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Ticks fauna and identification and characterization of tick-borne pathogens in red fox populations (*Vulpes vulpes*) from Serbia

Doctoral Dissertation

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УНИВЕРЗИТЕТ У БЕОГРАДУ
БИОЛОШКИ ФАКУЛТЕТ

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Фауна крпеља и идентификација и карактеризација крпељима преносивих патогена у популацијама лисице (*Vulpes vulpes*) у Србији.

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Realization of the doctoral dissertation entitled: "Ticks fauna and identification and characterization of tick-borne pathogens in red fox populations (Vulpes vulpes) from Serbia" was conducted in cooperation of Group for Medical Entomology, Institute for Medical Research, University of Belgrade and Chair of Animal Ecology and Zoogeography, Faculty of Biology, University of Belgrade.

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Title: Ticks fauna and identification and characterization of tick-borne pathogens in red fox populations (*Vulpes vulpes*) from Serbia

Abstract: Ticks have a great importance in human and veterinary medicine but general knowledge about tick fauna in different hosts in Serbia is lacking. Since research on ticks that parasitize foxes has not been done systematically, the goal of this study was to research the tick fauna in Red Fox population and identify the most important tick-borne pathogens that can be transmitted to other mammals, including humans. For the purpose of this study 129 red foxes and 113 ticks parasitizing on them were collected. A total of six tick species was identified. The most abundant species was *Ixodes ricinus* (69%), followed by *I. hexagonus* (11.5%), *I. canisuga* (5.3%), *I. kaiseri* (5.3%), *Dermacentor reticulatus* (4.4%) and *Haemaphysalis concinna* (4.4%). Spleen samples from 129 collected red foxes were individually tested for presence of following tick-borne pathogens: Anaplasmataceae*,* *Hepatozoon* spp., *Babesia* spp., *Borrelia burgdorferi* sensu lato, *Rickettsia* spp., *Coxiella burnetii*, *Francisella tularensis* and *Bartonella* spp. DNA of pathogenic microorganisms was detected in spleen samples of 94 out of 129 animals (72.9%). DNA of *Hepatozoon* spp. was detected in 79 analyzed animals (61.2%). *H. canis* was identified after sequencing and analyzing the data*.* DNA of *Babesia* spp. was detected in 38 analyzed animals (29.5%). Presence of two *Babesia* species was confirmed: *B. vulpes* in 37 samples (28.7%) and *B. canis* in one sample (0.8%). *Borrelia burgdorferi* s. l. complex revealed presence of the pathogen in 7 samples (5.4%) from 5 localities. The obtained sequences from sequencing of 5S-23S rRNA intergenic spacer region confirmed presence of three *B. burgdorferi* s. l. species: *B. burgdorferi* s. s., *B. lusitaniae* and *B. garinii*. Finally *Candidatus* Neoerlichia sp. (FU98) was confirmed in 6 Red Fox individuals.

Key words: Ticks, tick-borne pathogens, Red Fox, *Vulpes vulpes*, *Hepatozoon* spp, *Babesia* spp, *Borrelia* spp, Anaplasmataceae

Scientific field: Biology

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Наслов: Фауна крпеља и идентификација и карактеризација крпељима преносивих патогена у популацијама лисице (*Vulpes vulpes*) у Србији

Abstract: Крпељи имају велики значај у хуманој и ветеринарској медицини, али генерално недостају информације о фауни крпеља код различитих домаћинима у Србији. Будући да истраживање на крпељима који паразитирају на лисицама није рађено систематски, циљ ове студије је био да истражити фауну крпеља у популацији лисице, и да идентификује најважније патогене који преносе крпељи, а који се се могу пренети на друге сисаре, укључујући и људе. За потребе ове студије прикупљено је 129 лисица и 113 крпеља који паразитирају на њима. Идентификовано је укупно шест врста крпеља. *Ixodes ricinus* (69%), је била најбројнија врста, и прате је *I. hexagonus* (11.5%), *I. canisuga* (5.3%), *I. kaiseri* (5.3%), *Dermacentor reticulatus* (4.4%) и *Haemaphysalis concinna* (4.4%). Прикупљени узорци слезине од 129 лисица појединачно су тестирани на присуство следећих патогена који преносе крпељи: Anaplasmataceae*, Hepatozoon* spp., *Babesia* spp., *Borrelia burgdorferi* sensu lato, *Rickettsia* spp., *Coxiella burnetii*, *Francisella tularensis* и *Bartonella* spp. ДНК патогених микроорганизама је детектована у узорцима слезине код 94 од 129 анализираних животиња (72.9%). ДНК пореклом од *Hepatozoon* spp. је детектована у 79 анализираних животиња (61.2%). *H. canis* је идентификован након секвенцирања и анализирања података. ДНК *Babesia* spp. је детектована код 38 анализираних животиња (29.5%). Потврђено је присуство две врсте *Babesia*: *B. vulpes* код 37 узорака (28.7%), и *B. canis* у једном узорку (0.8%). Присуство *Borrelia burgdorferi* s. l. комплекса је откривено код 7 узорака (5.4%) на 5 локалитета. Секвенцирањем добијених секвенци 5S-23S rRNA интергенског региона потврђено је присуство три *B. burgdorferi* s. l. врсте: *B. burgdorferi* s. s., *B. lusitaniae* и *B. garinii*. Коначно, *Candidatus* Neoerlichia sp. (FU98) је потврђена код 6 јединки лисица.

Кључне речи: крпељи, крпељски преносиви патогени, лисица, *Vulpes vulpes*, *Hepatozoon* spp, *Babesia* spp, *Borrelia* spp, Anaplasmataceae

Научна област: Биологија

Ужа научна област: Ентомологија

УДК број:

Contents

1. Introduction

Ticks (Acari: Ixodida) are temporal ectoparasites of amphibians, reptiles, birds and mammals. They are obligatory hematophagous and feed exclusively on blood of their hosts. About 900 species of ticks have been described so far (Estrada-Peña, 2015). Ticks are distributed in all parts of the world, from polar to tropical regions; however, despite broad distribution they are quite restricted concerning the habitat type they inhabit. It's usually a wooded area, high grass, meadow, leaf piles and forest litter, or any similar habitat that could provide suitable microclimate conditions.
Ticks have a pronounced importance in human and veterinary medicine. In addition to the direct effect of their parasitic behavior, attachment and ingestion of blood of the host on which they parasitize, ticks are far more significant as vectors and reservoirs of causative agents of viral, bacterial, rickettsial or protozoal diseases, both in humans and in domestic animals (Estrada-Pena and Jongejan, 1999). They are second to mosquitoes in their importance as arthropode disease vectors worldwide and vectors that transmit the highest variety of infectious agents. The worldwide economic loss due to direct and indirect effects of ticks is estimated to billions of dollars annually (Jongean and Uilenberg, 2004) and it's expected that overall impact of these ectoparasites will increase in the coming decades.

1.1. Ticks - general characteristics

1.1.1. Origin and taxonomy of ticks

The precise origin of ticks, both temporal and spatial, is difficult to estimate due to very limited fossil evidence (Fig. 1). The oldest useful examples occur mostly in amber originating in the Cretaceous period (Grimalde et al., 2002). Some authors place the origin of ticks in the part of Gondwanaland that become Australia approximately 390 million years ago, suggesting ancient amphibians as primordial hosts (Barker and Murrell, 2008). Another theory by Nava *et al.* (2009) proposed mid-Cretaceous period as time when ticks evolved on amphibians or reptiles as hosts.

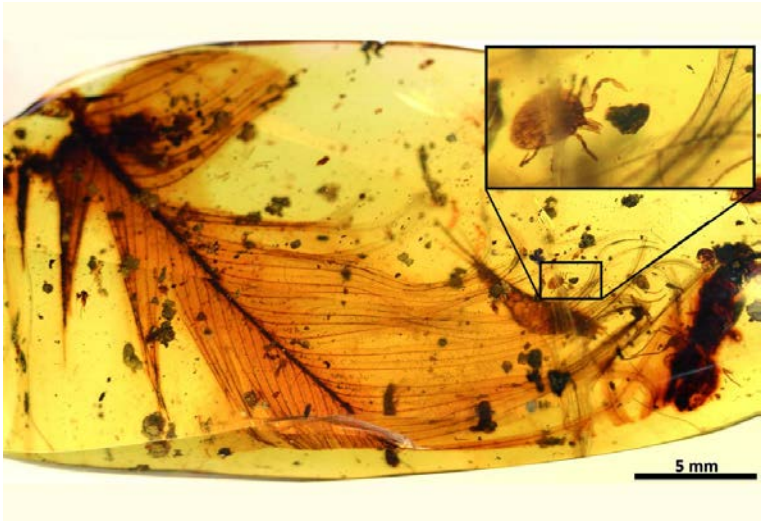


Fig. 1. About 99 million years ago, amber entombed a tick grasping a dinosaur feather. (Image: © Nature Communications/Peñalver et al.)

The taxonomic position of ticks is traditionally determined on the basis of their morphological, biological and ecological characteristics. Recently, with the introduction of molecular methods in the systematics and taxonomy, certain modifications to the previous classifications have been implemented. Molecular taxonomy has its place in cases when it impossible to rely on stabile morphological characters (damaged specimens, engorged ticks, immature stages), with morphologically similar species or when there are no experienced entomologists available to participate in the study. However, morphology-based taxonomy is still irreplaceable, and with addition of molecular tools will provide more homogenous and independent criteria for classification (Nava et al., 2009).
Ticks are classified into phylum Arthropoda, class Arachnida, subclass Acari, superorder Parasitiformes and order Ixodida (Nava et al., 2009) (Tab. 1).

Tab. 1. Classification of ticks

Regnum	Animalia
Phylum	Arthropoda
Classis	Arachnida
Subclassis	Acari
Ordo	Parasitiformes
Subordo	Ixodida

Almost 900 tick species have been described up to date, and they are divided into three families: Ixodidae (hard ticks), Argasidae (soft ticks) and the monospecific family Nuttallielidae, represented by the South African species of *Nuttalliella namaqua* (Fig. 2) (Guglielmone et al., 2010). These tick families differ in their morphology, diet and life cycle details.

Fig. 2. *Nuttalliella namaqua,* the single representative of family Nuttallielidae (Image: © Mans et al. 2011)

Fig. 3. Schematic illustration of representatives of families Argasidae and Ixodidae
Image: https://phil.cdc.gov/Details.aspx?pid=5993

Family Ixodidae, or hard ticks, includes 692 described species classified in two groups: *prostriata*, which includes genus *Ixodes,* and group *metastriata* with genera *Amblyomma*, *Bothriocroton*, *Haemaphysalis*, *Hyalomma*, *Rhipicentor*, *Nosomma*, *Margaropus*, *Dermacentor*, *Cosmiomma*, *Anomalohimalaya* and *Rhipicephalus* (Nava et al., 2009).
Hard ticks are easily distinguished from soft ticks by a number of discriminating characteristics (Fig. 3). The most obvious is sclerotized scutum on the dorsal part of the body, giving the ticks in this family their “hard” appearance. Capitulum in hard ticks is prominent and projects forward; in some species eyes are present on scutum, and they have a single relatively long blood meal per each life stage (Schmidt G & Roberts L., 2006).

Fig. 4. Life stages of hard ticks: *Ixodes scapularis* – female, male, nymph and larvae (left to right). Scale is in cm (Shapiro ED, 2014).

Sexual dimorphism is present in hard ticks. Scutum covers the whole dorsal part of the body in males, while in females, nymphs and larvae it reaches only to one third of the dorsal part of the body, enabling ingestion of large amounts of blood (Sonenshine, 1991). Certain hard tick species may be endophilic; however, most are exophilic and inhabit open habitats during the host-seeking period. They generally go through a three-host life cycle during which they take only one large blood meal at each stage (larva, nymph, adult) and then may remain attached to the host for more than seven days (Fig. 4). Females mate once in a lifetime, lay eggs after a meal, and then die. Males can take small amounts of blood, although in some species such as *Ixodes* sp. mating is possible without a previous meal in males so they usually don't feed (Parola & Raoult, 2001).

Family Argasidae or soft ticks are represented by 186 species classified in genera *Argas*, *Ornithodoros*, *Otobius*, *Nothoaspis* and *Antricola*. The body of soft ticks is covered with flexible cuticle lacking scutum and giving them "soft" appearance (Fig. 5). Capitulum is positioned more ventrally and is not visible from the dorsal view. Unlike the hard ticks, Argasidae are mostly endophilic. They spend most of their lives near their hosts (in bird and rodent nests, caves, underground pits, human settlements in rural areas, etc.) where they feed several times during their lives. They take a number of smaller meals while remaining attached to the hosts for a few minutes to a few hours (Gray, 2002). Females mate and lay eggs several times during their lives.

Fig. 5. Life stages of soft ticks: *Argas reflexus*a – unengorged females; b – unengorged males; c – nymphs (Buczek et al., 2018).

Due to the greater importance in human and animal health and the subject of this dissertation, the following chapters will be focused on family Ixodidae (hard ticks).

1.1.2. Morphology of hard ticks

The body of the tick consists of two regions – gnathosoma and idiosoma. Gnathosoma or capitulum bears mouth parts, while idiosoma, oval in shape, bears legs. Tick larvae have six legs while nymphs and adults have eight legs.
The capitulum of ticks is located in a cavity on the idiosoma. It consists of the basis capituli to which complex mouthparts are attached. The mouth parts are made of central rostrum, consisting of a hypostome and two chelicerae. Two palps are surrounding mouth parts (Schmidt G. & Roberts L., 2006). The basis capituli may differ in shape and in some species is a taxonomic character. In females, a pair of depressions with number of pores (called area porosa) is located in the dorsal area of bases of capituli. The secretory glands are located below area porosa, but little is known about the exact role of their secretions (Goethe et al., 1987). The two chelicerae are placed dorsally on the capitulum. They are mobile and their movements enable ripping and tearing of the the host's skin. Hypostome placed on the ventral side of the capitulum is dentated, which helps penetration and attachment of mouthparts into the skin of the host during feeding on blood. The chelicerae and hypostome form a narrow buccal canal, which transports blood from the host to the pharynx of the tick. Two palps surrounding the rostrum are covered in setae topped with chemo- and mechanosensory receptors that form a sensory field. On ventral and medial parts they are long and stout, protecting the mouthparts (Fig. 6) (Soneshine 2014).

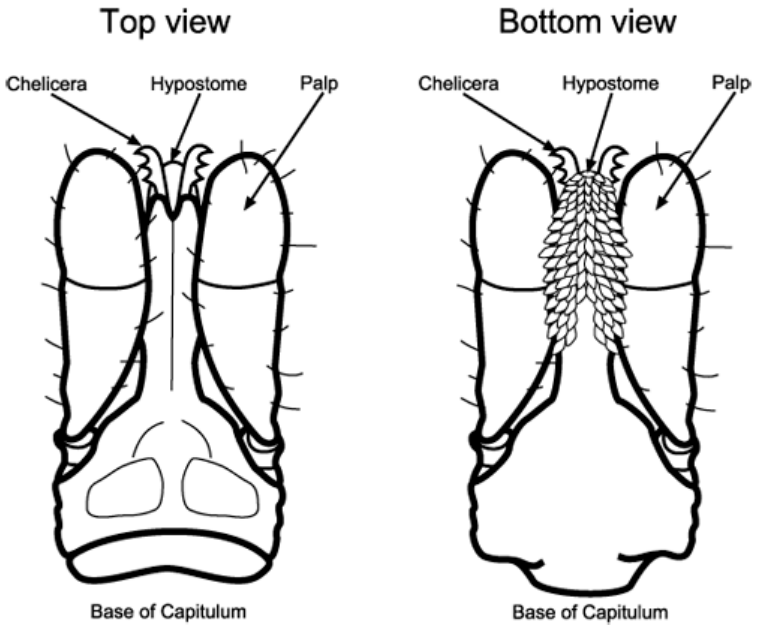


Fig. 6. Generalized mouthparts of a hard tick, based on a species of *Ixodes*. Illustration by: Scott Charlesworth, Purdue University.

The idiosoma of ticks consists of an anterior podosoma and a posterior opisthosoma. Ventral side of podosoma bears legs, along with the genital pore in adults. The dorsal side of podosoma is covered by scutum in immature stages and females, while in males the scutum covers the dorsal side of the entire idiosoma. In some genera simple eyes are present and placed along the lateral parts of the scutum. Aloscutum is located posterior to the scutum. It is flexible with superficial folds of cuticula and enables increasing of body size during ingestion of blood. The ventral side of the opisthosoma bears spiracles, openings of the respiratory system, the anus, grooves, plates, and other body structures (Fig. 7) (Soneshine 2014).

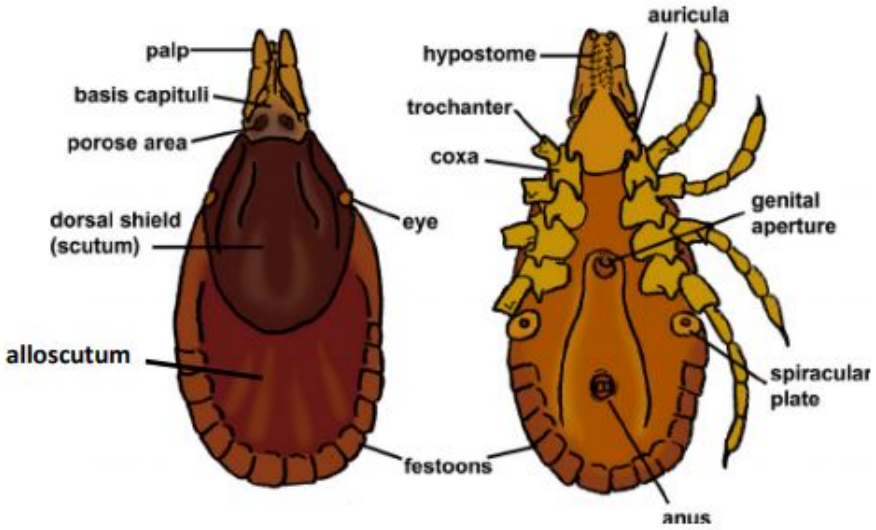


Fig. 7. Generalised morphology of a hard tick (Ixodidae). Image from Mathison and Pritt (2014)

Due to their parasitic behavior, ticks have developed specific morphological characteristics, mostly regarding the mouthparts and the digestive system. One of their adaptations to hematophagy and prolonged feeding is a special type of saliva produced in salivary glands. Tick saliva is a complex mixture of proteins, lipids and number of non-protein components originating from salivary glands and haemolymph of ticks, with vasodilatory, antihaemostatic and immunomodulatory roles (Mihaljica 2017). In some ixodid ticks, saliva product participates in the secretion of the cement which enables prolonged attachment of tick to the host (Fawcett *et al.*, 1986). Additional role of saliva, closely related to parasitic behavior and significant for vector role, is assistance in pathogen transmission to the host (Fawcett *et al.*, 1986; Wikel, 1999; Sauer *et al.*, 2000; Bowman and Sauer, 2004).

Digestive system of ticks starts with a buccal canal where the blood intake and outflow of saliva take place. Food is transferred to the muscular pharynx and strong pumping motions move host's blood to the esophagus and then to the midgut. The midgut fills most of the tick's body cavity; it has a central chamber and numerous diverticula that enable ingestion of large amounts of blood. The digestion surface is significantly increased and midgut is capable of peristaltic movements. Undigested food is transferred through short intestine to the rectal sac, where feces is mixed with products of the Malpigian tubes before elimination through the anus (Mehlhorn 1988).

1.1.3. Host specificity and life cycles of ticks

Unlike some other blood-feeding organisms, ticks are obligately hematophagous. They feed on a wide range of mammal, bird, amphibian and reptile species. Ticks show high diversity in levels of host specificity: some species including *Ixodes ricinus* are generalist and feed on more than 300 vertebrate species (Anderson, 1991), while others such as *Rhipicephalus sanguineus* are more specialized and feed usually on dogs, but in absence of primary hosts they may feed on other species. Strict specialists include species such as *Ixodes simplex* that parasitize only on bats, mostly only on bat species *Miniopterus schreibersii* (Kolonin 2007). Host specificity is a result of coevolution, and tick species coordinate their seasonal or daily dynamics with that of their hosts in order to increase probability of contact. Ticks unwillingly feed on non-primary hosts and in that case take smaller amounts of food which might affect the reproduction process. The specialization is mostly determined by the size of the host, as larva and nymph feed on smaller animals while adults parasitize bigger ones (Loye & Lane, 1988). The choice of the host is limited by the hunting position of tick, depending on drought tolerance and available energy of the ectoparasite (Rechav, 1979).

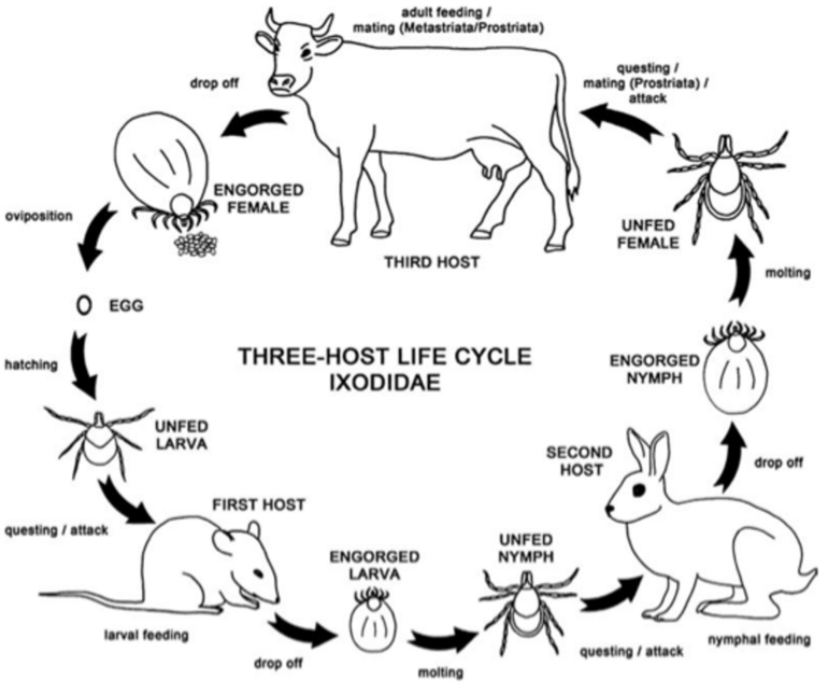


Fig. 8. Three host life cycle of hard ticks (Ixodidae) (Soneshine, 2014).

During their life cycle, hard ticks may feed on one, two or three different hosts. Depending on the number of hosts ticks feed on, they have one-, two- or three-host life cycle.
The majority of hard ticks have three-host life cycle. After hatching from the egg, larvae attach to the first host, feed until depletion, drop off the host and molt into nymphs. The unfed nymphs attach to second host, feed, drop off and molt into adult ticks. Adults quest for the third host, to which they attach, mate and females feed until depletion, digest food, lay eggs and die. Females can lay as much as 20000 eggs during several weeks. The next

generation of ticks starts with larvae hatched from the eggs. They stay close to the place the female has chosen and then seek for their first host (Fig. 8).
Many species belonging to the genera *Amblyomma, Anomalohimalaya, Bothriocroton, Haemaphysalis*, *Ixodes, Rhipicephalus, Dermacentor* and *Hyalomma* are obligate three-host ticks.
For some species the entire life cycle is completed on two or even a single animal (two- or one-host life cycle). Number of species from the Metastriata group have two- or one-host life cycle.
In the two-host life cycle, larvae remain on the host after feeding, molt to the nymph and feed on the same host. After feeding the nymph drops off and molts to the adult that quests for the second host to mate and feed on (Fig. 9). In the case of one-host ticks, engorged larvae and nymph stay on the same host and after adult tick completes the blood meal it drops off the host (Fig. 10) (Soneshine 2014).

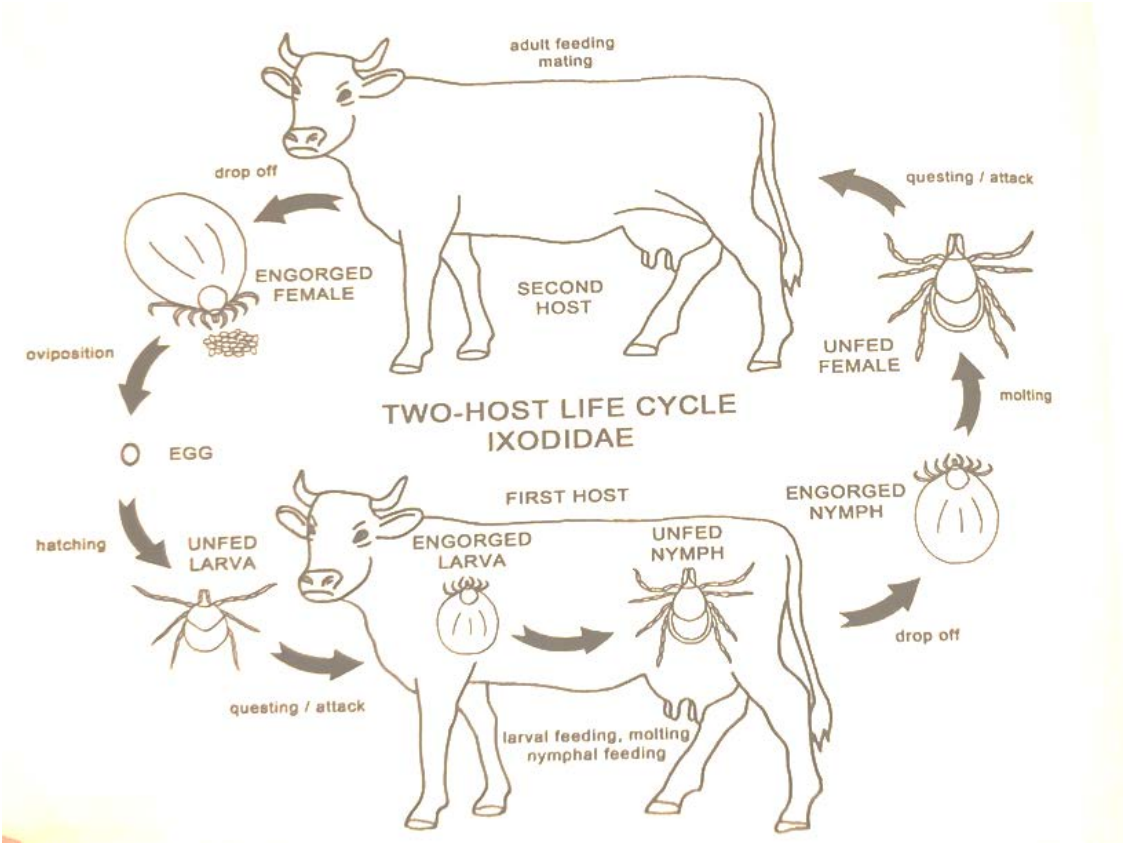


Fig. 9. Two-host life cycle of hard ticks (Ixodidae) (Soneshine, 2014).

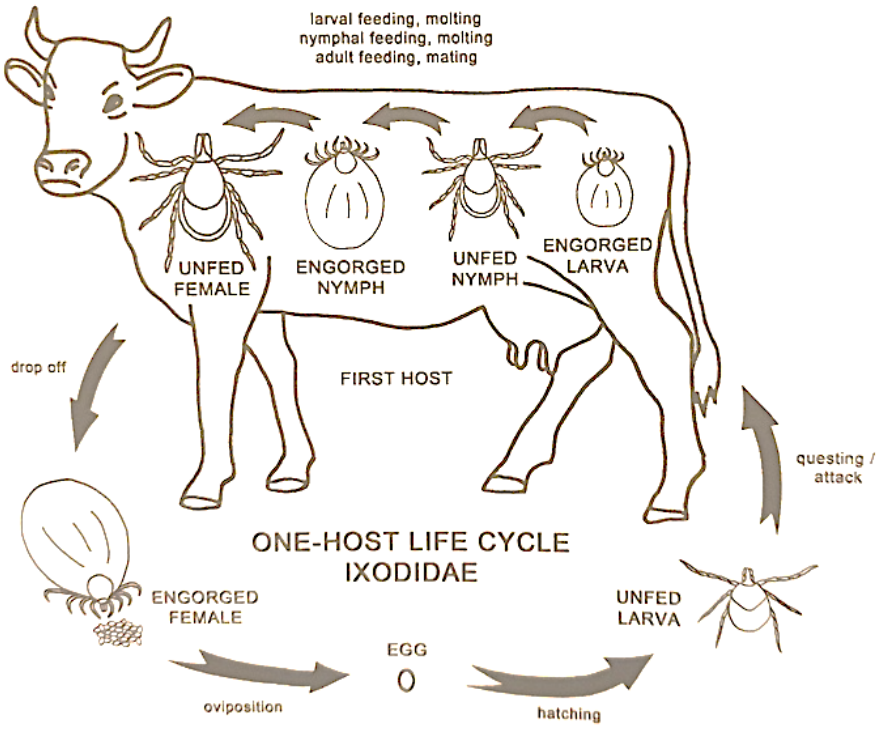


Fig. 10. One-host life cycle of hard ticks (Ixodidae) (Soneshine, 2014).

Hard ticks feed only once in each life stage, with the exception of males of some species belonging to prostriata group, which don't feed at all or rarely take small amounts of blood. Reproduction is closely related to feeding, and mating occurs on hosts in most cases (Milutinović *et al.*, 2012). Larvae of ixodid ticks feed usually for 2-4 days, nymphs for 4-6 and females for 5-15 days. Depending on the geographic region and climate conditions life cycle is usually complete within 2-3 years, but could last for up to 6 or more years (Kettle, 1984).

1.2. Ticks as ectoparasites

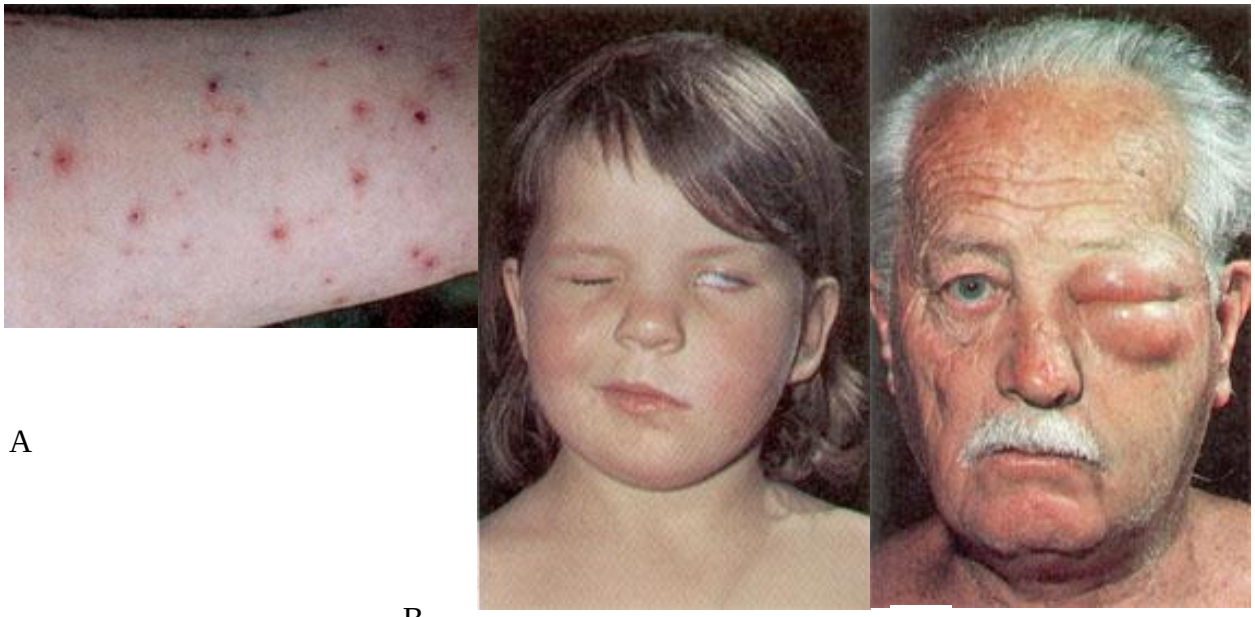
1.2.1. Direct effects of tick infestations

Parasitic behaviour of ticks may cause direct harmful effect on the host by feeding on blood. The direct effects of ticks are manifested as mechanical damage, local irritation, anemia, paralysis and intoxication, and in some cases secondary infections at the bite site caused by bacteria or fungi. It was noticed that saliva of certain tick species from North America (*Dermacentor andersoni, D. variabilis*), South Africa (*Ixodes rubicundus*, *Rhipicephalus eversti eversti*) and Australia (*I. holocyclus)* contains toxins causing various forms of paralysis or even death in livestock and humans (Mans et al., 2004). Massive infestations with ticks are frequent in wild and domestic animals and may cause anemia,

weight loss and reduction in the milk production and quality of hides, which all lead to high economic loss (Fig. 11). The majority of tick species are host-specific and tick-host relationship is the product of coevolution. Resistance of animal species to bite of specific tick species is genetically determined and depends on the capability of developing an effective immunological response to infestation. This is particularly important in the case of one-host species like *Boophilus* spp. While massive infestations with *Boophilus* ticks severely affect European cattle (*Bos taurus*) they have limited effect on Zebu cattle (*Bos indicus*), so it is more economical to use *Bos indicus* breeds (pure or cross-bred) instead of European cattle in areas where *Boophilus* ticks are abundant (Gothe, 1999).

Fig. 11. Massive infestation of cattle with ticks *Boophilus decoloratus.*
Photo credit: Anipedia, www.anipedia.org: JAW Coetzer and P. Oberem (Directors) In: *Infectious Diseases of Livestock*, JAW Coetzer, G.R. Thomson, N.J. Maclachlan and M-L. Penrith (Editors). F. Jongejan and G. Uilenberg, Vectors: Ticks, 2018.

Massive infestations in humans are rare and tick bites are usually individual. In most cases, due to anesthetic components of saliva, tick bites go unnoticed. However, they may be painful in species with very long mouth parts, such as the representatives of genera *Ixodes*, *Hyalomma* and *Amblyomma*, which penetrate much deeper than ticks with short mouth parts, such as the representatives of genera *Dermacentor*, *Haemaphysalis* and *Rhipicephalus*. Tissue damage caused by penetration of the parasite's mouth apparatus is not always easy to distinguish from damage caused by host's immune response (Fig. 12). The lesions may complicate if tick mouth parts are damaged during the manual removal and remain at the site of bite. In most ticks this rarely happens but in removal of females from genus *Ixodes* it may happen even in over 50% of cases.



A B C

Fig. 12. Direct effects of tick bites on humans. A-Local irritation on the site of tick bite, B - tick paralysis, C - severe allergic reaction to tick bite (Moorhouse, 1981).

1.2.2. Importance of ticks as vectors of pathogens

In addition to the evident direct effects on hosts, ticks are far more important as vectors of causative agents of disease in humans and animals. They can act as mechanical or biological vectors of pathogens, while for some infective agents they are reservoirs as well. Pathogens are sustaining and reproducing in ticks and transmit transstadialy to the next life stage, or transovarialy from females to eggs to the next generation (Urquart *et al.*, 1987). Ticks transmit a higher number of pathogenic microorganisms (protozoans, bacteria, viruses) than any other group of arthropode vectors (Balashov, 1972; Jongejan and Uilenberg, 2004).

The global importance of ticks as vectors is reflected in their wide distribution, diversity and complexity of pathogens they may transmit, as well as in the high impact on veterinary and human medicine. The specific biology and life cycle contribute to their extraordinary vector potential, and there is not a single molecular, cellular, physiological, anatomical or behavioral characteristic of ticks that does not contribute to their role as parasites and vectors.

Ticks as parasites of humans were already described in Ancient Greece (Sonenshine, 1991), while today over 30 species of ticks are known to parasitize on humans (Estrada-Peña and Jongejan, 1999). In the early 20th century it was determined that ticks may be vectors of bacterial diseases in humans (Dutton & Todd, 1905). It is estimated that approximately 10% of described tick species are vectors of pathogens causing various human and animal diseases (Jongejan and Uilenberg, 2004). After the discovery of Lyme disease, which is presently considered the most important vector-transmitted disease in Europe and North America, and its causative agent, the spirochete *Borrelia burgdorferi* sensu lato, during the 1980s (Johnson *et al.*, 1984), the studies on tick-borne diseases

were intensified and a significant number of expanding tick-borne pathogens have been recorded since (Vorou *et al.*, 2007).
Ticks differ from other arthropod vectors in various ways and they open up habitats for pathogens in areas where, for example, mosquitoes are not able to transmit diseases due to constraining abiotic factors (L'vov and Gostinshchikova, 1970). Their low mobility forces them into a way of life which makes them more vulnerable to a wide range of changing climatic conditions (questing on vegetation) or to specialized protective host responses (living in or near nests or the burrows of their hosts) (Randolph, 1998). They usually invest less energy in finding a host than flying vectors but can use their energy reserves to survive for relatively long periods, in some cases for many years (Oliver, 1989). Compared with other arthropod vectors, they also use more energy in the uptake of a usually large blood meal, which enhances the tick's potential as a vector (Randolph, 2004a). Unlike other blood-feeding arthropods, with some exceptions, every life stage of a tick feeds only once before moulting or oviposition (female argasids feed several times and lay eggs after each blood meal), but feeding occurs over a prolonged period, in some ixodid species up to 14 days (Kröberand Guerin, 2007). While insect vectors approach many different hosts, Argasidae feed on very few hosts and members of the Ixodidae on a maximum of three individual hosts per lifetime (Oliver, 1989). In order to use a tick as a vector, a pathogen must survive transstadially (from one life history stage to the next) or, more rarely, trans-ovarially from female to egg. Thus the pathogen has to maintain itself through the tick's developmental phases, which can last several months to years depending on the species and environmental conditions, and then through transmission to a new host. This means that a pathogen depends heavily on the development, survival and reproductive rate of its tick vector and the tick's developmental, stage-specific host relationships (Randolph, 1998).
Although certain soft ticks (Argasidae) transmit causative agents of diseases including tick-borne relapsing fever, African swine fever etc., the ticks from the family Ixodidae (hard ticks) have a far greater importance for human and veterinary medicine (Gray, 2002).

1.3. Ecology and epidemiology of tick-borne diseases

Many tick-borne diseases are natural focal infections, characterized by endemism and seasonal occurrence. In nature, the pathogens causing a tick-borne zoonosis are sustained by enzootic cycles, including not only pathogens but also vectors and wild animals as vertebrate hosts. In most cases, ticks, pathogens and wild vertebrate hosts have co-evolved forming an equilibrium, however domestic animals and humans may sporadically enter these cycles as dead-end hosts (Fig. 13)(Jongejan and Uilenberg, 2004).

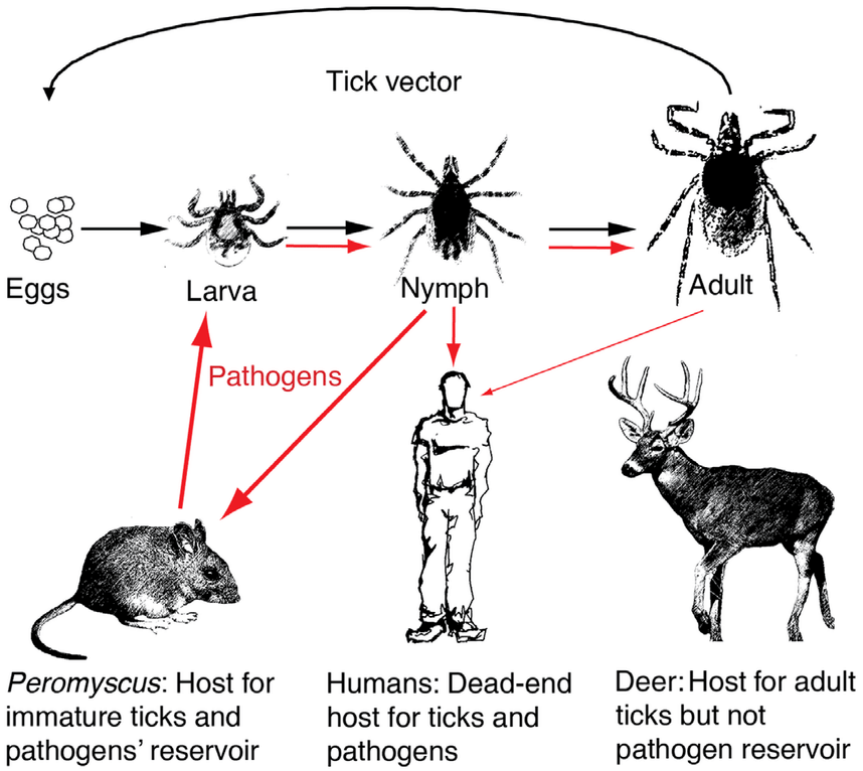


Fig. 13. Enzootic cycle of tick-borne pathogens (Long et al., 2019)

Transmission of the pathogen and its maintainance in nature are closely related to feeding of ticks. Ticks cause infection while feeding on infected animal and with transstadial and transovarial transmission. Infected tick can pass infection to the next host, in the case of ixodid ticks only during the next feeding at the subsequent life stage. Transstadial transmission is in the base of vector role of ixodid ticks (Randolph, 2004). In most cases hosts need to develop systemic infection in order to transmit the pathogen to ticks that feed on them. However, transmission of certain pathogens is possible even on hosts that don't develop systemic infection. This phenomenon called „cofeeding" transmission is described for *Borrelia burgdorferi* sensu lato, Crimean-Congo hemoragic fever, tick-borne encephalitis and some other viral diseases, and takes place between infected and uninfected ticks closely feeding on the host before it develops a systemic infection (Randolph et al., 1996, Raoult and Roux, 1997).

The main factors determining epidemiology of tick-borne diseases are the infection rate of reservoirs and ticks, as well as the population size of host species. They are caused by physiological and ecological factors, including: specificity of various stages of tick life cycle toward different hosts, duration of period of attachment to the host, environmental conditions as well as the state of host's immunity system (Lane, 1994; Mather and Howard, 1994). Spread of tick-borne diseases depends on zoogeographic distribution of both tick vectors and host reservoirs (Korch, 1994). Active dispersion of ticks is limited to distance of approximately 50 m (in the case of *Ixodes ricinus* 5 m), but ticks may travel passively, attached to hosts, to much greater distances. If an infected tick appears in a new habitat, the possible survival of the pathogen in the new environment depends on finding an adequate host (susceptible to infection, able to reproduce and transfer the pathogen to the new vector) (Sonenshine, 1991, 1993). It is important to note that a

zoonosis, and particularly tick-borne one, is a result of a synergy of two independent phenomena - risk caused by natural enzootic cycles and exposure of humans and domestic animals to that risk. The epidemiology of tick-borne diseases is influenced not only by the complex biology and ecology of pathogens, vectors and hosts, but also by anthropogenic activities. Humans directly influence tick dispersion by altering the conditions in their habitats (cultivation, timbering etc.) or transporting them to greater distances together with host animals. The increase in visits to areas inhabited by ticks leads to increase in number of tick bites and expansion of tick-borne diseases, particularly tick encephalitis and Lyme disease (Randolph, 2001). Monitoring the fluctuations of social, ecological, technological and microbiological factors influencing the expansion of tick-borne diseases is of crucial importance in defining the strategy for prevention, control and eradication of these diseases.

1.4. Tick–borne zoonoses

It is often assumed that all tick-borne diseases are zoonotic while anthropogenic diseases including mosquito-borne diseases such as malaria, caused by *Plasmodium falciparum* and *Plasmodium vivax*, do not appear to have evolved into zoonoses. The possible exception is the relapsing fever pathogen, *Borrelia duttoni*, which was long considered to be anthropogenic. Recent evidence, however, has shown that this species can infect domestic pigs and chickens and that it is probably also zoonotic (McCall *et al.,* 2007). Tick-borne diseases have been known since the second half of the 19th century (Hoogstraal, 1967; Hoogstraal, 1977) and represent some of the world's most rapidly expanding arthropod-borne diseases. Although known for many years, tick-borne encephalitis and Rocky Mountain spotted fever used to be either distributed locally and/or showed a low prevalence of infection (Wolbach, 1919; Chumakov and Seitlenok, 1940). The major impact of tick-borne diseases on public health in Europe and North America first became evident with detection of *Borrelia burgdorferi* as the causative agent of Lyme disease in the 1980s (Burgdorfer *et al.*, 1982; Granström, 1997) and the recognition of its medical significance, wide distribution and high prevalence (O'Connell *et al.*, 1998; Sood, 2002). Since then the number of recognized, medicinally important tick-borne diseases has increased significantly, undoubtedly due to the stimulus generated by the impact of Lyme disease. More than ten Rickettsia species pathogenic to humans have been described since 1984, and the notifiable tick-borne diseases in the United States of America increased from two in 1990 to five in 1998 (Paddock and Telford, 2011). Not only is the number of newly recognized tick-borne diseases increasing, but also the number of case reports. For instance, the reported numbers of Lyme diseases in the US increased by 101% in a 14 year period between 1992 and 2006 (Bacon *et al.*, 2008).

1.4.1. Viral tick-borne diseases

Ticks play an important role in the epizootiology and epidemiology of several viral tick-borne diseases circulating in Europe. As an intracellular agent, virus replication is closely connected to the host cell and can be maintained by transovarian transmission in ticks until the fourth generation without any contact with infected vertebrates (Mehlhorn, 1988;

Milutinović, 1992). Therefore, the epidemiology of these diseases is closely related to the ecology and biology of the vector species (Randolph *et al.,* 2000).
Ticks are natural vectors of tick-borne encephalitis virus (Tick-Borne Encephalitis-TBE) from the genus Flavivirus. Based on serological and molecular differences, three types of this virus have been registered: Central European, Siberian, and Far Eastern (Walner *et al.,* 1996; Ecker *et al.,* 1999). *Ixodes ricinus* as a dominant tick species throughout Europe is the most important vector of the Central European subtype, while *I. persulcatus*, a vector of the Siberian and Far Eastern subtype of TBE virus, is widespread in the wooded areas of the Urals, Siberia and Far Eastern Russia. It has been diagnosed in most European countries: France, Switzerland, Germany, Denmark, Norway, Sweden, Finland, Estonia, Lithuania, Latvia, Poland, the Czech Republic, Slovakia, Austria, Slovenia, Croatia, Bosnia, Serbia, Greece, Romania, Ukraine and Russia (Charrel *et al.*, 2004). Nuttall and Labuda (1994) pointed to the species *Clethrionomys glareolus* and *Apodemus flavicollis* as the most important reservoirs, although it is assumed that wild carnivores and some domestic ruminants play an important role in maintaining this virus in nature and its transmission to humans.
Louping ill virus (LIV), another member of the genus Flavivirus, causes encephalitis in sheep in the UK. Variants of this disease are also found in other European countries (Charrel *et al.*, 2004). In natural conditions, the infection mainly occurs in risk groups that are in direct contact with infected animals (farmers, veterinarians, etc.), although cases of the disease have been reported after tick bites. The principal vectors are *Ixodes ricinus* ticks.
Crimean-Congo hemorrhagic fever (CCHF) virus, which is also transmitted by ticks, belongs to the genus Nairovirus within the Bunyaviridae family. The CCHF virus is widespread throughout Africa, the Middle East, and Central and Southwest Asia. It was also found in some parts of Europe: Russia (Hoogstraal, 1979), Bulgaria (Vasilenko, 1973), Greece (Antoniadis and Casals, 1982), Serbia (Papa *et al.*, 2002b; Drosten *et al.*, 2002) and Albania (Papa *et al.*, 2002a).The CCHF virus survives transstadially and intersezonally in several tick species, and is transmitted transovarially by members of the *Hyalomma marginatum* complex. The 27 species and subspecies of ticks, reservoirs, and CCHF vectors include species of the genera *Boophilus* and *Hyalomma*, ticks of the *Hyalomma marginatum* complex, *H. anatolicum* (Koch, 1844), and *Rhipicephalus bursa*. Ticks of the genera *Haemaphysalis*, *Amblyomma*, *Dermacentor*, *Hyalomma*, and *Rhipicephalus* are mainly responsible for maintaining the enzootic focus of the CCHF virus circulation among ticks and wild and domestic animals (Kettle, 1984).
Russian spring-summer encephalitis (RSSE virus, Flavivirus group B) causes a complex of viruses of wide geographical distribution from East Germany to Siberia and the Far East. It is mainly associated with *Ixodes persulcatus*, but also with *Haemaphysalis concinna* (Santos Dias, 1963) and other tick species, and can be transmitted through other arthropods. In endemic areas such as wooded taiga, over 50% of the population may have antibodies without symptoms, while newcomers to these areas show clinical symptoms.
The causative agent of Powassan encephalitis is POWE virus of the genus Flavivirus group B, first isolated in 1958 in Canada. Cases of patients have also been registered in Russia, where *Ixodes persulcatus* and ticks of the genus *Haemaphysalis* (Gould *et al.*, 2001) appear as vectors.

1.4.2. Protosoan tick-borne diseases

Ticks are vectors of several diseases caused by protozoa. Among them, the most important are babesiosis infections that occur primarily in domestic animals (cattle, horses,

dogs). Infections of humans, which are increasingly registered, are caused primarily by species of the genus *Babesia*, while in animals the following species are also important: *Aegyptianella* and *Theilaeria*. *Ixodes ricinus* is the primary vector of *Babesia divergens*, a blood parasite and causative agent of bovine babesiosis (Joyner and Davis, 1967). The first case of human babesiosis caused by this causative agent was described in the former Yugoslavia in 1957 (Škrabalo and Deanović, 1957). Newly diagnosed cases are rare, usually in persons whose spleens were removed, and are generally fatal (Gray, 1991). A far more common cause of human babesiosis is *Babesia microti* whose vector is *Ixodes scapularis*. Several hundred cases of this disease occur annually in North America (Kjemtrup and Conrad, 2000). Using American and European isolates of *Babesia microti* HK, Gray *et al.* (2002) proved that *Ixodes ricinus* can be a successful vector of this pathogen in Europe. However, cases of human babesiosis caused by *Babesia microti* have not been registered in Europe so far.

1.4.3. Bacterial tick-borne diseases

Ticks have been known as vectors of human bacterial diseases (Rocky Mountain fever, relapsing fever, Q fever, tularemia) since the early 20th century. Their true importance to human health, in North America and Europe, was recognized by the discovery of *Borrelia burgdorferi* as the cause of Lyme disease in 1982. Rickettsiae are caused by obligate intracellular bacteria belonging to the genus *Rickettsia* and are among the oldest known arthropod-borne diseases (Sonenshine, 1993). The most well-known rickettsial disease is Rocky Mountain spotted fever, which is caused by *Rickettsia rickettsi*. It was first described as a disease in 1899 by Maxey, and *D. andersoni* was diagnosed as a vector ten years later (Riketts, 1909). It is based in Canada and North America. Boutonneuse fever was first described in 1910 in Tunisia (Conor and Bruch, 1910), and is caused by *R. conori*, which is widespread in Africa, the Mediterranean and parts of Southeast Asia.
Q-fever was first described in Australia in 1935 as a disease of unknown etiology (Marrie & Raoult, 1997). The causative agent of the disease (*Coxiella burneti*) was determined in 1937. This anthropozoonosis was later described in the United States and the Balkans, and today it is registered in more than 60 countries. Rabbits, rats and foxes are the most common reservoirs of this infection outside the settlements, and cattle and sheep in the rural environment. Liebisch (1980) considers that there are three epidemiological zones of Q-fever in Europe. In addition to the species *Dermacentor marginatus*, according to this author, the causative agents of Q fever are also transmitted by the following tick species: *Haemaphysalis punctata*, *Rhipicephalus sanguineus*, *Rhipicephalus bursa*, *Hyalomma marginatum* and *Hyalomma anatolicum*. The most important diseases caused by bacteria and transmitted by ticks are Lyme disease, caused by spirochaetes belonging to the *Borrelia burgdorferi* s.l. complex, human granulocytic anaplasmosis caused by *Anaplasma phagocytophilum* and tularemia caused by *Francisella tularensis*.

1.5. Red Fox (*Vulpes vulpes*) as a host for ticks and tick-borne pathogens

Among the twelve species of genus *Vulpes,* the Red Fox is the largest species. The Red Fox has an elongated body, relatively short legs, elongated and relatively narrow head with long ears (Fig. 14). Canines are slender, relatively long with concave rather than convex profiles, and they protrude from the mouth over the lower jaw. The forepaws have five digits, while the hind feet have only four and lack dewclaws. Fur has long guard hairs and dense, short, silky soft, pale grey undercoat. Although color of the Red Fox fur may show various color morphs, typical color is bright reddish-rusty with tinge of yellowish-brown. Coat on dorsal side of the body (stomach, chest and neck) always has lighter shades – from whitish to light gray color. The backs of the ears are dark brown or even black, while on the inner side very short hears are almost white. The tip of the tail and its central part has very long, bushy reddish-brown hairs. Tip of the tail is always white (Novikov, 1956; Geptner and Naumov, 1967).
As one of the most widely distributed members of the order Carnivora, body size and body weight vary significantly across distribution range. Research has shown that craniodental charactesistics vary significantly across the wide range of the species. These variations are affected by biogeographical and climate conditions and correspond to the Bergman's ecogeographical rule (Churcher, 1960; Davis, 1977; Meiri *et al.*, 2004; 2007; Szuma, 2008a; 2008b).

Fig. 14. Red Fox (*Vulpes vulpes*) (https://en.wikipedia.org/wiki/Red_fox#/media/File:Fox_-_British_Wildlife_Centre_(17429406401).jpg)

The variation in average body length (with head) across the species range is from 44 cm to 90 cm, while tail is 30-55 cm long. Shoulder height is in general between 35 cm and 45 cm. Adult animals have body mass up to 10 kg. Sexual dimophism is quite prominent. Males are 8-10% larger and heavier than females. For example, body weight of adult males is between 6 kg and 10 kg, while in females it ranges between 5 kg and 8 kg (Wandeler and Lüps, 1993; Macdonald and Reynolds, 2008). Head and skull are elongated with a relatively narrow nasal region. Zigomatic width is between 64 mm and 87 mm, while condilobasal length in both sexes varies between 115 mm and 165 mm (Novikov, 1956; Geptner and Naumov, 1967).

The Red Fox is a very mobile and agile animal. Its usual speed is 6–13 km/h, but it may run at speed of 50 km/h. Animals are capable of jumping over 2-metre-high fences or other barriers. Red Foxes are also good swimmers (Hoffmann and Sillero-Zubiri, 2016).

1.5.1. Ecology of Red Foxes

The Red Fox (*Vulpes vulpes*) is a medium-sized carnivore species which is characterized by one of the largest ranges in mammals. The native range includes almost entire Holarctic except for extreme boreal zone on the North and the extreme deserts (Fig. 15). In Europe, this species is present on large Mediterranean islands such as Cyprus, Sicily, Sardinia and Corsica (Mitchell-Jones *et al.*, 1999; Macdonald and Reynolds, 2008). Red Fox was reintroduced to several areas. At the beginning of 19th century Red Fox was introduced to Australia and in the late 1990s to Tasmania (Caley *et al.*, 2015; Hoffmann and Sillero-Zubiri, 2016). In South America this species was introduced only to Falkland Islands but its status here is still unclear (Hoffmann and Sillero-Zubiri, 2016). In North America species is reintroduced into the range of existing native populations. As European haplotypes were not found within the North American Red Fox populations at later date (Statham *et al.*, 2012) this introduction cannot be considered successful. The present total range was estimated to 70 million km^2. Regarding the altitudes, Red Fox lives from the coastal areas up to 4500 m a.s.l (Hoffmann and Sillero-Zubiri, 2016).

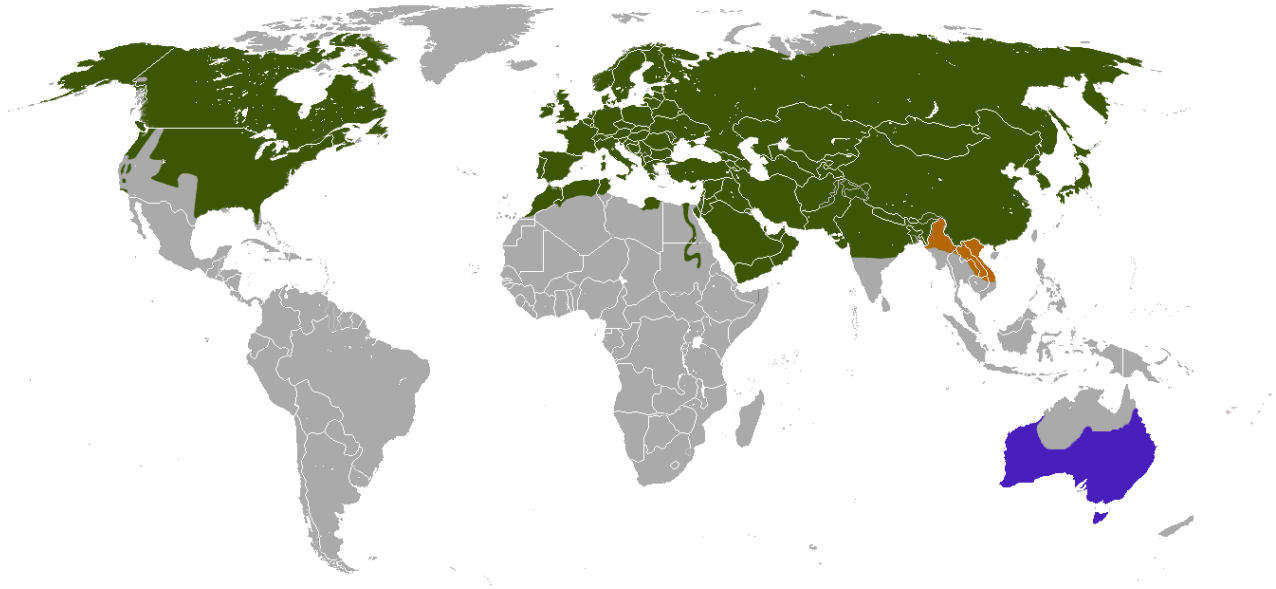


Fig. 15. Red Fox (*Vulpes vulpes*) range map: green = native, purple = introduced, orange = presence uncertain (https://en.wikipedia.org/wiki/Red_fox#/media/File:Wiki-Vulpes_vulpes.png)

Inside its vast range, the Red Fox inhabits very diverse habitats and ecosystems – from deserts in the South to forests, steppe and riparian ecosystems in the central part of its distribution and tundra and taiga in the North. In addition, Red Fox is particularly abundant

in anthropic transformed ecosystems and habitats, such as agricultural and urban areas. In Europe, the greatest abundance in populations is present in steppe, forest-steppe and mixed-forest biome (Lloyd, 1980). High densities are also present in mixed (ecotone) habitats. Red Fox is the commonest predator in urban (Harris, 1977; MacDonald, 1987; Scott *et al.*, 2014; Wandeler and Lüps, 1993) or suburban areas, particularly in agricultural landscape in vicinity of human settlements. Adaptation on these artificial habitats affected changes in Red Fox ecology, particularly in feeding strategy, activity and behavior (Harris and Smith 1987; Gloor *et al.*, 2001).

Red Fox is a flexible omnivore capable of using diverse available food resources – from vegetables and berries to insects and small vertebrates. Its feeding depends on habitat, altitude, longitude, seasons, age, sex and available food resources. These factors significantly affect the variations in diet (see Nentvichová *et al.*, 2010; Kidawa and Kowalczyk, 2011; Díaz-Ruiz *et al.* 2013; Hartová- Soe *et al.* 2017; Lanszki *et al.* 2019a). However, many studies across the species range show that small vertebrates are generally the main prey and most important food sources across habitats or seasons. Among the small vertebrates, vole species (Microtine) and mice from genus *Apodemus* are the most frequent food items in the Red Fox diet (see Jedrzejewski and Jedrzejewska, 1992; Lanszki *et al.*, 2007; Pagh *et al.*, 2015; Soe et al. 2017). These species are the basic food source within agricultural habitats (Soe *et al.* 2017). Plants have an important role in the Red Fox diet only in certain seasons and specific habitats. In summer and early autumn this food has higher occurrence and percentage of consumed biomass (Jedrzejewski and Jedrzejewska, 1992; Lanszki *et al.*, 2019b, Hartová-Nentvichová *et al.*, 2010). In urban habitats anthropogenic food resources and small rodents are the most frequent foods (Contesse *et al.*, 2003; Lavin *et al.*, 2003). In general, Red Foxes consume between 0.5 and 1 kg of food daily.

Red Fox is mainly a monogamous species. Contemporary molecular-genetics studies reveal a high level of polygamy within the populations or even mixed paternity within litters. Mating season varies across its range. In the temperate zone, mating season is in January and beginning of February (Macdonald and Reynolds, 2008). The gestation period is on average between 49 and 58 days. Fox cubs are usually born in mid-spring. Litter size is on average 4-6 cubs. Litter size is strongly related to available food resources and local population density (Elmeros *et al.*, 2003; Ruette and Albert, 2010). Young foxes reach adult proportions at the age of 6–7 months. They reach sexual maturity at the age of 9-10 months, and usually reproduce during next winter (Elmeros *et al.*, 2003; Macdonald and Reynolds, 2008; Ruette and Albert, 2010).

Population density of Red Fox varies significantly across its range. It is related to available food resources, habitats, health conditions (presence of parasites or diseases in the population), competitors and climate zone. Population density in Poland is 1.3-2 individuals/km^2 (Goszczyński *et al.*, 2008). In rural areas of Switzerland density is 3 individuals/km^2 (Meia, 1994), 1.17 in Wales (Harris and Rayner 1986; Macdonald and Newdick 1982), while in Italy density in an area varied seasonally from 0.39 to 2.01 individuals/km^2 in spring and from 0.54 to 4.3 individuals/km^2 in winter season (Pandolfi *et al.,* 1991). In some urban areas densities are significantly higher than in natural areas. In some cities in United Kingdom, Red Fox density is greater than 30 individuals/km^2 (Harris and Rayner 1986; Macdonald and Newdick 1982). In the last few decades abundance and densities in Europe have been increasing. This population change is affected by rabies vaccination carried out across the entire continent. Until now rabies has been almost eliminated. Increase of abundance and population density has been described in many European countries where oral rabies vaccination was administered (Breitenmoser *et al.*, 2000; Chautan *et al.*, 2000; Kauhala *et al.*, 2006).

1.6. Ticks parasitizing Red Foxes

Among mammals, Red Fox is one of the species best adapted to ecosystems altered by humans such as urban or agriculture ecosystems (Harris, 1977; Macdonald and Newdick, 1982; Gloor *et al*., 2001). This carnivore is also known as a very important reservoir for a number of zoonotic pathogens in Europe, including these transmitted by vectors such as mosquitoes and ticks, and as a host for various species of ectoparasites e.g. fleas, mites and ticks. Although ticks are not inducing the highest parasitic impact on foxes, they may, like in other wild animals, play an important role in epidemiology of tick-borne diseases and in the maintenance of tick populations in certain areas (Bengis *et al*. 2004).
The epidemiology of tick-borne diseases is closely connected to the ecology of the ticks as vectors, and for the evaluation of risk for animal and human health in a certain area it is necessary to have detailed information on presence, distribution and host-parasite relationship of exact tick species. For this reason, role of Red Foxes in transmission of tick-borne diseases has recently been in focus of researchers across Europe.
As a medium-sized mammal, Red Fox is host for nymphs and adults of more than 10 tick species in Europe known to harbor different pathogenic organisms (Fig. 16).

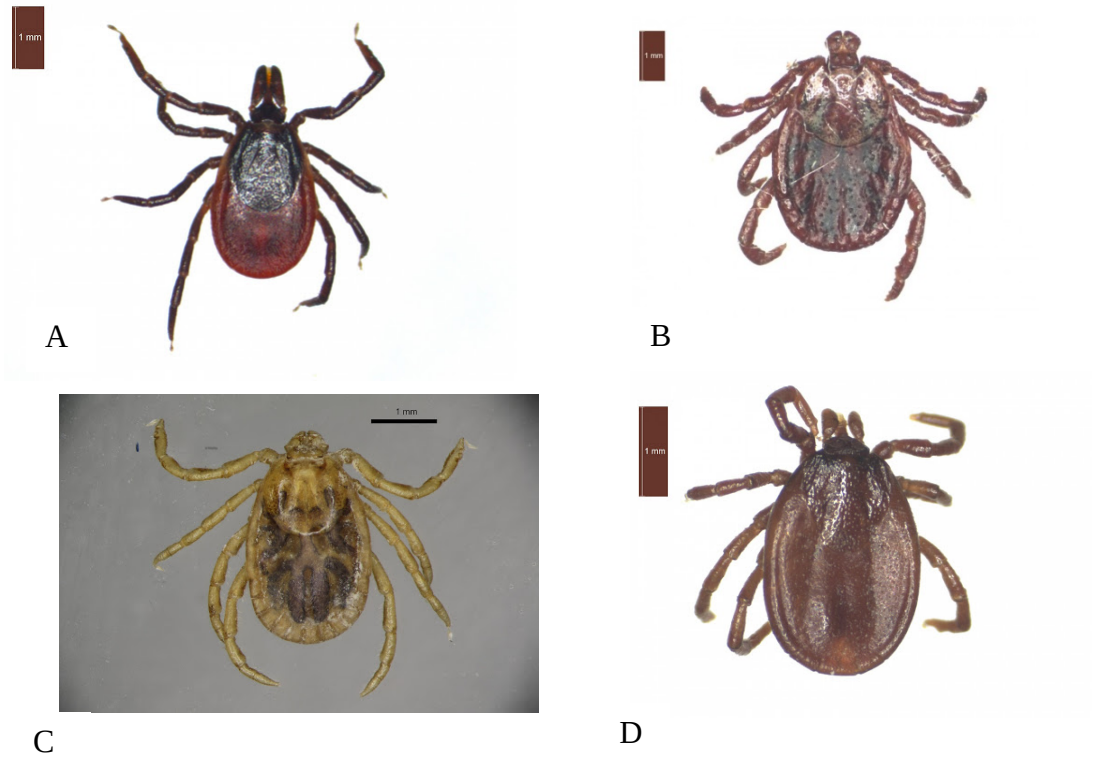


Fig. 16. The commonest tick species parasitizing Red Fox in Europe: A - *Ixodes ricinus* female, B - *Dermacentor reticulatus* female, C - *Haemaphysalis concinna* female, D - *Ixodes canisuga* female (Bristol University Ticks ID - http://www.bristoluniversitytickid.uk)

In Romania, dynamics of tick infestation were studied by Dumitrache and colleagues (2014). Ticks were collected from fur of 357 Red Foxes from 12 Romanian counties. In the total 5753 ticks were collected from 156 animals (prevalence 43.7%). All collected ticks belonged to five species: *Haemaphysalis punctata*, *Dermacentor marginatus*, *Ixodes hexagonus*, *Ixodes ricinus*, and *Ixodes crenulatus*. The highest prevalence was that of

ticks from genus *Ixodes* (*I. hexagonus* and *I. ricinus* 72.44% and 28.84% respectively) while lower prevalence was found for *Haemaphysalis punctata*, *Dermacento marginatus* and *Ixodes crenulatus* (0.64%, 7.05% and 7.7% respectively). Infestation with multiple tick species was registered in 24 Red Foxes. Most of them were infected with two tick species (22 Red Foxes) and only two animals with three tick species. Detected tick associations in Red Fox population in Romania were: *I. ricinus* + *I. hexagonus* (10 Red Foxes), *I. hexagonus* + *Dermacentor marginatus* (5 Red Foxes), *I. ricinus* + *I. crenulatus* (4 Red Foxes), *I. ricinus* + *D. marginatus* (2 Red Foxes), *I. hexagonus* + *I. crenulatus* (1 Red Fox), *D. marginatus* + *I. hexagonus* + *I. ricinus* (1 Red Fox), and *Haemaphysalis punctata* + *I. hexagonus* + *I. ricinus* (1 Red Fox) (Dumitrache *et al.*, 2014).

Contrary to the results from Romania, only two *Ixodes* species were found in a suburban population of the Red Fox from Great Britain - *I. hexagonus* and *I. canisuga*. Recorded prevalence was very high (Harris and Thompson, 1978). In specific urban habitats the most important factor affecting the recorded high prevalence in Red Fox populations was frequency of den usage. High tick infection had no significant effect on health of Red Foxes in urban areas in Great Britain (Harris and Thompson, 1978).

Several studies focused on the Red Fox as host for ticks in Central Europe (Hinaidy, 1971; 1976; Schöffel *et al.*, 1991; Lassnig *et al.*, 1998; Sréter *et al.*, 2003; Kočišová *et al.*, 2006). These studies have shown presence of six tick species in this region. Results of studies of ectoparasite fauna based on 100 examined Red Foxes from Hungary have shown relatively high to moderate infestation by ticks. Altogether four tick species have been found – *I. ricinus*, *I. canisuga*, *Haemaphysalis concinna*, and *Demacentor reticulatus* with prevalence 45%, 19%, 33% and 27% respectively (Sréter *et al.*, 2003). In Austria, three studies confirmed presence of five species - *I. ricinus*, *I. hexgonus*, *I. canisuga*, *H. concinna*, and *D. reticulatus* (Hinaidy, 1971; 1976; Lassnig *et al.*, 1998) with very low prevalence. Overall prevalence of recorded ticks was 13%, 4%, 1%, 2% respectively (summarized by Sréter *et al.*, 2003). In Slovakia, Kočišová *et al.* (2006) found only two species - *Ixodes ricinus* and *Demacentor reticulatus*, also with relatively low prevalence (17.9%, 3.8% respectively).

Higher prevalence was found in Western Europe than in Central Europe region (Schöffel *et al.*, 1991; Domingez *et al.*, 2004). In 100 examined Red Foxes from Germany, Schöffel *et al.* (1991) reported only ticks from genus *Ixodes*. The authors recorded three species - *I. ricinus*, *I. hexgonus*, *I. canisuga* with prevalence 27%, 18% and 15 % respectively (Schöffel *et al.*, 1991). In Spain the prevalence was higher, but number of individuals of recorded ticks was lower. In 26 examined Red Foxes only two tick species were identified - *I. ricinus* and *I. hexgonus*. Registered prevalence was high – 34.6% (*Ixodes ricinus*) and 30.8% (*Ixodes hexagonus*) (Domingez *et al.,* 2004). In a short article with the aim to detect presence of *Rickettsia* spp. and *Bartonella* spp. from the ticks from Red Fox in France only one species was found. From four foxes collected in southeastern part of country (2) and Corsica (1), only two foxes from suburbs of Marseille were infected with 50 ticks identified as *Rhipicephalus turanicus* (Marié *et al.,* 2012).

There are several recently published studies for the northern and northeastern part of the Mediterranean region (Aydin *et al.,* 2011; Chochlakis *et al.,* 2011; Keysary *et al.,* 2011; Vincenzo *et al.,* 2011; Psaroulaki *et al.,* 2014). In a study from Italy ticks were collected from road-killed wildlife species (including Red Fox) in southern Italy (Vincenzo *et al.,* 2011). From 81 collected Red Foxes six tick species were identified at larval, nymph or adult stage (*D. marginatus*, *R. bursa*, *R. turanicus*, *Haemaphysalis erinacei*, *I. canisuga*, *I. ricinus*) with very low prevalence. Authors described three tick associations in Red Foxes (*I. canisuga* + *R. turanicus*, *D. marginatus* + *R. turanicus*, *H. erinacei* + *I. ricinus*, + *R. bursa* + *R. turanicus*). Each association was found in only a single animal (Vincenzo *et al.,* 2011). Four tick species were recorded within the isolated Red Fox population from Cyprus Island (*Rhipicephalus sanguineus*, *R. turanicus*, *Ixodes ventalloi*, and I. *gibbosus*).

Most abundant ticks were *R. turanicus* followed by *I. ventalloi*, *I. gibbosus* and *R. sanguineus* (79.91%, 9.56%, 8.66% and 1.83%, respectively) (Chochlakis *et al.,* 2011). Another study from Cyprus closely confirmed the above results (Psaroulaki *et al.,* 2014). There is just a short recently published note about ticks and fleas from Turkey. Two tick species were identified in the sample of only three Red Foxes collected from traffic accidents (Aydin *et al.,* 2011). From a sample of seven Red Foxes (mostly immobilized) collected in Israel, three tick species were identified at the species level (*R. turanicus*, *Haemaphysalis adleri* and *H. parva*) with low prevalence. Additional one tick was identified as *Hyalomma* sp. (Keysary *et al.,* 2011).

In the North Balkan region (including Serbia) there are only three recently published studies focusing on ticks parasitizing on the Red Fox (Tomanović *et al.,* 2013; Jemeršić *et al.,* 2014; Stojanov *et al.,* 2014). In Croatia Jemeršić *et al.* (2014) reported five tick species in fox population from central and eastern part of the country (*I. ricinus*, *I. hexagonus*, *H. punctata*, *D. reticulatus*, *R. sanguineus*). Authors did not report prevalence. Studies of ticks as ectoparasites of Red Fox were carried out in a large part of the territory of Serbia by three groups of researchers. Red Fox was determined as host for eight identified species (Pavlović *et al.,* 2001; Tomanović *et al.,* 2013; Stojanov *et al.,* 2014). 70 ticks were collected from bodies of 58 Red Foxes culled at nine localities in the central and northern part of Serbia and identified to species level. There were five detected species: *I. ricinus, I. hexagonus, Ixodes canisuga, Haemaphysalis concinna and D. reticulatus*. Most abundant tick species was *I. ricinus* (62.86%), followed by *I. canisuga, I. hexagonus, H. concinna, D. reticulatus* (14.28%, 10%, 7.14%, 5.71% respectively) (Tomanović *et al.*, 2013). Stojanov *et al.* (2014) collected and inspected bodies of 23 hunted foxes at two localities (Pećini and Titel). Four tick species were recorded: *I. ricinus*, *D. marginatus*, *R. sanguineus* and *H. punctata* with prevalence 47.86%, 43.47%, 8.69% and 4.34% respectively. Besides the above-mentioned tick species, Pavlović *et al.* (2001) also reported presence of two additional species – *Ixodes kaiseri* and *Haemaphysalis inermis*. All those studies confirmed that *Ixodes ricinus* was most abundant tick in the Red Fox population in Serbia (Pavlović *et al.,* 2001; Tomanović *et al.,* 2013, Stojanov *et al.,* 2014).

1.7. Red Fox as reservoir for tick-borne pathogens

As Red Fox is a synanthropic, territorial wild carnivore species with a relatively small home range of individuals, acting as a host for a number of tick species known to harbor tick-borne pathogens important for human and animal health. Thus Red Fox plays a significant role in sustenance of the tick-borne pathogens in nature and epidemiology of tick-borne zoonoses. In Europe, Red Fox is the most studied wild carnivore species from the epizoological point of view, however knowledge is still scarce.

Several tick-borne pathogens like *Babesia* spp., *Hepatozoon canis, Anaplasma* spp., *Borrelia* spp. etc., have been detected in Red Foxes collected at localities across Europe.

Protozoa from genus *Babesia* are causing some of the most important diseases of domestic animals, while some also cause human diseases. In Europe, *Babesia canis*, *Babesia vogeli* (traditionally named as “large” babesial species), *Babesia gibsoni* and *Babesia vulpes*, previously known as *Babesia microt*-like (“small” babesial species) are associated with canine babesiosis, and *Babesia microti* and *Babesia divergens* with human babesiosis in Europe (Irwin, 2009, Beneth *et al.* 2015). The most significant vectors of babesiosis in Europe are tick species *Dermacentor reticulatus, Rhipicephalus sanguineus, Haemaphysalis* spp. and *Ixodes ricinus,* recorded parasitizing a number of

wild animals including Red Foxes, domestic animals and humans (Petra *et al.* 2018). Presence of *Babesia canis* in Europe is closely associated with the broad distribution of the main vector *Dermacentor reticulatus* (Földvári *et al.* 2016)*, Babesia gibsoni* is associated with vectors from the genus *Haemaphysalis*, while *Rhipicephalus sanguineus* s.l. have been proposed as potential vector (Baneth 2018). For *Babesia gibsoni* vertical transmission and direct infection with transfusion and dog fighting have been documented (Fukumoto *et al.* 2005; Birkenheuer *et al.* 2005). Based on the epidemiological studies, *Ixodes hexagonus, Ixodes canisuga, Ixodes ricinus* and *Dermacentor reticulatus* are candidate vectors for *Babesia vulpes* (Camacho *et al.* 2003; Najm *et al.* 2014). *Babesia divergens* and *Babesia microti,* which cause human babesiosis in Europe, are transmited by *Ixodes ricinus*, the most anthropophilic tick species in Europe (Hunfeld and Brade, 2004).

According to the previous studies, two *Babesia* species have been detected in Red Foxes in Europe, *Babesia canis* and *Babesia vulpes* previously known as *Babesia (Theileria) annae*, *Babesia* "Spanish dog isolate", *Babesia* cf. *microti.* While *Babesia vulpes* has been detected in Red Fox samples from several European countries - Spain (Checa *et al.,* 2018), Portugal (Cardoso *et al.,* 2013), Austria (Duscher *et al.,* 2014), Germany (Najm *et al.,* 2014), Bosnia and Herzegovina (Hodžić *et al.,* 2015a), Hungary (Farkas *et al.,* 2015), UK (Bartley *et al.,* 2016), Croatia (Dezek *et al.,* 2010), and Italy (Zanet *et al.,* 2014), with prevalence ranging from less than 1% to over 70%, *Babesia canis* have been detected only twice at global level. The first finding was in a single fox from Portugal (Cardoso *et al.,* 2013), and the second finding was also confirmed in one fox in Bosnia and Herzegovina (Hodžić *et al.,* 2015a). The high prevalence of *Babesia vulpes* and the absence of clinical symptoms in Red Foxes in Europe, indicate their role as reservoirs for this species of babesia (Checa *et al.,* 2018).

In Serbia, babesia was confirmed in wild and domestic carnivores. Canine babesiosis caused by *Babesi canis* and *Babesia gibsoni* has been described (Davitkov *et al.* 2015), while presence of *Babesia vogeli*, *Babesia vulpes* as well as zoonotic *Babesia microti* was confirmed in healthy, asymptomatic dogs (Gabrielli *et al.,* 2015). DNA of *Babesia canis* (4.2%) was detected in golden jackals (*Canis aureus*) (Sukara *et al.,* 2018).

Hepatozoon canis is another protozoan parasite widespread among domestic and wild carnivores in Europe (Baneth 2011). Infection with *Hepatozoon canis* in dogs is commonly subclinical but in predisposed animas can lead to severe disease (Baneth and Weigler 1997; Sakuma *et al.,* 2009). Autochthonous dog hepatozoonosis is present in many European countries with Mediterranean climate (Turkey, Bulgaria, Greece, Croatia, Italy, Portugal) (Mylonakis *et al.,* 2004; Ivanov and Tsachev, 2008; Beck *et al.,* 2009; Baneth, 2011; Aktas and Ozubek, 2017). The high prevalences of *Hepatozoon canis* are detected in foxes across the entire Europe (Hodžić *et al.,* 2015; Cardoso *et al.,* 2014; Farkas *et al.,* 2014a; Tolnai *et al.,* 2015; Karbowiak *et al.,* 2010). In Europe, *Hepatozoon canis* is transmitted mainly by *Rhipicephalus sanguineus* s.l. (Dantas-Torres & Otranto 2015) but presence of this parasite in regions considered free of *Rhipicephalus sanguineus*. s.l. points to other transmission routes (e.q. transplacental) (Hodžic *et al.,* 2018). So far in Serbia presence of *Hepatozoon canis* was confirmed in a healthy, asymptomatic dog from southern Serbia (Gabrielli *et al.,* 2015).

Same species of *Hepatozoon* and *Babesia* are often infecting both wild canids and domestic dogs. Based on the evolutionary approach it was suggested that these haematozoan pathogens have been transmitted to the dogs from wild canids (Penzhorn 2011). Therefore, there is a possibility for transmission of these protozoan pathogens from wild canids to domestic dogs in regions where ecological niches of wild and domestic canids are overlapped (Baneth 2011; Margalit Levi *et al.* 2018).

Regarding the bacterial tick-borne pathogens, Red Foxes were indicated as hosts for several species belonging to genera *Anaplasma, Ehrlichia, Borrelia, Rickettsia, Francisella, Coxiella, Bartonella.*

Presence of several tick-borne pathogens from the *Anaplasmataceae* family was detected in samples of Red Foxes collected in localities in Europe. *Anaplasma phagocytophilum* is an emerging tick-borne pathogen, distributed worldwide. It infects humans and a wide range of domestic and wild animals causing granulocytic anaplasmosis. *Ixodes ricinus* tick is considered the main vector of *Anaplasma phagocytophilum* in Europe*,* while wild animals are considered important reservoirs*.* The exact role of wildlife species as potential reservoirs is still to be determined (Stuen *et al.,* 2013)*.* Recent studies revealed presence of *Anaplasma phagocytophilum* in 16.6% of analyzed Red Foxes originating in Central Italy, 8.2% from Germany, 2.5% from Romania, 2.7% in Poland (Ebani *et al.,* 2011, Härtwig *et al.,* 2014; Dumitrashe *et al.,* 2015, Karbowiak *et al.*, 2009).

Anaplasma platys is an etiological agent of thrombocytic anaplasmosis in dogs. It is predominantly distributed in Americas, Africa, Asia and Australia, while occasional cases of diseases are registered in Europe although data on prevalence in ticks and wild animals are scarce (Otranto *et al.,* 2015)*.* The single worldwide study that reported presence of *Anaplasma platys* in Red Foxes with moderate prevalence of 14.5% was conducted by Cardoso and colleagues (2015) in Portugal.

Ehrlichia canis is causative agent of canine monocytic ehrlichiosis in domestic and wild carnivores. The main vectors are ticks belonging to *Rhipicephalus sanguineus* s.l. complex, however *Dermacentor variabilis*, *Dermacentor marginatus* and *Ixodes canisuga* were also proposed as potential vector species (André, 2018). DNA of *Ehrlichia canis* was detected in Red Foxes from Italy (31–52%), Portugal (2.29%) and Spain (16.6%) (Santoro *et al.,* 2016; Torina *et al.,* 2013; Ebani *et al.,* 2017; Millán *et al.,* 2016).

The role of foxes as potential reservoirs for newly discovered member of the family Anaplasmataceae, *Candidatus* Neoehrlichia sp., (FU98) was proposed recently (Hodžić *et al.,* 2015b).

Foxes were also recognized as reservoir hosts for the causative agents of Lyme borreliosis (Liebisch *et al.,* 1995; Dumutrashe *et al.,* 2015; Mysterud *et al.,* 2019). Causative agents of this disease of humans and some domestic animals are spirochaetes belonging to the *Borrelia burgdorferi* s.l. complex. Up to date, at least 21 species of borrelia distributed worldwide and belonging to Lyme borreliosis group, have been described and named (Steere *et al.,* 2016). Not all species have pathogenic potential for humans and animals. In Europe pathogenicity have been proven for *Borrelia afzelii, Borrelia garinii, Borrelia bavarensis, Borrelia burgdorferi* sensu stricto, while *Borrelia spielmanii, Borrelia bissetii, Borrelia valainiana* and *Borrelia lusitaniae* have been detected in human samples but their pathogenic potential is still unknown (Stanek *et al.,* 2012; Stupica *et al.,* 2015). The main vectors are ticks belonging to the genus *Ixodes,* mainly *Ixodes ricinus* (Estrada-peña *et al.,* 2018).

Although their presence was not confirmed by direct methods, seroprevalence studies showed that Red Foxes in Europe are exposed to members of the genus *Rickettsia* (Ortuño *et al.,* 2018), *Francisella tularensis* (Otto *et al.,* 2014) and *Coxiella burnetti* (Meredith *et al.,* 2015).

2. Objectives

Research within this doctoral dissertation aims to determine the role of ticks in enzootic cycles of tick-borne diseases as well as the importance of Red Foxes (*Vulpes vulpes*) as a reservoir of pathogens (causative agents from the genera: *Babesia*, *Borrelia*, *Rickettsia*, *Anaplasma*, *Ehrlichia*, *Francisella*, *Bartonella*, as well as *Coxiella burneti* and *Hepatozoon canis*) which are transmitted to other animals in natural and anthropogenic ecosystems in Serbia by these specific vectors.

Since research on ticks that parasitize foxes has not been done systematically, the goal is to investigate the fauna of these ectoparasites in the fox population in Serbia, and identify the most important pathogens that can be transmitted to other mammals, including humans.

In order to achieve the objectives, the following scientific tasks have been set:

• Sampling of tissues of hunted Red Foxes (spleen) and parasitizing ticks, from localities in the territory of the Republic of Serbia.

• Laboratory processing of collected samples (identification of tick species, sex and life stage, DNA extraction from tissue samples and collected ticks).

• Molecular detection of DNA pathogens: (*Babesia* spp., *Borrelia burgdorferi* s.l., *Rickettsia* spp., *Anaplasma* spp., *Ehrlichia* spp., *Francisella* spp., *Bartonella* spp., *Coxiella burneti* and *Hepatozoon canis*) in spleen samples of Red Foxes.

• Genotyping and characterization of detected pathogens by molecular methods (sequencing).

3. Material and Methods

3.1. Collection of samples

Samples included in the study were collected in cooperation with local hunters across Serbia during the hunting seasons in years 2010-2016.
Bodies of legally hunted Red Foxes (*Vulpes vulpes*) originated in 14 localities throughout Serbia (Veliko Gradište, Surčin, Obrenovac, Velika Plana, Svilajnac, Negotin, Despotovac, Rekovac, Kraljevo, Vrnjačka Banja, Trstenik, Blace, Niš, Bela Palanka) (Fig. 19.). Following data were recorded for each individual fox: sex, date of death and the most precise locality. Whenever possible the entire body of the animal was inspected for presence of ticks and all recorded ticks were removed with tweezers and placed into labeled tubes. Labels included information on animal from which ticks were collected from, date, location. All ticks collected from one animal were placed in a single tube containing 70% ethanol and transported to the laboratory of the Institute for Medical Research (IMR), Institute of national importance for the Republic of Serbia, University of Belgrade.
Animals were necropsied on the field and spleen samples were collected and transferred in the cold chain to the laboratory (IMR) where they were stored in a freezer (-80ºC) until further analysis.

3.2. Morphological identification of ticks

Morphological identification and determination of tick species and development stages were performed according to standard taxonomic keys (Pomerancev, 1948; Estrada Pena *et al.,* 2004). Individual ticks were placed on filter paper until ethanol in which they were preserved for the transportation from the field dried out, and then they were examined using stereo-microscope (58-06100, Bresser^{TM}) with up to 45X magnification (Fig. 17).

Fig. 17. Stereo-microscope used for morphological identification of ticks.

3.3. Molecular analysis

3.3.1. DNA extraction

3.3.1.1. DNA extraction from ticks

Prior to extraction of DNA, in order to eliminate superficial contamination of ticks, each ectoparasite was individually washed in 70% ethyl alcohol, rinsed in sterile water and dried on sterile filter paper.
Homogenate of each individual tick was prepared as follows: tick was placed into 1.5 ml plastic tubes and homogenized in 500 µl of PBS solution using sterile scissors in a laminar flow hood. DNA was extracted from 200 µl of tick homogenate by using the GeneJet Genomic DNA Purification Kit (Fermentas, Thermo Scientific) using protocol for DNA extraction from mammal tissue with modification of digestion time to 5 hours. Extracted DNA was stored at −20 °C until further testing.

3.3.1.2. DNA extraction from spleen samples

Spleen samples for DNA extraction were collected from few parts of the spleen in order to get the most representative sample. The small portion of frozen prepared spleen sample (up to 20 mg) was placed in 1.5ml sterile plastic tube and individually homogenized using micropestles (Micropestle, Eppendorf™). DNA was extracted from homogenized tissue by using the GeneJet Genomic DNA Purification Kit (Fermentas,Thermo Scientific) using protocol for DNA extraction from mammal tissue. Extracted DNA was stored at −20 °C until further testing.

3.3.2. Molecular identification of tick species

In order to confirm morphological identification, in case of ticks morphologically identified as members of species of subgenus *Pholeixodes* (*Ixodes canisuga* and *Ixodes hexagonus*) representative samples were forwarded to molecular analysis.
Molecular identification was performed using barcoding markers-genes for cytochrome c oxidase subunit I (cox1) and 16S rRNA at University of Veterinary Medicine, Budapest, Hungary. A parcel of DNA extracted from individual ticks had been shipped to the collaborative laboratory where amplification and sequencing were performed.
Following primers were used for amplification of barcoding sequences: the primers HCO2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) and LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) which amplify an approximately 710 bp long fragment of the cox1 gene; primers 16S + 1 (5′-CTG CTC AAT GAT TTT TTA AAT TGC TGT GG-3′) and 16S-1 (5′-CCG GTC TGA ACT CAG ATC AAG T-3′) which amplify an approximately 460 bp long fragment of the 16S rRNA gene of Ixodidae. Conditions of PCR reactions are as previously described (Hornok *et al.,* 2017).

3.3.3. Molecular detection and identification of tick-borne pathogens in spleen samples

In order to use resources and time in the most economic and efficient manner, DNA extracted from individual samples was pooled for initial screening.
Five individual samples of the extracted DNA were randomly combined into one pool in the laminar flow in order to exclude possibility of cross contamination of samples. Whenever the pools were positive for PCR reaction, the individual DNA samples included in that pool were subjected to repeated molecular analysis in order to determine individual positive animal.
All PCR reactions for initial screening of both pooled and individual samples were prepared in final volume of 25 µl, composed of: 6.5 µl of Molecular Biology Water, 1.5 µl of each primer (10 pmol/µl), 12.5 µl of PCR Master Mix (2X) (Thermo Fisher Scientific Inc.) and 3 µl of tested DNA.
Preparation of samples for sequencing: samples were performed in a final volume of 50 µl of a reaction mixtures composed of: 24.75 µl of Molecular Biology Water, 10 µl of 5X Reaction Buffer (7.5 mM MgCl2; pH 8.5), 1 µl of dNTPs (10 mM), 0.250 µl of Taq polymerase (5 u/µl, GoTaq G2 DNA Polymerase, Promega Corporation, USA), 4 µl of each primer (10 pmol/µl), and 6 µl of tested DNA.
All PCR reactions were performed in a Veriti Thermal Cycler device (Applied Biosystems). The obtained PCR products were electrophoresed on 2% agarose gel stained with ethidium bromide and visualized in a BioDocAnalyze device (Biometra GmbH).

Fig. 18. Veriti Thermal Cycler device (Applied Biosystems), PCR machine used for amplification of samples in this study.

3.3.3.1. Protocols for detection and identification of *Babesia* spp.

For initial detection of *Babesia* spp. in analyzed samples, the BabF(5'–GCGATGGCCCATTCAAGTTT–3') and BabR(5'–CGCCTGCTGCCTTCCTTAGA–3') primers were used to amplify a 146bp long fragment of the 18S ssrRNA gene (Theodoropoulos *et al.,* 2006). The amplification conditions were: initial denaturation step of 3 min at 95ºC, followed by 40 cycles of denaturation at 95ºC for 1 min, annealing at 52ºC for 1 min, and elongation at 72ºC for 1 min. Final extension was at 72°C for 10 min.
Positive samples were further processed for sequencing with PCR assay amplifying larger fragment of 18S rRNA gene (408bp) with the BJ1(5'–GTCTTGTAATTGGAATGATGG–3') and BN2 (5'–TAGTTTATGGTTAGGACTACG–3') primers (Casati *et al.,* 2006). The amplification conditions were: initial denaturation step of 2 min at 95ºC, followed by 35 cycles of denaturation at 95ºC for 1 min, annealing at 54ºC for 1 min, and elongation at 72ºC for 1 min. Final extension was at 72°C for 5 min.

3.3.3.2. Protocols for detection and identification of *Hepatozoon* spp.

Both initial screening and preparation for sequencing for identification of presence of *Hepatozoon* spp. in analyzed spleen samples were performed using the HepF_for(5'-ATACATGAGCAAAATCTCAAC–3') and HepR_rev(5'-CTTATTATTCCATGCTGCAG–3') primers, which amplify the 666-bp fragment of the 18S ssrRNA gene of *Hepatozoon* spp. (Inokuma *et al.,* 2002). The amplification conditions were: 95°C for 3 min; 40 cycles of denaturation at 98°C for 20 s, annealing at 57°C for 20 s, and elongation at 72°C for 20 s; final extension was at 72°C for 10 min. Positive samples were forwarded to sequencing.

3.3.3.3. Protocols for detection and identification of members of the family Anaplasmataceae

For initial screening of members of the family Anaplasmataceae primers EHR16SD (5'-TAGCACTCATCGTITACAGC-3') and EHR16SR (5'-GGTACCYACAGAAGAAGTCC-3') amplifying ~345bp long fragment of 16s rRNA gene were used. The amplification conditions were: 94°C for 3 min; 45 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 45 s, and elongation at 72°C for 1 s; final extension was at 72°C for 10 min.
Positive samples were preceded for initial sequencing. Based on preliminary results of sequencing positive samples were further tested for presence of *Candidatus* Neoerlichia sp. using the primers NeoeGroELFw (5'-CAGGTGAAGCACTAGATAAGTCCA-3') and NeoeGroELRV (5'-ACAGCAGCAACATGCAATCCA-3') targeting ~806 bp long fragment of groEL gene and 16SCNM_for (5'-GTGGCAGACGGGTGAGTAAT-3') and 16SCNM_rev (5'-TGCAGCACCTGTGTAAGGTC-3') targeting ~1053 bp long fragment of 16S rRNA gene. The amplification conditions for groEL gene were: 94°C for 3 min; 45 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 45 s, and elongation at 72°C for 1 s; final extension was at 72°C for 10 min. Positive samples were forwarded for sequencing using the previously described protocol.

3.3.3.4. Protocols for detection and identification of members of the complex *Borrelia burgdorferi* sensu lato

Detection of members of the *Borrelia burgdorferi* sensu lato complex in the analyzed spleen samples was performed with nested PCR targeting 5S-23S rRNA intergenic spacer fragment ~250 bp long. Two pairs of primers were used, external - RIS1 (5'-CTGCGAGTTCGCGGGAGA-3'), RIS2 (5'-TCCTAGGCATTCACCATA-3') and external RIS3 (5'-GGAGAGTAGGTTATTGCCAGG-3'), RIS4 (5'-GACTCTTATTACTTTGACC-3'). The amplification conditions were the same for both reactions: 94°C for 3 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and elongation at 72°C for 1 min; final extension was at 72°C for 10 min. Positive samples were forwarded for sequencing using the previously described protocol.

3.3.3.5. Protocols for detection and identification of *Rickettsia* spp.

Portion of citrate synthase gene approximately 380 to 397 bp long was amplified in order to detect *Rickettsia* spp. in spleen samples. Primers RpCS.877p (5'-GGGGGCCTGCTCACGGCGG-3') and RpCS.1258n (5'-ATTGCAAAAAGTACAGTGAACA-3') were used (Regnery *et al.,* 1991). The amplification conditions were: 95°C for 3 min; 35 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s, and elongation at 72°C for 1 min; final extension was at 72°C for 10 min.

3.3.3.6. Protocols for detection and identification of *Coxiella burnetii*

For detection of *Coxiella burnetii* in analyzed spleen samples, primers CB1 (5'-ACTCAACGCACTGGAACCGC-3') and CB2 (5'-TAGCTGAAGCCAATTCGCC-3') targeting 257bp long fragment of superoxide dismutase gene were used (Spyridaki *et al.* 1998). The amplification conditions were: 94°C for 5 min; 35 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s, and elongation at 72°C for 30s; final extension was at 72°C for 10 min.

3.3.3.7. Protocols for detection and identification of *Francisella tularensis*

An approximately 428bp long fragment of 17 kDa lipoprotein gene was amplified in order to detect presence of *Francisella turalrensis* in analyzed spleen samples. Primers TUL4-435 (5'-GCTGTATCATCATTTAATAAACTGCTG-3') and TUL4-863 (5'-TTGGGAAGCTTGTATCATGGCACT-3') were used Sjöstedt et al. (1997), while reaction conditions were 94°C for 5 min; 35 cycles of denaturation at 94°C for 1min, annealing at 55°C for 1min, and elongation at 72°C for 1min; final extension was at 72°C for 10 min.

3.3.3.8. Protocols for detection and identification of *Bartonella* spp.

Fragment of 16S-23S intergenic region, approximately 639bp long was targeted for detection of *Bartonella* spp. Primers Urbarto1 (5'-CTTCGTTTCTCTTTCTTCAA-3') and Urbarto2 (5'-CTTCTCTTCACAATTTCAAT-3') were used (Raoult *et al.* 2006). The amplification conditions were: 95°C for 5 min; 35 cycles of denaturation at 95°C for 1min, annealing at 45°C for 1min, and elongation at 72°C for 1min; final extension was at 72°C for 10 min.

3.3.4. Sequencing and sequence analysis

The purification and bidirectional sequencing (Sanger) of obtained PCR products were performed by commercial companies (Macrogen, Amsterdam, the Netherlands and Microsynth, Austria). Sequences were compared with previously published nucleotide sequences available in the GenBank® database using the BLAST tool (National Center for Biotechnology Information) (http://www.ncbi.nlm.nih.gov/BLAST), analysed using the FinchTV v1.5.0, software and aligned using Clustal W.
Representative sequences from this study have been deposited to the GenBank® database under the following accession numbers: MK043348 (16S rRNA), MK050781 (groEL)- *Candidatus* Neoehrlichia sp. (FU98), MK043031- *Borrelia burgdorferi* sensu stricto (s. s.), MK043032- *Borrelia garinii*, MK043041- *Borrelia lusitaniae*.

4. Results

4.1. Animals (Red Foxes - *Vulpes vulpes*) included in the study

Samples from 129 Red Foxes (*Vulpes vulpes*) (73 males and 56 females) were used in the analysis. The majority of samples (87/129, 67.5%) originated at three localities: Veliko Gradište (45/129, 34.9%), Surčin (29/129, 22.5%) and Svilajnac (13/129, 10.1%), while 42 animals (32.5%) were collected at the remaining 11 localities – Obrenovac (6/129, 4.6%), Velika Plana (7/129, 5.4%), Negotin (2/129, 1.6%), Despotovac (1/129, 0.8%), Rekovac (5/129, 3.9%), Kraljevo (3/129, 2.3%), Vrnjačka Banja (5/129, 3.9%), Trstenik (3/129, 2.3%), Blace (3/129, 2.3%), Niš (5/129, 3.9%), Bela Palanka (2/129, 1.5%).

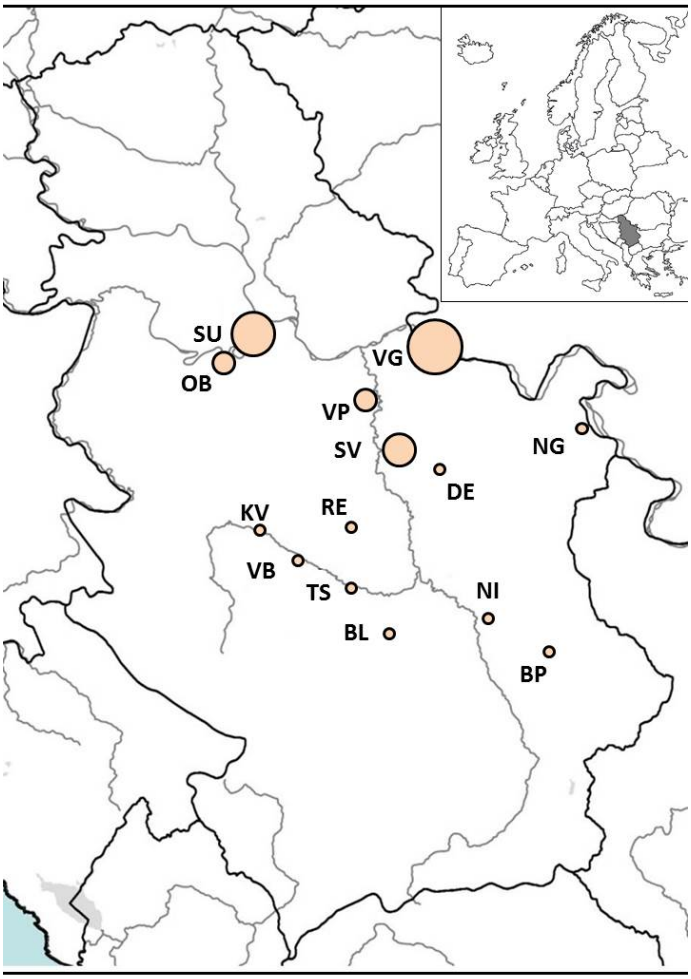


Fig. 19. Locations of collecting sites and size of sample collected at each site. The size of the circle correlate with the number of animals collected. Abrrevations SU-Surčin, OB-Obrenovac, VG-Veliko Gradište, VP-Velika Plana, SV-Svilajnac, DE-Despotovac, NG-Negotin, RE-Rekovac, KV-Kraljevo, VB-Vrnjačka Banja, TS-Trstenik, BL-Blace, NI-Niš, BP-Bela Palanka

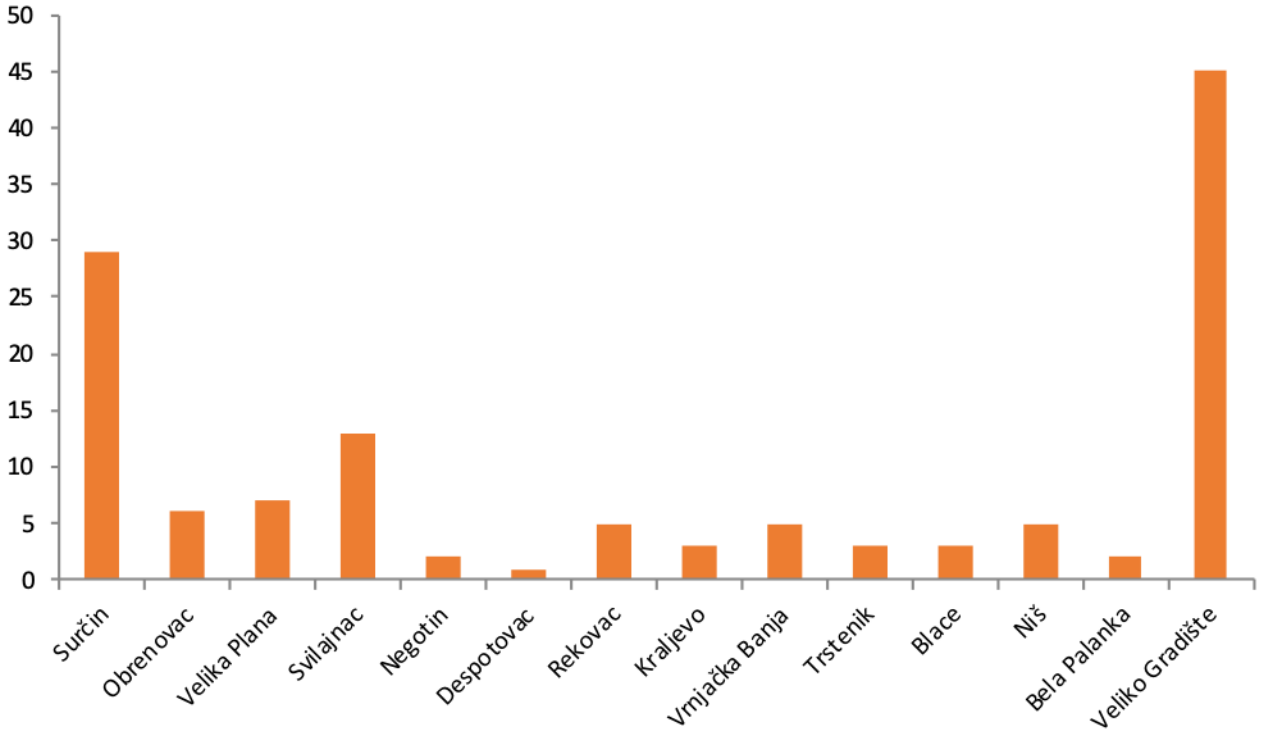


Fig. 20. Number of animals collected at each locality.

A total of 94 animals (72.9%) were collected in three years (2011 - 35 animals, 2013 - 36 animals and 2016 - 23 animals), while 27.1% of samples were collected during the remaining four years (2010 - 9, 2012 - 8, 2014 - 13, 2015 - 5) within the duration of the study.

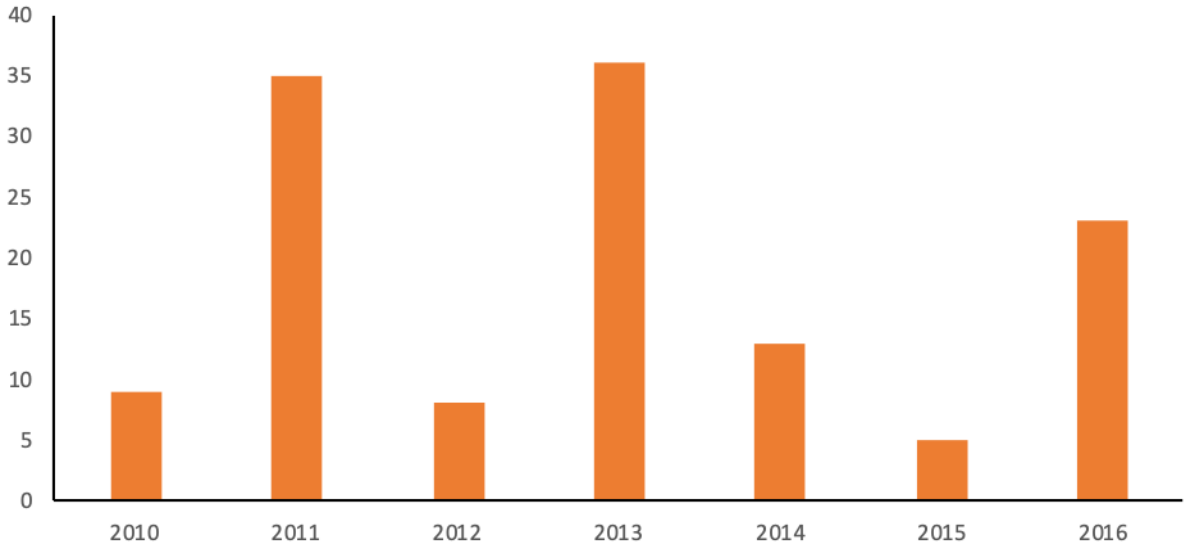


Fig. 21. Number of animals collected per year.

The majority of samples (108/129, 83.7%) were collected during winter months, in January (43), February (37) and December (28), while only few samples (1-6 animals) were collected during other months and no animals were collected in August (Fig. 22).

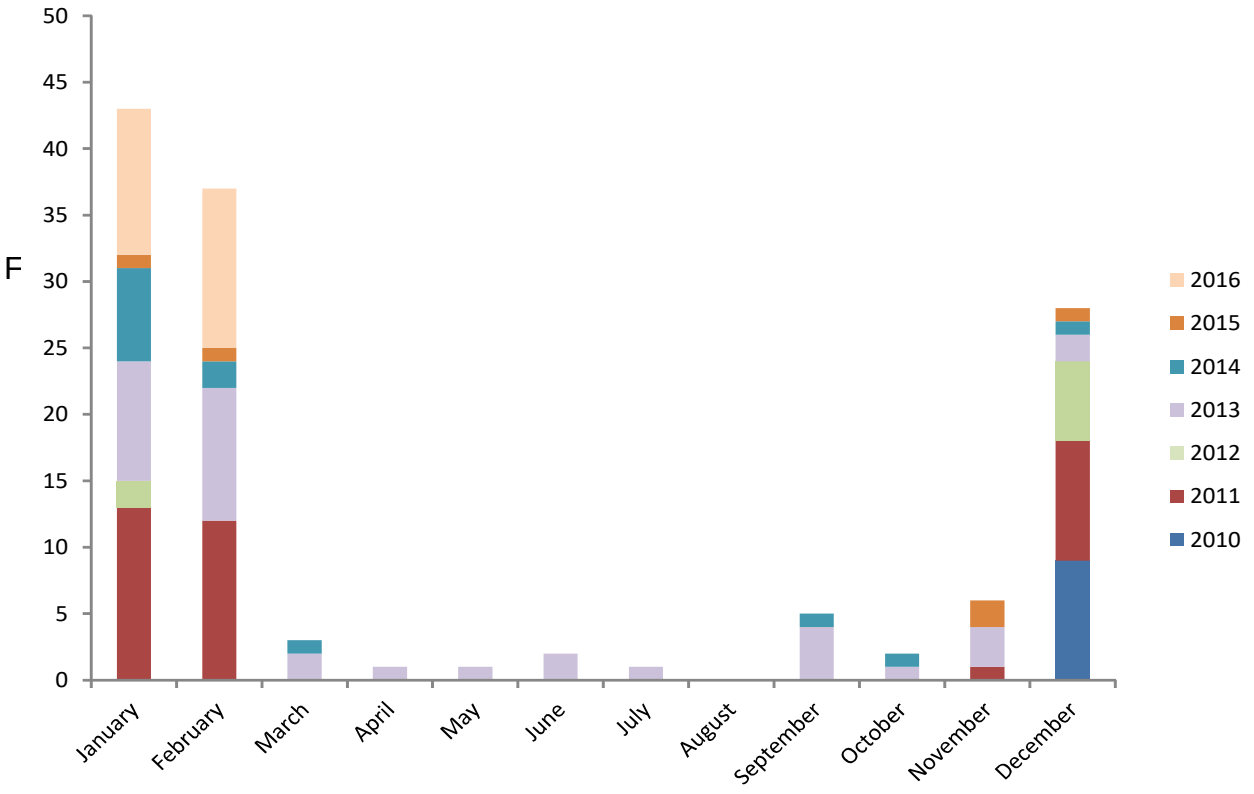


Fig. 22. Seasonal dynamics of collecting the Red Fox (*Vulpes vulpes*) samples

4.2. Ticks collected from analyzed Red Foxes

Total of 113 ticks were found in fur of 24 out of 129 collected animals. The number of ticks per animal ranged from 1 to 16, with the mean value of 4.7. Based on morphological keys, collected ticks were identified as following species: *Ixodes ricinus, Ixodes hexagonus, Ixodes canisuga, Haemaphysalis concinna* and *Dermacentor reticulatus.* Molecular identification of ticks classified as species belonging to subgenus *Pholeixodes* (*Ixodes canisuga* and *Ixodes hexagonus*) confirmed results for adult ticks of both species, while six out of nine nymphal ticks classified as *Ixodes canisuga* based on morphological keys were re-classified as *Ixodes kaiseri* based on analyzed sequences. Morphological identification of three nymphal ticks as *Ixodes canisuga* was confirmed. The most abundant species was *Ixodes ricinus* (78/113, 69%), followed by *Ixodes hexagonus* (13/113, 11.5%), *Ixodes canisuga* (6/113, 5.3%), *Ixodes kaiseri* (6/113, 5.3%), *Dermacentor reticulatus* (5/113, 4.4%) and *Haemaohysallis concinna* (5/113, 4.4%) (Fig. 23).

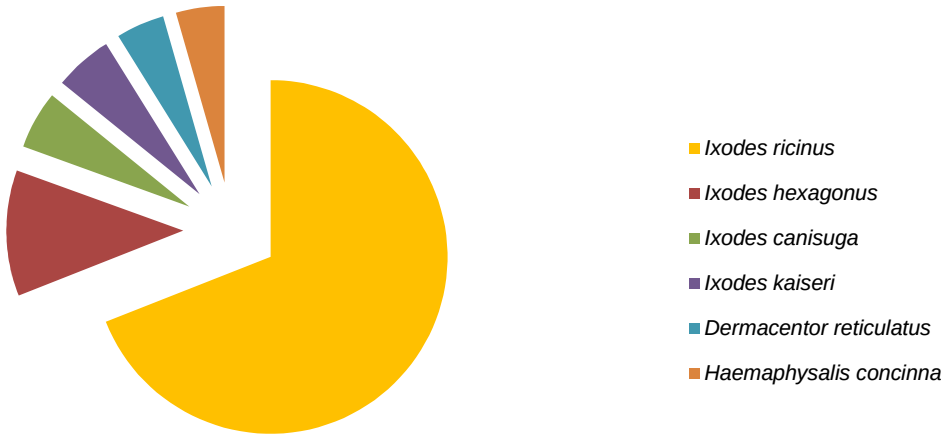


Fig. 23. Tick species found to parasitize Red Foxes (*Vulpes vulpes*) in Serbia as recorded in this study.

For three species (*I. ricinus, I. hexagonus* and *D. reticulatus*) only adult ticks were recorded, for *I. kaiseri* and *H. concina* only nymphs, while for *I. canisuga* both adults and nymphs were collected. No ticks of larval stage were observed (Fig. 24).

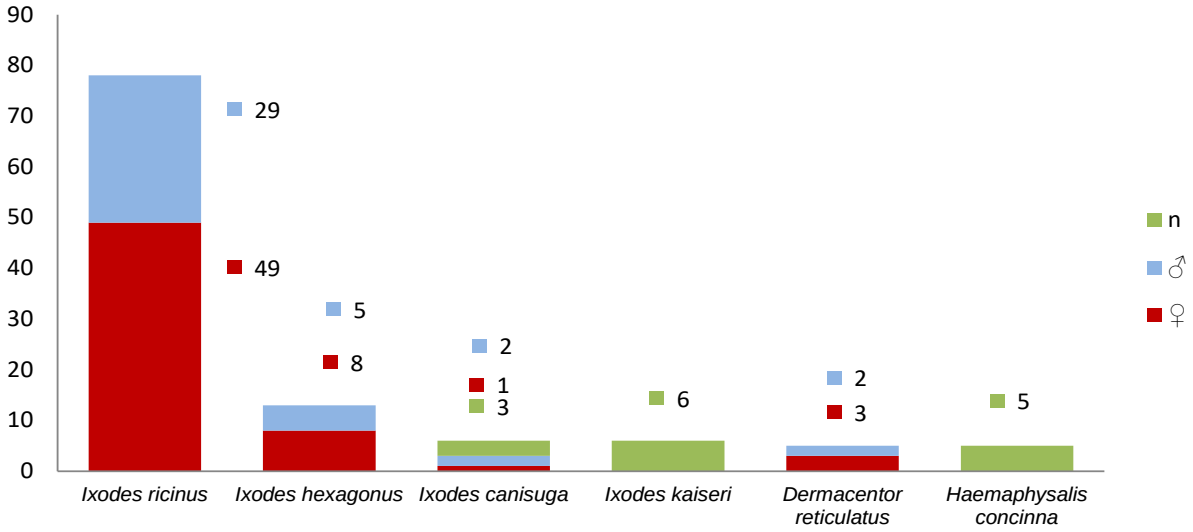


Fig. 24. Number of ticks belonging to each life stage of species founded to parasitize Red Foxes (*Vulpes vulpes*) from Serbia included in this study. n - nymphs

Seven parasitized animals (29.2%) were infested with two tick species each (*Ixodes ricinus/Ixodes hexagonus* – 1 animal (4.2%), *Ixodes ricinus/Ixodes canisuga* - 2 (8.4%), *Ixodes canisuga/Ixodes kaiseri* - 1 (4.2%), *Ixodes canisuga/Dermacentor reticulatus* - 1

(4.2%), *Ixodes hexagonus/Dermacentor reticulatus* - 2 (8.4%)) while 17 animals (70.8%) hosted only single tick species: *Ixodes ricinus* -12 (50%), *Ixodes hexagonus* – 2 (8.4%), *Ixodes kaiseri* – 1 (4.2%), *Haemaohysallis concinna* – 1 (4.2%), *Dermacentor reticulatus* – 1 (4.2%) (Fig. 25.).

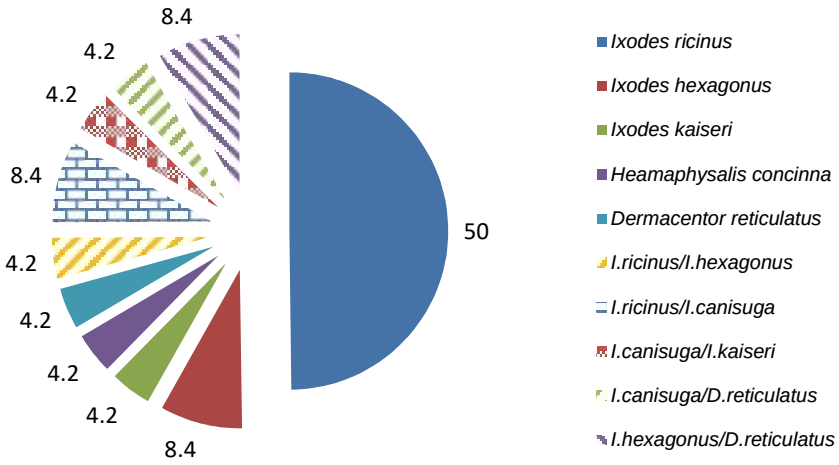


Fig. 25. Infestation and co-infestation of Red Foxes (*Vulpes vulpes*) with tick species. Numbers represent present of animals infested with single or multiple tick species.
Ticks were collected from animals at 6 out of 14 localities (Velika Plana, Veliko Gradište, Surčin, Niš, Despotovac, Svilajnac).

Ixodes ricinus was found to parasitize Red Foxes at five localities (Velika Plana, Veliko Gradište, Surčin, Niš and Svilajnac), *Ixodes hexagonus* and *Ixodes canisuga* at three (Velika Plana, Veliko Gradište and Surčin), *Ixodes kaiseri* at two (Surčin and Despotovac), while *Dermacentor reticulatus* and *Haemaohysallis concinna* were found at a single locality each (Surčin) (Tab. 2, Fig. 26).

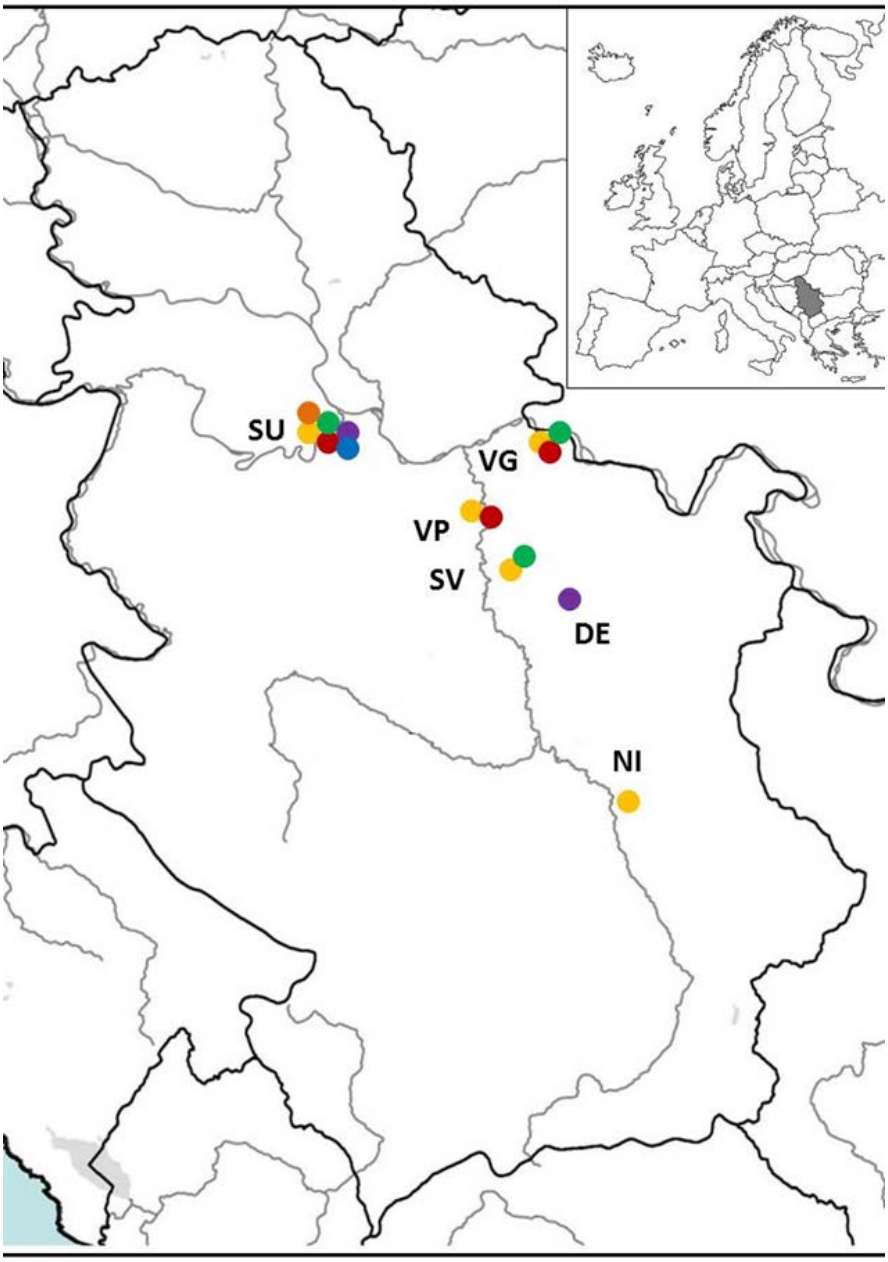


Fig. 26. Distribution of tick species found to parasitize Red Foxes (*Vulpes vulpes*) (yellow dot - *Ixodes ricinus*, red - *Ixodes hexagonus*, green - *Ixodes canisuga*, violet - *Ixodes kaiseri*, blue - *Dermacentor reticulatus*, orange - *Haemaphysalis concinna.* Abbreviations of collection sites: SU-Surčin, VG-Veliko Gradište, VP-Velika Plana, SV-Svilajnac, DE-Despotovac, NI-Niš).

Tab. 2. Number of ticks collected from Red Foxes at different localities in Serbia, by species and life stages.

Tick species	*Ixodes ricinus*		*Ixodes hexagonus*		*Ixodes canisuga*			*Ixodes kaiseri*	*Dermacentor reticulatus*		*Haemaphysalis concinna*
Life stage, sex	♀	♂	♀	♂	♀	♂	n	n	♀	♂	n
Veliko Gradište	25	14	1			1			1		
Surčin	5	3	2	4		1	3	5	2	2	5
Velika Plana	2		4	1							
Despotovac								1			
Svilajnac	2	3			1						
Niš	15	9									
Σ	49	29	7	5	1	2	3	6	3	2	5
	78		12			6		6	5		5

All six tick species were found at the locality of Surčin; four species (*Ixodes ricinus, Ixodes hexagonus, Ixodes canisuga* and *Dermacentor reticulatus*) were found at Veliko Gradište, two species (*Ixodes ricinus*, *Ixodes hexagonus* and *Ixodes ricinus, Ixodes canisuga*) at Velika Plana and Svilajnac respectively, while only single tick species were found on Niš and Despotovac localities (*Ixodes ricinus* and *Ixodes kaiseri,* respectively) (Fig. 26).

The majority of ticks (71/113) were collected during the winter months - January (12), February (53) and December (6), while the rest of ticks (42/113) were collected during March (19), April (9), July (5) and September (9).
Ixodes ricinus ticks were mostly found attached to foxes in February (44/78) and March (18/78), while smaller numbers were found in January (2/78), April (9/78) and December (5/78). *Ixodes hexagonus* ticks were found in January (6/13), February (6/13) and March (1/13), *Ixodes canisuga* in January (1/6), February (1/6), September (3/6) and December (1/6), *Ixodes kaiseri* in September (6/6), *Haemaohysallis concinna* in July (5/5) and *Dermacentor reticulatus* in January (3/5) and February (2/5) (Fig. 27).

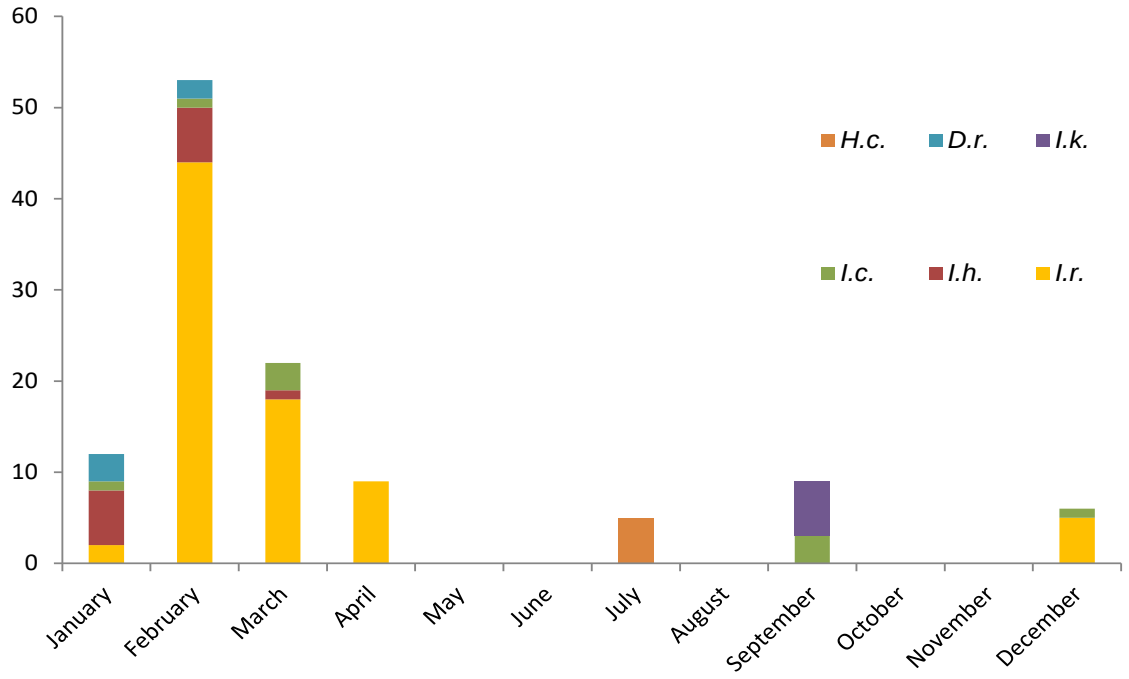


Fig. 27. Number of ticks of different species found to parasitize Red Foxes (*Vulpes vulpes*) by months when they were collected. Abrrevations: *H.c.- Haemaphysalis concinna, D.r. – Dermacentor reticulatus, I.k. - Ixodes kaiseri, I.c. – Ixodes canisuga, I.h. – Ixodes hexagonus, I.r. – Ixodes ricinus*

4.3. Presence of tick-borne pathogens in Red Foxes *(Vulpes vulpes*)

Spleen samples of hunted foxes were individually tested for presence of following tick-borne pathogens: members of the family Anaplasmataceae*, Hepatozoon* spp., *Babesia* spp., *Borrelia burgdorferi* sensu lato, *Rickettsia* spp., *Coxiella burnetii*, *Francisella tularensis* and *Bartonella* spp. DNA of pathogenic microorganisms was detected in spleen samples of 94 out of 129 animals (72.9%), in 40 out of 54 females (74.1%) and 54 out of 73 males (74%), originating at all study 14 localities. The majority of infected animals originated from three localities where the highest number of animals was collected – Veliko Gradište (30 infected/45 collected animals, 66.7%), Surčin (23/29, 79.3%) and Svilajnac (12/13, 92.3%) (Tab. 3).

Tab. 3. Number and prevalence of animals in which tick-borne pathogens were detected

Locality	Number of positive animals	Total number of animals	Prevalence %
Veliko Gradište	30	45	66.7
Surčin	23	29	79.3
Obrenovac	6	6	100
Velika Plana	6	7	85.7
Svilajnac	12	13	92.3
Negotin	1	2	50
Despotovac	1	1	100
Rekovac	3	5	60
Kraljevo	3	3	100
Vrnjačka Banja	2	5	40
Trstenik	1	3	33.3
Blace	1	3	33.3
Niš	4	5	80
Bela Palanka	1	2	50

PCR reactions used for initial screening were positive for *Anaplasmataceae, Hepatozoon* spp., *Babesia* spp., *Borrelia burgdorferi* sensu lato and negative for *Rickettsia* spp., *Coxiella burnetii*, *Francisella tularensis* and *Bartonella* spp.

4.3.1. Presence of *Hepatozoon* spp. in Red Foxes (*Vulpes vulpes*)

DNA of *Hepatozoon* spp. was detected in 79 out of 129 analyzed animals (61.2%), in 33 (58.9%, 33/56) female and 45 (61.6%, 45/73) male foxes. After sequencing and analyzing the data, all samples positive for the presence of DNA of *Hepatozoon* spp. were identified as *Hepatozoon canis.* Animals positive for the presence of DNA of *Hepatozoon canis* originated at 12 out of the 14 locations; they were absent at Negotin and Trstenik (Fig. 28). The majority of positive samples were collected from Veliko Gradište (28), Surčin (15) and Svilajnac (11) (Fig. 28, Tab. 4).

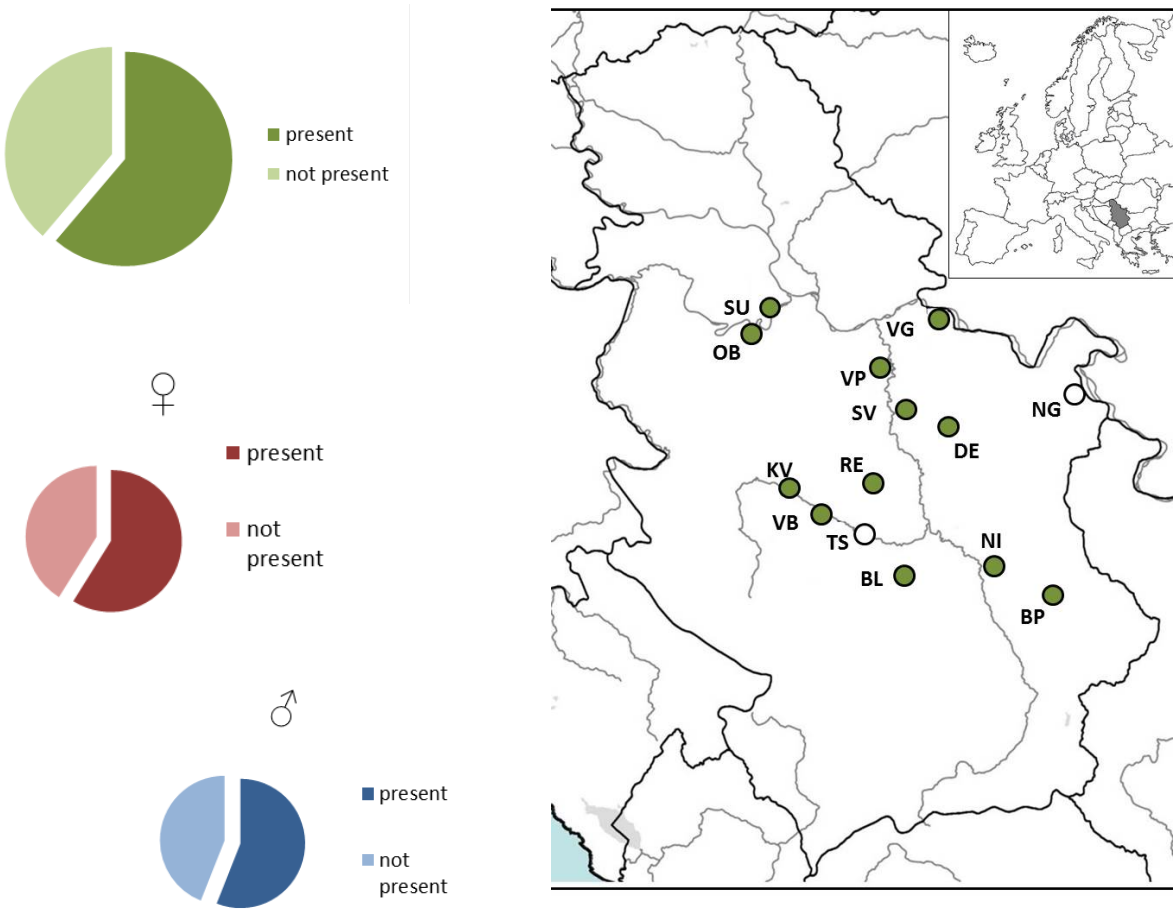


Fig. 28. Prevalence and distribution of *Hepatozoon canis* in analyzed Red Foxes (*Vulpes vulpes*). Green dot-pathogen present. Abrrevations SU-Surčin, OB-Obrenovac, VG-Veliko Gradište, VP-Velika Plana, SV-Svilajnac, DE-Despotovac, NG-Negotin, RE-Rekovac, KV-Kraljevo, VB-Vrnjačka Banja, TS-Trstenik, BL-Blace, NI-Niš, BP-Bela Palanka

Tab. 4. Number and prevalence of animals in which *Hepatozoon canis* was detected.

Locality	Number of positive animals	Total number of animals	Prevalence %
Veliko Gradište	28	45	62.2
Surčin	15	29	51.7
Obrenovac	5	6	83.3
Velika Plana	6	7	85.7
Svilajnac	11	13	84.6
Negotin	0	2	0
Despotovac	1	1	100
Rekovac	3	5	60
Kraljevo	3	3	100
Vrnjačka Banja	2	5	40
Trstenik	0	3	0
Blace	1	3	33.3
Niš	3	5	60
Bela Palanka	1	2	50

The highest number of positive animals were collected in 2011, 2013 and 2016, 23, 22 and 13 respectively, in years when the majority of animals were collected (Fig. 29). The prevalence of animals positive for the presence of *Hepatozoon canis* varied from 37.5% in 2013 (3 positive/8 collected) to 100% in 2010 (9/9) (Tab. 5). The majority of positive animals were collected during winter months January, February and December, 29, 23 and 19 respectively, when most of the samples were collected (Fig. 30).

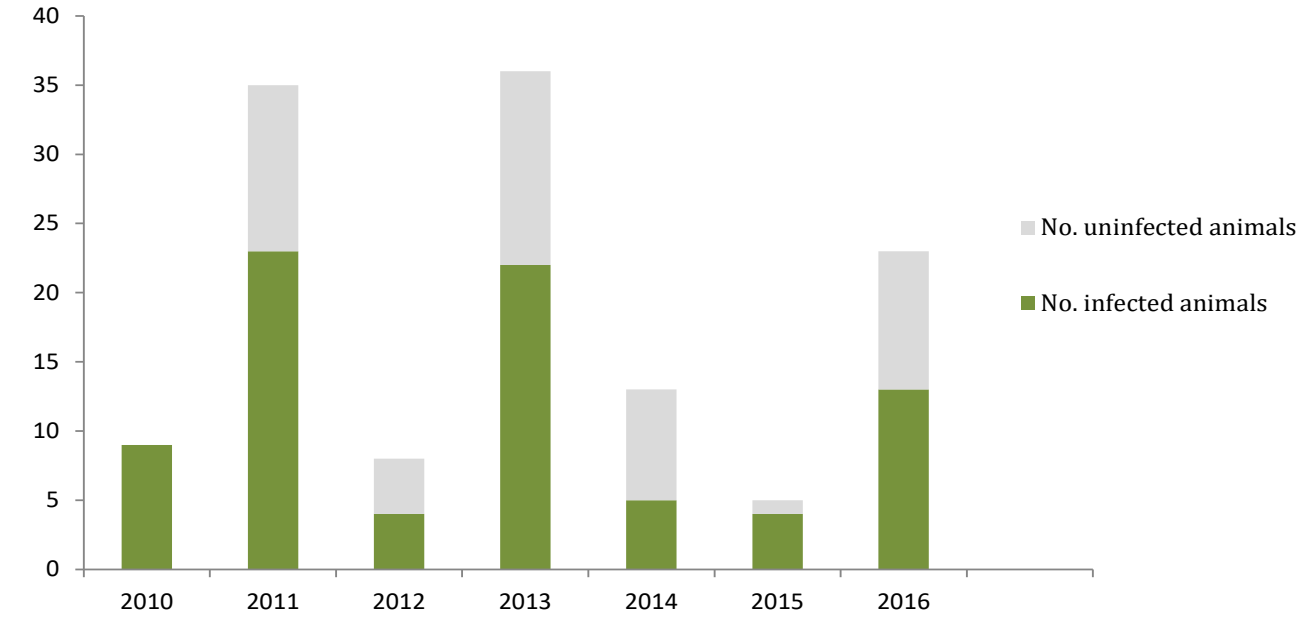


Fig. 29. Number of collected animals and animals positive for presence of *Hepatozoon canis* in each study year.

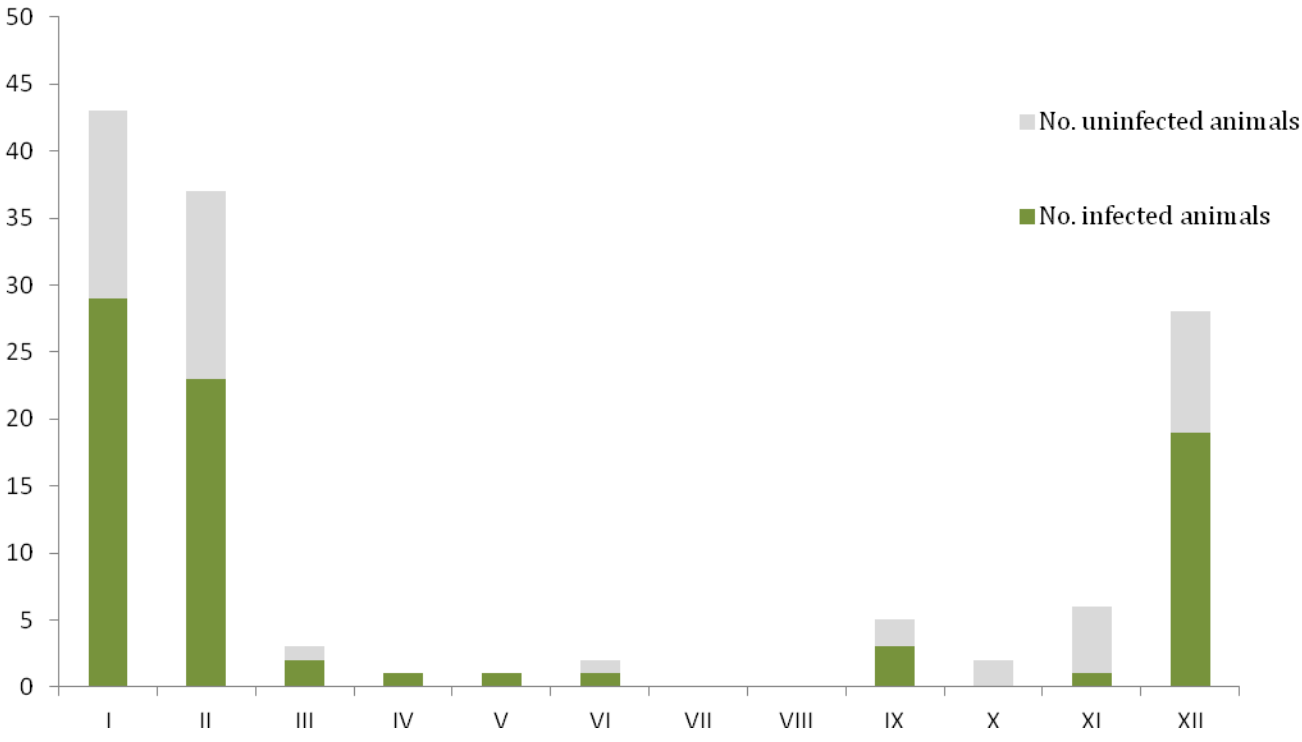


Fig. 30. Cumulative number of animals and animals positive for presence of *Hepatozoon canis* in each month of the study.

Tab. 5. Number and prevalence of animals positive for the presence of *Hepatozoon canis.*

year / month	2010	2011	2012	2013	2014	2015	2016	Σ	Prevalence %
January		9		9	3	1	7	**29**	64.4
February		9		6	1	1	6	**23**	62.2
March				1	1			**2**	66.7
April				1				**1**	100
May				1				**1**	100
June				1				**1**	50
July									0
August									0
September				3				**3**	60
October									0
November						1		**1**	16.7
December	9	5	3			1		**18**	64.3
Σ	**9**	**23**	**3**	**22**	**5**	**4**	**13**	**79**	
Prevalence %	100	65.7	37.5	61.1	38.5	80	56.5	61.2	

Sequences of samples positive for *Hepatozoon canis* showed high levels of similarity, and nine representative sequences were deposited to the GenBank under accession numbers MH699884-MH699892. When the 18S rRNA sequences obtained in this study were compared with the sequences available in GenBank, they have shown 100% similarity to the sequences from different hosts originating in several European countries, e.g., foxes from Spain (AY150067), Croatia (HM212626), Italy (GU371448, GU371447), Romania (KM096414), and Slovakia (KX887327, KX887323); golden jackals from Hungary (KJ572976), Romania (KX712129, KX712127), and Austria (KX712123); dogs from Croatia (FJ497022, FJ497021, FJ497020) and Turkey (KY247115, KY247114, KY247113, KY247112, KY247111); *R. sanguineus* ticks from Turkey (KY197000, KY196999).

4.3.2. Presence of *Babesia* spp. in Red Foxes (*Vulpes vulpes*)

DNA of *Babesia* spp. was detected in 38 out of 129 analyzed animals (29.5%). After sequencing and analyzing the obtained sequences, the presence of two *Babesia* species was confirmed: *Babesia vulpes* in 37/129 samples (28.7%) and *Babesia canis* in 1/129 sample (0.8%). DNA of *Babesia vulpes* was detected in 14 female (14/56, 25%) and 23 male (23/73, 31.5%) animals. Foxes infected with *Babesia vulpes* were collected at 9 out of 14 localities (Fig. 31, Tab. 6), while *Babesia canis* was detected in a male fox collected at Surčin locality in February 2011 (Fig. 31).

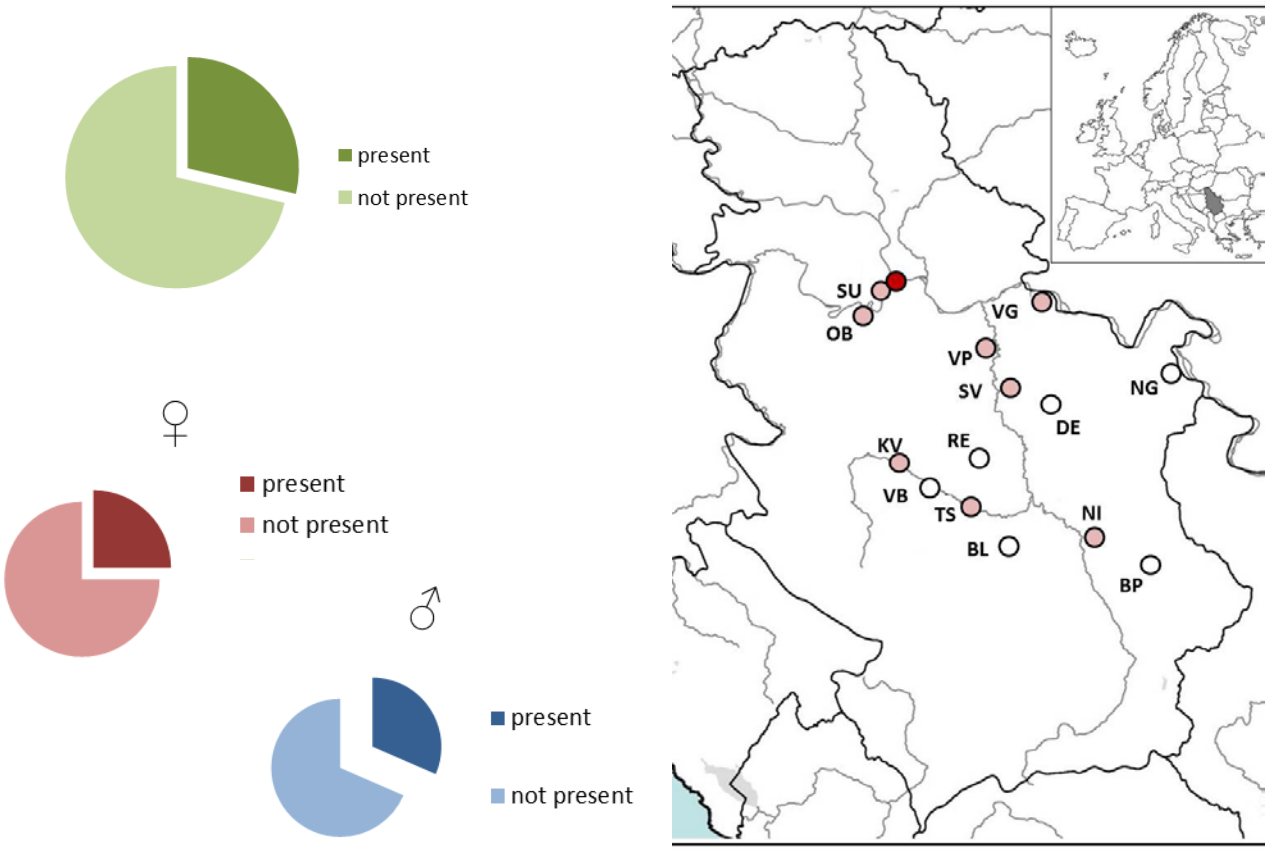


Fig. 31.Prevalence and distribution of *Babesia vulpes* and *Babesia canis* in analyzed Red Foxes (map: pink dot - *Babesia vulpes,* red dot - *Babesia canis*). Abrrevations SU-Surčin, OB-Obrenovac, VG-Veliko Gradište, VP-Velika Plana, SV-Svilajnac, DE-Despotovac, NG-Negotin, RE-Rekovac, KV-Kraljevo, VB-Vrnjačka Banja, TS-Trstenik, BL-Blace, NI-Niš, BP-Bela Palanka

Tab. 6. Number and prevalence of animals in which *Babesia vulpes* was detected.

Locality	Number of positive animals	Total number of animals	Prevalence %
Veliko Gradište	9	45	20
Surčin	15	29	51.7
Obrenovac	2	6	33.3
Velika Plana	3	7	42.9
Svilajnac	3	13	23.1
Negotin	0	2	0
Despotovac	0	1	0
Rekovac	0	5	0
Kraljevo	1	3	33.3
Vrnjačka Banja	0	5	0
Trstenik	1	3	33.3
Blace	0	3	0
Niš	2	5	40
Bela Palanka	0	2	0

The highest number of positive animals were collected in 2013, 2014 and 2016 - 13, 7 and 8, respectively (Fig. 32). The prevalence of animals positive for presence of *Babesia vulpes* varied from 5.8% in 2011 (2 positive/35 collected) to 60% in 2015 (3/5) (Tab. 7). The majority of positive animals were collected during winter months (January, February and December - 11, 11 and 6, respectively), in period when most samples were collected overall (Fig. 33).

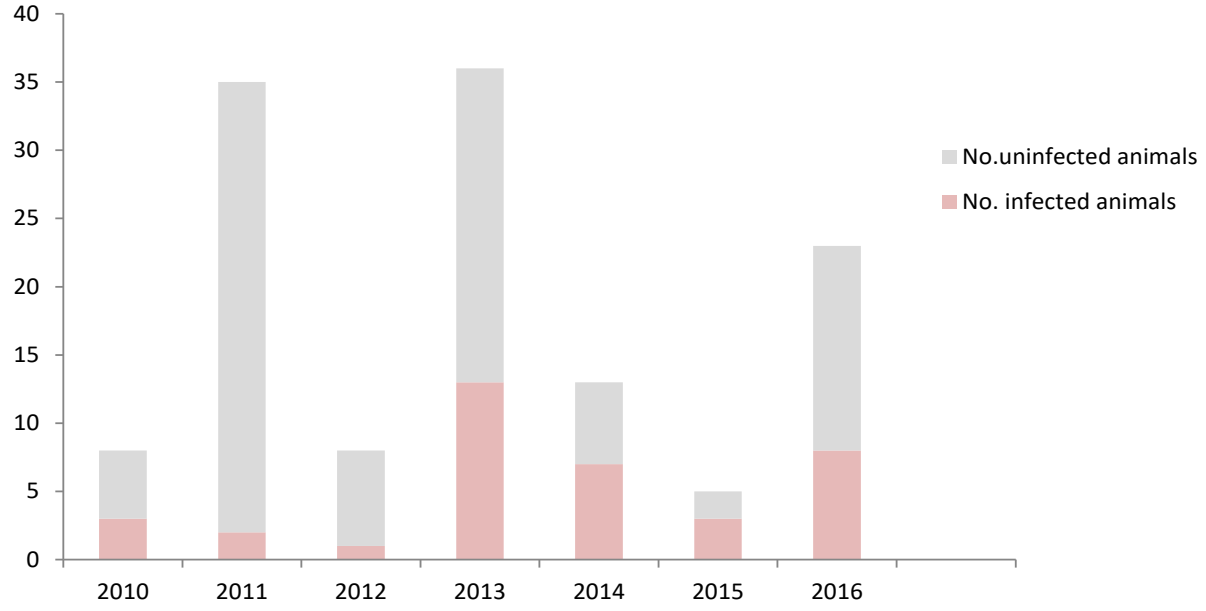


Fig. 32. Number of collected animals and animals positive for presence of *Babesia vulpes* in each year of the study.

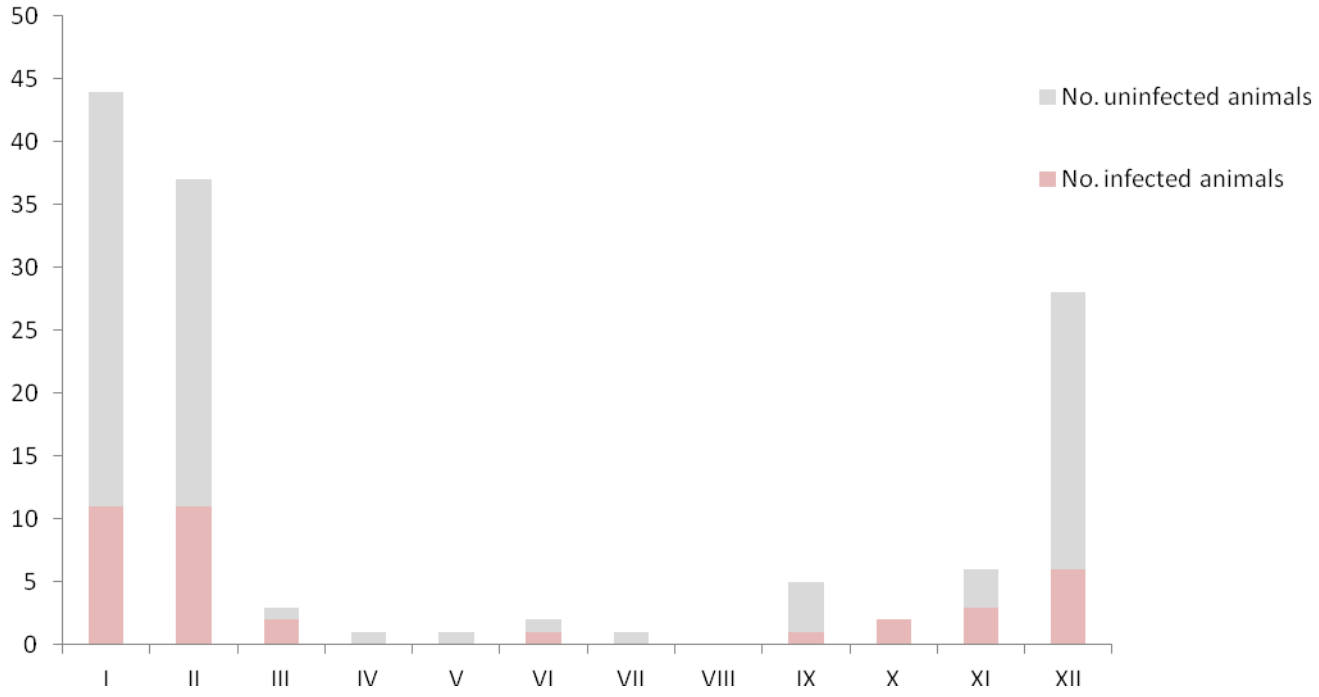


Fig. 33. Cumulative number of collected animals and animals positive for presence of *Babesia vulpes* in during each month of the study.

Tab. 7. Number and prevalence of animals positive for the presence of *Babesia vulpes.*

year / month	2010	2011	2012	2013	2014	2015	2016	Σ	Prevalence %
January		1		2	4		4	**11**	25.6
February				5	1	1	4	**11**	29.7
March				1	1			**2**	66.7
April									0
May									0
June				1				**1**	50
July									0
August									0
September				1				**1**	20
October				1	1			**2**	100
November				2		1		**3**	50
December	3	1	1			1		**6**	21.4
Σ	**3**	**2**	**1**	**13**	**7**	**3**	**8**	**37**	29.5
Prevalence %	33.3	5.8	12.5	36.1	53.8	60	34.8	29.5	

The 18S rRNA sequences of *Babesia vulpes* obtained in this study (representative sequences were deposited under accession numbers MH699381-MH699396) show complete mutual similarity, and 100% identity is evident when compared with the previously deposited sequences available in GenBank from foxes originating from different countries (e.g., Italy MG451839, Austria KY693667, Slovakia KX761397, Spain KT223483).

4.3.3. Presence of *Borrelia burgdorferi* sensu lato in Red Foxes (*Vulpes vulpes*)

Initial molecular detection of DNA in members of *Borrelia burgdorferi* s. l. complex revealed presence of the pathogen in 7 samples (5.4%) originated from 2/73 (2.7%) male and 5/56 (8.9%) female animals. Red Foxes positive for presence of *Borrelia burgdorferi* s. l. were collected ar 5 localities (Surčin, Obrenovac, Veliko Gradište, Negotin and Niš). Sequencing of 5S-23S rRNA intergenic spacer region was successful for four samples and analysis of the obtained sequences confirmed the presence of three *Borrelia burgdorferi* s. l. species, namely: *Borrelia burgdorferi* s. s. (locality Veliko Gradište), *B. lusitaniae* (localities Surčin and Negotin) and *B. garinii* (locality Surčin), (Figure 34, Table 8).

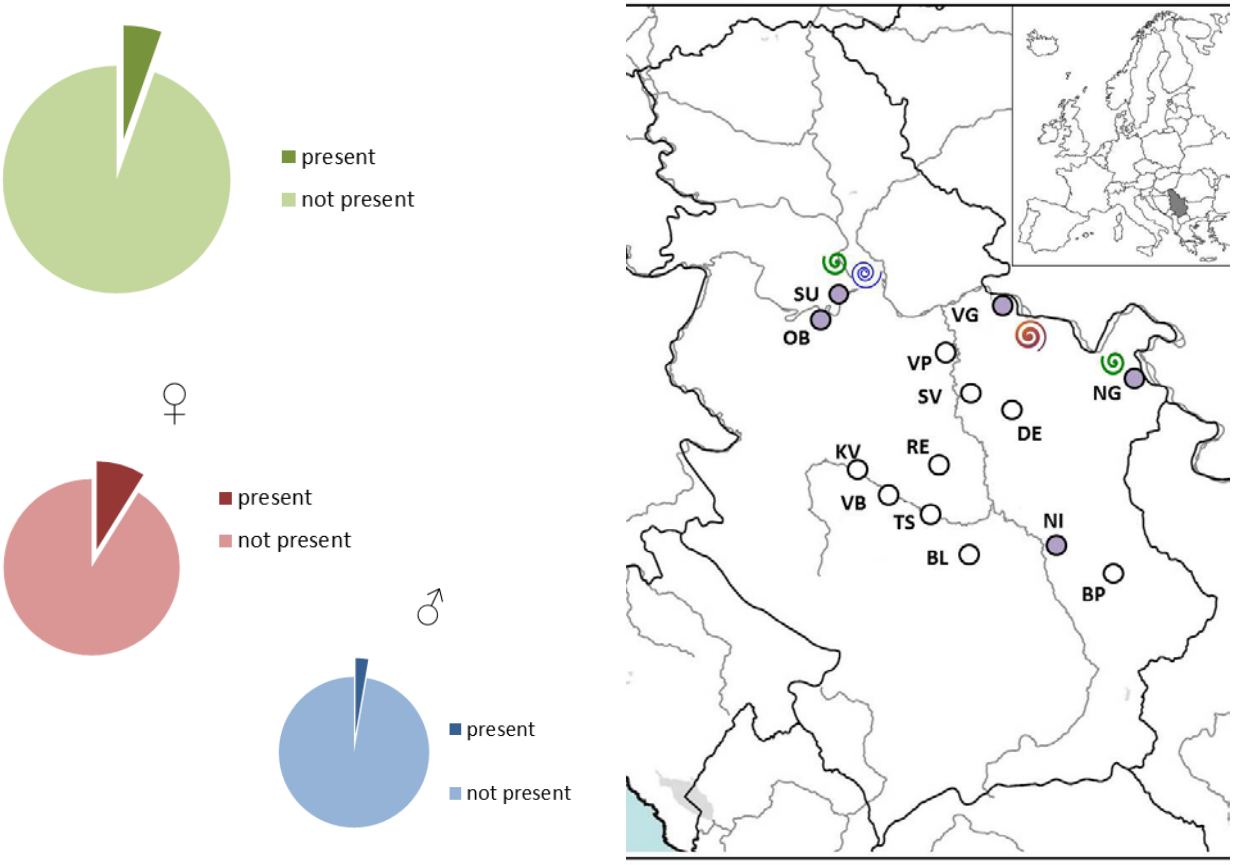


Fig. 34. Prevalence and distribution of *Borrelia burgdorferi* sensu lato in analyzed Red Foxes (*Vulpes vulpes*) (map: green spiral - *Borrelia lusitaniae,* red spiral - *Borrelia burgdorferi* sensu stricto, blue spiral - *Borrelia garinii*). Abrrevations SU-Surčin, OB-Obrenovac, VG-Veliko Gradište, VP-Velika Plana, SV-Svilajnac, DE-Despotovac, NG-Negotin, RE-Rekovac, KV-Kraljevo, VB-Vrnjačka Banja, TS-Trstenik, BL-Blace, NI-Niš, BP-Bela Palanka

Tab. 8. Number and prevalence of animals in which *Borrelia burgdorferi* sensu lato was detected.

Locality	Number of positive animals	Total number of animals	Prevalence %
Veliko Gradište	2	45	4.4
Surčin	2	29	6.9
Obrenovac	1	6	16.7
Velika Plana	0	7	0
Svilajnac	0	13	0
Negotin	1	2	50
Despotovac	0	1	0
Rekovac	0	5	0
Kraljevo	0	3	0
Vrnjačka Banja	0	5	0
Trstenik	0	3	0
Blace	0	3	0
Niš	1	5	20
Bela Palanka	0	2	0

The majority of animals were collected in 2016 (4), while single positive animals were collected in 2010, 2013 and 2014 (Fig. 35). The prevalence of animals positive for the presence of *Borrelia burgdorferi* s. l. varied from 2.8% in 2013 (1 positive/36 collected) to 17.4% in 2016 (4/23).

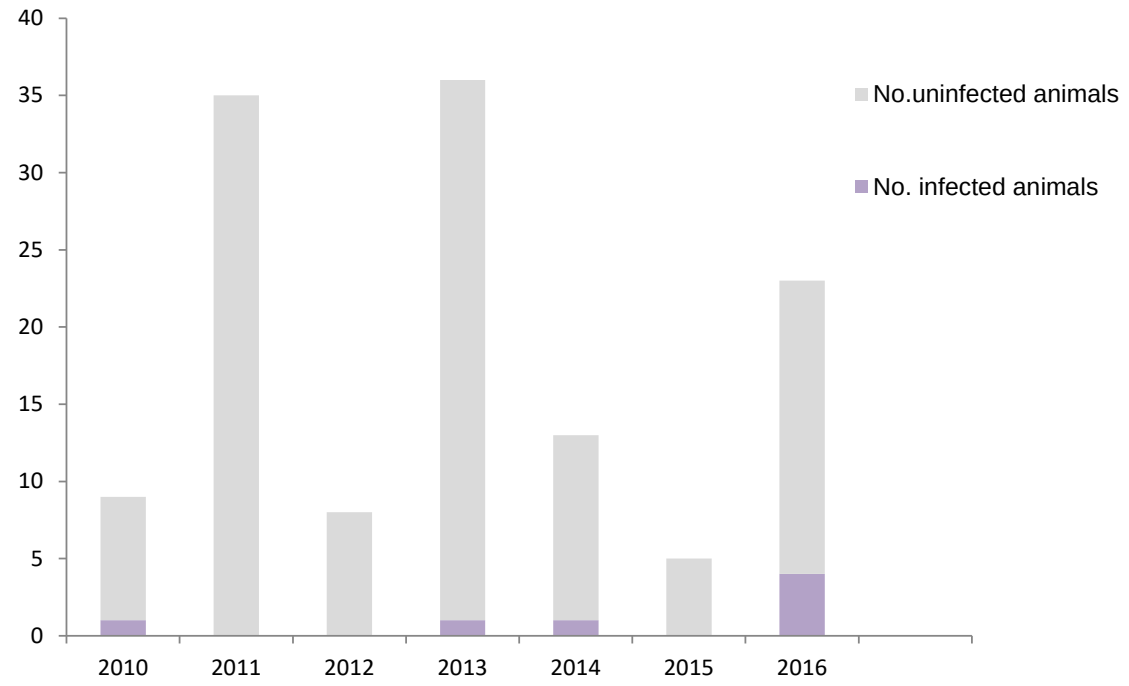


Fig. 35. Number of uninfected animals and animals positive for presence of *Borrelia burgdorferi* sensu lato in each year of the study.

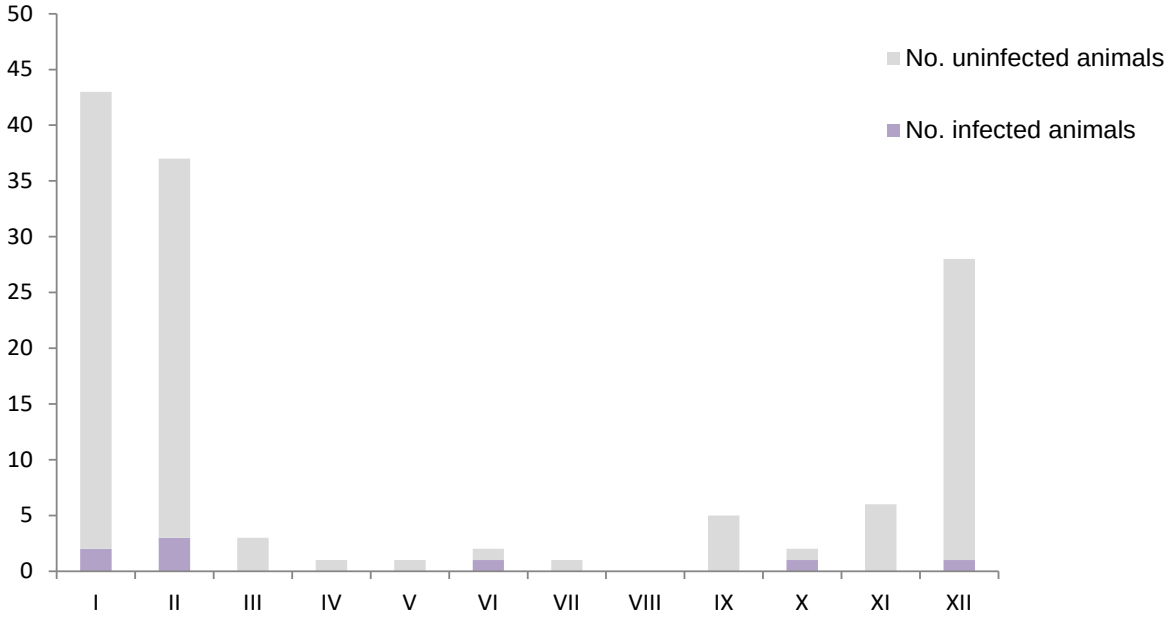


Fig. 36. Cumulative number of collected animals and animals positive for presence of *Borrelia burgdorferi* sensu lato during each calendar month of the study.

4.3.4. Presence of members of the family Anaplasmataceae in Red Foxes *(Vulpes vulpes)*

Initial screening of spleen samples for presence of members of family Anaplasmataceae, with primers specific for 16S rRNA gene fragment (~345 bp), showed 21 positive results, and these samples were forwarded for the sequencing. Obtained sequences were compared with the previously published sequences deposited at the GenBank®, and 8 sequences showed partial coverage with 93-99% similarity to *Candidatus* Neoehrlichia spp. sequences. Samples were further tested with primers specific for longer fragments of *groEL* (~806 bp) and 16S rRNA (~1053 bp) genes of *Candidatus* Neoehrlichia spp. Out of 8 tested samples, amplification was successful for 6 samples, (4.7%) originating from 4/73 (5.5%) male and 2/53 (3.8%) female animals. Red Foxes positive for presence of *Candidatus* Neoehrlichia spp. were collected by hunters at 3 out of 14 study localities: Surčin, Veliko Gradište and Svilajnac (Fig. 37, Tab. 9).

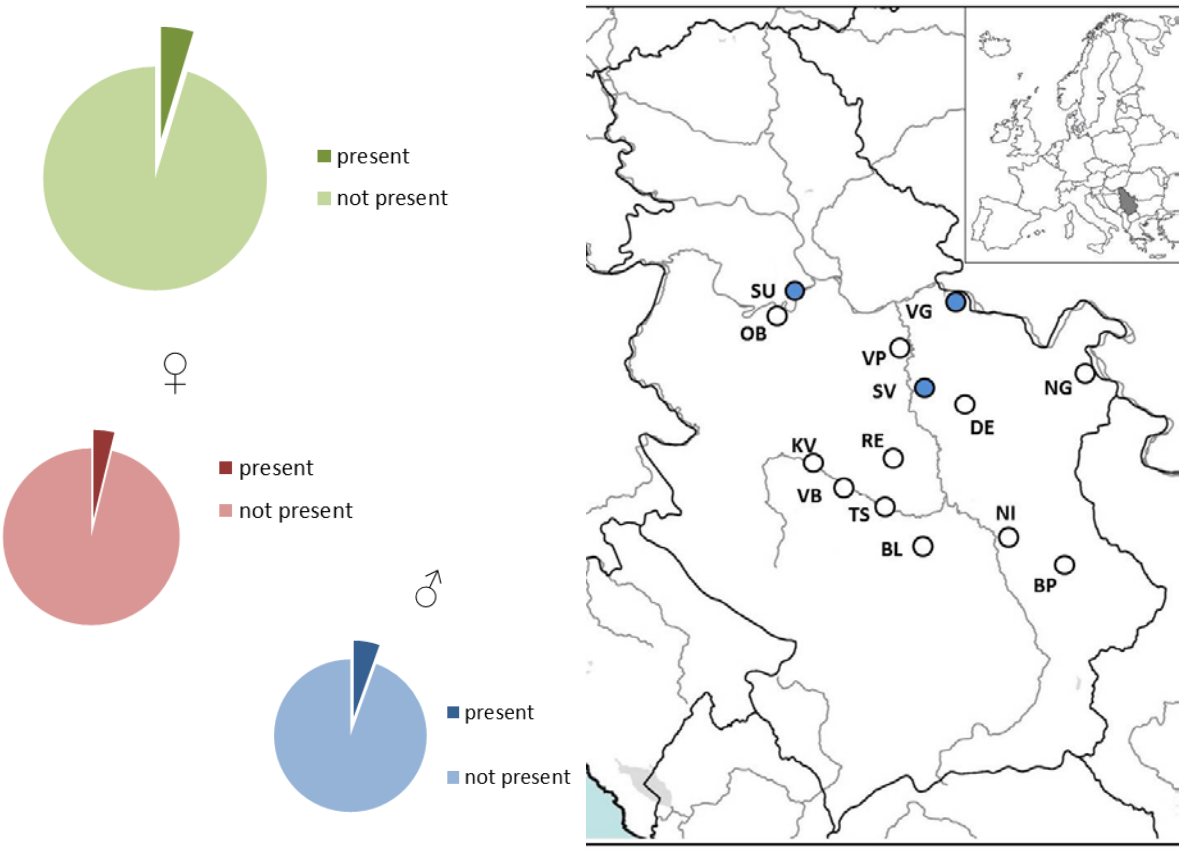


Fig. 37 Prevalence and distribution of *Candidatus* Neoerlichia sp. (FU98) in analyzed Red Foxes (*Vulpes vulpes*) (map: blue dots – pathogen present). Abrrevations SU-Surčin, OB-Obrenovac, VG-Veliko Gradište, VP-Velika Plana, SV-Svilajnac, DE-Despotovac, NG-Negotin, RE-Rekovac, KV-Kraljevo, VB-Vrnjačka Banja, TS-Trstenik, BL-Blace, NI-Niš, BP-Bela Palanka

Tab. 9. Number and prevalence of animals in which *Candidatus* Neoerlichia sp. (FU98) was detected.

Locality	Number of positive animals	Total number of animals	prevalence %
Veliko Gradište	3	45	6.7
Surčin	2	29	6.9
Obrenovac	0	6	0
Velika Plana	0	7	0
Svilajnac	1	13	7.7
Negotin	0	2	0
Despotovac	0	1	0
Rekovac	0	5	0
Kraljevo	0	3	0
Vrnjačka Banja	0	5	0
Trstenik	0	3	0
Blace	0	3	0
Niš	0	5	0
Bela Palanka	0	2	0

The sequences of *Candidatus* Neoehrlichia sp. (*groEL* and 16S rRNA), obtained in this study showed 100% similarity to the sequences of *Candidatus* Neoerlichia sp. (FU98) from Red Foxes from Austria (GenBank® accession numbers: KT833357, KT833358), Raccoon Dogs (*Nyctereutes procyonoides*) from Poland (MG670107, MG670109) and a European Badger (*Meles meles*) from Hungary (KX245423, KX231830).

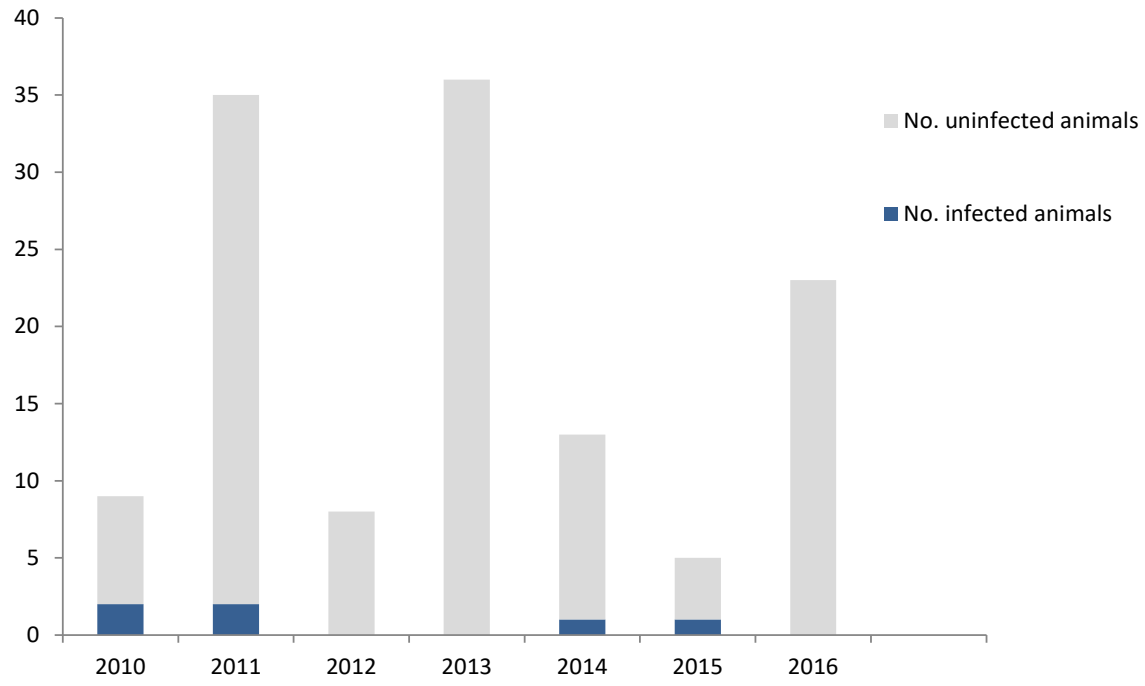


Fig. 38. Number of uninfected animals and animals positive for presence of *Candidatus* Neoerlichia sp. (FU98) in each year of study.

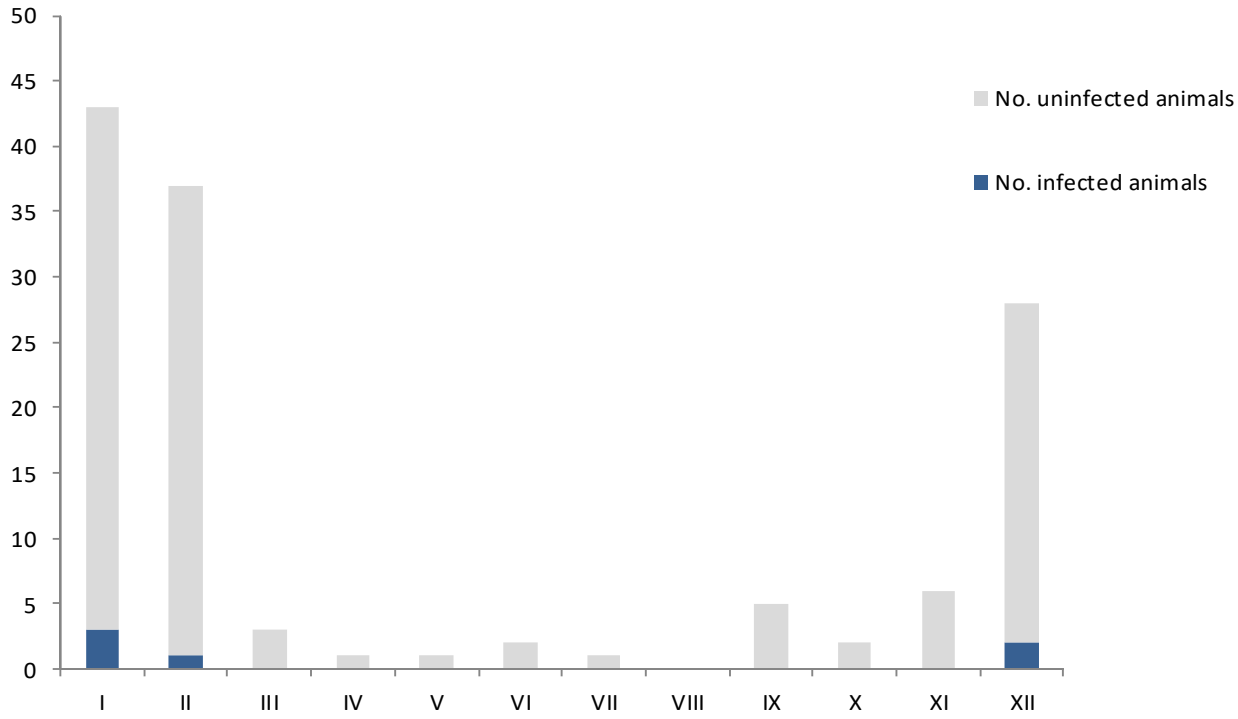


Figure 39. Cumulative number of collected animals and animals positive for presence of *Candidatus* Neoerlichia sp. (FU98) during each calendar month of the study.

4.3.5. Presence of co-infections by detected pathogens in Red Foxes (*Vulpes vulpes*)

Presence of DNA of different pathogenic species was detected in 34 animals (26.3%) from 8 localities. The highest number of animals harboring multiple pathogens was detected in Veliko Gradište and Surčin (11), followed by Svilajnac (3), Obrenovac, Niš, Velika Plana (2) and Kraljevo (1). Double infections were present in 32 animals (24.8%), while triple infections were detected in two animals (1.5%) (Fig. 40).

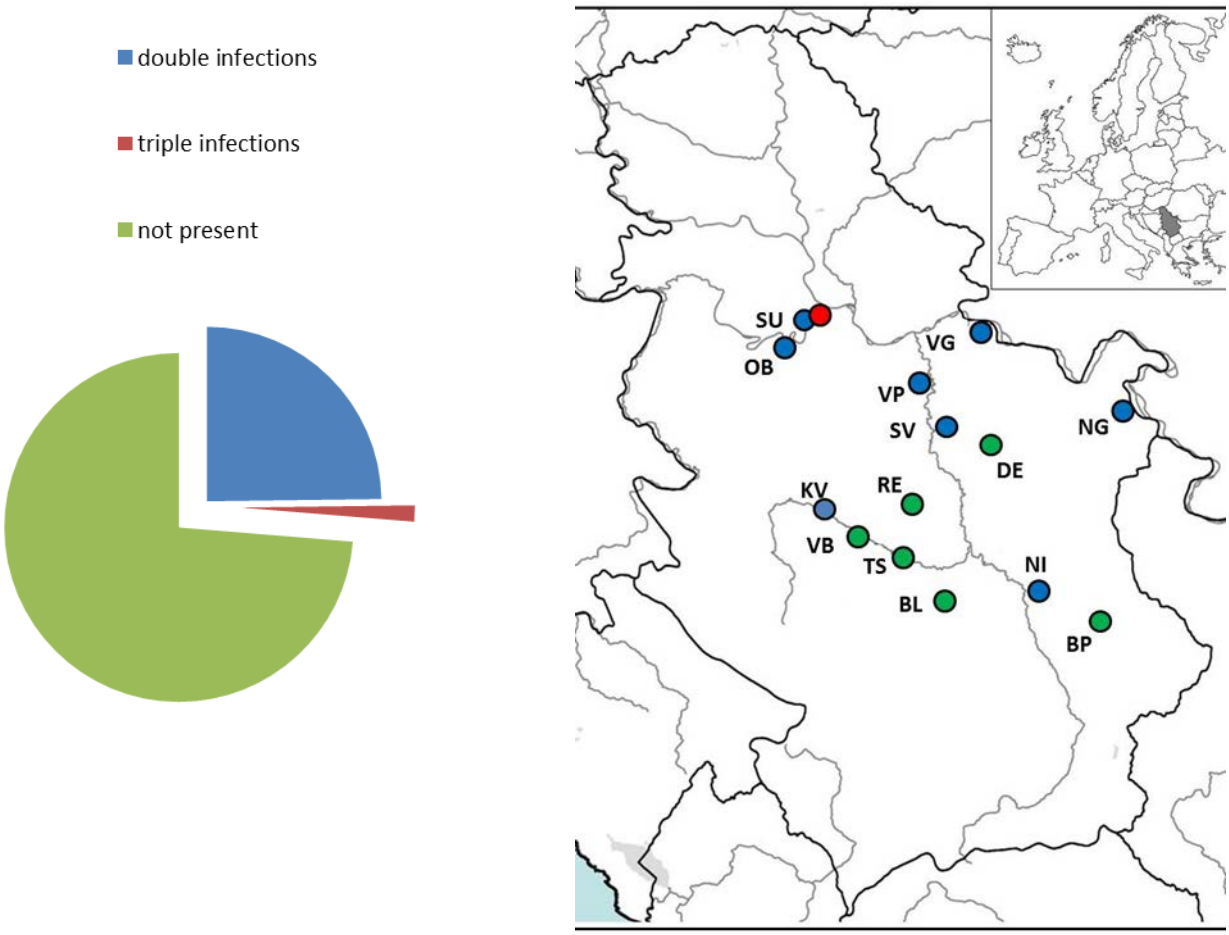


Fig. 40. Prevalence and distribution of multiple infections in analyzed Red Foxes (*Vulpes vulpes*). Abrrevations SU-Surčin, OB-Obrenovac, VG-Veliko Gradište, VP-Velika Plana, SV-Svilajnac, DE-Despotovac, NG-Negotin, RE-Rekovac, KV-Kraljevo, VB-Vrnjačka Banja, TS-Trstenik, BL-Blace, NI-Niš, BP-Bela Palanka

The majority of multiple infections included *Hepatozoon canis*. *Babesia vulpes*/*Hepatozoon canis* was present in 26 (20.2%) foxes, one animal harbored *Babesia canis/Hepatozoon canis* (0.8%), four *Hepatozoon canis*/ *Candidatus* Neoerlichia sp. (FU98) (3.1%), one *Hepatozoon canis*/*Borrelia burgdorferi* s. l. (0.8%), one *Babesia vulpes*/*Hepatozoon canis*/ *Borrelia* burgdorferi s. l. (0.8%) and one *Babesia vulpes*/*Hepatozoon canis*/ *Candidatus* Neoerlichia sp. (FU98) (0.8%). Three animals harbored *Babesia vulpes/Borrelia burgdorferi* s. l. co-infection (Fig. 41).

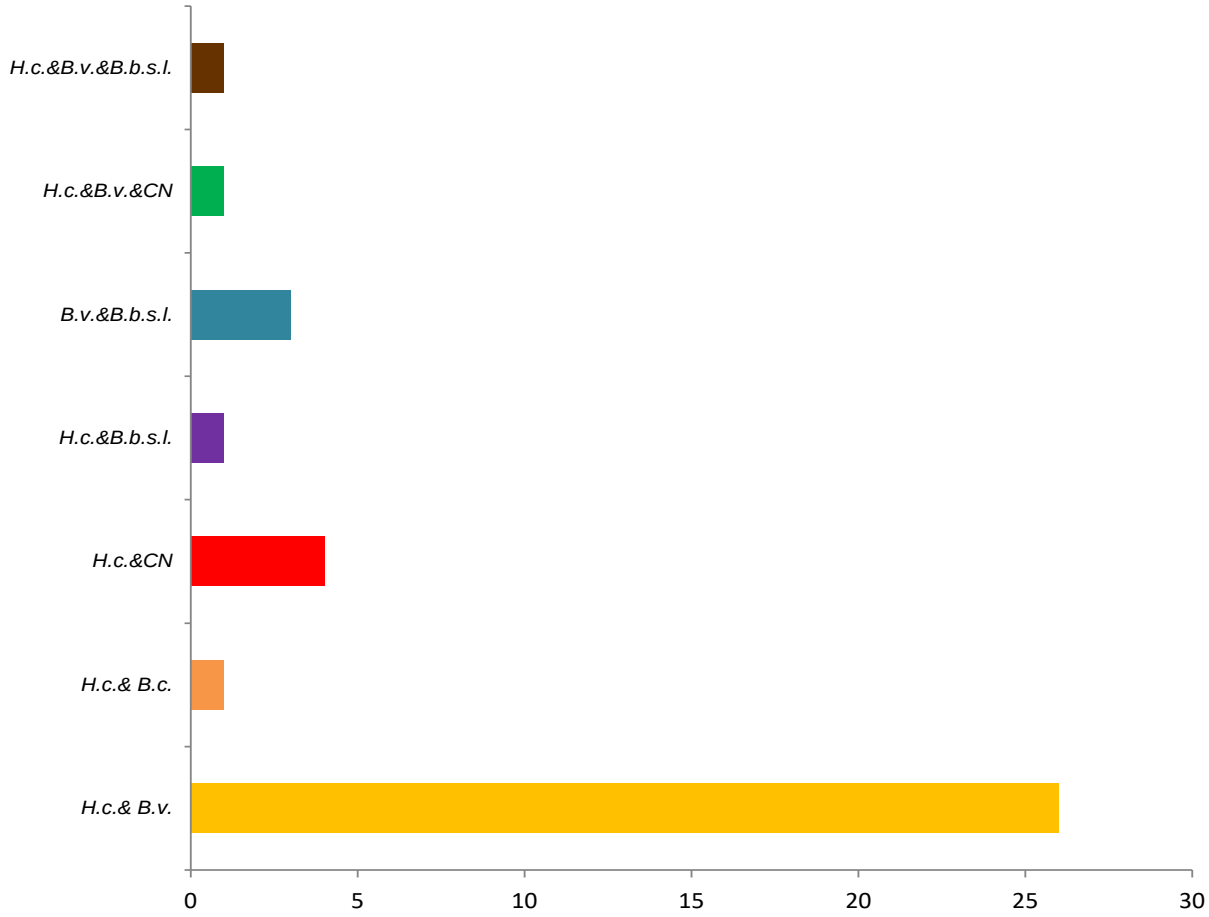


Fig. 41. Number of Red Foxes (*Vulpes vulpes*) infected with different types of multiple infections analyzed in the study. Abrrevations – H.c. – Haemaphysalis concinna, B.v. – Babesia vulpes, B.b.s.l. – *Borrelia burgdorferi* sensu lato, CN – *Candidatus* Neoerlichia sp. (FU98),

Multiple infection by *Babesia vulpes*/*Hepatozoon canis* was recorded in animals from seven localities (Veliko Gradište, Svilajnac, Surčin, Kraljevo, Obrenovac, Niš and Velika Plana), *Babesia canis/Hepatozoon canis* in a fox from Surčin, *Hepatozoon canis*/ *Candidatus* Neoerlichia sp. (FU98) in animals from Veliko Gradište and Svilajnac, *Hepatozoon canis*/*Borrelia burgdorferi* s. l. in a fox from Veliko Gradište, *Babesia vulpes*/*Hepatozoon canis*/ *Borrelia burgdorferi* s. l. and *Babesia vulpes*/*Hepatozoon canis*/ *Candidatus* Neoerlichia sp. (FU98) in single foxes from Surčin, and *Babesia vulpes/Borrelia burgdorferi* s. l. sensu lato in animals from Negotin and Surčin (Fig. 42).

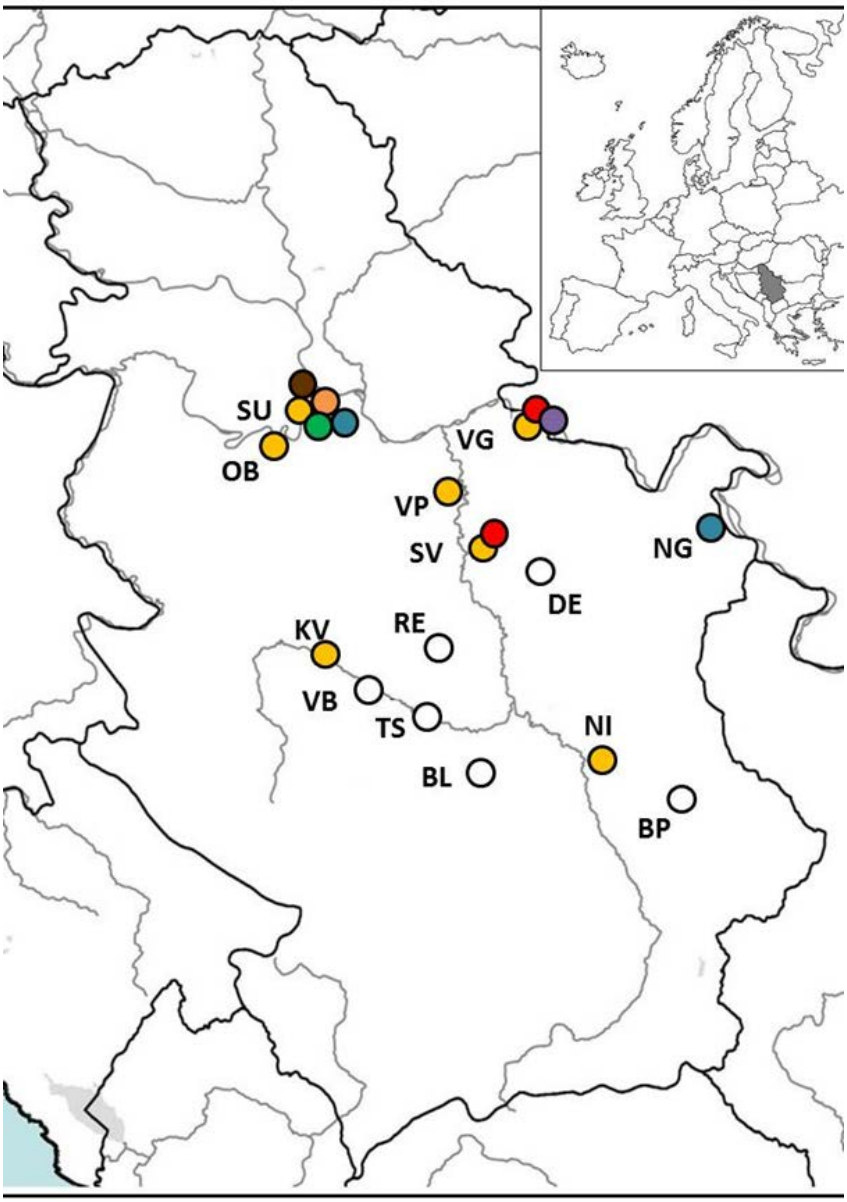


Fig. 42. Distribution of Red Foxes (*Vulpes vulpes*) infected with different types of multiple infections analyzed in the study (yellow dot - *Hepatozoon canis/Babesia vulpes*, orange dot - *Hepatozoon canis/Babesia canis,* red dot - *Hepatozoon canis/Candidatus* Neoerlichia sp. (FU98), violet dot – *Hepatozoon canis*/*Borrelia burgdorferi* sensu lato, blue dot - *Babesia vulpes/ Borrelia burgdorferi* sensu lato, green dot – *Hepatozoon canis/Babesia vulpes*/*Candidatus* Neoerlichia sp. (FU98), brawn dot - *Babesia vulpes*/ *Hepatozoon canis / Borrelia burgdorferi sensu lato.* Abrrevations SU-Surčin, OB-Obrenovac, VG-Veliko Gradište, VP-Velika Plana, SV-Svilajnac, DE-Despotovac, NG-Negotin, RE-Rekovac, KV-Kraljevo, VB-Vrnjačka Banja, TS-Trstenik, BL-Blace, NI-Niš, BP-Bela Palanka

5. Discussion

This research represents the first complex and systematic study of tick fauna in Serbia in the Red Fox (*Vulpes vulpes*) as host, and of the role of this carnivore as host for tick-borne pathogens. Among the five canid species distributed in Europe, Red Fox is the best-studied from the epizoological point of view, however knowledge is still scarce. Results of recent studies indicate that Red Fox is a host and a reservoir for a number of ticks and tick-borne pathogen species (Dumitrashe *et al.*, 2014; Tolnai *et al.*, 2015; Hodžić *et al.*, 2018).

For this study Red Foxes were collected within approximately half of total territory of Serbia (14 localities), during the period 2010-2016. A total of 129 Red Foxes have been collected and inspected for ticks. All 113 recorded ticks were identified to species level.

Tick fauna in Red Fox population in Serbia has been studied at 14 localities (Veliko Gradište, Surčin, Obrenovac, Velika Plana, Svilajnac, Negotin, Despotovac, Rekovac, Kraljevo, Vrnjačka Banja, Trstenik, Blace, Niš and Bela Palanka). Most of the localities are situated in the central part of country. There were six recorded tick species (*Ixodes ricinus, Ixodes hexagonus, Ixodes canisuga, Ixodes kaiseri, Dermacentor reticulates* and *Haemaphysalis concinna*). These results are consistent with the earlier published results by Tomanović *et al.* (2013). Their sample included 58 collected and inspected Red Foxes and five tick species recorded in Red Fox as a host (Tomanović *et al.*, 2013). Results from another recently published research from Serbia showed presence of different tick species. Stojanov *et al.* (2014) recorded four tick species in the Red Fox. Only one of them (*Ixodes ricinus*) was also confirmed in this study. Three species (*Dermacentor marginatus, Rhipicephalus sanguineus* and *Haemaphysalis punctata*) recorded by Stojanov *et al.* (2014) were not found during this study and are therefore absent from our list of tick fauna. These differences might be explained by habitat differences. Studies carried out by Stojanov *et al.* (2014) were focused on two localities in the northern part of Serbia (Vojvodina) dominated by agricultural land (>80%). In the region of Central Serbia habitats are more mosaic, with a higher percentage of forest within the studied localities (Tomanović *et al.,* 2013; this study).

Ticks were also collected from Golden Jackals (*Canis aureus*) at almost the same localities (Titel, Surčin, Smederevo, Smederevska Palanka, Velika Plana, Veliko Gradište, Svilajnac, Bela Palanka, Negotin and Zaječar) (Sukara, 2019). In the total, three tick species (*Ixodes ricinus, Dermacentor reticulates* and *Haemaphysalis concinna*) were found at seven localities (Titel, Surčin, Smederevo, Smederevska Palanka, Veliko Gradište, Svilajnac, Bela Palanka). All three species described as tick fauna in the Golden Jackal population from Serbia (Sukara, 2019) are also common ticks for the Red Fox population (Stojanov *et al*., 2014; Tomanović *et al.*, 2013; this study).

Regarding the geographic point of view, the largest range is that of *Ixodes ricinus*. It was confirmed in all recent studies on mesocarnivora in Serbia. This tick species was found in all localities, both on foxes and jackals. *Ixodes ricinus* also had the highest prevalence and was the most abundant tick species in all studies carried out in Serbia (Stojanov *et al.*, 2014; Tomanović *et al.*, 2013; Sukara, 2019; this study). *Ixodes hexagonus* and *Ixodes canisuga* shared the second place among the widespread ticks in fox population in Serbia. According to the results of this study prevalences of those tick species were 11.5% and 5.3% respectively. Both tick species were collected from five out of 14 studied localities on Red Foxes. In the research provided by Tomanović *et al.* (2013) the abundances of these ticks were opposite. *Ixodes canisuga* was more abundant than *Ixodes hexagonus* (14.28% and 10% of collected ticks, respectively). These differences between studies could be influenced by differences in sample size. Tomanović *et al.*

(2013) collected 70 ticks from 58 Red Foxes while in this study sample size was larger (113 recorded and identified ticks).

All six tick species were recorded at Surčin site, including *Haemaphysalis concinna* and *Dermacentor reticulatus* that were not recorded at other localities. These tick species are able to tolerate lower humidity of habitats and therefore occupy landscapes dominated by agricultural land, such as the Surčin site.

Our study reported presence of *Ixodes kaiseri* for the first time in Serbia. Molecular identification used to confirm morphological determination of tick species belonging to subgenus Pholeixodes (*Ixodes canisuga* and *Ixodes hexagonus*) has shown positive results for adult ticks of both species, while six out of nine nymphal ticks classified as *Ixodes canisuga* based on morphological keys were re-classified as *Ixodes kaiseri* according to analyzed sequences. For the remaining three nymphal ticks, morphological identification as *Ixodes canisuga* was confirmed.

Ixodes kaiseri is known to parasitize mostly carnivores and is present in North Africa (Arthur, 1957), Middle East (Theodor and Costa, 1967), Turkey (Orkun and Karaer, 2018) and China (Sheng *et al.*, 2019). In the region, *Ixodes kaiseri* have been previously reported in Romania (Filipova and Uspenskaya, 1973), recently reconfirmed in Romania and recorded for the first time in Germany and Hungary (Hornok *et al.*, 2017b). Due to morphological and ecological similarities to other *Ixodes* species parasitizing wild carnivores, *Ixodes hexagonus* and *Ixodes canisuga,* the new records in Serbia, Hungary and Germany might be a result of previous misidentification. However a more recent arrival of the species cannot be completely ruled out.

Similar tick fauna was found in Red Fox populations in the neighboring countries (Croatia, Hungary and Romania) (Dumitrache *et al.*, 2014; Jemeršić *et al.*, 2014; Sréter *et al.*, 2003). Five tick species were identified in Romania (*Haemaphysalis punctata, Dermacentor marginatus, Ixodes hexagonus, Ixodes ricinus,* and *Ixodex crenulatus*) and Croatia (*Ixodes ricinus, Ixodes hexagonus, Haemaphysalis punctata, Dermacentor reticulatus, Rhipicephalus sanguineus*) while four species (*Ixodes ricinus*, *Ixodes canisuga*, *Haemaphysalis concinna*, and *Demacentor reticulatus*) were recorded in Hungary. Just like in Serbia, the tick species with highest prevalence in Hungary was *Ixodes ricinus* – 45% (Sréter *et al.*, 2003) while in Romania it was *Ixodes hexagonus* with prevalence of 72.44% (Dumitrache *et al.,* 2014). *Ixodes ricinus* has shown second highest prevalence in Romania, followed by *Ixodes canisuga* – 28.84% and 19% respectively (Dumitrache *et al.*, 2014; Sréter *et al.*, 2003). Similar high prevalence (69%) for dominant tick species *Ixodes ricinus* was found in Serbia as well. Unfortunately Jemeršić *et al.* (2014) did not report prevalence for ticks recorded in the Red Fox population from Croatia. Therefore we cannot discuss prevalence with data from Croatia.

In general, *Ixodes ricinus* is a common tick parasite on Red Fox, with highest prevalence among all tick species recorded in Europe (Hinaidy, 1971; 1976; Schöffel *et al.*, 1991; Lassnig *et al.*, 1998; Dominuez, 2004; Kočišová *et al.*, 2006; Perrucci *et al.*, 2016). In comparison to the available recently published data from the rest of Europe, relatively low prevalence were found in Austria, north Italy and Slovakia (19%, 6% and 17.9% respectively) (Kočišová *et al.*, 2006; Lassnig *et al.*, 1998; Schöffel *et al.*, 1991; summarized by Sréter *et al.*, 2003) and moderate to high in Germany and Spain (27% and 34.6% respectively) (Schöffel *et al.*, 1991; Dominuez, 2004). In those countries *Ixodes hexagonus* had the second-highest prevalence in Austria, Germany and Spain, with very low prevalence in Austria (4%), moderate in Germany (18%) and high in Spain (30,8%) while in Slovakia the second most important species was *Demacentor reticulatus*, also with low prevalence (3.8%) (Hinaidy 1971; 1976; Schöffel *et al.*, 1991; Lassnig *et al.*, 1998; Dominuez 2004; Kočišová *et al.*, 2006). Different pattern was reported from Great Britain (Harris and Thompson, 1978). Authors of that study recorded only two species (*Ixodes hexagonus* and *Ixodes canisuga*), with high prevalence.

A completely different tick fauna was reported for North and Northeastern Mediterranean region (Chochlakis *et al.*, 2011; Keysary *et al.*, 2011; Psaroulaki *et al.*, 2014; Vincenzo *et al.*, 2011). Six tick species (*Dermacentor marginatus, Rhipicephalus bursa, Rhipicephalus turanicus, Haemaphysalis erinacei, Ixodes canisuga, Ixodes ricinus*) were reported in the Red Fox population from Italy (Vincenzo *et al.*, 2011), four tick species from Cyprus (*Rhipicephalus sanguineus, Rhipicephalus turanicus, Ixodes ventalloi,* and *Ixodes gibbosus*) (Chochlakis *et al.*, 2011; Psaroulaki, *et al.*, 2014), three in Israel (*Rhipicephalus turanicus, Haemaphysalis adleri* and *Haemaphysalis parva*) (Keysary *et al.*, 2011) and only a single one (*Rhipicephalus turanicus*) in France (Marié *et al.*, 2012). These differences in tick fauna composition between Serbia and the general continental part of Europe with Mediterranean region is influenced by differences in climate, habitats and ecosystems.

Considering the count vector capacity and importance in human and animal medicine, *Ixodes ricinus* is the most significant species found to parasitize Red Foxes in Serbia. It is the confirmed vector for a number of viral, bacterial and protozoal pathogens (tick-borne encephalitis virus, *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum, Francisella tularensis, Rickettsia helvetica, Babesia divergens, Babesia microti* etc.) (Raoult and Roux 1997; Gorenflot *et al.*, 1998; Randolph 2001). Being the most widely distributed European tick species that shows very aggressive and unselective behavior toward potential hosts, it represents a huge hazard for public health. Like in the rest of Europe, *Ixodes ricinus* is the most abundant and the most widely distributed tick species in Serbia (Petrović 1979; Milutinović 1992; Milutinović and Radulović 2002) and the tick species most frequently found to parasitize humans. According to the earlier studies, *Ixodes ricinus* is found to harbor number of zoonotic pathogen species (*Borrelia afzelii, Borrelia gariniii, Borrelia bavarensis, Borrelia burgdorferi* sensu stricto, *Borrelia lusitaniae, Anaplasma phagocytophilum, Babesia venatorum, Babesia microti, Rickettsia monacensis, Rickettsia helvetica, Borrelia myamotoi, Anaplasma ovis, Francisella tularensis, Coxiella burnetii, Candidatus Neoerlichia* micurensis*,* Tick borne encephalitis virus, Crimean Congo hemorrhagic fever virus) (Milutinović *et al.*, 2008, Radulović *et al.*, 2011, Milutinović *et al.*, 2012, Tomanović *et al.*, 2013, Potkonjak *et al.*, 2016, Ćakić *et al.*, 2019).

Another anthropophilic tick species, *Dermacentor reticulatus*, was found to parasitize Red Foxes in Serbia. The predominant hosts of this tick are wild mammals, horses, cattle, goats, dogs, cats, and occasionally it may be found on birds (Milutinović *et al.* 2012). In Serbia, *Dermacentor reticulatus* is mainly distributed in the northern parts of the country as well as in Belgrade region (Petrović 1979; Milutinović 1992; Tomanović 2009). Our results match this observation as the single locality where this tick species was found is Surčin. *Dermacentor reticulatus* is a vector of several zoonotic pathogens *Babesia microti*, *Rickettsia sibirica, Francisella tularensis, Coxiella burnetti* and Omsk hemorrhagic fever, while several other pathogenic species have been detected (*Borrelia burgdorferi* sensu lato, *Babesia divergens, B. bigemina, Rickettsia slovaka, R. conorii, R. helvetica. R. raoultii, A. phagocytophilum, Bartonella henselle*) (Obsomer *et al.* 2013). Previous research in Serbia determined presence of *Rickettsia raoulti, Anaplasma phagocytophilum, Coxiella burnetii, Borrelia garinii* in this tick species (Tomanović *et al.* 2013, Potkonjak *et al.,* 2016, Sukara *et al.,* 2018).

Rarely parasitizing humans, but still considered anthropophilic, the relict tick species *Haemaphusalis concinna* was found to parasitize Red Foxes sampled in this study. It is distributed in Central and Eastern parts of Europe and Asia, while in Serbia it is mainly present in the northern parts of the country. In this study it was found only at a single locality, Surčin, which is a mainly agricultural area at the very edge of Belgrade administrative region. *Haemaphusali concinna* is a vector for *Francisella tularensis*, TBE virus and *Rickettsia sibirica*, pathogens *causing* diseases in humans (Estrada Peña and

Jongean 1999). Previous studies in Serbia have reported *Coxiella burnetii, Anaplasma ovis, Anaplasma phagocytophilum* and *Babesia canis* in samples of this tick species (Tomanović *et al.* 2013).

The medical importance of endophilic *Ixodes hexagonus, Ixodes kaiseri* and *Ixodes canisuga* is limited, as they barely ever parasitize humans (Arthur 1953; Liebisch and Liebisch 1996).

In order to determine the role of Red Foxes as hosts of tick-borne pathogens, spleen samples were tested for the presence of: members of the family Anaplasmataceae, *Hepatozoon* spp., *Babesia* spp., *Borrelia burgdorferi* sensu lato, *Rickettsia* spp., *Coxiella burnetii*, *Francisella tularensis* and *Bartonella* spp. DNA of pathogenic microorganisms was detected in spleen samples of 94 out of 129 animals (72.9%) collected from all 14 localities. The majority of infected animals originated from three localities with the highest number of collected animals– Veliko Gradište (30 infected/45 collected animals, 66.7%), Surčin (23/29, 79.3%) and Svilajnac (12/13, 92.3%). The following pathogens were detected – *Hepatozoon canis, Babesia canis, Babesia vulpes, Borrelia burgdorferi* sensu stricto, *Borrelia garinii, Borrelia lusitaniae, Candidatus* Neoerlichia sp. (FU98).

Within this study, *Hepatozoon canis* was recorded for the first time in Serbia. It is apicomplexan form which is in focus of several studies in Europe (Criado-Fornelio *et al.*, 2007; Majlathova *et al.,* 2007; Deždek *et al.,* 2010; Gabrielli *et al.,* 2010; Grzegorz *et al.,* 2010; Cardoso *et al.,* 2014; Duscher *et al.,* 2014; Najm *et al.,* 2014; Hožić *et al.*, 2015; Imre *et al.,* 2015) due to its current range increase with in Europe. This parasite may be transmitted from wild canids to domestic dogs (Conceicao-Silva *et al.,* 1998). The Red Fox is an important reservoir in nature. Due to PCR methodology, the body of knowledge about presence, transmission, reservoirs, importance and emergency of *Hepatozoon canis* significantly increased in the last few decades.

According to our results, *Hepatozoon canis* is a widely present pathogen with high prevalence in the Red Fox population from Serbia. Molecular detection showed that *H canis* is present in almost all analyzed localities (12 out of 14). Presence of pathogen was not confirmed only in Negotin and Trstenik. Reason for that could be the small sample size (2 and 3 respectively; Tab. 3). Detected prevalence of *H canis* in analyzed animal samples was high. Of the 129 collected and analyzed spleens, 79 showed positive result on presence of *H canis* DNA. At the local scale prevalence was also high with significant variations of values (from 33.3% in Blace up to 100% in Despotovac). Due to the small sample size at locality level, some of highest prevalence values could be a result of a small number of analyzed samples. However, the results from Veliko Gradište and Surčin confirmed high prevalence of *H. canis* among foxes. In Veliko Gradište 45 spleens were examined on presence of *H. canis* and 28 of them were positive (62.2%). In Surčin 15 out of 29 analyzed foxes (51.7%) were positive too. No doubt that those results confirm high prevalence of *H. canis* in the Red Fox population throughout the territory of Serbia.

Hepatozoon canis used to be considered a tropical and subtropical parasite with only the southern part of Europe included in its range. Up to now this apicomplexan protozoan parasite was recorded in the Red Fox population from several European countries including countries of Central Europe (Criado-Fornelio *et al.,* 2007; Majlathova *et al.,* 2007; Deždek *et al.,* 2010; Gabrielli *et al.,* 2010; Grzegorz *et al.,* 2010; Cardoso *et al.,* 2014; Duscher *et al.,* 2014; Farkas *et al.,* 2014; Najm *et al.,* 2014; Hožić *et al.,* 2015; Imre *et al.,* 2015). There is no general recorded pattern in prevalence of *H. canis* in the Red Fox population across Europe. Low prevalence was found in the Red Fox population from Hungary, Poland, Romania and Italy - 7.8% 11.6%, 12.6% and 13.4% respectively (Gabrielli *et al.,* 2010; Grzegorz *et al.,* 2010; Farkas *et al.,* 2014; Imre *et al.,* 2015). Moderate prevalence were recorded in neighboring countries - Croatia (23%) and Bosnia and Herzegovina (38.3%) (Deždek *et al.,* 2010; Hožić *et al.,* 2015). High prevalence were reported for Slovakia, Germany, Austria, Portugal and Spain - 44.4%, 45.2%, 58% 75.6%

and 90% respectively (Criado-Fornelio *et al.,* 2007; Majlathova *et al.,* 2007; Cardoso *et al.,* 2014; Duscher *et al.,* 2014; Najm *et al.,* 2014). High prevalence (61.2%) was also recorded in the Red Fox population from Serbia. According to the prevalence values, results from this study are similar to those from Portugal (Cardoso *et al.,* 2014) and Austria (Duscher *et al.*, 2014).

According to the literature data, *Rhipicephalus sanguineus* is considered the main tick vector for this apicomplexan blood parasite (Baneth *et al.,* 2001; 2007). As *Rhipicephalus sanguineus* was recorded in Serbia in relatively low prevalence (8.69%) (Stojanov *et al.,* 2014), it may be possible that other tick species is/are responsible for transmission as well. We can suppose that some of other species might be important vectors. That hypothesis may be supported by low prevalence of *Rhipicephalus sanguineus* in the Red Fox population in Serbia, and with fact that prevalence of *Hepatozoon canis* in the Red Fox population is high. Some studies confirmed that other ticks are also vectors (de Miranda *et al.,* 2011; Otranto *et al.,* 2011; Hornok *et al.,* 2013). But situation with vectors is a little a bit more complicated as *Rhipicephalus sanguineus* as the main vector is not abundant in Europe, and on other hand the *Ixodes ricinus,* which demonstrated highest abundance and widest range (Dominuez, 2004; Hinaidy, 1971; 1976; Kočišová *et al.,* 2006; Lassnig *et al.,* 1998; Perrucci *et al.,* 2016; Schöffel *et al.,* 1991) was not recognized as a suitable vector for this apicoplexan pathogen (Giannelli *et al.,* 2013). In order to answer these questions related to possible vectors, further investigation must be focused on presence of *Hepatozoon canis* in different tick species collected from hosts (e.g. Red Fox, Golden Jackal, Gray Wolf or even domestic dogs).

Nine representative DNA sequences of *Hepatozoon canis* from Serbia have shown 100% similarity to samples from the rest of Europe. This similarity was recorded in analyses of isolated DNA from *Hepatozoon canis* from Red Foxes from Spain, Croatia, Italy, Romania, Austria, Golden Jackals from Hungary, Romania, Austria, as well as domestic dogs from Croatia and Turkey and even with most important vector (*Rhipicephalus sanguineus*) from Turkey (Juwaid *et al.,* 2019).

We detected two species from genus *Babesia*– *Babesia vulpes* and *Babesia canis.* DNA of *Babesia canis* was detected in a single fox (0.8%) shot at the Surčin site. Although *Babesia canis* is widely distributed in Europe and closely connected to the distribution of the main vector, tick *Dermacentor reticulatus* (Petra *et al.,* 2018), our finding represents just the third molecular confirmation of presence of *Babesia canis* in any Red Fox population worldwide (Cardoso *et al.,* 2013; Hodžić *et al.,* 2015). Recently, Davitkov and colleagues (2015) confirmed with molecular methods that *Babesia canis* is a causative agent of babesiosis in Serbian dogs (Davitkov *et al.,* 2015) and presence of this pathogen has been determined in spleen samples of nine Golden Jackals (*Canis aureus*) collected from four localities Surčin, Svilajnac, Veliko Gradište and Smederevo (Sukara *et al.,* 2018). *Dermacentor reticulatus*, the main vector of *Babesia canis*, was found to parasitize foxes and jackals in Serbia (Tomanović *et al.,* 2013; Sukara *et al.,* 2018), and presence of *Babesia canis* in this tick species have been previously confirmed in localities at the northern part of the country (Tomanović *et al.,* 2013). Natural habitats of these two canid species overlap (Penezić and Ćirović, 2015), and it is highly probable that they share mutual ectoparasite species. Shared ectoparasites might explain the presence of *Babesia canis* in a fox in Serbia, however further studies are necessary in order to reveal the exact role of Red Foxes in enzootic cycles of *Babesia canis*.

Babesia vulpes was detected in spleen samples of 37 out of 129 analyzed animals, with relatively high prevalence – 28.7%. Positive samples originated at 8 out of 14 localities, indicating that the pathogen is widespread in population of Red Fox in Serbia. The prevalence among localities was in range 20-51.7% while the prevalence of animals positive for presence of *Babesia vulpes* varied from 5.8% in 2011 (2 positive/35 collected) to 60% in 2015 (3/5). However, such high differences may be caused by variation in

sample size of analyzed animals per year. *Babesia vulpes* is common in fox populations in Europe (Duscher *et al.,* 2014; Checa *et al.,* 2018) and found with relatively high prevalences. Hodžić and colleagues (2015) reported that 31% of analyzed Red Foxes from Bosnia and Herzegovina were infected with *Babesia vulpes,* Farkas and colleagues (2015) detected 20% of positive animals in Hungary, in Romania 20.1% of Red Foxes were positive (Daskalaki *et al.,* 2018), while Deždek and colleagues (2010) reported prevalence of 5.2% for analyzed Red Foxes in Croatia. Based on the relatively high prevalence rates in foxes in Europe and only a single described clinical case in fox from Canada (Clancey *et al.*, 2010) it may be assumed that fox may act as a reservoir species for *Babesia vulpes.*

Pathogenicity of *Babesia vulpes* in dogs has been previously confirmed (Solano-Gallego *et al.* 2016) and for now this pathogen has been confirmed by molecular methods in a single clinically healthy dog from Serbia (Gabrielli *et al.,* 2015). Potential vectors of *Babesia vulpes* – tick species *Ixodes hexagonus, Ixodes canisuga, Ixodes ricinus* and *Dermacentor reticulatus* were also found to parasitize foxes in Serbia (Tomanović *et al.,* 2013) and babesiosis in dogs caused by this pathogenic *Babesia* species should not be ruled out.

Red Foxes analyzed in this study were positive for presence of three *Borrelia* species: *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* and *Borrelia lusitaniae.* DNA of *Borrelia burgdorferi* sensu lato was detected in seven animals (5.4%) collected from five localities (Surčin, Obrenovac, Veliko Gradište, Negotin and Niš), but sequencing was successful for four samples and based on similarities of obtained sequences with previously published in GenBank three *Borrelia* species were detected : *Borrelia burgdorferi* s. s. in a sample from locality Veliko Gradište, *Borrelia lusitaniae* in samples from localities Surčin and Negotin and *Borrelia garinii* in a sample from locality Surčin. If was shown that foxes are appropriate reservoirs for *Borrelia burgdorferi* sensu lato (Liebisch *et al.*, 1995, Gern *et al.,* 1998), however the data on prevalence of borrelia in foxes are still scarce. DNA of *Borrelia burgdorferi* sensu lato was detected in the skin of foxes in Germany, with prevalences of 7% and 24% (Liebisch *et al.*, 1995, Heidrich *et al.*, 1999), as well as in Romania where prevalence in analyzed heart tissue was 1.42% (Dumutrache *et al.,* 2015). The prevalence detected in this study is within the range of data from other European studies, but borrelia DNA was detected for the first time in spleen samples of foxes. *Borrelia* species detected in this study along with *Borrelia afzelii, Borrelia valaisiana* and *Borrelia bavariensis,* were previously detected in ticks in Serbia (Milutinović *et al.,* 2008, Ćakić *et al.,* 2019), however except for isolation of *Borrelia* strains from *Apodemus* mice (Stajković *et al.* 1993) this is the first information on presence of borrelia in animal tissue, indicating Red Foxes as potential reservoir in the investigated area. Further studies are necessary in order to elucidate the exact role of this carnivore species in enzootic cycles of borrelia.

Initial screening with primers specific for members of the family Anaplasmataceae resulted in 21 positive spleen samples. After sequencing, 8 sequences showed partial coverage with 93-99% similarity with *Candidatus* Neoehrlichia spp. sequences. Additional analysis of two additional longer fragments, *groEL* (~806 bp) and 16S rRNA (~1053 bp) genes of *Candidatus* Neoehrlichia spp, was successful for six samples originating from four male and two female animals collected at Surčin, Veliko Gradište, and Svilajnac localities. Based on the 100% identity of sequences obtained in this study, with sequences from Red Foxes from Austria, Raccoon Dogs (*Nyctereutes procyonoides*) from Poland and a European Badger (*Meles meles*) from Hungary (KX245423, KX231830) the agent was identified as *Candidatus* Neoerlichia sp. (FU98).

Currently, two candidates are proposed to be classified in the Anaplasmataceae family: *Candidatus* Neoehrlichia mikurensis and *Candidatus* Neoehrlichia lotoris. *Candidatus* Neoehrlichia mikurensis was identified for the first time in Netherland in ticks, then also in small mammals from China, while the first human case was detected in Sweden in 2010

(Schouls *et al*., 1999, Pan *et al.,* 2003, Wellinder-Olson *et al.,* 2010). *Ixodes ricinus* tick is proposed as the main vector, while rodents are suggested as reservoirs. Candidatus Neoerlichia lotoris, agent closely related to *Candidatus* Neoerlichia micurensis, was isolated for the first time in North America from tissue of Racoon (*Procyon lotor)* and ticks from the genus *Ixodes* are indicated as main vectors (Dugan *et al.,* 2005).
Recently, another agent closely related to *Candidatus* Neoerlichia lotoris, but clearly distinct from *Candidatus* Neoerlichia micurensis, was initially described in Red Foxes from Austria (Hodžić *et al.,* 2015) and then confirmed in a fox from the Czech Republic (Hodžić *et al.,* 2017). It was designated as *Candidatus* Neoerlichia sp. (FU98). Later it was also detected in different animal species in Europe, a Raccoon Dog from Poland [44] and European Badger from Hungary [45]. Moreover, Hornok *et al.,* (2018) identified a strain similar to *Candidatus* Neoerlichia sp. (FU98) in dogs from Hungary. The recordings of these agents in Red Foxes from Serbia indicate its broader distribution in Europe. The pathogenic potential of this agent is still unclear and further research is needed, as well as delineation of the exact role of canid species in enzootic cycles of *Candidatus* Neoerlichia sp. (FU98).
The present study is the first systematic study of Red Foxes as hosts for ticks and tick-borne pathogens in Serbia. Obtained results indicate that this wild canid species is an important host for a number of tick species, including the ones with anthropophilic behavior and significant vector potential for zoonotic pathogens, such as *Ixodes ricinus, Dermacentor reticulatus, Haemaphysalis concinna.* The study reports the first finding of *Ixodes kaiseri* for the tick fauna of Serbia. Presence of DNA of seven tick-borne pathogens in analyzed Red Fox samples does not necessarily mean that foxes are their competent reservoirs, but it indicates a role in the enzootic cycles of these pathogens. The high prevalence of *Hepatozoon canis* detected in this study indicates that routes of transmission other that tick bite might be included in the preservation of the pathogen, for example infection by ingestion of the tick during grooming or by transplacental transmission to cubs, and further research toward clarification of this question is needed. Another important question that is opened with this research is the exact role of Red Foxes in maintenance of causative agents of two diseases with great importance for animal and human health – *Babesia canis* and *Babesia vulpes*, which cause babesiosis in domestic dogs, and *Borrelia burgdorferi* s.s. and *Borrelia garinii* , the proven agents of Lyme disease, the most important tick-borne disease in the Norther hemisphere. It will be important to determine whether Red Foxes have a role in circulation of the most dominant *Borrelia* species in Serbia, *Borrelia lusitaniae*.

6. Conclusions

This research represents the first complex and systematic study of tick fauna in Serbia in the Red Fox (*Vulpes vulpes*) as a host, and of the role of this carnivore as host for tick-borne pathogens. Based on the results of the study following conclusions were made:

1. Red Fox is a host for a number of tick species. In this research, six tick species have been found to parasitize in Red Fox: *Ixodes ricinus, Ixodes canisuga, Ixodes hexagonus, Ixodes kaiseri, Dermacentor reticulatus,* and *Haemaphysalis concinna*. Some of them show anthropophilic behavior and significant vector potential for zoonotic pathogens, i.e. *Ixodes ricinus, Dermacentor reticulatus, Haemaphysalis concinna.*
2. The study reports the first finding of *Ixodes kaiseri* for the tick fauna of Serbia.
3. The most prevalent tick species parasitizing Red Foxes analyzed in this study was *Ixodes ricinus* (69%), followed by *Ixodes hexagonus* (11.5%), *Ixodes canisuga* (5.3%), *Ixodes kaiseri* (5.3%), *Dermacentor reticulatus* (4.4%) and *Haemaphysalis concinna* (4.4%). As majority of ticks belonged to adult stage, Red Fox is an important host for this life stage of hard ticks in Serbia.
4. Presence of pathogenic microorganisms transmitted by ticks was detected in spleen samples of 72.9% animals (74.1% females and 74% males), originating in all 14 studied localities. DNA of seven tick-borne pathogens was identified – *Hepatozoon canis, Babesia canis, Babesia vulpes, Borrelia burgdorferi* sensu stricto, *Borrelia garinii, Borrelia lusitaniae, Candidatus* Neoerlichia sp. (FU98)
5. Within this study, *Hepatozoon canis* was recorded for the first time in Serbia. DNA of *Hepatozoon canis* was detected in high prevalence in analyzed Red Foxes (61.2%). This pathogen is widespread in Serbia, as animals positive for the presence of *Hepatozoon canis* DNA were recorded at 12 out of 14 investigated localities.
6. Sequences of samples positive for *Hepatozoon canis* obtained in this study showed high levels of mutual similarity, and they have shown 100% similarity to the sequences from different hosts originating in several European countries, e.g., foxes from Spain, Croatia, Italy, Romania, and Slovakia; Golden Jackals from Hungary, Romania, and Austria; dogs from Croatia and Turkey; and *R. sanguineusi* ticks from Turkey. The high level of homology indicates recent dispersal of the pathogen.
7. Two species from genus *Babesia: Babesia vulpes* and *Babesia canis* were detected during this research in the Red Fox - *Babesia canis* in a single fox (0.8%) shot at the Surčin site, while *Babesia vulpes* was detected with a relatively high prevalence – 28.7% at 8 out of 14 investigated localities, indicating that the pathogen is widespread in Red Fox population in Serbia. Our discovery represents just the third molecular confirmation at global scale of presence of *Babesia canis* in any Red Fox population.
8. Three species from the Lyme borreliosis group (*Borrelia burgdorferi* sensu lato) have been detected in Red Fox spleen samples – *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* and *Borrelia lusitaniae.* It was shown that foxes are competent reservoirs for borrelia, however our study was the first time that borrelia DNA was detected in spleen samples of foxes.
9. Except for isolation of *Borrelia* strains from *Apodemus* mice in the early 90s, this is the first information on presence of borrelia in animal tissue in Serbia, indicating Red Foxes as a potential reservoir in the investigated area.

10. Newly described agent *Candidatus* Neoerlichia sp. (FU98) was detected for the first time in Serbia in spleen of analyzed Red Foxes. The recordings of these agents in Red Foxes from Serbia indicate their broader distribution in Europe. The pathogenic potential of *Candidatus* Neoerlichia sp. (FU98) is still unclear and further research is necessary, as well as delineation of the exact role of Red Foxes and other canid species in the enzootic cycles of *Candidatus* Neoerlichia sp. (FU98).
11. Presence of DNA of *Rickettsia* spp., *Coxiella burnetii*, *Francisella tularensis* and *Bartonella* spp. wasn't detected in analyzed spleen samples of Red Foxes.

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Biography

Salem Emhemed Husayn Juwaid was born in Misurata, Libya in 1971 where he graduated elementary school and graduate in 1989 at .Health Institute Misurataon. He enrolled bachelor study at Faculty of Medical Technology, Public Health Department in 1991. At same Faculty Salem graduated in 1994 with score good. Master study enrolled at Faculty of Medical Technology, Public Health Department in 2005. In 2008 Salem graduated with master thesis entitled “Prevalence the sandfly and its roles in the transmission of leishmaniasis in Misurata , Libya” with score very good. At Faculty of Biology University of Belgrade he enrolled PhD study in 2013 (study program Entomology). Research related with his thesis has been focused on tick fauna on the Red Fox as host and presence of ticks-borne diseases in red fox population in Serbia. During his study at Faculty of Belgrade Salem cooperated on his research topics with Animal Ecology and Zoogeography laboratory, Invertebrate Zoology and Entomology laboratory at Faculty of Biology, University of Belgrade as well as at Medical Entomology laboratory at Institute for Medical Research, University of Belgrade.

During 1990-2009 Salem Emhemed Husayn Juwaid was employed at Central Hospital, Misurata (Libya) as laboratory technician. In period 2009-2013 he has teaching position at Nutrition Department, Faculty of Medical Technology in Misurata. From 2011 to 2013 he was head of Nutrition Department, Faculty of Medical Technolog, Misurata

Изјава о ауторству

Потписани Salem Emhemed Husayn Juwaid
број индекса B3054/2013

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да је докторска дисертација под насловом
Фауна крпеља и идентификација и карактеризација крпељима преносивих патогена у популацијама лисице (*Vulpes vulpes*) у Србији (Tick fauna and identification and characterization of tick-borne pathogens in Red Fox populations (*Vulpes vulpes*) from Serbia)

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Име и презиме аутора Salem Emhemed Husayn Juwaid
Број индекса B3054/2013
Студијски програм Екологија

Наслов рада Фауна крпеља и идентификација и карактеризација крпељима преносивих патогена у популацијама лисице (*Vulpes vulpes*) у Србији (Tick fauna and identification and characterization of tick-borne pathogens in Red Fox populations (*Vulpes vulpes*) from Serbia)

Ментори Проф. др. Душко Ћировић и др Снежана Томановић

Потписани Salem Emhemed Husayn Juwaid

Изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла за објављивање на порталу **Дигиталног репозиторијума Универзитета у Београду.**
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