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Absence of blood parasites in the Alpine Accentor *Prunella collaris*

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Abstract. Total of 83 birds Alpine Accentor *P. collaris* were investigated for occurrence of blood parasites *Haemoproteus* sp. and *Leucocytozoon* sp. During the years 2001-2009 birds were captured in their natural habitats in alpine areas of the Slovak high mountains. The blood smears of all individuals were examined by microscopical scanning and 50 individuals were tested for blood parasites using PCR. None of the investigated birds was found to be infected with blood parasites.

Key words: Prunella collaris, blood parasites, *Haemoproteus, Leucocytozoon*, high mountains, Slovakia

Introduction

Hematozoa have been found in 68% of bird species examined to date (Krone *et al.* 2001). Infections of avian blood hemosporidian parasites are characterized by a high prevalence in bird populations, i.e. with a high proportion of infected individuals (Valkiūnas 1997). Wild birds throughout the world except Antarctica are commonly hosts of haemoprotozoans such as *Haemoproteus* sp., *Leucocytozoon* sp., *Plasmodium* sp. (Valkiūnas 1997) and *Trypanosoma* sp. (Rintamäki *et al.* 1999). Besides these taxa, a number of other genera of protozoan blood parasites - e.g. the *Babesia, Atoxoplasma* and *Hepatozoon* and larval stages of nematodes (microfilariae) have also been described in birds, but their occurrence in peripheral blood smears are rare (Kučera 1982). The blood parasites can be detected microscopically on stained blood smears. In this case, the infection is characterized by the presence of gametocytes in erythrocytes, or sometimes in leukocytes (Valkiūnas 1997). Recently, the molecular techniques are used to determine the presence of hemosporidia in the blood of birds. The techniques are based on the amplification of parasite DNA from blood samples (Perkins and Schall 2002, Bensch *et al.* 2004, Ricklefs *et al.* 2004). Mosquitos, hippoboscid flies, biting midges or tabanids are the most commonly cited intermediate hosts for haemoprotozoans (such as *Plasmodium, Haemoproteus, Leucocytozoon*; Valkiūnas 1997).

The *Prunella collaris* Scopoli, 1769 is a strictly alpine species, breeding above the treeline at altitudes of 1,800-4,000m a.s.l. (Cramp 1988, Dyrcz and Janiga 1997). In Slovakia, the blood parasites in passerine birds were investigated by Kučera (Kučera 1981a,b,c, Kučera 1982) and Hauptmanová *et al.* (2006). Kučera (1981c) mentioned on positive findings of *Haemoproteus* in *P. collaris*. The published note looks to be, at least, questionable because the quotation is in a bibliographic study and no more detailed data or original references are specified.

Material and Methods

From 2001 to 2009, the Alpine Accentors were captured in their natural habitats in the alpine areas of the Slovak high mountains - The High, Low, Belianske and Western Tatras and Veľká Fatra. Trapping was carried out throughout the year including the winter months. Birds were captured using ornithological mist nets or ornithological folding traps. After the trapping, standard morphometric measurements were taken for each individual. Captured birds were identified as juveniles or adults (age ≤ 2 months). Adult birds were sexed by the presence of a cloacal protuberance in males (Nakamura 1990) or since 2005 by PCR detection of sex.

The blood was taken by puncture from vena brachialis and a drop of blood was transferred on the slide and thin blood smear was made. The blood smears were air dried, then fixed by 96% methanol and dried again. The dried smears were stained with a combination of stains May-Grünwald and Giemsa-Romanowski, using a method according to Pappenheim (Lucas and Jamroz 1961). The smears were examined microscopically under 400× and 1000× magnification for the presence of blood parasite gametocytes.

During the blood collection, a sample of blood was absorbed on a paper tampon and retained for subsequent PCR analysis, which was used for the determination of sex of the birds according to the methodology by Griffiths *et al.* (1998) and for the presence of blood parasites in the blood samples.

Genomic DNA was extracted from dry blood spots on paper tampons (after blood collection) using the QIAamp DNA Mini Kit (Qiagen, Germany).

PCR amplification was carried out according to studies by Bensch *et al.* 2000, Waldenström *et al.* 2004, Hellgren *et al.* 2004). Based on the sequence homology between aligned sequences of the blood parasites, initial primers were Haem NFI (5'-CATATATTAAGAGAAITATGGAG-3') and Haem NR3 (5'-ATAGAAAGATAAGAAATACCATTC-3') used to amplify parasite mitochondrial DNA (gene of the cytochrome b, 617 bp large fragment) from both genera of *Haemoproteus*, and *Leucocytozoon*. For the second PCR, the following primers were used: for *Haemoproteus* sp. HaemF (5´-ATGGTGCTTTCGATATATGCATG-3´) and HaemR2 (5´-GCATTATCTGGATGTGATAATGGT-3´) to amplify a 480 bp large fragment, for *Leucocytozoon* sp. HaemFL (5´-ATGGTGTTTTAGATACTTACATT-3´) and HaemR2L (5´-CATTATCTGGATGAGATAATG-3´) to amplify a 478 bp large fragment. The first PCR (using HaemNFI–HaemNR3 primers) was performed in using 20 µl reaction volumes, which included 50 ng of total genomic DNA, 1x reaction buffer (15mM Tris-HCl, (pH 8,2 at 25° C) 30mM KCl, 5mM $(NH_4)_2SO_4$, 2,5 mM $MgCl_2$, 0,02% BSA), 200 µM of each dNTP, 0,5µM of each primer and 0,5 U DynaZyme DNA polymerase (Finnzymes OY). The PCR amplification protocols were as follows: initial denaturation 94° C for 10 min, then 20 cycles of 94 ° C for 30 s, 50° C for 30 s and 72° C for 1 min, finally 72° C 10 min.

The product of the first PCR was taken (2 µl) as the template for the second PCR, 2 µl for *Leucocytozoon* sp. (HaemFL–HaemR3L) and 2 µl for *Haemoproteus* sp. (HaemF–HaemR2). These PCR's were performed separately in 20 µl volumes with the same proportions of reagents as in the initial PCR reactions. The thermal profile of the PCR was identical to the initial PCR but performed for 35 cycles. The PCR products were visualized on a 2 % agarose gel stained with ethidium bromide.

Results

Of all 83 captured birds of *P. collaris* (Table 1), 40 were males, 23 females and 20 juveniles (juveniles until two months were not sexually differentiated). None of the examined individuals was found to be infected by microscopic examination. 50 individuals were tested for blood parasites using PCR, but no blood parasites were detected in the examined samples.

Month	Number of examined birds	Number of infected birds
March	5	0
April	3	0
May	11	0
June	3	0
July	20	0
August	10	0
September	22	0
October	9	0
Total	**83**	**0**

Table 1. Number of examined birds *P. collaris* during the months.

Discussion

The blood parasites are most frequently determined microscopically in peripheral blood smears. Kučera (1981c) suggested, that more than 70% of chronical infections by blood parasites may be overlooked, if the intensity of infection is low. Some species of blood parasites are difficult to detect in blood smears (Waldenström *et al.* 2004). Polymerase chain reaction is generally more sensitive than traditional microscopic procedures (Perkins *et al.* 1998; Richard *et al.* 2002). Durrant *et al.* (2006) estimated a 10 times lower sensibility of blood smear examination in comparison with PCR. Despite the use of both methods simultaneously in most samples, blood parasites were not detected in *P. collaris*.

Martínez-Abraín *et al.* (2004) suggested that some sort of biological interaction might occur between some ectoparasite groups (e.g. highly mobile species such as wingless flies and fleas whose role as blood parasite vectors is unclear) and dipteran blood parasite vectors. This hypothesis is supported by the fact that Alpine swifts, which are highly infested with ectoparasites, are apparently free of haemoparasites (González-Solís and Abella 1997, Merino and Mínguez 1998, Tella *et al.* 1998).

Rytkönen *et al.* (1996) found that the *Poecile montanus* from Finnish coniferous forests were free of blood parasites. However, Rintamäki *et al.* (2000) found positive prevalence for the same tit population sampled in the later seasons. This shows that the absence of blood parasites in this species was caused by an inappropriate sampling date. However, our data was sampled in all seasons over the course of several years, therefore we should have detected any seasonal fluctuations in prevalence of hemosporidia.

Absence of blood parasites has been commonly attributed to the absence or scarcity of appropriate vectors (Bennett *et al.* 1992a,b; 1995; Tella *et al.* 1996; Sol *et al.* 2000; Martínez-Abraín and Urios 2002), especially in marine, saline, arid, open, alpine or high latitude environments (Martínez-Abraín *et al.* 2004). It has been described that many species can be apparently free of blood parasites (e.g. Blanco *et al.* 1998, Rytkönen *et al.* 1996, Tella *et al.* 1995, Figuerola *et al.* 1996, Blanco et al. 1998, Sodhi *et al.* 1996, Engström *et al.* 2001). An alternative explanation for the absence of blood parasites could be either a low host density or insufficient time for the co-evolution of the host, vectors and parasites (Bennett *et al.* 1992a, Rytkönen *et al.* 1996, Valera *et al.* 2003).

Since *P. collaris* spends most of its life in high mountain environments, which are generally inhabited by a low number of flying arthropods due to low temperatures, low oxygen and frequent winds,

we suggest that a poor density or absence of potential vectors could be the cause of the absence of blood parasites in the Alpine Accentor.

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