 (36.07) | 9.475 | 61 | 0.002 |
| | W2 | 22 (36.07) | 39 (63.93) | | 61 | |
| | W1 | 39 (63.93) | 22 (36.07) | 10.48 | 61 | 0.001 |
| | W3 | 6 (25.00) | 18 (75.00) | | 24 | |
| | W1 | 39 (63.93) | 22 (36.07) | 10.232 | 61 | 0.001 |
| | W4 | 44 (38.60) | 70 (61.40) | | 114 | |

## 7. COMPARISON OF METHODS USED FOR THE DETECTION OF *BORRELIA BURGDORFERI* S.L. IN *IXODES RICINUS*

Among the ticks tested with DFM, 44 (20♂ and 24♀) were also tested with nPCR. Nymphs were not included because of their small size. The κ values between the three assays showed different levels of agreement. When comparing all ticks tested by nPCR (n = 214) on the two genes, agreement was substantial κ = 0.785 (p = 0.001) 95% CI: [0.703, 0.868]. The number of matches in the results (agreements): was 191 (89.25% of observations). The same substantial agreement was found between the two nPCRs when comparing samples detected with DFM as well (n = 44), where κ = 0.665 (p = 0.001), 95% CI: [0.445, 0.886]; number of matches: 37 (84.09% of cases).

The κ values when comparing the results of DFM with $nPCR_{5S\text{-}23Sigs}$ and TPM with $nPCR_{flab}$ were respectively: moderate agreement κ = 0.435 (p = 0.004) 95% CI: [0.153, 0.716], number of matches: 33 (75.00% of cases) and fair agreement κ = 0.335 (p = 0.024), 95% CI: [0.056, 0.613], number of matches: 30 (68.18% of cases).

The results of comparing the three tests in 44 *Ixodes ricinus* ticks are presented in Table 18.

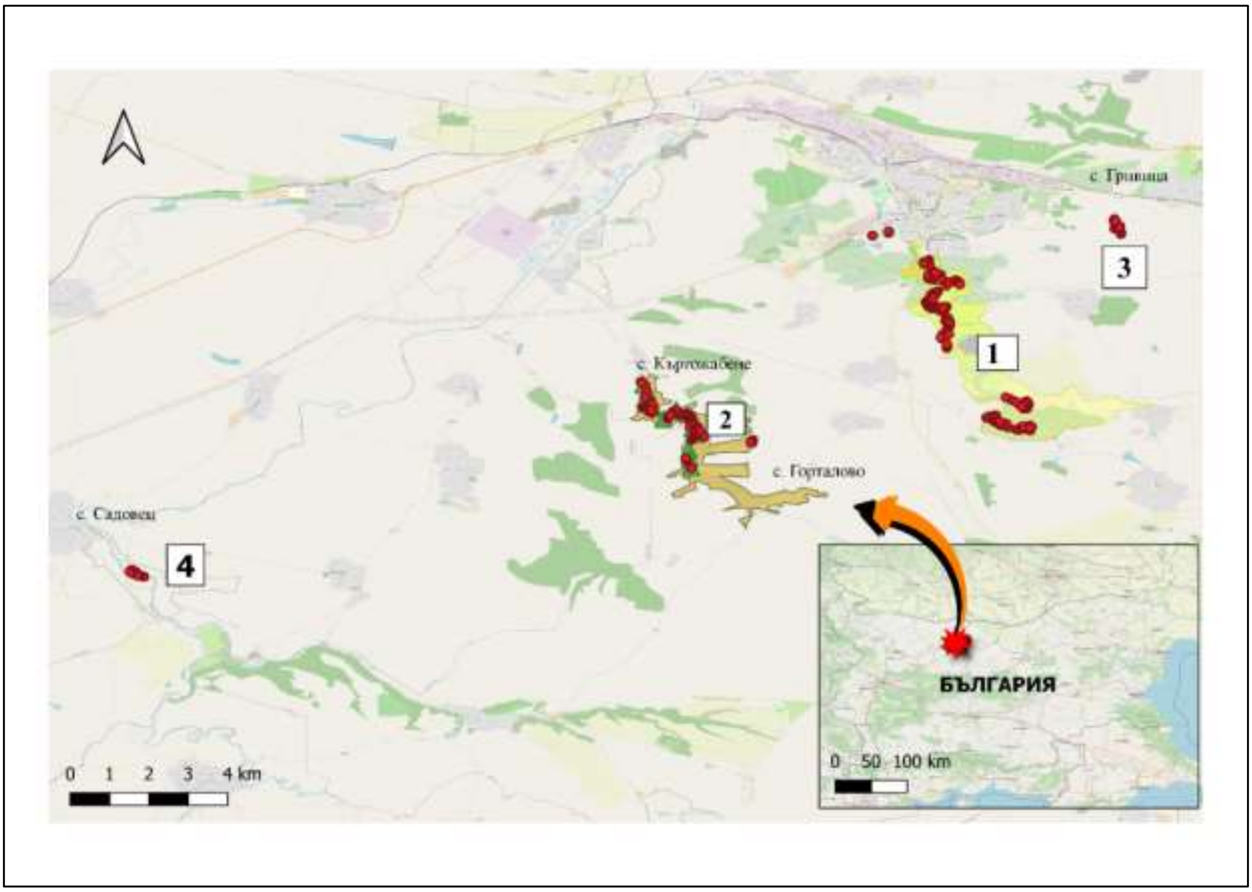
*Table 18. Comparison of DFM and PCR analysis in the detection of B. burgdorferi sensu lato in ticks I. ricinus (n = 44) collected from the Pleven region. ☒- indicates the method used.*

| | **Results by the following method** | | | **Total number of positive tick** |
|---|---|---|---|---|
| | DFM | PCR *5S-23S IGS* | PCR *FlaB* | |
| **Positive ticks on each method** | 15 | 14 | 19 | |
| **Negative ticks on each method** | 29 | 30 | 25 | |
| **Positive ticks by only one method** | | | ☒ | 3 |
| | | ☒ | | 0 |
| | ☒ | | | 4 |
| **Positive ticks by two of the methods** | | ☒ | ☒ | 13 |
| | ☒ | ☒ | | 9 |
| | ☒ | | ☒ | 10 |
| **Positive ticks in all three methods** | ☒ | ☒ | ☒ | 10 |

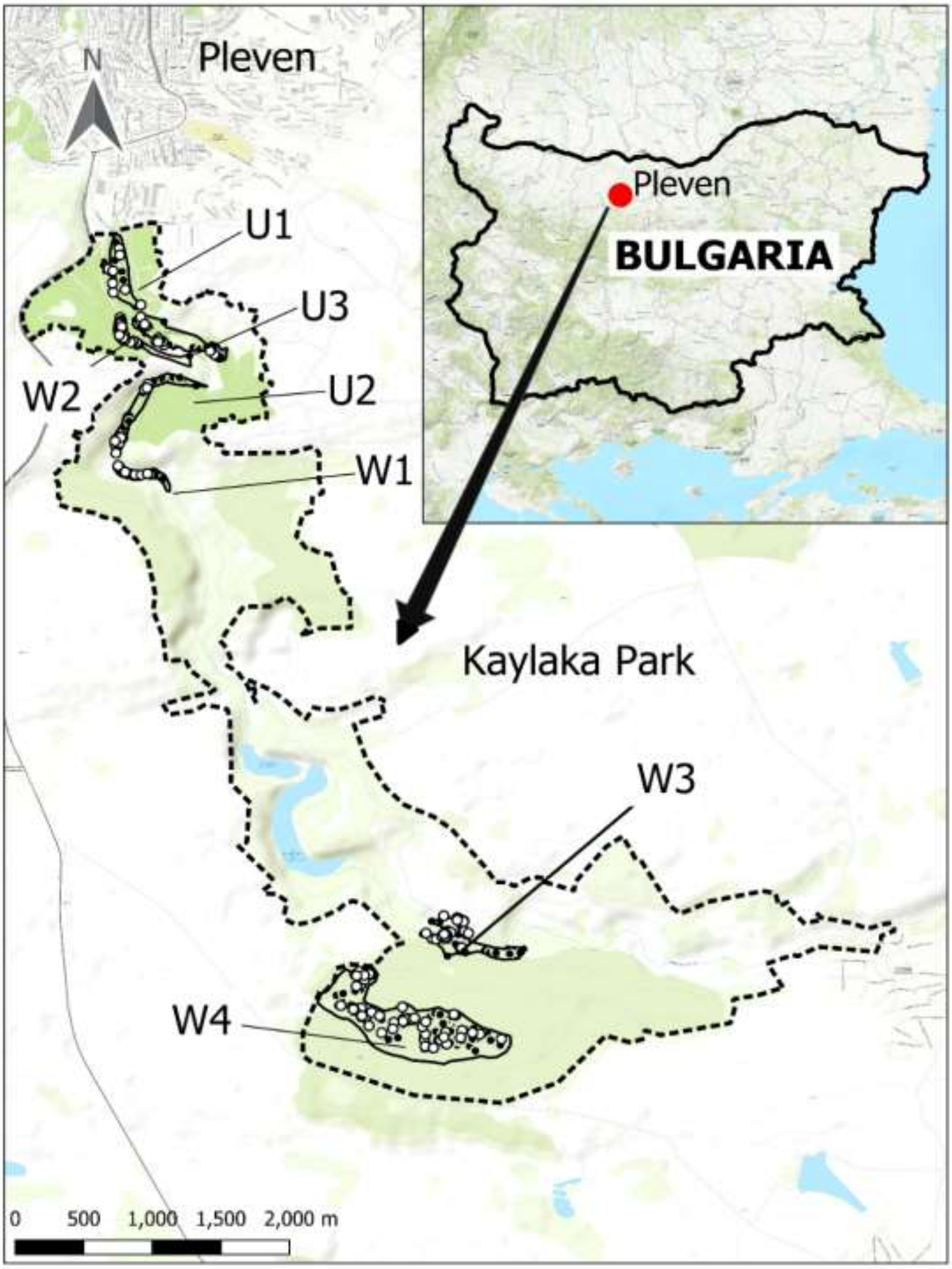
Since the different used methods for detection of *Borrelia burgdorferi* sl, have several limitations, advantages and disadvantages, we have taken into account the positive ticks in which *Borrelia* was detected regardless of the method of testing (DMF and/or PCRs positive tick).

# 8. EPIZOOTIOLOGICAL MAPPING

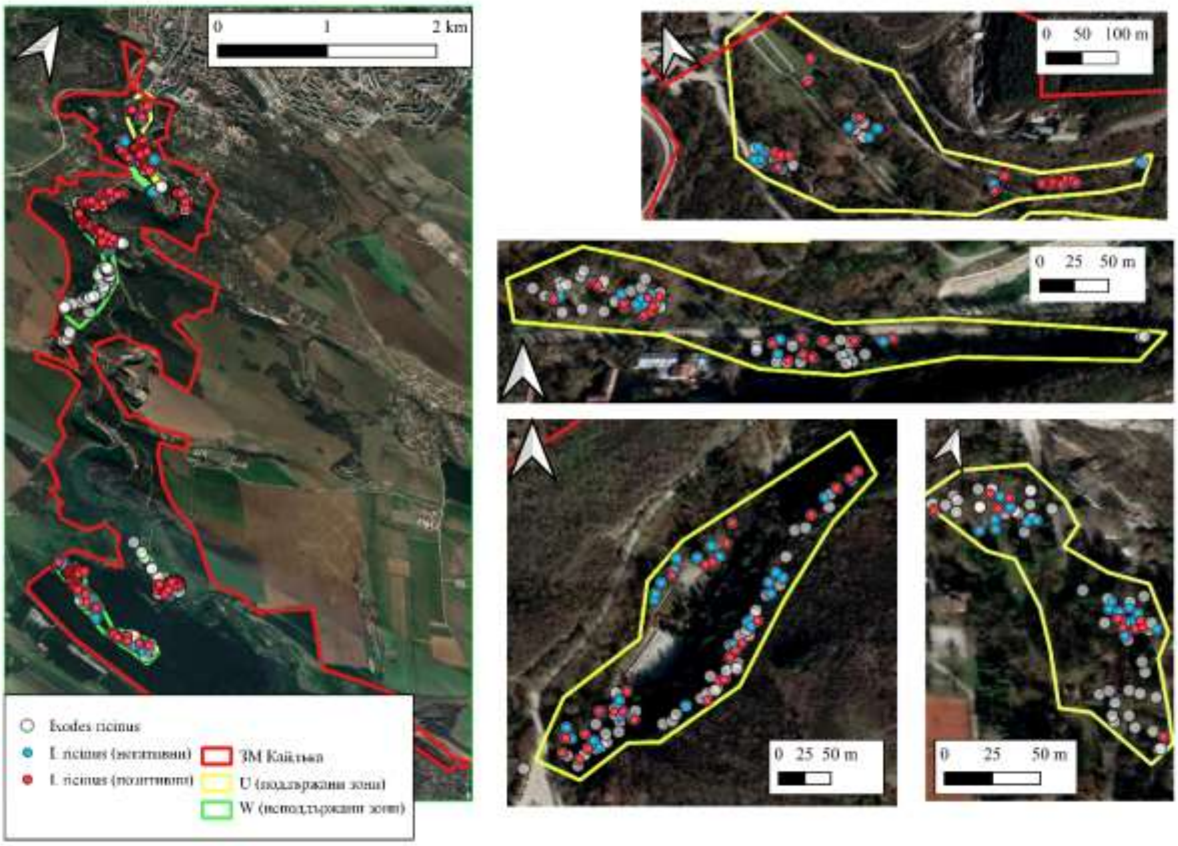
Because of the result of the overall survey results, epizootological maps were created, covering several regions of Pleven distrinct.



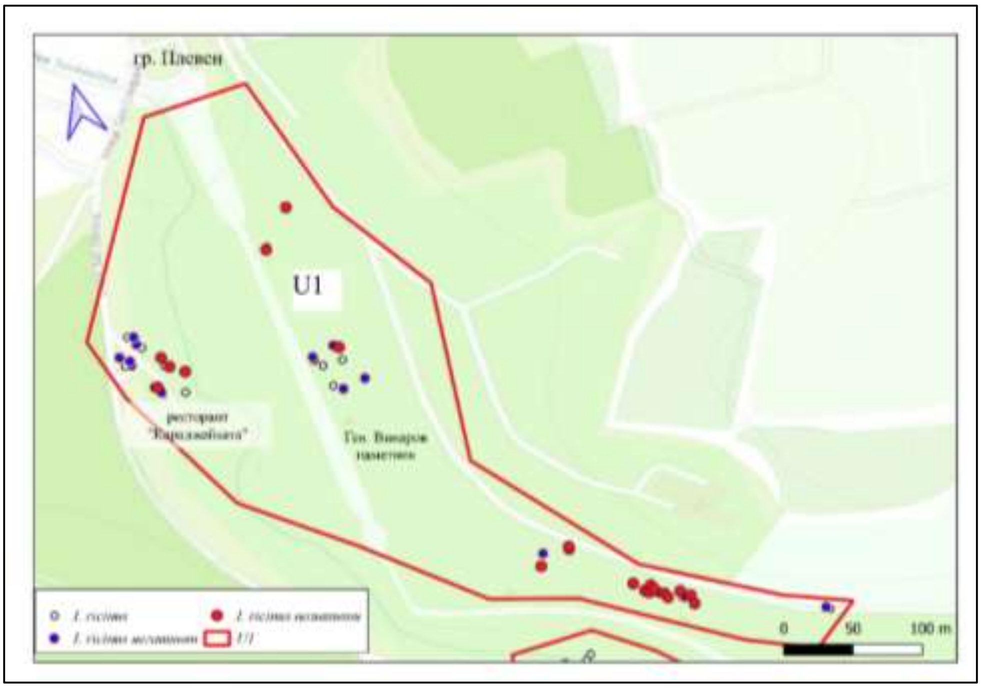

***Map 1.*** *Areas of study in Pleven region. 1)PA "Kaylaka"; 2) "Karst Gorge of the River Chernelka"; 3) the Village of Grivitsa – "Tachova Cheshma"; 4) Village of Sadovets – "Garvan Dol".*



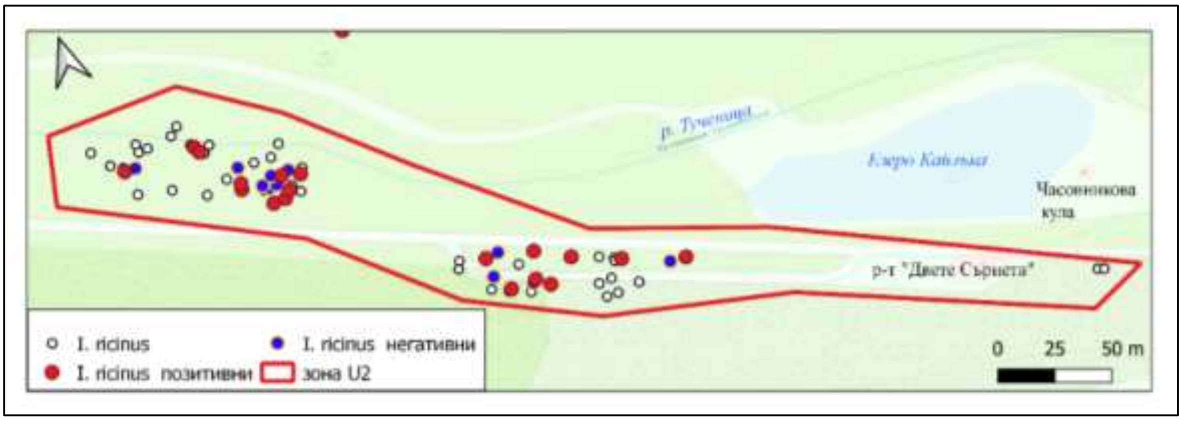

*__Map 2__. Location of the PA "Kaylaka"and sampling sites Urban (U1 - U3) and Wild (W1 - W4) in Bulgaria. The boundaries of the PA "Kaylaka"protected area are represented by a dotted line. All sample collection areas are represented by a solid line. Each collected tick is marked with dark circle. Ticks positive for Borrelia are represented by a white circle.*



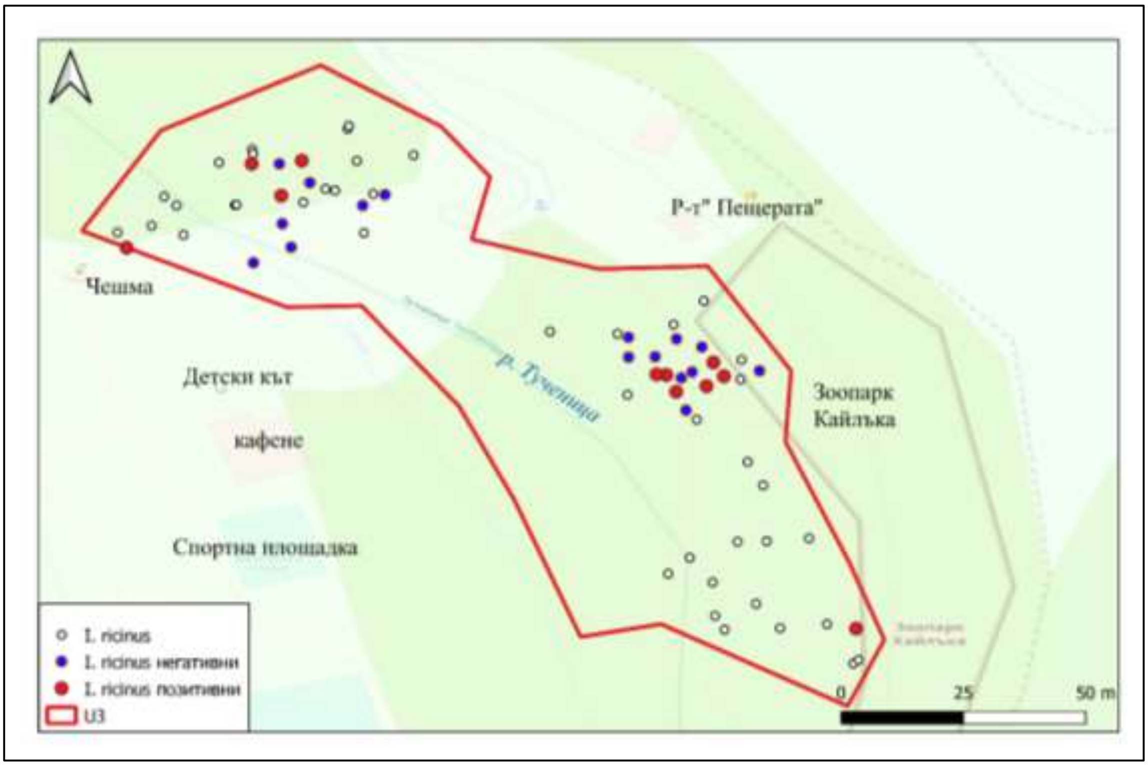

***Map 3.*** *Map of the PA "Kaylaka"and urbanized areas with foci of Ixodes ricinus and their infection with Borrelia burgdorferi s.l. On the left is presented the area of the PA "Kaylaka", on the right are presented the urbanized areas U1 - U4.*



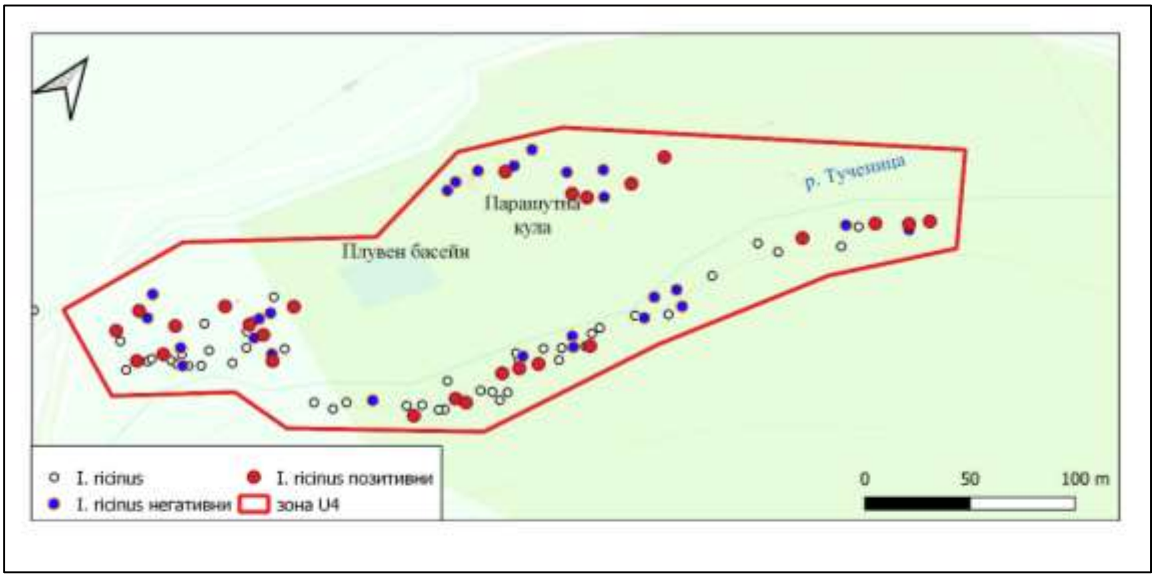

***Map 4***. *Urbanized area U1 in PA "Kaylaka".*



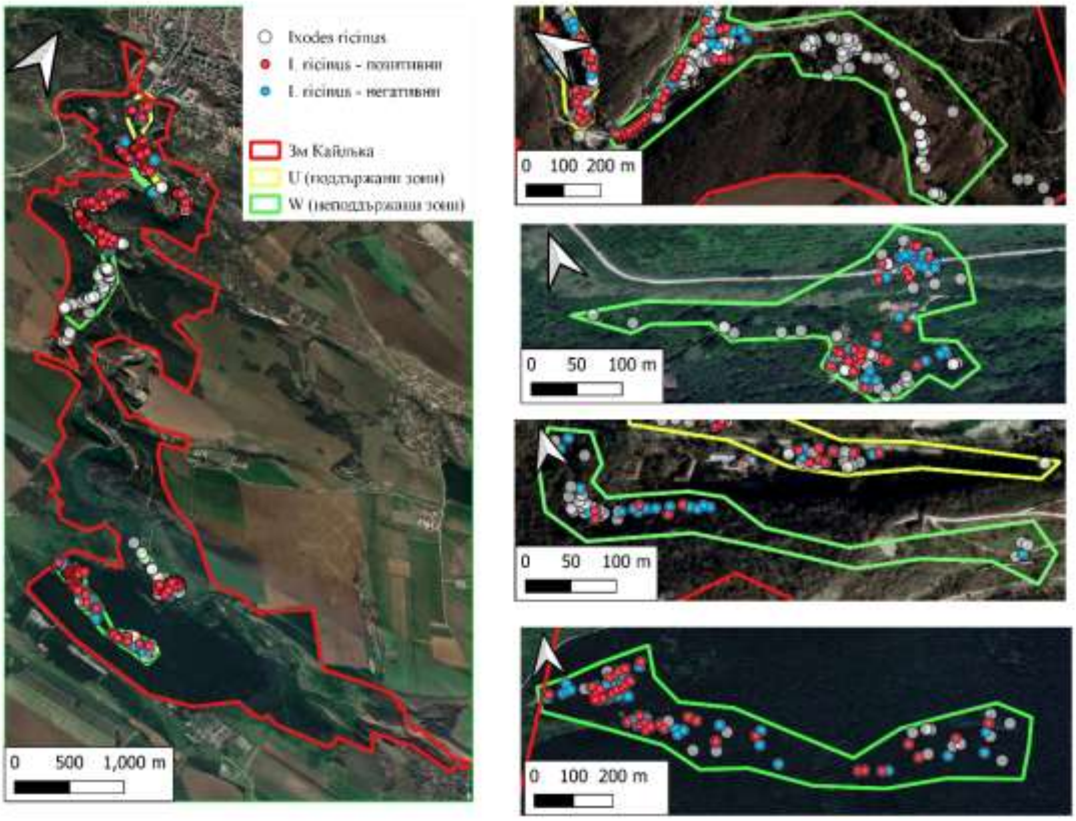

***Map 5***. *Urbanized area U2 in PA "Kaylaka".*



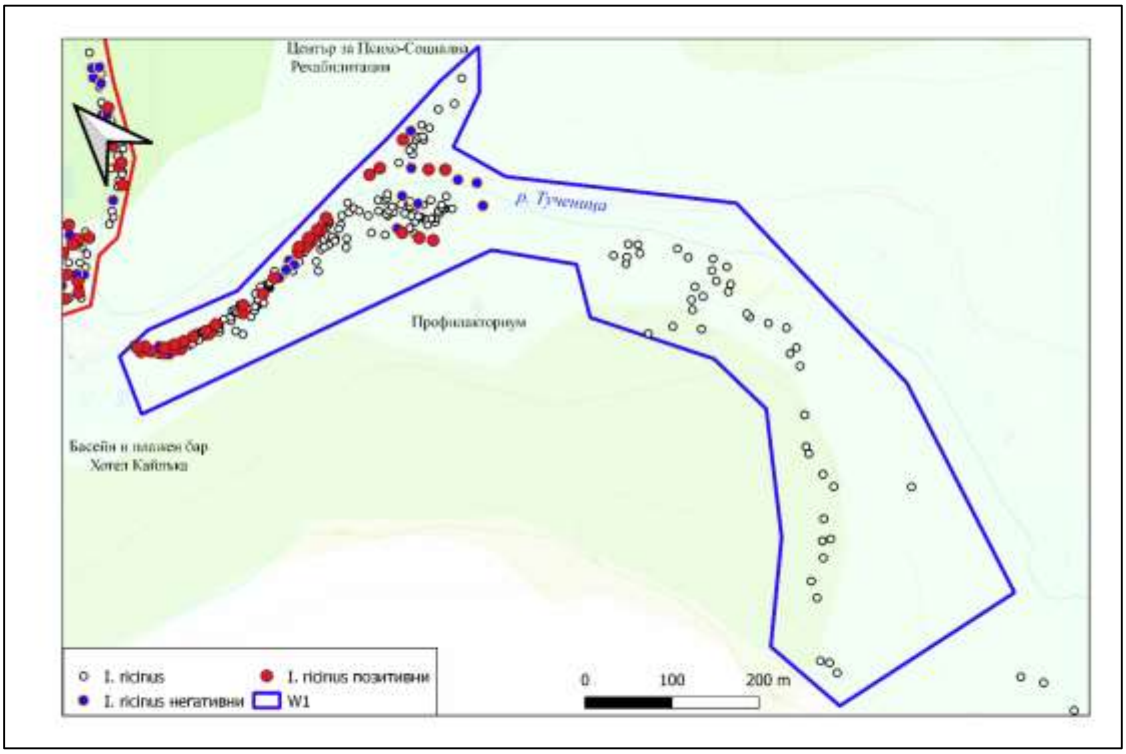

***Map 6****. Urbanized area U3 in PA "Kaylaka".*



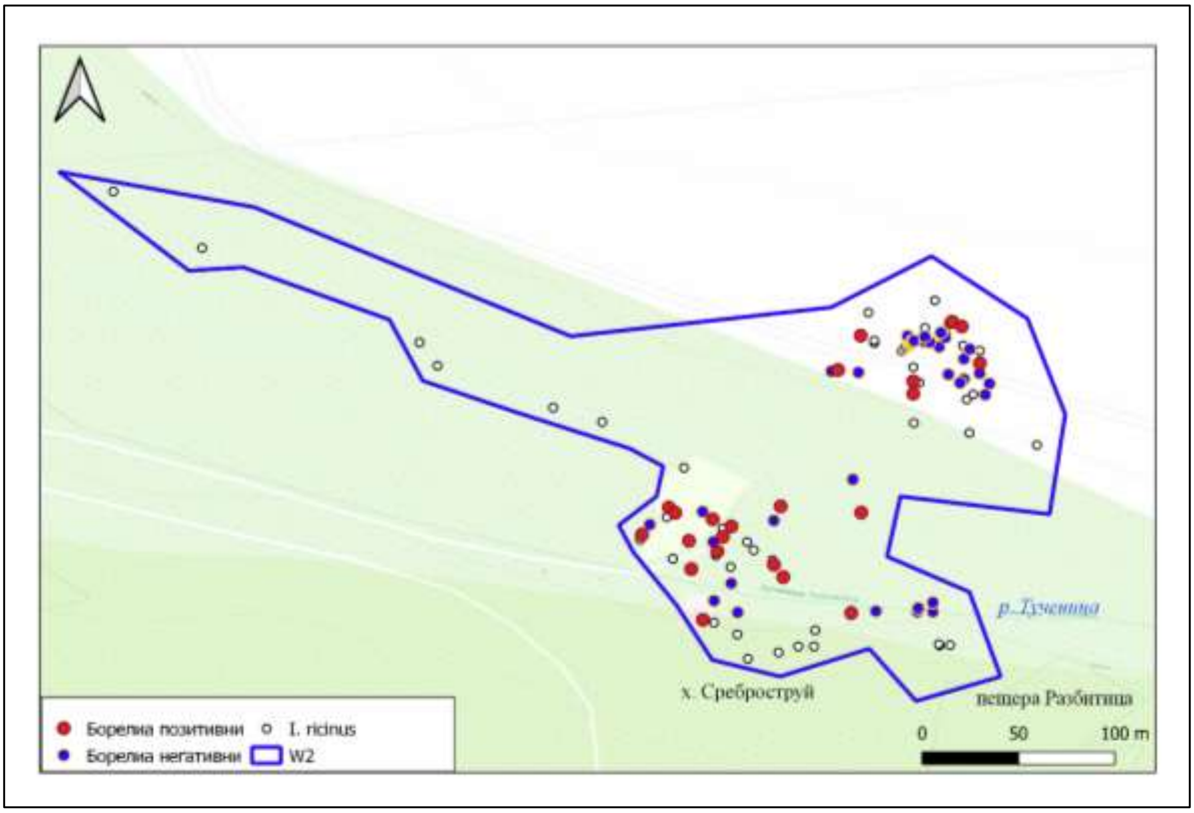

***Map 7****. Urbanized area U4 in PA "Kaylaka".*



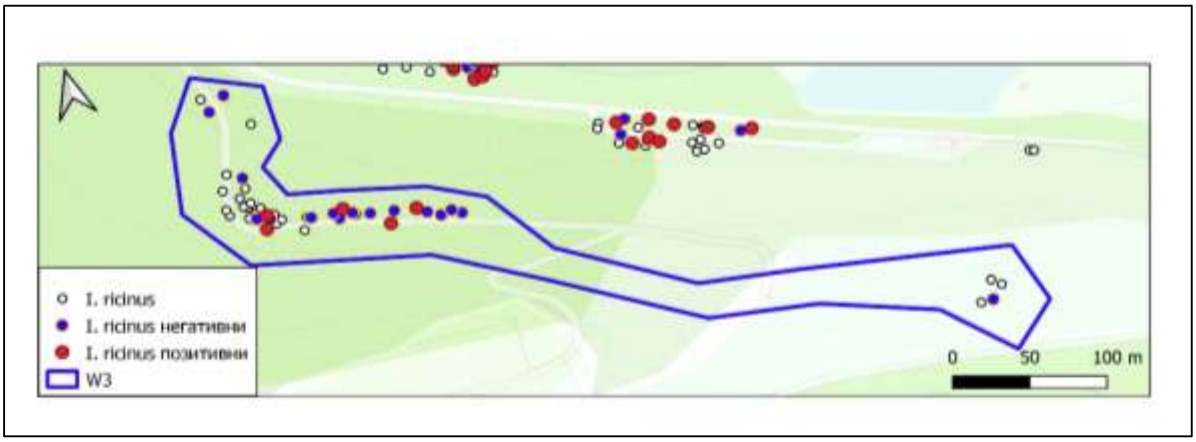

***Map 8***. *Map of the PA "Kaylaka"and wild areas with foci of Ixodes ricinus and their infection with Borrelia burgdorferi s.l. On the left is presented the area of the PA "Kaylaka", on the right are presented the urbanizedwild areas W1 - W4.*



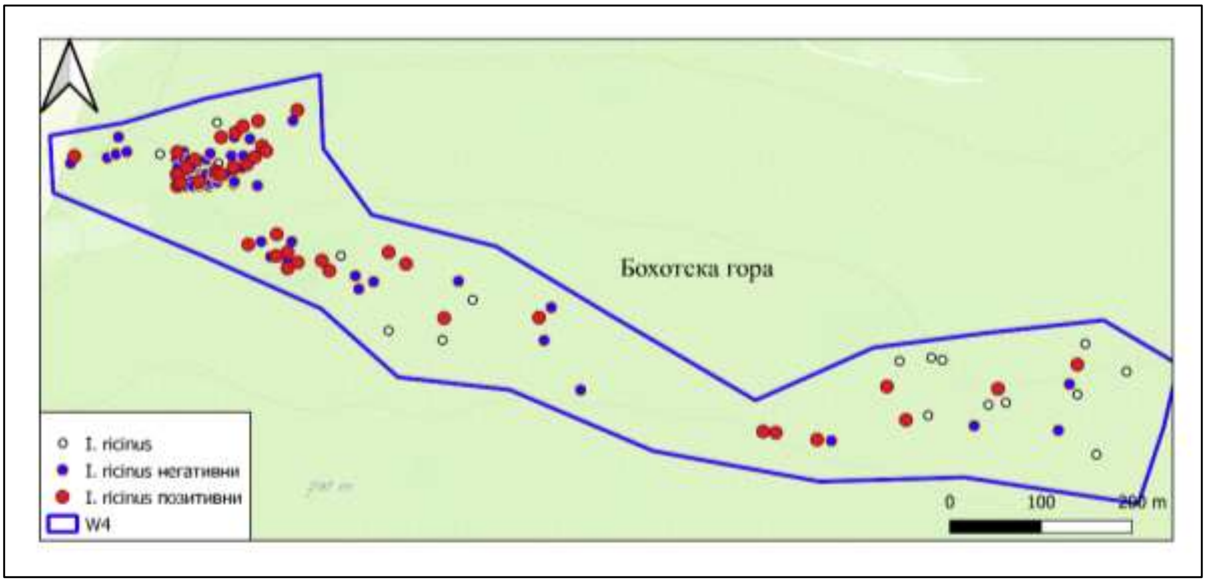

***Map 9***. *Wild area W1 in PA "Kaylaka".*



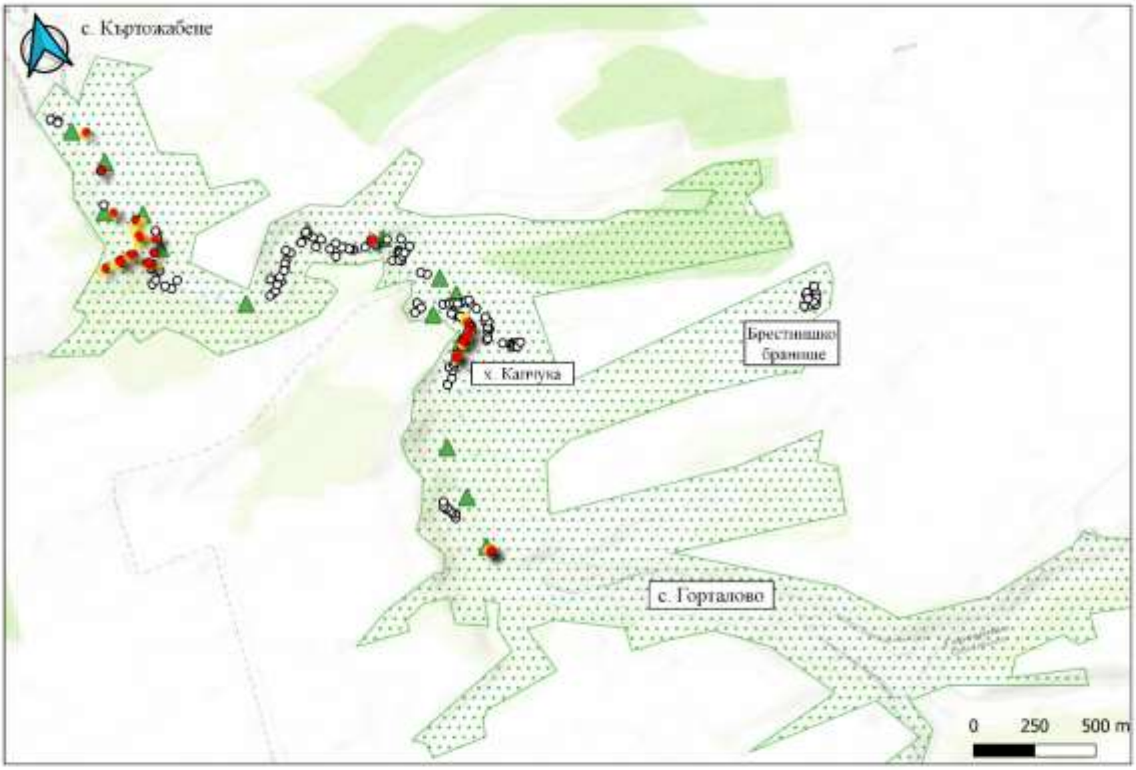

***Map 10***. *Wild area W2 in PA "Kaylaka".*




***Map 11***. *Wild area W3 in PA "Kaylaka".*




***Map 12***. *Wild area W4 in PA "Kaylaka".*




***Map 13***. *Map of the "Karst Gorge of the River Chernelka". All collected I. ricinus ticks are marked with ○; positive for B. burgdorferi s.l. with ●; negative with ●; Recreation areas with ▲.*

# V. DISCUSSION

## 1. RETROSPECTIVE EPIDEMIOLOGICAL STUDY

The literature review shows that LB is the most prevalent tick-borne infection in the world. In Europe, LB is recorded in all countries. A high rate of tick-borne *Borrelia burgdorferi* infection (nymphs over 10%, imago over 20%) has been recorded in Central Europe (Austria, Czech Republic, Southern Germany, Switzerland, Slovakia, Slovenia). The first focused studies on the morbidity in Bulgaria date from the 1990s. Some authors present the regions in Bulgaria as follows: having an incidence of less than $10\%_{000}$ (Vidin, Silistra, Sofia, Kyustendil, Blagoevgrad, Smolyan, Kardzhali, Yambol); with an incidence of up to $20\%_{000}$ - (Pernik, Pleven, Lovech, Gabrovo, Plovdiv, Pazardzhik, St. Zagora, Sliven, Burgas, Dobrich, Razgrad) and with an incidence of up to $30\%_{000}$ (Vratsa, V. Tarnovo, Targovishte, Rousse, Shumen, Varna). Fourteen Lyme borreliosis zones have been formed, depending on the geographical-landscape conditions and administrative units in the country. Pleven and the district fall within the so-called Pleven-Lovech zone. As the data from the retrospective epidemiological study show, the incidence in the Pleven region has remained at the level of up to $20\%_{000}$ over the last 20 years. This indicates that the factors (biological and social) influencing the epizootic and epidemic process continue to be active. The link between the reservoir host and humans is the infected *Ixodes* spp. ticks. From the exophilic ticks collected during the fieldwork, it was found that they are the predominant species in the Pleven region.

The other transmissible zoonosis is the Mediterranean spotted fever. Endemic outbreaks of MSF exist in countries of the Mediterranean basin. It is caused by *Rickettsia conorii conorii*. As early as the beginning of the last century, the dog tick *Rhipicephalus sanguineus* was identified as the reservoir and vector of this disease. In the last decade, the incidence has ranged between $2.96\%_{000}$ and $19.35\%_{000}$. Endemic areas are Pazardzhik, Plovdiv, Sliven, Haskovo, Burgas, Varna.

In the Pleven region, cases of MSF are registered sporadically. It is suspected that the infected people have been residing in endemic areas where they have been bitten by a tick. The circulation of the tick *Rhipicephalus sanguineus* s. l., according to flagging findings, suggests the maintenance of a local epizootic, but the influence of the geographical factors on *R. conorii* is also relevant here. It is reflected on the ranges of the disease vectors. *R. sanguineus* s. l. have high biological plasticity, making them the most widespread ticks in the world.

The results of our survey confirm the fact that of the tick-borne diseases in the Pleven region, Lyme borreliosis is the most important. There are physico-geographical conditions, vectors and reservoirs that maintain the epidemiological process continuity. Systematic monitoring of tick infectivity is needed to improve preventive and anti-epidemic measures.

# 2. COLLECTION OF IXODID TICKS

## 2.1. Choosing a method for collecting ticks

The observation of ticks in medical and veterinary entomology began in 1902 when *Ixodes ricinus* were collected from sheep to understand their impact on sheep health. Initially, efforts were focused on collecting ticks directly from their hosts, but subsequently methods were developed to collect them during the active questing period.

From biotopes in nature, non engorged ticks of exophilic species in the active questing stage were collected. This was done in a number of different methods: by dragging a bright (white) cotton blanket, a piece of fuzzy fabric, a flag, by baiting a person or a chemical substance such as $CO_2$. The specific sample collection methodology varies greatly depending on the purpose of the study. Although ticks are removed from the environment during collection campaigns, these are not considered to have an effect on reducing or controlling tick populations.

The efficiency of each method can also vary depending on tick species, developmental stage, and host-seeking behavior. Ticks classified as ambush (questing) ticks climb to the top of vegetation and wait to attach to a host passing by. In these questing ticks the useful methods of collection that provide a large surface area for contact with vegetation.

To determine the presence of ticks as well as their infectivity with *Borrelia burgdorferi* s.l. in the defined areas, we focused on individuals that were collected in the developmental stages outside the host. Collection of ticks from selected animal species may lead to underestimation of species diversity and tick abundance in a given area. Tick infectivity with spirochaetes in fed individuals also cannot be estimated. Harvesting ticks from an LB-infected host will significantly increase the infection rate in the samples examined due to the transmission of the pathogen during co-feeding.

The most commonly used techniques for collecting questing ixodid ticks are dragging (dragging) and flagging (flagging) (Figure 25). Only questing hungry ticks are captured by these methods. Both methods use a piece of white cotton cloth. In dragging, the operator drags the cloth behind him. In flagging, the cloth is secured on a flag-like handle and is passed by flapping or sliding over foliage or vegetation. The two methods are often used interchangeably in the literature, but are in fact different sample collection techniques motivated by different objectives.

## 2.2. Hard Ticks Species

*Ixodes ricinus* accounted for the largest proportion of Ixodid tick species detected by flagging. It is also the most synanthropic species, accounting for about 90% of cases found on humans. *I. ricinus* was found in all study areas in the Pleven region. According to Gladnishka et al. 92 - 96% of ticks removed from humans in Bulgaria for the period 2016 - 2021 were identified as I. *ricinus*. The study included cases of people from Sofia who visited the National Reference Laboratory for Tickborne/Vector-borne Infections, *Listeria* and *Leptospira*. The epidemiological significance of I. *ricinus* as a vector of various transmissible diseases is high due to the fact that various pathogens have been isolated from it. Estrada-Peña describes 20 different pathogens in *Ixodes ricinus*, highlighting that it is the main vector of *Borrelia burgdorferi* s.l. for Europe.

Based on a metaanalysis of 115 studies published during the period 1990-2021, tick species I. *ricinus* collected from urban green spaces have been reported in the literature from 24 countries in Europe, including Bulgaria. Most of the publications related to urban areas report the simultaneous presence of all life stages, covering different European regions. The ratio of sexually mature forms to nymphs and larvae is highly variable. In the review by Hansford et al. on the distribution of I. *ricinus* in urban areas, the proportion of males (49.6%) and females (50.4%) is in agreement with our results (49% males and 51% females). The presence of larvae was reported in only one-third of all surveys, and it was not specified how they were collected. Some publications indicate nymphs as the largest proportion of ticks collected, whereas in our study they accounted for 17%.

For the first time in Bulgaria, a questing female tick of the species *Ixodes frontalis* was collected (Figure 26). It was captured in the vicinity of Hotel "Spartak", located in PA "Kaylaka". In Bulgaria, one individual of this species has been described, taken by a White-throated Thrush (*Turdus torquatus*), in the Lyulin Mountains. Collection in spring by flagging indicates that it successfully overwintered and molted from nymph to adult female in the area. Its medicinal importance is not well understood, but it has a proven role in the enzootic cycle of *Borrelia* spp. *Borrelia afzelii, Borrelia garinii* and *Borrelia turdi* have been detected by PCR. *Ixodes frontalis* is an ornithophilic species and commonly inhabits bird nests. Publications related to its capture by flagging are exclusively rare in Europe, although the species is considered ubiquitous.

Relatively few species of ixodid ticks belonging to the Metastriata are of medical importance to humans. In the course of the study, members of the genera *Rhipicephalus*, *Dermacentor* and *Haemaphysalis* were mainly collected by flagging in the Pleven region.

*Figure 22. Ixodes frontalis, female. Ventral view. Digiscopy, Leica DM750, magnification x40; Nikon D7200; Nikkor 50mm 1.8. A. Blazhev 29.04.2021.*

The two individuals identified as *Haemaphysalis punctata* were caught in different localities - the first in "Tachova Cheshma" near the village of Grivitsa, and the second one in the grassy area of the zoo in the urbanized part of PA "Kaylaka". Its discovery in the vicinity of the village of Grivitsa is not unusual, due to the fact that there are rabbits, hedgehogs and various small rodents, which are its main hosts. The individual captured in the grassy parts of the zoo in the PA “Kaylaka” may have been caught both by natural inhabitants in the park and by the animals kept there.

No specimen of the genus *Hyalomma* has been found in Pleven region during the survey period, neither by flagging nor by host removal.

The possibility of animal-to-human transmission of ticks is of concern in rural as well as suburban and urban areas. Urban parks and suburban green spaces that are recreational areas for families, sportsmen and pets are suitable environments for contact with blood-sucking arthropods such as ticks. However, studies on the abundance and seasonal dynamics of ticks in urban and suburban parks used for recreational activities are insufficient in Bulgaria.

## 2.3. Effect of temperature and relative humidity on the questing activity of *Ixodes ricinus*

A unimodal pattern of tick-questing activity was found during the study. The pattern was confirmed by both the density of questing ticks and TF (Figure 6 and Figure 7). The highest questing activity was observed in the period from late April to early June. This pattern was expected for the territory of the Pleven region. The main tick-questing activity during the spring period was a logical consequence of the initial retrospective study of tick-borne infections in Pleven District. The results showed that recorded cases of LB and tick bites were mainly in the period March - July and only single case in autumn. The climate in northern Bulgaria is characterised by hot, dry and prolonged summers until mid-October, after which temperatures drop to critical levels for tick activity. Minimal autumn tick activity probably exists, but weather conditions are not suitable for tick collection (rain, high winds and impassability in some areas at this time of year).

The biggest challenge for terrestrial arthropods is to maintain water balance, a fact that is particularly important for the survival of ticks and especially for the *I. ricinus* as it is extremely sensitive to temperature and relative humidity compared to other hard tick species. Similar observations were made in the present study. The increase in relative humidity had a positive effect on the activity in questing *I. ricinus*, as the maximum activity expressed by TQI was reached up to 60%. The decrease in TQI at elevated relative humidity seemed contradictory at first glance, possibly due to the longer search activity and greater chance of attachment to hosts. Because of the larger number of ticks that were attached to a host, the number of questing ticks on the grass surface was depleted and results in their lower amount of flagging. Compared to the temperature, the TQI increased and reached its highest values at 20 °C, after which the TQI falls (Figure 2a). The rise in temperature had a negative effect on the survival of ticks, as well as their activity. Therefore, after June, we did not find ticks in the park area.

In studies carried out in Europe there was variation in density depending on the season, with some evidence of unimodal activity with spring peaks as in our studies and others with bimodal patterns and second peaks later in the year.

Schulz et al. found that thirteen of twelve sampling sites in Germany showed a unimodal pattern of activity for all developmental stages, characterised by a single peak of activity beginning in early March to late May. Only one of the sampling sites had a bimodal activity pattern. A second smaller peak of activity was found for nymphs and larvae in autumn, while no second peak was observed in adult forms.

Supporting the unimodal activity found in the Pleven region are the results obtained from the registered cases of LB in the district. The highest incidence was recorded in the months of June-July (Figure 4), which coincides with the maximum activity of I. *ricinus* in the period from April to the end of May. Symptomatology of LB does not always occur immediately after tick bite. Testing for *B. burgdorferi* s.l. in patients is based on the detection of antibodies against the pathogen (IgM and/or IgG) in serum. The test is performed at least 30 days after the tick bite. Therefore, a delay is detected in the peaks of tick activity and registered cases in the PHI-Pleven. On the other hand, the analysis of registered cases in Bulgaria shows a bimodal activity with a smaller peak in October (Figure 4).

This suggests that a wide range of urban green spaces may be suitable environments for the development of tick populations, whose activity may occur at different times during the year.

*Ixodes ricinus* is an exophilic, three-host tick species restricted to areas with moderate to high rainfall and vegetation that retains high humidity. During periods without hosts, I. *ricinus* needs relative humidity of at least 81-85% for its survival. Our results showed that I. *ricinus* was most active in host searching at t = 20 ºC and RH = 60%.

We found that the density and frequency of questing ticks decreased when temperatures rise above 20°C. Qviller et al. obtained similar results when examining the density of I. *ricinus* in the coastal region of western Norway. In this part of Europe, activity is highest at 15-17°C. However, ticks have been shown to be active and abundant at much higher temperatures in other locations. For example, in southern England, positive geotropism of *Ixodes ricinus* starts at 24°C at 80% RH or at 18°C at 71% RH. The decrease in tick density with increasing temperature, as found in the guideline study, could theoretically be related to RH becoming limiting at higher temperatures. In a controlled laboratory study that tested the activity of I. *ricinus* at 25°C and 60% RH, 25°C and 85% RH, and 15°C and 85% RH, suggests that optimal search conditions are closer to 15°C at 85% RH. Other studies have found both negative and positive correlations with temperature. The negative correlation with temperature in a more continental climate in Sweden compared to the positive correlation in an Atlantic climate in Ireland suggests that differences in temperature response are also related to other climate factors. High temperatures can cause desiccation stress in dry/continental regions, such as summer in North Bulgaria.

## 3. *IXODES RICINUS* DISTRIBUTION IN PA “KAYLAKA”

The risk of a person contracting a tick-borne infection is directly related to the number and prevalence of the actively seeking ticks. One of the objectives of this study was to compare and estimate populations of Ixodid ticks (Ixodidae) in urbanized (maintained) and wild (unmaintained) areas in the PA “Kaylaka”.

The increased risk of tick bites in urban parks and gardens has been studied in many European countries. In Bulgaria, the first studies of tick populations in urban environments have been conducted in Sofia, in the Borisova Garden.

Stable tick populations have been observed in many urban parks in Europe, and various pathogens have been shown to cause disease in humans and domestic animals. Parks and gardens during the spring-summer season are sites that are visited by many people, and therefore a complete tick bite risk assessment is necessary to prevent this serious threat to public health.

During the survey, we confirmed the presence of ticks in all surveyed areas in the PA "Kaylaka", mainly on I. *ricinus*, with no significant differences in their abundance throughout the park. *Ixodes ricinus* is the dominant species of the tick fauna in Europe and is also the most studied species.

Lack of differences in *I. ricinus* density between urban and wild areas in the protected area of PA "Kaylaka" are most likely due to the large number of small and medium-sized mammals and plenty of birds inhabiting the park area. Most of the ticks collected in the urban areas were found near places where there was food waste, near bars, restaurants, playgrounds and trash cans. These places usually attract small rodents as well as stray dogs, which are the hosts in the different stages of the ticks.

PA "Kaylaka" is located in the canyon of the small river Tuchenitsa, and is surrounded on both sides by rock formations, which provides relatively constant humidity in the park. On the other hand, the park is bordered by wooded areas inhabited by various animals, delivering excellent conditions for the life cycle of the *I. ricinus* ticks in all stages of its development.

An analysis of the literature related to the study of tick populations in urban and suburban parks and gardens indicates that the predominant species is *Ixodes ricinus*. In general, it is described as the most synanthropic species, probably due to its wide range of hosts, including humans. In the review on the x expansion of *Ixodes ricinus*, Medlock discusses the role of deer and roe deer as central to its expansion in parts of Europe. The reasons for this are largely unconfirmed, but are thought to be due to a combination of events such as: behavioural adaptations of deer in relation to their tolerance of human presence; laws prohibiting their hunting, (including their care, through feeding); changes in agricultural practice; and the reduction of predators.

An interesting fact is that 87% of the ticks collected in the PA "Kaylaka" area were mature. The small number of captured nymphs and the lack of larvae can be explained by the sampling method. Nymphs, due to their significantly smaller size, are more sensitive to water loss and possess lower absolute energy reserves than adults. Because of this, few nymphs climb to the top of vegetation, reducing the chance of being captured by flagging. It is likely that many of the nymphs were found in the lower layers of vegetation that remain untouched by the flag. Another possible cause is seasonal nymph activity. It is possible that nymph activity is greater in the autumn, when no collecting campaigns have taken place, due to the adverse weather conditions.

The region analysed in this study has not been subjected to hard tick analysis. This study contributes to the understanding and mapping of tick distribution areas and can contribute to the design of an effective comprehensive surveillance strategy. These data also allow for the identification of the periods of higher tick abundance in the study area, thus providing guidance on precautions to be observed by the maintenance staff during these periods.

### 3.1. Ticks frequencies and Ticks Questing Index in PA "Kaylaka"

Tick frequency (TF) is a indicator used to assess a person's risk of coming into contact with a tick, at a given time, in a given area. We assumed that it was a more convenient indicator compared to tick density, as the time of collection may vary in parts of the same area. This index indicates the frequency of ticks in a given area - number per unit time. On the other hand, TF is an index that gives a relative idea of the rate at which searching ticks are collected by flagging in a given area.

Comparison of TF and TQI in the two types of areas in the PA “Kaylaka”revealed similar values. This indicates that the risk of tick bite during the tick active period in the urbanized and wild areas of the park is the same. After comparing the zones with each other (each with each separately), statistically significant differences were found for TF.

The lowest mean values for TF were calculated for the area at the beginning of the park - U1. Three of the remaining areas (U3, W1, W2 and W4) show statistically significant higher values with those in U1. In zone U4, the frequency of I. *ricinus* was also higher compared to U1, but not significant ($p = 0.055$). Figure 32 shows that the TF values increase from U1 to U4, i.e. from the urban park area to the unmaintained areas of the PA “Kaylaka”. As noted in the “Materials and Methods” section, in all urbanised areas (U1-U4), the lawn areas are equally well maintained. The difference in frequencies between U1 and U3 is likely due to the presence of a zoo in the U3 area. Despite the deworming of the zoo animals, the availability of food in the feeders attracts a variety of natural inhabitants to the park. This increases the density of hosts and therefore the number of the associated parasites in a given area. The differences with the three wilderness areas are not unusual, as U1 is the closest part to the city and representatives of wild fauna typical of the rest of the park and the entire PA “Kaylaka” can be observed much less frequently.

The highest mean TF values were reported for areas W4 ($0.92 \pm 0.21$), followed by U4 ($0.84 \pm 0.76$) with a protected area-wide mean of $0.61 \pm 0.46$. Bohotska Gora (W4) is the most distant zone in relation to the town of Pleven. It is the most remote area of the region and lacks acaricide treatment. Therefore, the difference in the frequency of capture in U1 and U2 relative to W4 is significant.

Area U4, as seen on Map 3, is adjacent to the unmaintained natural areas of the park. It is also one of the narrowest parts of the river Tuchenica canyon. On the one hand, the local climatic conditions are favourable for tick development, the humidity persists longer, and on the other hand, the passage of various wildlife from the rest of the park is more frequent, likely to increase the tick abundance in this area.

No significant differences in TF were found between different wild areas, but when comparing the densities of I. *ricinus* estimated by TQI a difference was observed between W1 and W3. This difference in densities is due to the location of zone W3. It is located on the western rock ledge above the canyon. This part is more open, less forested and consequently drier, compared to W1, which is richly forested with a variety of tree species (abandoned arboretum) providing suitable conditions for the existence of I. *ricinus*.

Falco and Fish studied recreational areas for the risk of acquiring a tick bite and possibly Lyme disease in parks in New York. They use an index called “encounter distance”. It measures the distance that the first tick will catch on the flag. Their results also show that in areas of parks that are used more often by citizens, there is a lower risk for tick bites, as more walking distance is required to catch a tick.

It is difficult to compare the two indices, but in both studies the aim was to determine the risk to humans. With an encounter distance, it is possible to catch a tick on the flag in the first step, but the next questing tick will be after many meters away. For this reason, we chose the risk to be assessed by the capture number per flagging time. On the other hand, the time spent staying or walking in a habitat inhabited by ticks significantly increased the risk. Although there were no differences in densities between urban and wild areas, comparing the number of ticks per unit time showed that in wild areas there was a higher risk of tick bites.

Finding fewer ticks in frequently used urban areas could result from ticks attaching to domestic dogs and humans and being removed from the questing population. Additionally, this may be due to the more frequent maintenance of green areas, such as mowing and exporting green waste. On the other hand, fewer ticks in high-use areas may be due to habitat alteration, such as buildings, roads and maintained lawns, which may discourage wild mammal hosts or reduce tick survival.

When comparing the density of NQI nymphs, no differences were found between urbanized and wild areas in the PA "Kaylaka". Numerous studies have shown that nymphs are the source of infection in humans for various tick-borne pathogens. This is due to their relatively small size which makes them difficult to detect, and thus they remain attached to humans longer before being removed. This increases the possibility of transmission of pathogens from the nymphs to the host. The seasonal phenology of nymphs of I. *ricinus* differs between geographical areas, but in continental Europe it is bimodal and consists of a large spring peak followed by a smaller autumn peak. From the perspective of public health, understanding the environmental factors that cause interannual variation in NQI, and hence in the risk of infection, is important for developing control strategies to reduce the incidence of tick-borne diseases.

Since no statistically significant differences between urbanized and wild unsupported areas were obtained for any of the tick questing indices, it cannot be stated that the risk of tick bites to humans is different in the two areas. Based on these data, we cannot conclude that there is a difference in the distribution and density of tick populations between the two types of areas in the PA "Kaylaka".

These results indicate that regardless of the type of area surveyed, there is a high risk of being bitten by ticks and therefore infected with tick-borne diseases. The urbanised part of the PA "Kaylaka" attracts many citizens, tourists and visitors, therefore more effective measures need to be applied to reduce tick numbers in urban areas.

# 4. DARK-FIELD MICROSCOPY FOR THE DETECTION OF *BORRELIA BURGDORFERI* S.L

Since the discovery of *Borrelia burgdorferi* by Willey Burgdorfer in 1982 by DFM, various methods have been used for the identification of *Borrelia* in the LB group. One of the cheapest, fastest and easily applicable method remains DFM. For this reason, pilot studies for the presence of spirochaetes have been performed by this method. The accuracy of the method has been discussed and compared with other detection techniques in various publications. All microscopic techniques (immunofluorescence, dark-field or phase-contrast microscopy) can detect the presence of spirochaetes, but cannot identify the species of the bacterium in question. Detection is based on typical morphological features. On the other hand, *Borrelia* of the Reverse Fever group have also been detected in ticks of the genus *Ixodes,* for example *B. miyamotoi*. Spirochaetes from both groups cannot be differentiated by DFM. For this purpose, PCR techniques are used by amplifying genome regions characteristic of one or the other group of borrelia.

In the present study, 32.94% infectivity of *Borrelia burgdorferi* s.l. of ticks collected from different regions of Pleven district was found by DFM. Out of the four main areas in which samples were collected, only in “Garvan Dol” no infected ticks were found. It should be noted that in the same area the number of examined ticks was the lowest, only 9 individuals. In all other areas, *Borrelia* were observed. The highest percentage of infestation by DFM was found in ticks collected from the PA “Chernelka” (46.84%), followed by the PA “Kaylaka” (32.77%) and “Tachova Cheshma” (21.74%).

## 5. DETECTION OF *BORRELIA BURGDORFERI* S.L. IN *IXODES RICINUS* BY NPCR

In the identification of *Borrelia* of the LB group in ticks, the most commonly used method is PCR. Many PCR-based tests have been developed. Due to their high conservativeness as markers the *16S* rDNA, the intergenic spacer of the *5S-23S* rDNA and the *flaB* gene have been most commonly used for this purpose. In the course of the present study, one structural gene, *flaB*, and the non-coding region of the *5S-23S rRNA intergenic spacer* were used.

Due to the use of different methods for the detection of *Borrelia burgdorferi* s.l., we considered as positive the ticks in which *Borrelia* was detected, regardless of the method used. The agreement between $nPCR_{23S/5S}$ and $nPCR_{FlaB}$ with DFM was moderate and good, respectively, according to Cohen's Kappa test.

When evaluating the methods, the highest number of borrelia-positive ticks was detected with $nPCR_{FlaB}$ (19/44). Using $nPCR_{23S/5S}$, 14 of 44 were positive. It is possible that amplification of the flagellin gene could also detect *Borrelia* from Relapsing Fever group, as this gene is relatively conserved in the genus, whereas the highly variable *5S-23S* duplicated region has been widely used to subtype *Borrelia burgdorferi* s.l. species.

*Borrelia burgdorferi* was detected by nPCR in three ticks found to be negative by DFM. On the other hand, 4 of the 44 DFM-positive ticks were not confirmed by either of the two nPCRs. Nefedova et al. in their study compared the two methods, DFM and PCR, where by PCR they detected 10% more infected ticks than DFM. However, they indicated that 8% of highly DFM-positive ticks did not yield a PCR product.

In a study by Alekseev et al. for the presence of *B. burgdorferi* s.l. by DFM and PCR in ticks collected from the Baltic region of Russia the results of the two methods were compared. Of the DFM-positive ticks (n = 472), only 64.8% amplified a PCR product corresponding to *B. burgdorferi* s.l. In DFM-negative (n = 159) ticks, the absence of Borrelias DNA was confirmed in 79.81%. The authors assumed that DFM is more sensitive than PCR for the detection of *Borrelia*. The detection of *Borrelia* DNA in nearly 20% of negative dark-field samples contradicts their results. To explain the inconsistency, they give the following reasons: 1) DNA degradation during storage; 2) the microorganisms observed in DFM may represent species other than *B. burgdorferi* s.l.

Possible reasons for failed amplification may be due to purely laboratory errors, which may be caused by a variety of reasons and occur during the preparation of a multicomponent sample for PCR. On the other hand, a probable reason for the lack of amplification could also be the low amount of *Borrelia* in the ticks. Similar differences in assay methods were obtained when comparing the results of DFM, cultivation in BSK II medium and PCR using a primer set based on the flagellin gene of *B. burgdorferi*.

An alternative explanation for the discrepancy in the detection of *Borrelia* by PCR and DFM may be the fact that a portion of the intestinal contents was used for DFM and the spirochaetes may have been absent or not detected there. The failure to detect these spirochaetes by DFM may be due to their limited distribution in this fragment of the tick midgut. Better results with PCR compared with DFM were also found in a study of actively searching ticks from Finland, in which DFM, cultivation of *B. burgdorferi* s.l. and PCR were compared.

Microscopic techniques used as standard procedure in the past do not allow differentiation between genospecies and, if not used correctly, can lead to underestimation of the rate of infection with *B burgdorferi* s.l. On the other hand, previously reported prevalence rates of LB spirochaetes among *I. ricinus* could also be unrealistically high due to misidentification of Relapsing Fever group spirochaetes (*B. miyamotoi*), using microscopy.

In conclusion, PCR methods are extremely precise and finding growing applications in various fields of biology and medicine. Dark-field microscopy is a convenient, inexpensive and accessible method suitable for pilot studies on the distribution of *Borrelia* sp. in tick fauna. Despite the lack of complete consistency of the results used together or separately, they provide a good insight into the distribution of LB in *Ixodes ricinus* populations.

## 6. SURVEY OF THE PROTECTED AREA PA “Kaylaka”FOR THE PRESENCE OF *BORRELIA BURGDORFERI* S.L. IN *IXODES RICINUS* TICKS

In the present study, most of the ticks collected and tested were from the area of the PA “Kaylaka”. This area is located next to the city, easily accessible, visited daily by many citizens and characterized by rich flora and fauna. This allows the collected material to be processed and systematized quickly and efficiently. The study of urban, roadside parks and gardens for arthropods, vectors of various vector-borne diseases, has been extremely relevant in the last two decades. Much of this has focused specifically on the ixodofauna and in particular on *I. ricinus* and its associated infections.

Studies in Bulgaria on the infectivity of *I. ricinus* with LB in urban parks were conducted in 2001 only in the Borisova garden area in Sofia, where a slightly lower tick infectivity of 45% was found. No other similar study performed in the country was found in the available literature. Analogous studies of *I. ricinus* for the presence of *B burgdorferi* s.l. in other countries of the Balkan Peninsula showed large differences in tick infectivity. When examining different tick species, of which 30 *I. ricinus* from Bosnia and Herzegovina no positive ones were reported for *B. burgdorferi* s.l.. Relatively low tick infectivity was shown by studies from Romania, with an overall prevalence of *Borrelia* of 18%. One of the regions studied was Giurgiu, where an increased infectivity of 19.8% was observed in adult ticks. Also in Romania, in a survey of 12 221 I. *ricinus* for different genotypes of *B. burgdorferi* s.l. by PCR with the intergenic spacer of the 23S-5S gene, the authors reported an overall prevalence of 1.4%, with an average local prevalence between 0.75% and 18.8%. In the European part of Turkey, the infection rates were 38.7% in Istanbul and 11.4% in the Kirklareli area. High tick infectivity was reported in studies from Serbia. Milutinović et al. a study of 18 different areas in Serbia found 42.5%, while in other surveys from Serbia 49% of the collected ticks were positive for *Borrelia burgdorferi* s.l.

Rauter and Hartung in their meta-analysis classify areas according to tick infection rates with low infection rates (nymphs ≤ 11%; adults ≤ 20%) and high infection rates (nymphs >11%; adults >20%), and extremely high (>30%). Their analysis showed that the average infection rate was 13.7% out of a total of 112 579 ticks tested in Europe over the period from 1984 to 2003. Another meta-analysis based on detection of *Borrelia burgdorferi* sl mainly by PCR (2010-2016) showed that the overall mean prevalence of *B. burgdorferi* sl in I. *ricinus* ticks reached 12.3% for Europe. The results of this study identify PA "Kaylaka" as an extremely high infection area.

Despite the increasing number of LB cases both in Europe and in Pleven , the tick populations in the PA "Kaylaka" have not yet been examined for infection with *Borrelia burgdorferi* sl. In the present study, we confirmed the presence of LB spirochaetes by DFM and nPCR in both maintained urban and wild areas of the park. Between urban and wild areas, there was a higher percentage of positive I. *ricinus* in maintained areas, but the difference was not significant. In a study from Germany (Bonn), where they compared the infectivity of ticks collected from 3 different types of areas (urban parks, private garden areas and suburban parks) they also found no statistically significant differences in infectivity between the different areas.

The ticks collected from W1 (63.93%) have the highest percentage of positives for *B. burgdorferi* s.l. . This area is highly favourable for the survival of *Ixodes ricinus*, as it occupies a site with dense vegetation forming small fragmented wooded areas, with different sized meadow patches. On the other hand, moisture from the river helps tick populations to survive and maintain the enzootic cycle of *B. burgdorferi* s.l. Forest fragmentation is one of the main factors considered favouring the development and spread of *I. ricinus*. This type of mosaic in vegetation increases the number of suitable habitats for different competent hosts, which also leads to an increase in tick-associated populations. This is supported by the fact that we recorded a significantly higher percentage of infected ticks from W1 compared to those collected from the area of the Srebrostrui hut (W2) and the Bohot forest (W4). Areas W2 and W4 are in woodland with less grassland, i.e. low fragmentation.

Of the maintained areas, U2 has the highest percentage positive *I. ricinus* for *B. burgdorferi* s.l. (57.58%). This is one of the most visited areas in PA "Kaylaka". The high infection rate can be explained by the fact that: 1) the area is highly fragmented; 2) the area contains restaurants that attract stray dogs as well as local animals (rodents and birds). Similar results for urban parks, with a high incidence of infection in local populations of I. *ricinus*, have also been found in Helsinki. The authors demonstrate that infected tick populations of *I. ricinus*, highly infected with *B. burgdorferi*, can exist even in urban environments not populated by large mammals. The case of zone U2 is similar, because despite the presence of large mammals (deer, wild boar) in the territory of the PA "Kaylaka", they enter the maintained areas extremely rarely.

The review by Herrmann and Gern (2015) discusses the impact of *B. burgdorferi* s.l. on the behaviour of *I. ricinus*. It is noted that infected ticks have a longer questing period and more resistance to desiccation. Since ticks caught by flagging are only questing ticks, the higher infectivity in zones W1 and U1 is most likely also due to this fact.

The high incidence of Borrelia-infected ticks potentially exposes people using this area for recreation to the risk of being bitten by an infected tick. Further research on the reporting of tick bite cases among visitors to PA "Kaylaka" may be useful here. In such high-risk locations, regular maintenance of lawns through treatment against blood-sucking arthropods, as well as installation of information signs about the presence of ticks, may be useful in reducing human exposure to ticks. Collecting data on tick populations and their infectivity with various pathogens in these types of suburban parks can help alert public health campaigns, especially if visitors in such areas have limited knowledge of ticks and the pathogens they can have

Although tick-borne infections have been the subject of intensive research worldwide, few studies have been conducted in Bulgaria. For the first time, this study addresses issues related to both the ixodofauna and the distribution of spirochaetes of the LB group in the Pleven region.

The current study provides a basis for further research involving the relationship between pathogens in ticks, microclimate and host distribution analysis. In this study, ticks of the species *Ixodes ricinus* are potential or recognized carriers of a wide range of animal/human pathogens (*Rickettsia* spp., *Anaplasma phagocytophilum*, *Coxiella burnetii*, *Bartonella clarridgeia*, *Babesia* spp., *Erlichia* spp., etc.), which reinforces the need to establish and implement control programmes in the area aimed at reducing both tick numbers and the impact of environmental factors conducive to tick development and distribution. Ecological analysis based on a geographical information system (GIS) allows the analysis of information on the specific suitable habitats for these tick species in the area, providing a useful basis for control interventions.

Mapping of ticks and Borrelia-infected vectors in the area would be a useful tool in decision-making by health authorities to determine the possible risk for specific transmissible pathogens. This study would also assist in the selection of adequate pest control strategies to protect public health.

# VI. SUMMARY CONCLUSIONS

1. A retrospective analysis of data from the National Statistical Institute and the Regional Health Inspectorate of Pleven shows that cases of tick-borne infections are registered annually in the Pleven region. The highest number of cases is of LB, and according to this measure Pleven ranks 9th in the country.

2. The field surveys carried out demonstrated the presence of various exophilic species of the ixodofauna, with *Ixodes ricinus* being the most widespread species and the main vector of LB in Europe.

3. The methods used for collecting ticks from their natural habitats and their mapping are suitable for assessing the distribution of *Borrelia burgdorferi* s.l. and appropriately describe the epizootic situation in the study area.

4. The established pattern of questing activity by I. *ricinus* that spans the spring-summer season with a peak in the months of April-May is unimodal:
   - The maximum host-seeking activity of I. *ricinus* was at 20ºC.
   - The highest number of ticks was collected at 60% relative humidity

5. The presence of spirochaetes of the Borrelia burgdorferi s.l. complex was detected in their vectors from different areas in the Pleven District.

6. New, previously undescribed foci with high densities of I. ricinus have been identified, in some of which tick infections have extremely high values for the carriage of spirochaetes.

7. When comparing areas where measures against ticks are applied with those of the natural type, no difference was found in the presence and distribution of ticks.

8. There was no difference in the infectivity of *I. ricinus* ticks with *B. burgdorferi* s.l. between the studied urban and wild areas.

9. GIS-based ecological analysis is suitable for summarizing information on specific habitats for tick species in the area, which can be used as a model for control interventions.

10. The assessment of the risk of infecting the population in the Pleven region based on the analysis of the infectivity of the examined ticks, as well as the creation of maps with data on identified foci with tick populations and their infection levels with B. *burgdorferi* s.l. could be successfully applied as a model and strategy for the management and prevention of the considered infectious diseases, as well as for complex studies in other regions of the country.

# VII. CONTRIBUTIONS

Based on the research conducted in this dissertation, the results obtained and their discussion, the following contributions can be stated:

## 1. ORIGINAL CONTRIBUTIONS

1. A comprehensive retrospective epidemiological, socio-demographic study of population samples with confirmed LB was conducted.
2. For the first time in the Pleven District, a survey of ixodid tick populations was conducted.
3. For the first time the frequency of questing ticks was used as an index to compare different *I. ricinus* populations.
4. For the first time in the Pleven District, I. Ricinus populations were examined for *B. burgdorfer*i sensu lato complex carriage.
5. For the first time in Bulgaria, the population density and frequency of Ixodes ricinus ticks was studied.
6. For the first time in Bulgaria, a questing female of Ixodes frontalis was captured and identified.

7. An original contribution of the study was the identification of *I. ricinus* ranges with extremely high infection rates with *B. Burgdorferi* s.l.
8. For the first time in Bulgaria, a comparison of tick populations collected from areas with regular maintenance of grasslands with natural unmaintained areas was performed.

## 2. APPLIED SCIENCE CONTRIBUTIONS

1. A risk assessment of the population in the Pleven area has been carried out based on an analysis of the infectivity of the examined ticks.
2. Maps have been created with data on the identified foci of tick populations and their infection with *B. burgdorferi* s.l.
3. The conducted study can be used as a model and strategy for the management and prevention of the examined infectious diseases, as well as for complex studies in other regions of the country.

## 3. CONFIRMATORY CONTRIBUTIONS

1. The epidemiological importance of *Ixodes ricinus* in the enzootic cycle of *Borrelia burgdorferi* sensu lato was confirmed.
2. The most common exophilic Ixodid ticks of medical importance for humans in the Pleven District were confirmed.
3. The unimodal activity pattern of *Ixodes ricinus* has been confirmed for the territory of Pleven District.
4. The transstadial transmission route of *Borrelia burgdorferi* s.l. was confirmed.
5. The influence of temperature and relative humidity on the activity of *Ixodes ricinus* ticks was confirmed.

# VIII. APPENDICES

## 1. LIST OF SCIENTIFIC PUBLICATIONS IN RELATION TO THE DOCTORAL THESIS

1. Blazhev A, Karcheva M, Tsenova A, Blazheva S, Kostov K. Serological tests for lyme borreliose. General Medicine. 2018;20(3):20-4. **URL:** https://www.scopus.com/inward/record.uri?eid=2-s2.0-85056886355&partnerID=40&md5=b1f59de2508382a8321e0545dfb2fbc9 **ISSN**: 1311-1817 **Q4**

2. Blazhev A, Karcheva M, Yordanova N, Kostovska M. Tick-borne infections in the pleven region - environmental and epidemiological aspects. General Medicine. 2018;20(1):30-5. **URL:** https://www.scopus.com/inward/record.uri?eid=2-s2.0-85047321879&partnerID=40&md5=28a2d98113bcaa719dd159ed08b0ca99 **ISSN**: 1311-1817 **Q4**

**3.** Blazhev A, Stanilov I, Miteva LD, Atanasova M, Blazheva S, Stanilova S. Prevalence of *Borrelia burgdorferi* Sensu Lato in *Ixodes ricinus* Ticks Collected from Kaylaka Park in Pleven, Bulgaria. Microorganisms. 2022 Apr 3;10(4):772. DOI: 10.3390/microorganisms10040772 **ISSN: 2076-2607; IF = 4.926; SJR = 0.86; Q2**

# 2. LIST OF PARTICIPATIONS IN SCIENTIFIC CONFERENCES RELATED TO THE DISSERTATION

## 2.1. Participation in scientific conferences in Bulgaria

1. A. B. Blazhev, M. D. Karcheva, M. A. Atanasova, K. S. Ilieva, S. O. Blazheva, K. G. Kostov. Study of the Hard Tick Populations in Pleven Region and Their Infectivity with *Borrelia Burgdorferi* Sensu Lato. XII National Conference on Medical Biology Tsigov Chark 8-10 September 2017
2. A. B. Blazhev, M. D. Karcheva, K. S. Ilieva, K. G Kostov Tick-Borne Infectious Diseases in the Pleven Region; XII National Conference on Medical Biology Tsigov Chark 8-10 September 2017
3. A. Blazhev, Ts. Doichinova, S. Blazheva, M. Atanasova, M. Karcheva Study of *Ixodes ricinus* populations in Pleven region for *Borrelia burgdorferi* infection s.l.. Third International Conference on Zoology, Zoonoses and Epidemiology October 21 – 23, 2019, Hissar, Bulgaria.
4. N. Emilova, A. Akisheva, P. Makaveeva, A. Blazhev, M. Karcheva. Sero Epidemiological Study Of Lyme Disease Susceptible Persons In Pleven Region. Black Sea Symposium for Young Scientists in Biomedicine 2018 with a Special Jubilee Session on Healthcare Management. April 12, 2018 – April 15, 2018. Medical University Prof. Dr. Paraskev Stoyanov, Varna.
5. A. Blazhev , S. Blazheva, Ts. Doichinova, M. Karcheva, M. Atanasova. The estimation scale of endangerment with tick attacks in Kaylaka park, Pleven. XIII Conference of Medical Biology Sept 13-15, 2019; Varna: Varna Medival Forum; 2019. p. 29-30.

6. A. Blazhev , S. Blazheva, Ts. Doichinova, M. Karcheva, M. Atanasova. Hot spots infested with Ixodid ticks (Acari: Ixodidae) around Pleven, Bulgaria and their role as vectors of Lyme borreliosis. JBCR. 2019;12 supl 2:140-1.
**7.** A. Blazhev, Ts. Doichinova, M. Karcheva, S. Blazheva, M. Atanasova. Hotspots infested with Ixodid ticks (Acari: Ixodidae) around Pleven, Bulgaria and their role as vectors of Lyme borreliosis. Jubilee scientific conference - 45 years of the higher school in Pleven. 31.10.2019 - 02.11.2019

### 2.2. Participations in scientific forums abroad

Blazhev A, Karcheva M, Blazheva S, Ilieva K, Atanasova M. Foci of hard ticks (Ixodidae) in areas with increased outdoor activity near to the town of Pleven, Bulgaria. 35th Balkan Medical Week 25th-27th September 2018 Athenes Greece

### 2.3. Science Projects:

№4/2017 Study of *Borrelia burgdorferi* Sensu Lato infection in ixodid ticks in Pleven region. MU-Pleven

№20/2019 Investigation of the distribution of *Borrelia burgdorferi* Sensu Lato among ticks of the species *Ixodes ricinus* in the protected area “Kaylaka” MU-Pleven