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Occurrence of blood parasites and intensity of infection in *Prunella modularis* in the montane and subalpine zone in the Slovak Carpathians

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Abstract

The objective of this study was to obtain primary information on the occurrence of blood parasites and intensity of infection in the Dunnock *Prunella modularis* in the montane region of Slovakia. Altogether 109 birds were examined during the years 2006–2010. The occurrence of *Haemoproteus* sp., *Leucocytozoon fringillinarum* and *Trypanosoma* sp. was documented. Blood parasite prevalences of 45% by microscopic examination and 55% by PCR diagnostics were found. The prevalence of *Leucocytozoon* sp. was found to be dependent on host sex with males showing a significantly higher intensity of infection with *Leucocytozoon*. Adult birds showed significantly higher infection prevalence than subadults. The prevalences of both *Leucocytozoon* sp. and *Haemoproteus* sp. were significantly dependent on bird age. The intensity of infection with *Haemoproteus* and *Leucocytozoon* was positively correlated and higher intensity of infection was confirmed in adult birds then in subadults birds. The prevalence of blood parasites in our samples was significantly higher in comparison to most other studies from different countries of Europe conducted at lower altitudes, indicating that the montane zone is especially favourable for the transmission of *Leucocytozoon* and *Haemoproteus* species.

Keywords

Prunella modularis, *Leucocytozoon*, *Haemoproteus*, blood parasites, Slovakia

Introduction

The aim of our study was to determine the occurrence of blood parasites in the Dunnock *Prunella modularis*, a common European passeriform bird species (Davies 1992) in the montane and subalpine zone in the Slovak Carpathians. The blood parasite fauna of this species has not yet been thoroughly studied and records of blood parasites from the Dunnock are scarce (Dymowska and Żukowski 1968; Peirce and Mead 1976, 1977, 1978; Merino *et al*. 1997; Palinauskas *et al*. 2005; Benedikt 2006; Hauptmanová *et al*. 2006). There is also only limited information about the occurrence and prevalence of blood parasites in the Dunnock from the Slovak territory (Kučera 1981a,b,c 1982; Hauptmanová *et al*. 2006).

The Dunnock prefers mountain forests, scrubs along the forest edge or in forest glades and extends up to the scrub just above the tree-line, but it remains below the higher, more open rocky areas (Davies 1992). This bird species is common in all favourable habitats especially in the mountain regions of Slovakia and it is a very common species in the higher mountain zone (1,400–1,600 m a.s.l.) of the High Tatras (Ferianc 1979).

The Dunnock in Slovakia avoids cold winters by migrating south to Central and Southern Europe. Birds breeding in mountainous regions move to lower areas (Ferianc 1979, Davies 1992).

We used two methods for the detection the blood parasites – the traditional light microscopy of blood smears and the PCR detection. PCR analysis proved to be more sensitive in detecting blood parasites (e.g. Perkins *et al*. 1998, Bensch *et al*. 2000, Jarvi *et al*. 2002, Križanauskienė *et al*. 2006). Especially at early stages of infections parasites can be overlooked in blood smears (Jarvi *et al*. 2003). In chronic infections, e.g. chronic malaria, detectability in smears is very low (Feldman *et al*. 1995).

Materials and methods

The birds were captured between 2006–2010 in the characteristic habitats of the Dunnock from the montane to alpine zone of several mountain ranges in the Slovak Republic

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(Fig. 1). The birds were captured using ornithological mist-nets. Weight and basic morphometric measures were taken. The birds were classified as adults (>1 year of age) or subadults (<1 year of age). Adult birds were sexed by PCR based sex detection according to Griffiths *et al*. (1998).

The blood collection was carried out using the branchial vein puncture. After the preparation of blood smears, bleeding was stopped by wound compression with paper swabs. Blood spots on the paper swabs were air dried and saved later for PCR analyses.

The blood smears were fixed with methanol and in the laboratory stained using combined method according to Pappenheim (Lucas and Jamroz 1961). The blood smears were examined using the BX41 light microscope (Olympus, Tokyo, Japan) at 400× and 1000× magnification for the presence of blood parasite gametocytes. A digital camera (Moticam 1000) with imaging software Motic Images Plus 2.0 ML (Motic China Group Co.) was used to examine slides, prepare illustrations, and take measurements. Each blood smear was examined for the presence of blood parasites by observing 100 microscope view fields at both magnifications. All detected parasites were photographed and measurements of the fully grown gametocytes were carried out.

Intensity of infection was estimated by counting the number of parasites per 10,000 erythrocytes and expressed as percentage of infected erythrocytes. The intensity for *Haemoproteus* was classified into three levels: low – if fewer than 0.1% erythrocytes were infected, as medium if 0.1–0.5% erythrocytes were infected and as high if over 0.5% erythrocytes were infected and for *Leucocytozoon*: low – if fewer than 0.02% erythrocytes were infected, medium – 0.02–0.1% erythrocytes were infected, high – more than 0.1% erythrocytes were infected (according to Hauptmanová *et al*. 2006). Intensity of infection was also classified as low in those cases where the parasite was only detected by PCR analysis. The prevalence was calculated as the proportion of individuals infected with particular haematozoan parasite (Peirce 1981, Jurášek and Dubinský 1993). Microscopic screening was used to identify parasite species and for the classification of infection intensity level. The blood smears from all birds were examined by light microscopy for the presence of intracellular and extracellular blood parasites and PCR analysis for the presence of *Plasmodium*, *Haemoproteus* and *Leucocytozoon*.

Genomic DNA was extracted from avian dried blood spots using the QIAamp DNA Mini Kit (QIAGEN, Germany) according to standard protocol. *Leucocytozoon* species were identified by nested PCR using two sets of primers for cyt b gene (HAEMF/HAEMR2 and HAEMFL/HAEMR2L) by Hellgren *et al*. (2004).

DNA sequencing analysis was carried out in 5 randomly chosen positive samples where the presence of *Leucocytozoon* sp. was confirmed both by microscopical examination and PCR analysis. Positive fragments were sequenced with the one primer (HAEMFL) in 5´-3´ direction with Beckman GeXP sequencer. Sequences were subjected to the BLAST analysis (http://blastn.ncbi.nlm.nih.gov/Blast.cgi) with equivalent

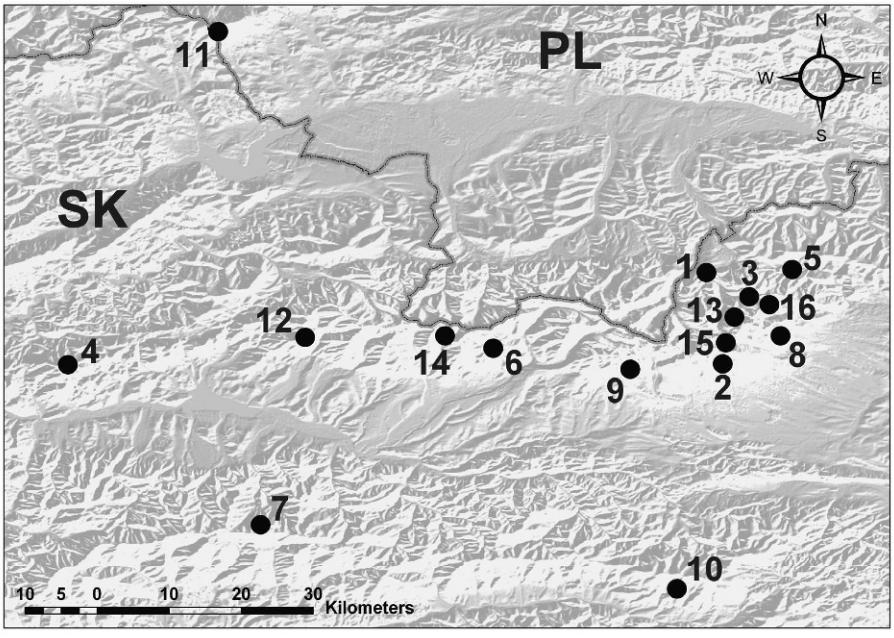

Fig. 1. Capture sites of *P. modularis*: **1** – Tatranská Javorina (1,000 m a.s.l.); **2** – Sliezky dom (1,575 m a.s.l.); **3** – Zadné Meďodoly (1,430 m a.s.l.); **4** – Veľký Choč (1,543 m a.s.l.); **5** – Monkova dolina (930 m a.s.l.); **6** – Kamenistá dolina (1,270 m a.s.l.); **7** – Chopok (1,637 m a.s.l.); **8** – Skalnatá dolina (1,742 m a.s.l.); **9** – Mlynická dolina (1,618 m a.s.l.); **10** – Kráľova hoľa (1,460 m a.s.l.); **11** – Babia hora (1,598 m a.s.l.); **12** – Červenec (1,439 m a.s.l.); **13** –Zbojnícka chata (1,935 m a.s.l.); **14** – Račková dolina (1,503 m a.s.l.); **15** – Čierna Javorová dolina (1,527 m a.s.l.); **16** – Dolina Bielych plies (1,733 m a.s.l.)

records for Haemosporida sequences available in GenBank. Our samples were most highly identical with *L. fringillinarum* by 81–93%. Phylogenetic relationships between positive samples and *L. fringillinarum* were estimated using the program Mega 5.05 and similarity was evaluated by Maximum Likelihood Tree, Tamura Nei Model.

Statistical analyses were carried out using R statistics (R Development Core Team 2009).

Results

Altogether 109 Dunnocks were examined for the presence of blood parasites. Occurrence of *Haemoproteus* and *Leucocytozoon* was confirmed both by light microscopy and PCR analysis. Infection by *Trypanosoma* sp. was also confirmed in blood smears. Other species of protozoan parasites such as *Plasmodium* sp., *Atoxoplasma*, *Babesia*, *Haemogregarina*, *Hepatozoon* and microfilariae were not found.

Species identification of haematozoan parasites

Species identification of blood parasites was possible when fully-grown parasites were present on the slides. *Haemoproteus* sp. could not be identified to species level. According to the morphological data, the recorded species was not identical with any described species of *Haemoproteus* (Valkiūnas 2005) and its precise identification will require further morphological and molecular analyses. Gametocytes of *Leucocytozoon* suitable for species identification were found in 36 out of 39 individuals with positive result from light microscopy and by using morphological (the mode, size and position of nuclei of infected cell) and morphometric characteristics of the gametocyte (Valkiūnas 2005) it was identified as *Leucocytozoon fringillinarum* Woodcock, 1910. The measurements of gametocytes are shown in the Table I.

Morphological description of the gametocytes of L. fringillinarum

Only round gametocytes were observed in our blood smears. The nucleus of the host cell was deformed and pressed to the periphery of the cell. It never exceeded ½ of the gametocyte perimeter and often appeared in the typical cap-shaped form. The cytoplasm of the microgametocyte was pale (grey-violet), whereas the cytoplasm of the macrogametocytes showed intense blue-violet coloration. The parasite's nucleus was round or ellipsoid and nucleolus was occasionally visible. If the host cell cytoplasm was preserved, it was visible as fragmented projections around the parasite body.

DNA sequencing analysis

DNA sequencing analysis was carried out in 5 randomly chosen *Leucocytozoon* positive samples, where the presence of *Leucocytozoon* sp. was confirmed both by microscopical examination and PCR analysis. The nucleotide BLAST analysis of the sequenced cyt b gene showed close identity with *Leucocytozoon* sp. in all analysed samples (Fig. 2).

Intensity of infection

The intensity of infection by *Haemoproteus* and *Leucocytozoon* was positively correlated (Spearman's rho = 0.275, p = 0.004). The intensity of infection was higher in adult birds then in subadults both for *Haemoproteus* (Mann-Whitney Z = –3.549, p<0.001) and *Leucocytozoon* (Mann-Whitney Z = –2.72, p = 0.007). In adult birds, the intensity of infection was significantly higher in males for *Leucocytozoon* (Mann-Whitney Z = –2.18, p = 0.029). Intensity of infection with *Trypanosoma* sp. was not classified.

Prevalence

Fewer infections were confirmed by microscopic examination in comparison to PCR analysis. Comparison between the prevalence of the blood parasites of genus *Haemoproteus*, *Leucocytozoon* and *Trypanosoma* found with microscopical examination and PCR diagnostic is given in Table II.

The prevalence of *Leucocytozoon* sp. was statistically dependent on host sex (G = 3.84, df = 1, p-value = 0.05). Dependence of *Haemoproteus* sp. prevalence on sex was not confirmed. Adult birds showed higher infection prevalence than subadults. The prevalence of any haematozoan parasite

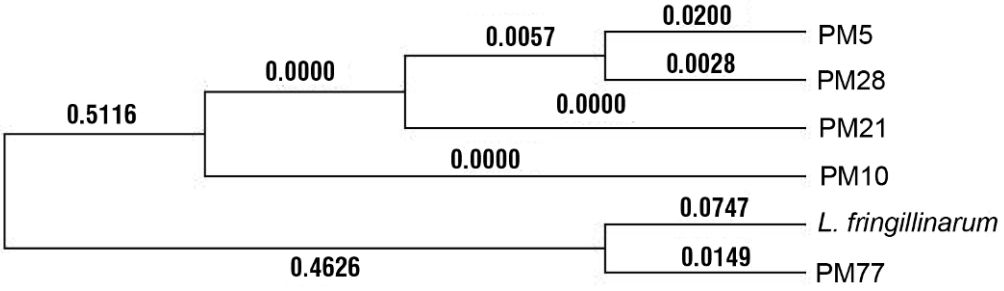

Fig. 2. Dendogram showing genetic relationship among 5 samples of blood parasites – *Leucocytozoon* sp. from *P. modularis* from Slovak Republic with *L. fringillinarum*. The Maximum Likelihood Tree method based on Tamura Nei Model was used for the analysis

Table I. Measurements of *L. fringillinarum* from the blood smears of *P. modularis*. All measurements are given in micrometers

	Gametocytes (macrogametocytes); n = 53				
	Gametocyte length	**Gametocyte width**	**Length of gametocyte nucleus**	**Width of gametocyte nucleus**	**Length of nucleus of host cell**
Range	9.5–12.3	7.7–10.7	2.2–3.9	1.1–2.8	7.3–16.2
Mean	10.7	8.9	3.1	2.0	12.5
SD	0.6	0.7	0.4	0.3	2.1

Table II. Prevalence of blood parasites in *P. modularis* based on microscopical/PCR examination. H – *Haemoproteus*, L – *Leucocytozoon*, T – *Trypanosoma*, MI – mixed infection at least two species

	Total no. of examined birds	**Total no. of positive birds (microscopy/PCR)**				**Total no. of infected birds**	**Prevalence %**
		H	**L**	**T**	**MI**		
2006	3	0/1	1/1	0/-	0/1	1/1	33.3/33.3
2007	31	3/11	13/19	5/-	1/11	16/19	51.6/61.3
2008	64	10/11	21/29	1/-	5/6	26/34	40.6/53.1
2009	5	0/0	2/2	0/-	0/0	2/2	40.0/40.0
2010	6	2/3	2/4	0/-	0/3	4/4	66.7/66.7
Total	109	15/26	39/55	6/-	6/21	49/60	45.0/55.0
Prevalence total %		13.7/23.9	35.8/49.5	5.5/-	5.5/19.3		

Table III. Number of infected adult and subadult birds

	Leucocytozoon		***Haemoproteus***		***Haemoproteus*, *Leucocytozoon***	
	uninfected	**infected**	**uninfected**	**infected**	**uninfected**	**infected**
Adults	32	47	53	26	27	52
Juveniles	22	8	30	0	22	8

Table IV. Comparison of blood parasite prevalence in *P. modularis* from several European countries. H – *Haemoproteus* sp., L – *Leucocytozoon* sp. (G-Test *prevalence significantly lower than in our results, p <0.05, **p<0.01)

Examined	**Positive**		**Prevalence**		**Country**	**References**
	H	**L**	**H**	**L**		
75	5	0	6.7%**	0**	Great Britain	Peirce and Mead 1976, 1977, 1978
1	1	0	100.0%	0	Spain	Merino *et al*. 1997
55	5	0	9.1%*	0**	Poland	Dymowska and Żukowski 1968
136	13	8	9.6%**	5.9%**	Central Europe	Kučera 1981b, c
28	1	0	3.6%*	0**	Czech Republic	Benedikt 2006
26	3	0	11.5%	0**	Slovakia, Poland	Hauptmanová *et al*. 2006
12	7	0	58.0%	0%	Czech Republic	Haas 2011

species was statistically dependent on bird age (G = 9.65, df = 1, p-value = 0.00189). This relationship was confirmed both for *Leucocytozoon* (G = 8.31, df = 1, p-value = 0.00394) and *Haemoproteus* (G = 15.30, df = 1, p-value = 0.0000915; Table III).

We have not found any significant spatial or temporal patterns in prevalence (such as annual or seasonal prevalence, differences between capture sites). There was no significant relationship between the body size of the birds and haematozoan infection prevalence or infection intensity.

Discussion

Our results have confirmed that PCR analysis is more sensitive in detecting blood parasites than traditional light microscopy of blood smears, as mentioned in other studies (e.g. Perkins *et al.* 1998, Bensch *et al*. 2000, Jarvi *et al*. 2002, Križanauskienė *et al*. 2006).

Although several *Haemoproteus* species have been recorded in the Dunnock such as *H. fringillae* (Dymowska and Żukowski 1968, Peirce and Mead 1976, 1978), *H. danilewskii*

(Dymowska and Żukowski 1968) and *H. orizivorae* (Peirce and Mead 1976), our findings are not morphologically identical with any of these species. *Haemoproteus* sp. from our samples is being subjected to further description.

Our measurements of *L. fringillinarum* are slightly different from the results of Shurulinkov and Golemansky (2003) and Valkiūnas (2005). The results of DNA sequencing analysis have shown 90% identity with sequences of *L. fringillinarum* sequences from the GenBank. We suppose, that these differences could be caused by the fact that the parasite DNA was collected from different host species. Relatively low percentual similarity of our DNA sequences to *L. fringillinarum* sequences in GenBank (~90%) could be caused by intraspecific variability of this parasite. Recent molecular analyses of avian malaria parasites based primarily on mitochondrial sequences have revealed a wealth of genetic diversity among parasite lineages that is not apparent in their morphology (Bensch *et al*. 2000, Perkins and Schall 2002, Ricklefs and Fallon 2002), therefore the low similarity is not exceptional. Parasite lineages are also known to be quite different depending on geographic regions (Ricklefs and Fallon 2002).

The intensity of infection was classified as low in most cases, similarly to other studies. Infection with blood parasites can often be low or subclinical, but relapses occur if the birds are under stress or suffering from concomitant infections (Atkinson and van Riper 1991, Graczyk *et al*. 1994, Waldenström *et al*. 2002). Similarly to the results of Svobodová and Votýpka (1998), we have also recorded a higher infection intensity in adult birds than in subadults. Some studies have presented the opposite result for *L. fringillinarum* (Allander and Bennett 1994) or no significant relationship between haemoparasite prevalence and age of birds (Deviche *et al*. 2001).

In this study we recorded a 55% prevalence of blood parasites in the Dunnock. *L. fringillinarum* infections were more common than *Haemoproteus* sp. (*L. fringillinarum* 55% and *Haemoproteus* sp. in 23.9% of birds). According to Valkiūnas (2005), at least 50% of bird species investigated for blood parasites were infected with *Haemoproteus* sp. and 30% of bird species with *Leucocytozoon* sp. In several passeriform birds of Central Europe, Kučera (1981a) has recorded a 12.5% mean prevalence of *Haemoproteus* and 4% of *Leucocytozoon*.

The haematozoan prevalence is known to vary with geographical and climate conditions (Benedikt 2006). Although we have not confirmed any significant geographical variation between our sites, the prevalence of blood parasites in our samples was significantly higher in comparison to most other studies from different countries of Europe (Table IV). Especially the prevalence of *Leucocytozoon* sp. was markedly higher than in all European studies in comparison. This could be explained by differences in vector availability. All our capture sites were located in montane or subalpine zone which comprise favourable habitats for the *Leucocytozoon's* main vector – the blackflies (Simuliidae) (Illéšová *et al*. 2008, Bulánková and Zaťovičová 2006). Higher prevalence of simuliid-transmitted parasites such as *Leucocytozoon* was reported in montane regions and boreal biomes in North America, independent of host identity (Greiner *et al*. 1975). Murdock (2009) has recorded that *Leucocytozoon* was the most dominant hemosporidian parasite in the avian community in alpine environment too. Climate conditions of Central European mountain ranges are similar to those of northern Europe, where a higher prevalence of parasites transmitted by simuliid vectors has also been reported (Scheuerlein and Ricklefs 2004). This is supported by the fact that according to our information, the geographically closest capture sites (Kučera 1981b,c; Benedikt 2006; Haas 2011; Hauptmanová *et al*. 2006) were located at lower elevations than our capture sites.

Our results show a significantly higher intensity of infection in males than females and a positive correlation between the infection with *Haemoproteus* and *Leucocytozoon*. The prevalence of *Leucocytozoon* was also higher in males than females, which could not be confirmed for *Haemoproteus* sp. Higher prevalence and infection intensity in males could be anticipated as many authors have discussed that higher testosterone levels and other factors such as stress during courtship period have a significant immuno-suppressing role in males (Folstad and Karter 1992, Zuk and McKean 1996). This was confirmed by Madsen *et al*. (2007), who recorded significantly higher levels of testosterone in males infected with *Haemoproteus*. In contrast with our results, Allander and Bennett (1994) did not find a significant difference in the mean intensity of *H. majoris* between males and females nor any correlation between the occurrence of the most common blood parasite species.

Our results show a significantly higher prevalence of haemoparasites in adult birds than in subadults, confirming the predictions of Appleby *et al*. (1999), Garvin and Greiner (2003). The same relationship has been reported in the works of Svobodová and Votýpka (1998), Madsen *et al*. (2007). However, some studies concerned specifically with blood parasites in the Dunnock have not found any significant association between prevalence and host age (Kučera 1981b, Hauptmanová *et al*. 2006). The increase in prevalence with age could be explained by higher mortality of young birds (Valkiūnas 2005), deterioration in the immune function of adults (Cichoñ *et al*. 2003), or increased exposure to the vectors (Ahmed and Mohammed 1978). Several authors have confirmed the relationship between host body weight and prevalence of blood parasites (Scheuerlein and Ricklefs 2004, Marzal *et al*. 2008), but this effect is not always present and similarly to our study, other past studies have reported no direct relationship between the occurrence of blood parasites and host body weight (Hauptmanová *et al*. 2002, Deviche *et al*. 2001, Palinauskas *et al*. 2005). Clarification of this relationship is often complicated in wildlife field studies due to many uncontrolled variables (Madsen *et al*. 2007).

Relapses of increased parasitaemia are known to occur during the breeding period (Valkiūnas 2005) and after spring migration (Allander and Bennett 1995; Phalen *et al*. 1995; Rintamäki *et al*. 1998, 1999; Dawson and Bortolotti 2000;

Dyrcz *et al*. 2005; Garvin *et al*. 2006). Although the prevalence of haemoparasites in our samples was rather high in comparison with other studies on the Dunnock (Peirce and Mead 1976, 1977, 1978; Merino *et al*. 1997; Dymowska and Żukowski 1968; Kučera 1981b, c; Benedikt 2006; Hauptmanová *et al*. 2006), we have not confirmed any significant seasonal variation in parasitaemia, in contrast to the report of Hauptmanová *et al*. (2006) from other parts of Slovakia.

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