



Micronucleus Frequency in Rodents with Blood Parasites

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Abstract: Some parasites cause a genotoxic effect and induce micronuclei in cells of their rodent hosts. The rodents are frequently used for assessment of environmental pollution; therefore, it is crucial to know whether the genetic damage is due to xenobiotics presenting in their environment or is a result of parasite infection. The purpose of this study was to assess the potential link between blood parasites and micronucleus frequencies in erythrocytes of wild rodent populations from anthropogenic impacted and background regions. Individuals of Macedonian mouse *Mus macedonicus* Petrov & Ružić, 1983, yellow-necked mouse *Apodemus flavicollis* (Melchior, 1834) and common vole *Microtus arvalis* (Pallas, 1778) from heavy metal contaminated region and individuals of striped field mouse *Apodemus agrarius* (Pallas, 1771) and *A. flavicollis* from area with low anthropogenic impact have been studied. In all *Apodemus* populations, we registered non-identified bacterial infections with *Bartonella* spp., in *M. arvalis* – infections with *Babesia microti*-like piroplasms and *Bartonella* sp., and in *M. macedonicus* – infection with trypanosomes (*Trypanosoma musculi*) and *Bartonella* sp. No statistically significant difference ($p \geq 0.05$) between the average micronucleus frequency of the infected and non-infected animals inhabiting both polluted and unpolluted regions has been proven. The results show that the observed blood parasites cannot be associated with the induction of micronuclei in erythrocytes of the studied rodents. This provides justification to include rodents with the recorded protozoan and bacterial infections in biomonitoring studies for assessment of genotoxicity in contaminated areas.

Key words: micronuclei, rodents, haemoparasites, *Trypanosoma*, *Babesia*, *Bartonella*

Introduction

The small mammals accumulate a lot of pollutants presenting in the ecosystems because of their body size and high metabolic rate (DAMEK-POPRAWA & SAWICKA-KAPUSTA 2003, SÁNCHEZ-CHARDI et al. 2007, PETKOVŠEK et al. 2014) and thus they are very well suited for environmental risk assessment. In addition, rodents are important as abounding preys and are essential in the food chain. Therefore, they are preferred in many biomonitoring studies

(CABARCAS-MONTALVO et al. 2012, MITKOVSKA et al. 2012, PETKOVŠEK et al. 2014, HUSÁRIKOVÁ et al. 2015, POWOLNY et al. 2019).

Diverse biomarkers are applied to evaluate the rate of genotoxicity in polluted areas. The micronucleus evaluation is an *in vivo* genotoxicity test used for detection of natural mutagens (BOLOGNESI & HAYASHI 2011, HAYASHI 2016). It has been effectively applied for small mammals (CABARCAS-MONTALVO et al. 2012, MITKOVSKA et al. 2012, HUSÁRIKOVÁ et al. 2015) to assess damage to DNA and chromosomes.

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Rodents are hosts to different infectious agents and are vectors of many transmissible diseases. There is evidence that some parasites can cause a genotoxic effect and induce micronuclei in their host cells. SALAZAR et al. (2013) reported that a significant increase in micronucleus frequency was observed in hamsters infected with *Taenia solium* cysticerci, suggesting that the presence of *T. solium* tapeworms in the small intestine induces genotoxicity. Earlier, HERRERA et al. (2003) associated also the observed DNA damage with *T. solium*. The genotoxic potential of tropical liver fluke (*Fasciola gigantica*) infection in experimentally infected rabbits has been proved after using the standard comet assay and micronucleus test on the isolated hepatocytes and the whole blood from the infected rabbits (KHAN et al. 2016).

Although studies showing the genotoxic effect of blood parasites are extremely scarce, there are some reports of infection-related DNA damage. MANNA et al. (1991) reported that protozoan species could act as a mutagen to treated mice and investigated the mutagenic potential of *Entamoeba histolytica*. It was observed earlier that the human haemoflagellate *Leishmania donovani*, the causative agent for Kala-Azar (MANNA & SARKAR 1989), and the insect intestinal flagellate, *Herpetomonas* sp. (MANNA & PANDA 1989), when intraperitoneally injected into mice, induced a strikingly high frequency of bone marrow chromosome aberration, micronucleated erythrocytes and sperm head abnormality. All these studies showed the mutagenic potential of the mentioned parasitic species in experimentally treated mice system. CABARCAS-MONTALVO et al. (2012) suggested that their contradictory results obtained for the mean micronucleus frequency in *Mus musculus* (unexpectedly high values in the control region) may be due to protozoan infection. However, the authors did not explain how a blood parasite could induce micronuclei in the animals. It was also unclear whether the observed micronuclei were influenced by the presence of parasites reported as micronuclei.

It has been reported that parasites can damage host's DNA either by directly releasing its products during the infection or by inducing inflammatory responses (SALAZAR et al. 2013) mediated by the production of free radicals such as reactive oxygen and nitrogen species, leading to genetic and epigenetic processes that regulate cell proliferation, thereby influencing carcinogenesis (HERRERA & OSTROSKY-WEGMAN 2001).

Considering the above information, it is crucial to know whether the genetic damage is due to

the xenobiotics presenting in the environment or is a result of parasite infections.

The purpose of this study was to assess the potential link between blood parasites and micronucleus frequencies in erythrocytes in wild rodent populations from anthropogenic impacted and background regions. This will answer the question whether rodents with blood parasites can be used in biomonitoring studies.

Materials and Methods

Materials

Totally, 139 specimens of different rodent species from one heavy-metal polluted (lead-zinc smelter near Plovdiv, Bulgaria – LZS) and from one unpolluted area (Strandzha Nature Park – NPS) were caught (Table 1). To avoid intraspecific differences related to age, only adult specimens were examined. The age was determined according to the criteria of molar root development and growth (FELTEN 1952).

Table 1. Studied rodent species

Region	Species	Number
Lead-zinc smelter (LZS)	<i>Mus macedonicus</i>	37
	<i>Apodemus flavicollis</i>	23
	<i>Microtus arvalis</i>	7
Strandzha Nature Park (NPS)	<i>Apodemus flavicollis</i>	41
	<i>Apodemus agrarius</i>	31

Sampling

The field study was carried out during years 2013–2016. Sherman live traps were situated at nightfall, left active overnight and collected the next morning. The mice were transported to the laboratory, where their sex and weight were determined. All procedures with investigated animals were done according to the DIRECTIVE 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes. Peripheral blood samples for whole blood smears (two from each individual) were obtained from the tail vein by cutting the tip of the tail (app. 0.03 cm).

Observation for micronuclei (MN) and blood parasites

Thin whole blood smears were done on clean glass slides immediately after sampling, dried at room temperature and fixed with absolute ethanol for 20 min. They were stained with fluorochrome dye acridine orange (AO) according to HAYASHI et al. (1983): 0.1% AO solution supplemented in Sørensen's phosphate buffer, rinsed and cover-glassed imme-

diately before evaluation with fluorescence microscopy (Leica DM 1000) equipped with appropriate for AO filter (I3). The images were processed with a photo camera and Image J software. Only erythrocytes with intact cellular and nuclear membrane were scored. On each slide, only areas with uniform spread in monolayer without overlapping cells were targeted. All slides being scored by one person to avoid interobserver variability. The frequency of MN and blood parasites was scored at a magnification of 1000 \times . As a result of AO metachromasia, the cytoplasm of the immatures polychromatic erythrocytes (PCEs) emits red fluorescence, unlike greenish cytoplasm of mature normochromatic erythrocytes (NCEs) and the yellow-green or yellow of leucocytes nuclei. Analyses consisted of count and detection of erythrocytes with MN according to the criteria of FENECH et al. (2003) applicable to mammals nuclear-free erythrocytes: (a) MN should be smaller than one-third of main nucleus (before its disruption); (b) MN colour should be yellowish-green and its intensity should be the same as the one of cells nuclei such as leukocytes; (c) MN should have an oval shape and should be focused at the same plane as the cell. The average MN frequency (MNF), which represents the number of cells with MN per minimum 2000 accounted erythrocytes (both PCEs and NCEs) expressed as percentage, were calculated for each animal tested. The mean MN frequency for infected and non-infected individuals was calculated for each investigated rodent population and for all observed haemoparasites.

For each individual, the presence of blood parasites, which are identified by morphological features, is recorded. The total parasites prevalence (%) for each investigated population and frequencies of different haemoparasites were calculated.

Statistical analysis

Statistical analysis of the data was performed by GraphPad Prism 4.0 statistical software. Data was tested for normal distribution using Kolmogorov-Smirnov test. Since data was not normally distributed, significance of differences between the infected and non-infected rodent groups were tested using Mann-Witney non-parametric test. The results were expressed as mean \pm standard deviation. The differences were considered significant at $p \leq 0.05$.

Results

The fluorescent observation of blood smears stained with AO indicated MN in some circulating erythrocytes and different species of haemopara-

sites in investigated rodent populations (Fig. 1). We registered bacterial infection with *Bartonella* spp. in all investigated populations – in *Apodemus* populations (both *A. flavicollis* and *A. agrarius*) in both contaminated and protected areas as well as in *Mus macedonicus* and in *Microtus arvalis*. Infection with *Babesia microti*-like piroplasms was observed mainly in *Microtus arvalis* and only in one individual of *A. agrarius*. Trypanosome infection (*Trypanosoma musculi*) was detected only in *M. macedonicus*. Mixed infections with two haemoparasites were observed but no animals were infected with three parasites simultaneously. The diversity and prevalence of blood parasites are presented in Table 2 and Fig. 2. The blood parasites were distinguished according to their morphological characteristics (which is enough for the aim of this study). Usually PCR is required for accurate species identification of parasites.

Infections with *Bartonella* spp.

A non-identified bacterium belonging to the genus *Bartonella* (class Alphaproteobacteria, order Rhizobiales, family Bartonellaceae) was the most frequently recorded haemoparasite that occurred in all investigated rodent populations at the two sites – LZS and SNP. Out of all animals studied, *Bartonella* sp. was found in 25.18% of the animals. The highest prevalence of this parasite was observed in *Mus macedonicus* (43%) (Table 2), where the mixed infection with *Trypanosoma musculi* was observed in 18.92% of the individuals. The mean number of bacteria per single infected erythrocyte was quite variable, but higher values were observed in *M. macedonicus* (25-30 per cell).

Infections with *Babesia microti*-like piroplasm

Infection with *Babesia microti*-like piroplasm (phylum Apicomplexa, class Sporozoa, order Piroplasmida) was detected in two species only – *Microtus arvalis* from LZS and *Apodemus agrarius* from SNP (only one infected individual). The prevalence of infection was extremely high in *M. arvalis* (57%) compared to *A. agrarius* (3%). Coinfection with *Bartonella* sp. was observed in 50% of infected voles. The parasites mostly exhibited the ring- and pear-shaped forms (Fig. 1D) (the morphological terminology according to MEHLHORN & SCHEIN 1984). Single parasites were usually observed in the infected erythrocytes. The regular form of four cells – “Maltese cross”, characteristic for “small” *Babesia* spp., was not recorded. The *Babesia* differentiation from *Plasmodium* was carried out according to PETERSEN & AHMED (2016).

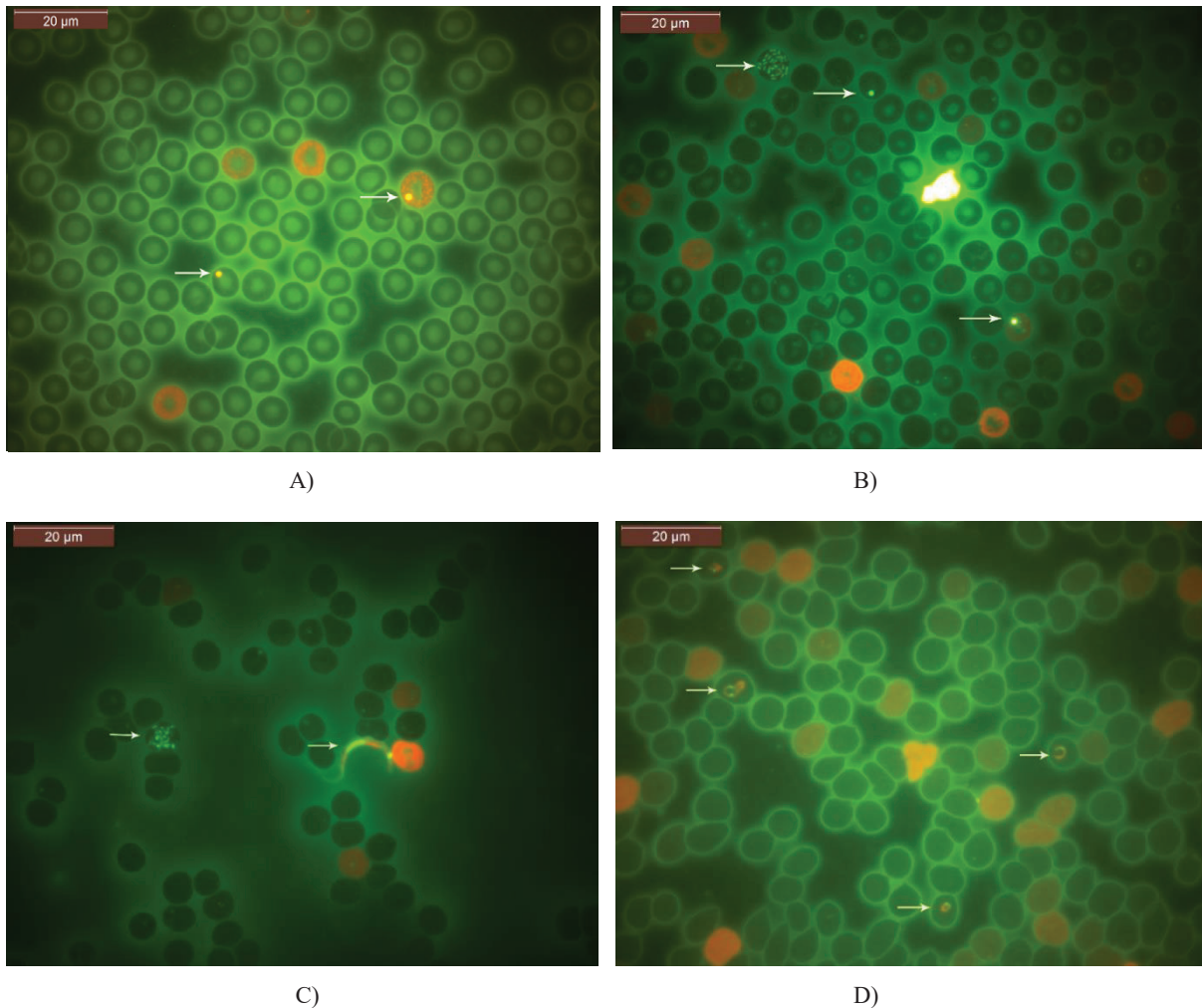


Fig. 1. Microphotographs of acridine orange-stained peripheral blood film of investigated rodents, showing (A) micronucleated cells in *A. flavicollis* without haemoparasites; (B) *Bartonella* sp. (upper arrow) and micronuclei in *M. macedonicus*; (C) *Trypanosoma musculi* (right arrow) and *Bartonella* sp. (left arrow) in *M. macedonicus*; (D) *Babesia microti*-like in *M. arvalis* (magnification 1000 \times , in imersion)

Infections with *Trypanosoma musculi*

Infection with *Trypanosoma* (phylum Euglenozoa, order Trypanosomatida) was detected at one site only – LZS, in *Mus macedonicus*. The prevalence of infection in this species was 21.62%. All observed parasite forms possess the morphological characteristics of the subgenus *Herpetosoma* (*Stercoraria* section), to which *T. lewisi*-like parasites belong. They are as follows: free flagellum, “C shape” of the parasite with size about 25 ± 5 μm , clear visible undulating membrane, subterminal kinetoplast with oval shape and an anterior located nucleus. Considering these morphological characteristics and the genus *Mus* as a host, we assume that the observed species was *Trypanosoma musculi*.

Micronuclei (MN) and haemoparasites

The mean MN frequency was calculated for infected and non-infected animals in all investigated popu-

lations and for each haemoparasite, except for the smallest sample of *M. arvalis* with *Bartonella* sp. (2 individuals) and *A. agrarius* with *Babesia microti*-like (1 individual) (Table 3). The results of the statistical comparisons of MN frequency between infected and non-infected individuals are presented in Fig. 3. No reliable difference was established ($p \geq 0.05$) between the average MN frequency of infected and non-infected animals.

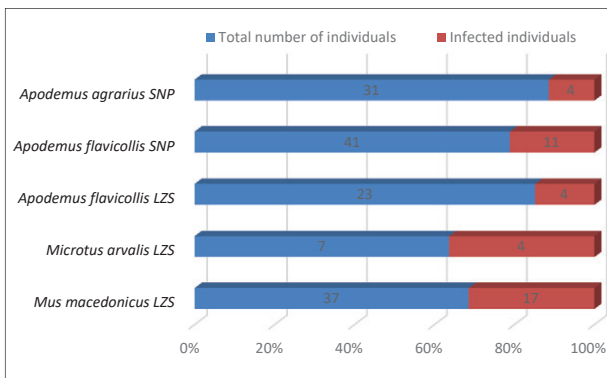
Discussion

Micronuclei are biomarker of genotoxicity and DNA damage as a result of clastogenic or aneuploidic disorders in chromosomes (HAYASHI 2016). It is well-established that MN mainly originate from acentric chromosome fragments, acentric chromatid fragments or whole chromosomes that fail to be included in the daughter nuclei at the completion of

Table 2. Infection of rodents with blood parasites in the studied areas of Bulgaria.

Parasites	Species	Study area	n	Prevalence (n, %)
<i>Bartonella</i> sp.	<i>Mus macedonicus</i>	LZS	37	16 (43.24%)
	<i>Microtus arvalis</i>	LZS	7	2 (28.57%)
	<i>Apodemus flavicollis</i>	LZS	23	4 (17.39%)
		SNP	41	11 (26.83%)
	<i>Apodemus agrarius</i>	SNP	31	3 (9.68%)
<i>Babesia microti</i> -like piroplasm	<i>Microtus arvalis</i>	LZS	7	4 (57.14%)
	<i>Apodemus agrarius</i>	SNP	31	1 (3.23%)
<i>Trypanosoma musculi</i>	<i>Mus macedonicus</i>	LZS	37	8 (21.62%)

n – number of individuals


Fig. 2. Ratio of infected individuals from the total number of animals in the investigated populations.

telophase during mitosis because they did not attach properly with the spindle during the segregation process in anaphase (FENECH et al. 2011). These displaced chromosomes or chromosome fragments are eventually enclosed by a nuclear membrane and, except for their smaller size, are morphologically similar to nuclei after conventional nuclear staining. Therefore, the MN test is widely used in monitoring in both contaminated and protected areas.

Acridine orange method is more efficiently and accurate for micronuclei determination compared to the Giemsa method (POLARD et al. 2011). At the same time, it has a high diagnostic capacity to detect also different parasites in blood smears because of its reading speed and sensitivity compared with the common bright field microscopy using Giemsa staining and the ability to detect DNA/RNA through staining in different colour. The technique was found to be accurate, simple and rapid for screening haemoprotozoans and representatives of the order Rickettsiales, and is recommended as an appropriate for detecting also cases of low-level parasitemia (RAVINDRAN et al. 2007). It is recommended for a fast diagnosis, especially in countries with endemic areas where diseases like malaria,

sleeping sickness, Lyme disease, babesiosis and spirochetemia occur.

Bartonella spp. are arthropod-borne bacterial pathogens that typically cause persistent infection of erythrocytes and endothelial cells in their mammalian hosts, including humans (DEHIO 2005). The high variability in the number of bacteria per infected cell that we observed and the reported high morphological similarity of all *Bartonella* spp. (BREITSCHWERDT & KORDICK 2000) suggest that the investigated rodents were infected with different *Bartonella* spp. This haemoparasite, first reported here for Bulgaria, was the most common in our study of all investigated populations but this is not surprising, since many European studies had reported *Bartonella* infections in rodents (KARBOWIAK et al. 2009, 2010, TURNER et al. 2014).

Although small mammals can demonstrate a high *Bartonella* prevalence (40-60%), infections are self-limiting and do not usually result in clinical disease (TURNER et al. 2014). We did not find any data in the available literature for inducing micronuclei in the presence of *Bartonella*. This fact, together with the lack of significant differences between MN of infected and non-infected individuals, suggests that this haemoparasite does not induce genotoxicity in host cells.

Babesia parasites, the causative agent of babesiosis, are transmitted by ticks and are able to invade the red blood cells of the vertebrate hosts causing a febrile disease with extensive erythrocytic lysis leading to anaemia, icterus as well as haemoglobinuria and can be fatal. In recent years, it has become evident that certain *Babesia* spp. are of zoonotic importance (PETERSEN & AHMED 2016).

The protozoans of the genus *Babesia* were recorded in European thick as well as in wild and domesticated animals in Poland, Germany, Czech Republic, Slovenia, Switzerland and Hungary (GREY 2004, KARBOWIAK 2004, TURNER et al. 2014). The

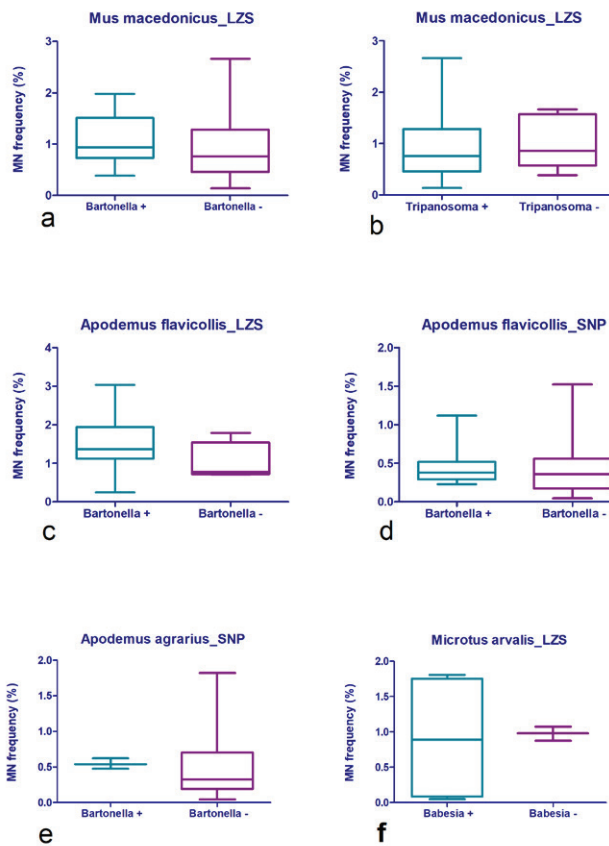


Fig. 3. Average MN frequencies of infected and non-infected animals of the studied species in the impact and background regions. Bottom and top of the box represent 25 and 75% percentile values, respectively, with median values within the box. Error bars indicate minimum and maximum values. Asterisk indicates a significant difference at $p \leq 0.05$ (LZS – Lead-Zinc smelter; SNP – Strandzha Nature Park).

Table 3. Mean micronuclei (MN) frequencies for infected and non-infected individuals in all investigated populations (LZS – Lead-Zinc smelter; SNP – Strandzha Nature Park; “+” – infection with haemoparasite; “-“ – lack of infection; \pm SD – standard deviation).

Investigated populations	Haemoparasites	MN Frequency \pm SD, %
<i>Mus macedonicus</i> LZS	<i>Bartonella</i> +	1.1 \pm 0.50
	<i>Bartonella</i> -	0.98 \pm 0.7
	<i>Trypanosoma</i> +	0.98 \pm 0.70
	<i>Trypanosoma</i> -	1.0 \pm 0,51
<i>Microtus arvalis</i> LZS	<i>Babesia</i> +	0.91 \pm 0.52
	<i>Babesia</i> -	0.98 \pm 0.10
<i>Apodemus flavicollis</i> LZS	<i>Bartonella</i> +	1.0 \pm 0.52
	<i>Bartonella</i> -	1.5 \pm 0.69
<i>Apodemus flavicollis</i> SNP	<i>Bartonella</i> +	0.47 \pm 0.26
	<i>Bartonella</i> -	0.39 \pm 0.38
<i>Apodemus agrarius</i> SNP	<i>Bartonella</i> +	0.55 \pm 0.07
	<i>Bartonella</i> -	0.49 \pm 0.33

importance of rodents as a reservoir of this haemoparasite has been extensively assessed by KARBOWIAK (2004). In Europe, various rodent species (KARBOWIAK et al. 2005, 2010) have been reported as a host of this parasite. The only report from Bulgaria is by SEBEK (1977) for *Apodemus sylvaticus*. Both species, in which we have recorded *Babesia* – *M. arvalis* and *A. agrarius*, are known as hosts of this parasite in Europe (KARBOWIAK 2004, 2009) but our study reports their presence in Bulgaria for the first time. According to previous studies, the most important role as *B. microti* reservoir is played by *Microtus voles* (KARBOWIAK 2004) and this has been confirmed in our study where the prevalence of infection in *Microtus arvalis* was higher (57.14%). Similar to previous surveys (KARBOWIAK et al. 2005), no visible pathological symptoms were manifested for the infected animals but a high degree of splenomegaly was observed during post-mortem examination of animals.

Trypanosomes are digenetic protozoans of the order Trypanosomatida of the phylum Euglenozoa (ADL et al. 2012). They cause important diseases from medical and veterinary point of view. It is known that different species of trypanosomes interact uniquely with their vertebrate hosts via different evasion strategies. They use unique immune evasion mechanisms to evade or modulate immunity of endothermic and ectothermic vertebrates as described first for trypanosomes (OLADIRAN & BELOSEVIC 2012). The registered species of *Trypanosoma musculi* in our study was a trypomastigote form, which lives in extracellular fluids and circulates freely among red blood cells in the genus *Mus* as a host. We have already reported the first record for this parasite in *Mus macedonicus* from Bulgaria (MITKOVSKA et al. 2014, DIMITROV et al. 2015). Although this species refers to non-pathogenic parasite transmitted by fleas, several atypical human infections caused by rodent trypanosomes were reported in recent years (TRUC et al. 2013). As for the micronuclei, only the preliminary data of HABIŁA et al. (2014) indicate that *T. evansi*, *T. b. brucei* (that have been implicated in Surra disease) and mixed infection in laboratory conditions with both parasites induced the formation of micronucleated polychromatic erythrocyte in the bone marrow cells of Wistar albino rats. These data give an evidence of possible genotoxic effects in trypanosomiasis. We did not observe clastogenic effect *in situ* of *T. musculi*, probably because the infection is usually persistent in natural rodent populations with a chronic level of parasitemia or because of the different invasion strategy of this haemoparasite.

The biology of infectious disease in natural host-parasite systems must include genetics, evolutionary ecology, immunology and epidemiology. That is why the host-parasite interactions are complex and their nature depends on many factors some of which are fitness, condition, susceptibility, genetics and the immune system of host (TURNER et al. 2014). The wild animals from LZS in our study are exposed in their natural habitats to air pollutants and heavy metals from industrial activity. It is supposed that environmental pollution has the potential to change the ecological interaction between species and the relationship between a parasite and its host (EEVA & KLEMOLA 2013). Mechanisms of host defence usually are negatively influenced by the environmental pollution, making a host more sensitive to parasite infection and leading to a deterioration of resistance to parasite infections (TERSAGO et al. 2004). Alternatively, there may be an increasing population of proper hosts and intermediate hosts (LAFERTY & KURIS 1999). Our results show the higher prevalence of parasites, including coinfections, in rodent populations of *M. macedonicus* and *M. arvalis* from the polluted region, probably due to the increased susceptibility to parasites at the presence of heavy metals. At the same time, the prevalence of *Bartonella* in *A. flavicollis* from the unpolluted region was found to be higher (26.83%) than this from the polluted region (17.39%). In accordance with our findings, ZÁBOJNÍKOVÁ (2017) have not recorded any significant variation in the concentration of heavy metals of infected rodents and have not detected any indication suggesting either the ability of a parasite to affect the host's metabolism or the opposite dependence when highly contaminated individuals are more vulnerable to infection by a parasite. The mean MN frequencies were higher in the examined rodent populations from the polluted region compared with these from SNP. This fact was established in our previous study (MITKOVSKA et al. 2012) and is due to many years of heavy metal contamination resulting in high accumulation of Pb and Cd in the liver of rodents (DIMITROV et al. 2016). However, our results do not show any significant differences between MN frequencies of infected and non-infected individuals from all investigated rodent populations, both in polluted and non-polluted region. This illustrates the lack of a clear link between the presence of registered parasites and the value of the mean MN frequency. The results show that the observed parasites cannot be associated with the induction of micronuclei *in situ* in erythrocytes of the studied native rodent's populations. Some of the highly infected animals have too low values for

this parameter, others have higher. In many animals with high mean MN frequencies, no blood parasites were detected at all.

Conclusions

Our study confirms that rodents of the examined species are hosts to various blood parasites. We have observed intracellular and intercellular protozoan infections with piroplasms (*Babesia*-like) as well as trypanosome (*T. musculi*) infections and bacterial intracellular infection (*Bartonella* spp.) in the investigated species. The most susceptible to blood parasites is *Mus macedonicus* inhabiting a polluted region where we have observed coinfections with two blood parasites. The results show that the observed parasites cannot be associated with the induction of micronuclei *in situ* in erythrocytes of the studied rodents. This provides justification to include rodents with recorded protozoan and bacterial infections in biomonitoring studies for assessment of genotoxicity in areas with anthropogenic pollution. The data in this study may be useful in future biomonitoring studies using a micronucleus test to assess genotoxic effects, both in heavy metal contaminated areas and protected areas.

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