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Occurrence of Haemoparasites of the Genus Hepatozoon (Adeleorina: Hepatozoidae) in the Marsh Frog (Pelophylax ridibundus Pallas, 1771) in Bulgaria

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Abstract. The distribution of species of *Hepatozoon* from anurans, specifically frogs, and their host-parasite relationships are of great interest. Due to the specifics of the ontogeny of frogs, they form a link between aquatic and terrestrial ecosystems. These amphibians are important in food chains, ensuring the normal functioning of biocenosis. In this study, we present data on the presence of *Hepatozoon* sp. in the marsh frog, *Pelophylax ridibundus* (Pallas, 1771) for the first time in Bulgaria. The blood smears of 137 individuals were investigated by fluorescence microscopy after staining with acridine orange. In three of five studied localities, the presence of apicomplexan haemoparasite from the genus *Hepatozoon* was revealed. Prevalence and parasitaemia values were different in frogs populations inhabiting the Chaya River (27.5% and 11.0%, respectively), Tsalapitsa Rice Fields (6.4% and 9.9%, respectively) and nature wetland Zlato pole (10.0% and 8.1%). It was found that the morphology and morphometric parameters of the parasite gamonts are closest to *Hepatozoon magna*, but molecular tools are required to confirm the genus and species determination. Our findings revealed that acridine orange is appropriate dye for detecting haemoparasites of the genus *Hepatozoon*.

Key words: amphibia, blood parasite, *Hepatozoon, Pelophylax ridibundus.*

Introduction

Apicomplexan haemoparasites of genus *Hepatozoon* Miller 1908, of the family *Hepatozoidae* (Wenyon, 1926) can infect a wide range of vertebrate animals that act as intermediate hosts (Smith, 1996). The gamont stages parasitize red blood cells in reptiles, amphibians, as well as leukocytes in birds and mammals. They are transmitted by a variety of arthropods, especially mosquitoes and ticks, which they use as definitive hosts (Smith, 1996). Their life cycle comprises roughly four stages: merogony and gamogony in the vertebrate host, and fertilization and sporogony in the arthropod host. Gamonts are usually intraerythrocytic and are variable in shape; some with broad shape, others with narrow and elongate shape (Rajabi et al., 2017).

More than 45 species of *Hepatozoon* have been described from amphibians around the world (Smith, 1996). Despite the wide geographical distribution of *Hepatozoon* species the data about this blood parasite found in frogs from certain geographical areas such as Europe and the Balkans are few compared to reports from Africa, North America, and Asia (Smith, 1996; Netherlands et al., 2014, 2018). Currently, despite the biological significance of the genus *Hepatozoon* the ecological and biological characteristics of the species are not completely understood in many areas due to the lack of information (Korzh & Zadorozhnyaya, 2013).

The distribution of blood parasites from anurans, specifically frogs, and their host-parasite relationship are especially important. The frogs form a link between aquatic and terrestrial ecosystems due to their specific ontogeny. These amphibians are important in food chains, preying on a variety of invertebrates, serving as prey for different animal groups, and ensuring the normal functioning of biocenosis (Toledo et al., 2007). At the same time, amphibians are among the most threatened vertebrate groups in the world due to the climate change, habitat destruction, and emerging diseases (Ferreira et al., 2020).

The marsh frog *P. ridibundus* is the anuran with the widest distribution in Bulgaria but the presence of parasites in its blood still remains unstudied. In the last decade, infection with *Hepatozoon magna* in *P. ridibundus* were reported from Russia (Peskova et al., 2018), Ukraine (Korzh & Zadorozhnyaya, 2013), and Iran (Rajabi et al., 2017). Previously, *Hepatozoon ridibundae* was detected in marsh frogs inhabiting Saudi Arabia (Shazly, 2003).

In Bulgaria, there are only three reports on representatives of species of *Hepatozoon*, one in passerine birds (Shurulinkov, 2003) and two in dogs (Ivanov & Kanakov, 2003; Tsachev et al., 2008). The current study is the first record of *Hepatozoon* sp. in amphibians from Bulgaria and the purpose of this paper is to present data about infection prevalence in established *Hepatozoon* reservoirs and morphological characteristics of the observed parasite in the blood of *P. ridibundus*.

Materials and Methods

Totally 137 sexually mature individuals of marsh frogs (*P. ridibundus*) were collected in five sites (Table 1) of Southern Bulgaria: 1) the Chaya River (synonyms: Chepelare River, Assenitsa River) near the confluence with the Maritza River (N42°15'61″, E24°89′73″); 2) the Vacha River (N42°03'43″, E24°47'04″), south of the town of Krichim, inside "Izgoraloto Gyune" Reserve; 3) the Tsalapitsa Rice Fields (N42°23'30″, E24°58'42″); 4) the natural wetland Zlato Pole (N42°02'12", E25°42'55") located several kilometers away from the town of Dimitrovgrad, south of the Zlato Pole village; and 5) the Veleka River near the confluence with the Black Sea (N42°03′40″, E27°57′56″). The last three localities are a part of the protected by Natura 2000 wetlands in Southern Bulgaria: protected area "Orizishta Tsalapitsa" (BG0002086), protected area "Martvitsata Zlato Pole" (BG0002103) and "Strandzha" Nature Park (BG0001007).

Table 1. Distribution, sampling period and number of *P. ridibundus* examined.

Site		*P. ridibundus*	Sampling time
1	Chaya River	40	April 2018
2	Vacha River	20	April 2018
3	Tsalapitsa Rice Fields	47	June 2017, May 2018
4	Wetland Zlato Pole	10	September 2018
5	Veleka River	20	June 2018

The field studies were conducted during 2018, except in the Tsalapitsa Rice Fields, where the marsh frogs were collected in 2017-2018 (Table 1). The frogs were captured nocturnally in the water using active collecting and a flashlight. Analyses were done in laboratory conditions in line with the ethical standards for research work with live animals according to Beaupre et al. (2004). Animals were transported from the site of capture to the laboratory in buckets full of water. Blood was taken through a cardiac ventricular puncture using small, heparinized needles after frogs were anaesthetized with ether according to Stetter (2001). Thin blood smears were prepared, air dried and fixed in absolute methanol. The presence of *Hepatozoon* was investigated on a Leica DM 1000 LED fluorescence microscope equipped with a special I3 filter (Leica Microsystems, Germany) suitable for AO, with a lens 40 × or 100 × below immersion. The samples were observed immediately after AO staining according to Hayashi et al. (1983). AO prepared first as a 0.1% aqueous solution, which may be available for several weeks, stored at 4 °C. 0.24 mM AO was dissolved in 1/15 M Sörensen phosphate buffer (pH 6.8) and used as working solution. Fixed blood smears were stained with this solution for 3 minutes at room temperature and then the slides were rinsed in the buffer. AO binds with nucleic acids and fluoresces. The fluoresces can easily be observed in contrast to the dark background. DNA bound with the AO fluoresces a bright yellow or apple green while RNA fluoresces red.

To assess the infection, we used prevalence, which characterizes the number of infected individuals as a percentage of the total number of examined frogs, and parasitaemia. Parasitaemia, a measure of infection intensity, of intracellular *Hepatozoon* sp. stages, and in this case was determined by counting the number of parasites per 2000 erythrocytes in randomly selected fields of view of the microscope. ImageJ software was used for microphotographs analysis and measurements. *Hepatozoon* species were identified based on comparative analysis of morphometric parameters of developmental stages (Smith, 1996). All measurements (i.e., mean length and width of the body and nucleus of parasite for established reservoirs) were given in µm. Additionally, the shape index as length/width ratio were calculated for gamonts and their nuclei.

The data were processed statistically using Graph Pad Prism 5 (GraphPad Software, Inc). One-way ANOVA was performed in order to assess significant differences among studied reservoirs.

Results

The presence of haemoparasites of genus *Hepatozoon* were established in the blood of *P. ridibundus* in three of five studied locations: Chaya River, Tsalapitsa Rice Fields and wetland Zlato Pole. The values of prevalence and parasitaemia of these reservoirs were present in Table 2, except for parasitaemia of Zlato Pole, where only one individual with *Hepatozoon* sp. infection was registered (parasitaemia=8.1%).

Table 2. Indices of infection with *Hepatozoon* in erythrocytes of *P. ridibundus*.

Reservoir	Prevalence (%)	Parasitaemia (%)			
		Mean	Min	Max	SD
Chaya River	27.5 (11/40)	11.0	0.5	39.0	7.9
Tsalapitsa Rice Fields	6.4 (3/47)	9.9	0.7	27.5	7.0
Wetland Zlato pole	10.0 (1/10)	-	-	-	-

Table 3. Morphometric characteristics of gamonts of *Hepatozoon* in erythrocytes of *P. ridibundus*. *Legend:* n - number of infected frogs.

	Parameters (µm)	Chaya River (n=11)				Tsalapitsa Rice Fields (n=3)				Wetland Zlato Pole (n=1)			
		Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD
Gamont	Length	24.08	36.85	30.45	2.90	23.17	36.92	31.38	2.68	25.07	32.71	30.07	1.98
	Width	3.88	6.43	5.25	0.55	3.82	5.68	4.70	0.40	3.56	5.09	5.09	0.47
	Shape Index	4.15	8.13	5.85	0.74	4.80	9.09	6.72	0.79	4.92	8.52	7.11	7.11
Nucleus	Length	3.99	5.88	4.86	0.51	3.39	5.94	4.44	0.65	3.44	6.06	4.49	0.64
	Width	3.47	5.03	4.36	0.38	3.09	4.23	3.76	0.33	2.46	4.37	3.42	0.36
	Shape Index	0.80	1.35	1.12	0.14	0.87	1.58	1.19	0.17	1.00	2.21	1.33	0.28

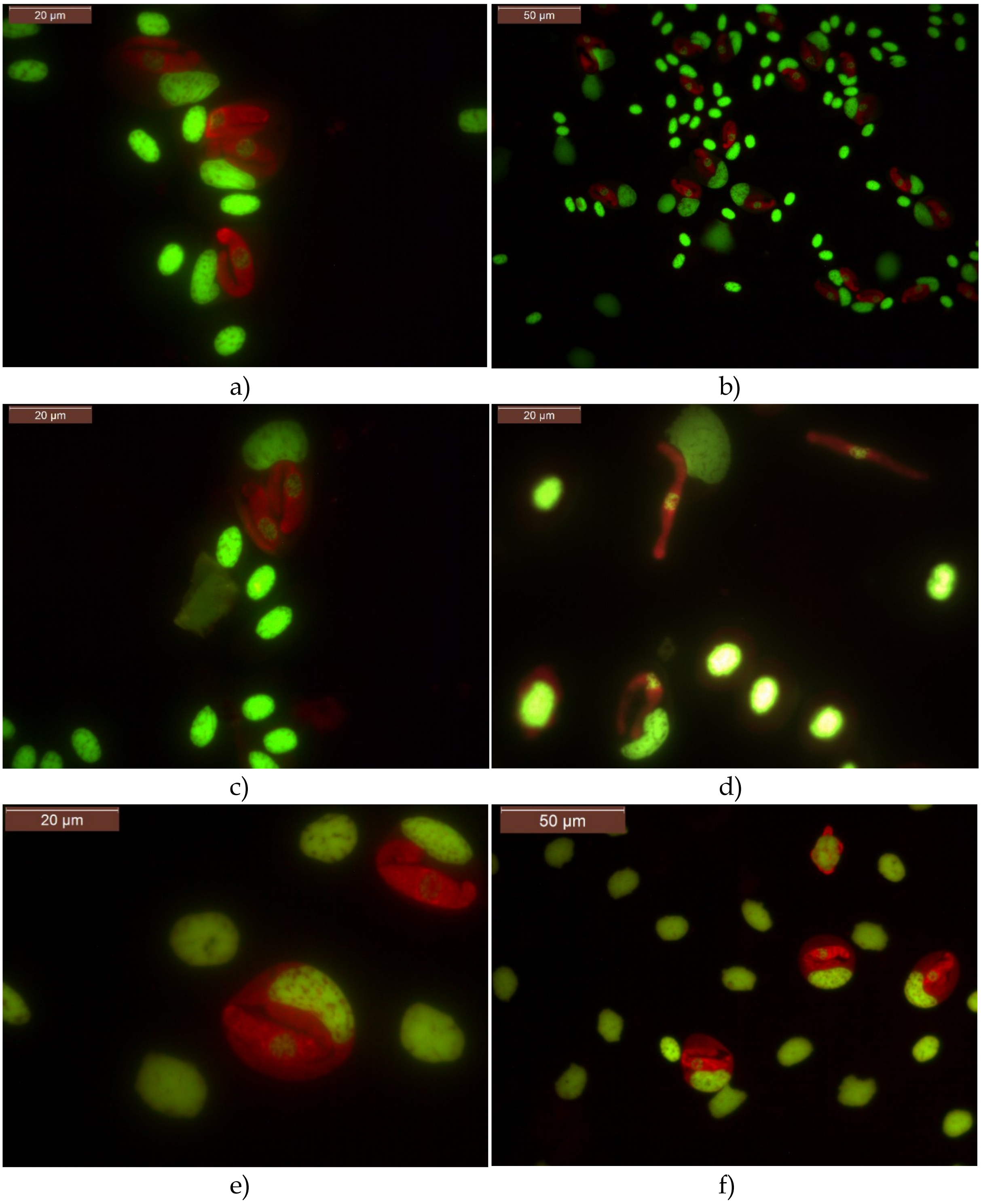

Fig. 1. Photomicrographs of *Hepatozoon* sp. observed from *P. ridibundus*: a) gamonts from Chaya River; b) individual with high value of parasitaemia; c) double infection in one erythrocyte; d) extracellular forms; e - f) red cytoplasm of host cells and increased size of nuclei of infected erythrocytes (a,c,d,e - magnification 1000 ×, in immersion; b, f - magnification 400 ×).

Extracellular forms, as well as intracellular forms (i.e., mature and young gamonts) were found. Most of observed gamonts were found within erythrocytes. Table 3 provides information on the length and width of gamonts, the length and width of their nucleus, and the shape index. The intracellular gamonts have cells bent in the middle so that the "tail" is pressed against the main body exceeding half of the body length (Fig. 1). The body shape was elongated and well differentiated in the anterior with broader rounded end and narrower posteriorly. The parasites occupy a maximum area in the host erythrocytes and were placed either with the concave border of the host cell nucleus, and or sometimes at one pole of the host cell.

Double infection (Fig. 1c) and extracellular forms have been observed only in infected erythrocytes of animals with high parasitaemia value. The extracellular forms of *Hepatozoon* (trophozoites) have an elongated worm-like body; one end of the body is narrower, the other is wider and rounded (Fig. 1d). The mean length (L) of the trophozoites is similar to gamonts, but the width (W) is less (Chaya River: L=30.6 µm, W=3.37 µm, L*nucleus*=4.652 µm, W*nucleus*=3.147 µm; Tsalapitsa rice fields: L=32.99 µm, W=3.654 µm, L*nucleus*=5.415 µm, W*nucleus*=3.436 µm; Wetland Zlato Pole: L=30.62 µm, W=3.37 µm, L*nucleus*=4.65 µm, W*nucleus*=3.147 µm).

The nuclei of parasites and host cells are well differentiated due to the staining with acridine orange. The parasite nucleus occupies almost the entire width of the cell body and has a granular structure. The host cell nucleus was displaced laterally or to one pole and appeared enlarged and frequently lobed (Fig. 1e). Erythrocytes infected with *Hepatozoon* sp. stages were increased in size, and their cytoplasm changed its color to red after staining with acridine orange (Fig. 1e, 1f).

We did not find any significant differences among the three reservoirs for the length of gamonts. However, the mean values of all other parameters (i.e., gamont width, nucleus length, nucleus width, and shape index for gamonts and nuclei) differed statistically between the three reservoirs ($P < 0.0001$).

Discussion

Amphibians are among the most threatened vertebrate groups in the world and anurans have the highest rate of population decline due to human activities, including climate change, habitat destruction, pollution, and emerging diseases (Wake & Vredenburg, 2008). Frogs are known to host a wide variety of haemoparasites, including intracellular apicomplexans from the genus *Hepatozoon* (Smith et al., 1996; Netherlands et al., 2014, 2018; Ferreira et al., 2020).

The prevalence and levels of parasitaemia of *Hepatozoon* sp. infection among marsh frogs, *P. ridibundus,* in Bulgaria was studied for the first time. Our results revealed that prevalence of *Hepatozoon* sp. in the blood of marsh frogs was higher in Chaya River (27.5%) compared to Zlato Pole and Tsalapitsa Rice Fields (10.0% and 6.4% respectively), but lower than values reported from Korzh & Zadorozhnyaya (2013) from two different biotops in Ukraine (44.4% and 66.6%). The individual frog with the highest parasitaemia, at 39.0%, was recorded in Chaya River (Table 2). The mean parasitaemia (%) for this site was 11.0±7.9, which represents a wide variability of this term. A comparison with similar studies showed various levels of parasitaemia (%) of *Hepatozoon* species in the blood of *P. ridibundus*: 10.8±1.2 and 17.3±1.8 from two different populations in Russia (Peskova et al., 2018); 2.8 in Iran (Rajabi et al., 2017); 5.8±1.8 and 10.1%±3.6 from two different populations in Ukraine (Korzh & Zadorozhnyaya 2013).

Based on the morphometrics of the gamont stages, initially we supposed the presence of *Hepatozoon magna* in blood smears of *P. ridibundus.* However, the length of gamonts were much greater in our study (i.e., 30.45±2.90, 31.38±2.68, and 30.07±1.98 µm for the three reservoirs) compared with data from other authors. Rajabi et al. (2017) reported *Hepatozoon magna* from *P. ridibundus* in Iran with gamonts that were remarkably similar in morphology as compared with those observed in our study, but with lower body length of 18.03±1.59 µm. For *H. magna* from *P. ridibundus* in Russia, Peskova et al. (2018) reported also lesser length of gamonts (16.3±0.28 and 17.2±0.58 for two studied sites), but greater width (7.1±0.16 and 7.6±0.12) compared to our results (5.25±0.55, 4.70±0.40 and 5.09±0.47 respectively). The results of Korzh & Zadorozhnyaya (2013) from two localities in Ukraine for length (35.31± 0.48 and 32.18± 0.82) and width (4.16± 0.07 and 3.68± 0.12) of gamonts observed in marsh frog are closest to the morphometrics of the gamonts from our study. Based on morphometric characteristics, the authors considered the larger parasite as *Hepatozoon magna*, and the smaller as *Hepatozoon* sp. In our study, we did not find any significant differences between the three established reservoirs only for the length of gamonts, but the mentioned authors described great variability of this parameter. *Hepatozoon* spp. are more challenging to differentiate than any of the other adeleorinid genera due to low host specificity for either or both their invertebrate and vertebrate hosts (Smith, 1996). Subtle differences in the morphology of species also makes them difficult to identify and differentiate from blood stages alone (Ferreira et al., 2020). Thus, despite the great morphological similarities with *H. magna*, the *Hepatozoon* detected in the blood of *P. ridibundus* in our study should be considered as *Hepatozoon* sp. for the present time, and the genus and species should be determined in a further analysis using molecular tools.

The fluorescent observations showed that infected erythrocytes and their nuclei are enlarged, and often lobed, compared to uninfected erythrocytes, which has also been observed in similar studies of *Hepatozoon* infection in marsh frogs (Korzh & Zadorozhnyaya, 2013; Rajabi et al., 2017; Peskova et al., 2018).

The presence of *Hepatozoon* gamonts in our research was discovered in blood smears stained with the fluorescent dye acridine orange (AO). The AO dye was found to have a high diagnostic capacity to detect different parasites like protozoans and rickettsiales in blood smears because of its higher speed of reading and sensitivity when compared with common bright field microscopy using Giemsa staining, as well as providing the opportunity to detect DNA/RNA through staining in different colors. In addition, for the microscopic observation of intraerythrocytic parasites of Giemsa-stained slides an oil emulsion objective (100 ×) is required, but the dry high lens (40 × or 50 ×) was sufficient for the AO method (Kong & Chung, 1995). The AO technique is recommended for a fast diagnosis, especially in countries with endemic areas of malaria, sleeping sickness, Lyme disease, babesiosis, and spirochetemia, and it is appropriate for detecting even cases of low-level parasitaemia (Ravindran et al., 2007; Kimura et al., 2018). Despite that the parasites reported in this study are quite large and would be easily detectible with more traditional bright-field staining techniques, the use of the AO fluorescent staining allows easy detection of a wide range of parasites in the same blood sample, including other adeleorinid blood parasites that present as much smaller intraerythrocytic forms (e.g. *Babesiosoma stableri*).

Conclusion

Our study revealed the presence of apicomplexan haemoparasite from the genus *Hepatozoon* in erythrocytes of marsh frog, *P. ridibundus*, for the first time in Bulgaria. Three of five studied populations from South Bulgaria were reservoirs of this infection. Prevalence and intensity of *Hepatozoon* was higher in Chaya River (27.5% and 11.0% respectively), followed by wetland Zlato Pole (10.0% and 8.1%) and Tsalapitsa Rice Fields (6.4% and 10.0%). Based on gamont morphology and morphometric characteristics, we suggest that the observed species is the closest to the *Hepatozoon magna* but should be considered as *Hepatozoon* sp. until confirmation in further studies, including molecular detection of the genus and species. Differences in the erythrocyte morphology of the marsh frog, when infected, were identified. Our findings revealed that acridine orange is appropriate dye for detecting haemoparasites of the genus *Hepatozoon*.

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