Research Article

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Haemosporidian parasites and leukocyte profiles of pre-migratory rosy starlings (*Pastor roseus*) brought into captivity

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Abstract: Immune response to pathogens such as avian malaria (*Plasmodium* spp.) and related haemosporidian parasites (*Haemoproteus* spp. and *Leucocytozoon* spp.) can lead to increases in energy demands of birds. Migrations are energetically costly for birds and require resources that could be otherwise invested in immune defence against pathogens. Here, we examined the prevalence and intensity of haemosporidian parasites (Apicomplexa, Haemosporida) in rosy starlings (*Pastor roseus*), an irruptive migrant on the Balkans, during their pre-migratory period. We further evaluated if these infections affected the stress levels of birds brought into overnight captivity, using leukocyte profiles (ratios of heterophils to lymphocytes, or H/L ratios). Haemosporidian parasites were detected based on microscopic examination, PCR and sequencing. We found high prevalence of haemosporidian parasites (83.9%) in rosy starlings (n=31), with predominant infections of *Haemoproteus pastoris* (cytochrome *b* lineage LAMPUR01) (80.1%). All birds had high heterophil/lymphocyte ratios (mean = 3.86), indicating the transition to captivity was stressful. However, there was no added stress from blood parasite infection during captivity, since infected birds did not have significantly higher heterophil/lymphocyte ratios than uninfected birds, despite all birds facing the same stress conditions. There was a negative correlation between intensity of *H. pastoris* and monocyte proportions, which

may indicate an association with infection, or an artefact of the stress-induced shift in leukocyte profile following captivity. Our findings suggest that blood parasites have minimal additional impact on rosy starlings during an acute stress scenario (overnight captivity), arguing that they carry little cost to the energetically challenging migratory flight.

Keywords: irruptive migrant, leukocytes, *Haemoproteus pastoris*, intensity, immune reaction

1 Introduction

Migratory birds are exposed to multiple and various pathogen agents during their journeys that could challenge their immune system [1]. Moreover, migration is a crucial period of the life of birds that is energetically demanding and could have strong selective pressure [2]. Therefore, a trade-off between investment in immune defence and resources for migratory flight is possible [3]. In particular, blood parasites may impose certain costs to birds during the migratory journey [4]. Avian malaria and related haemosporidian parasites (Apicomplexa, Haemosporida) are widespread and taxonomically diverse blood parasites that can have numerous impacts on their hosts [5, 6]. These pathogens need blood-feeding vectors (Diptera) to maintain transmission among potential avian hosts [7]. Chronic infections are common in wild birds and are suggested to be persistent for the entire life-span of their hosts [5]. Chronic parasite infections are generally considered more benign, but can have a cumulative impact on avian fitness and survival [8]. In fact, recent evidence suggests that avian haemosporidian parasites can negatively influence breeding success [9, 10], moulting [11], fat accumulation [12] and importantly, optimal migration of the infected birds [13].

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The rosy starling (*Pastor roseus*, Fig. 1) breeds in steppe, semi-deserts and open grasslands of the temperate zones, ranging from Eastern Europe to north-western China, and spends the winters mainly in India and Sri Lanka [14, 15] (Fig. 1). The species is an irruptive migrant in which movements are often governed by ephemeral and localized abundance of gregarious invertebrate food organisms, which are concentrated in dry, open and sometimes rocky terrain [14]. In Bulgaria, the species appears in May when the breeding starts and disappears immediately after the breeding season, usually in the beginning of July. The population of breeding rosy starlings in Bulgaria depends on the irruptive migration of the species every year. Breeding colonies are mostly observed along the rocky coasts of the Black Sea and inland cliffs or in stone quarries in areas with high abundance of their prey and mulberry fruit (*Morus* spp.) [16]. Compared to regular migrants, irruptive migrants such as rosy starlings show much greater year to year variations in the proportions of individuals that migrate, and greater variations in the timing, directions and distances of movements.

While the ornithological literature is rife with studies investigating the prevalence and effects of blood parasites on breeding birds (e.g. 17, 18-22), less studied is the impact of blood parasites during the migratory phase of the life cycle [4, 23, 24]. Further, no studies have asked these questions specifically about *irruptive* migrants, which may show differences in migratory preparations or energetic requirements than typical long-distance migrants. The rosy starling offers such an opportunity. Here we collected rosy starlings during their pre-migratory period (following breeding season) to quantify haemosporidian parasite loads. In addition, since during this procedure the captured birds were brought into short-term captivity (a stressful scenario), we further evaluated whether blood parasite infections impacted the level of stress this caused, as measured by the birds' heterophil-lymphocyte ratios [25]. We surmised that if blood parasites are costly, then infected birds would show greater stress reactions (to captivity) than uninfected birds.

Figure 1. Distribution map of rosy starlings (*Pastor roseus*) modified from BirdLife International. Breeding range is marked in yellow and non-breeding (wintering) range in blue. Location of bird capture site (Kaliakra cape) is indicated.

2 Methods

2.1 Animal capture and collection of blood samples

In total, 31 adult rosy starlings were captured with mist nets at Kaliakra cape, northeast Bulgaria (43°22'N, 28°27'E; Figure 1) one week before the colony departed their breeding site. Age, sex and moulting were identified according Svensson [26]. As part of this investigation, the birds were caught in the dusk, transported to a laboratory, kept overnight in aviary (2m x 3m x 2m) and blood sampled on the next day. Before sampling, they were placed in individual cotton bags for c. 5-20 min. ringed, measured, sampled and then released. These procedures were used for logistical reasons, but from the birds' perspective, they likely imparted considerable stress (e.g. 27). However, since all birds in this study were treated the same way, this allowed us to ask if this "stress challenge" impacted parasitized birds more so than those without blood parasites (see below).

The whole blood (c. 30 – 50µl) was withdraw after puncture of the ulnar vein with sterile needle (27G) in heparinised capillary tube. Two blood drops were used to prepare two thin blood smears on ready to use clean glass

slides. The rest of the blood was stored in 500µl SET buffer (0.05 M tris, 0.15 M NaCl, 0.5 M EDTA, pH 8.0) for further DNA analyses. The blood smears were fast dried with a battery fan, fixated in 100% methanol for 5 minutes and air-dried again. Fixated blood smears were stained with freshly prepared 10% solution of Giemsa (Sigma-Aldrich) for one hour and afterwards rinsed with tap water and air-dried [5].

2.2 Examination of the blood smears, parasite identification and intensity

Microscopic examination was performed on a Zeiss Axio Imager M2 light microscope equipped with ProgRes C7 plus digital camera and imaging software ProgRes CapturePro v2.8.0. For detection of parasite prevalence, the blood films were examined for 10–15 min at low magnification (630 x), and then at least 100 microscopic fields were screened at high magnification (1000 x). If no parasites were observed the bird was classified uninfected. The identification of parasite species followed the guidelines proposed by Valkiūnas [5]. Intensity values were calculated as a percentage based on the exact counts of infected erythrocytes per 1000 red blood cells when intensity was high (i.e. >0.1%) and per 10,000 cells when intensity was light (i.e. <0.1%).

Figure 2. Digital images of leukocytes (A - F) and *Haemoproteus pastoris* infected erythrocytes (A, D). A – heterophil, long arrow; microgametocyte of *H. pastoris* infecting erythrocyte, short arrow; macrogametocyte of *H. pastoris* infecting erythrocyte, arrowhead. B – eosinophil, long arrow. C – basophil, long arrow. D – lymphocyte, long arrow and two macrogametocytes of *H. pastoris* infecting erythrocytes, arrowheads. E, F – monocytes, long arrows. Scale bar = 20 µm

We use the stained blood smears to evaluate leukocyte proportions, which can be used to assess stress levels in vertebrates, because of the way in which specific cell types (heterophils and lymphocytes) shift in circulation following a stressor [25]. For this we examined digital photographs of sections of the smear; a 630 x immersion oil objective, ProgRes C7 plus camera and ProgRes® CapturePro 2.8.8 was used to take digital images of leukocytes. The identification of the five types of the leukocytes: heterophils, lymphocytes, monocytes, eosinophils, and basophils (Fig. 2) followed the criteria proposed by Campbell and Ellis [28]. Each image was taken in the way that at least one leukocyte is in the frame. Proportions of heterophils, eosinophils, lymphocytes, monocytes and basophils were calculated based on 100 leukocytes from each blood smear. Finally, we calculated the ratio of heterophils to lymphocytes based on these proportions, which served as an index of the stress level of each bird [25].

2.3 Molecular diagnosis of parasites

Molecular diagnosis and identification of the haemosporidian parasites was performed from the 31 complementary blood samples stored in SET buffer. Total DNA was extracted via proteinase K and ammonium acetate extraction method [29]. The proportion of the extracted DNA was evaluated by NanoDrop 2000 (Thermo Scientific) and diluted to approximate proportion of 25–50 ng/μl. We used nested PCR protocol to amplify a portion (c. 480 bp) of the parasite mitochondrial cytochrome b (cyt b) gene of the genera *Haemoproteus*, *Plasmodium* and *Leucocytozoon* [30]. Two subsequent PCRs were run in 25 μl volume with the initial primers HaemNFI (5'-CATATATTAAGAGAAITATGGAG-3' I = a universal base, inosine) and HaemNR3 (5'-ATAGAAAGATAAGAAATACCATTC-3'). The second PCR was run either with primers HaemF (5'-ATGGTGCTTTTCGATATATGCATG-3') and HaemR2 (5'-GCATTATCTGGATGTGATAATGGT-3') to detect *Haemoproteus* spp. and *Plasmodium* spp. or the primers HaemFL (5'-ATGGTGTTTTAGATACTTACATT-3') and HaemR2L (5'-CATTATCTGGATGAGATAATGGIGC-3') for *Leucocytozoon* spp. The thermal profile of the first PCR started with 2 min of denaturation at 94° C, followed by 20 cycles at 94° C for 30 s, 50° C for 30 s, 72° C for 45 s, and ended with an elongation step at 72° C for 10 min. The second PCR had the same thermal profile except the increased total cycles to 35. Two percent agarose gels stained with GelRed Nucleic Acid Stain were used

to visualize amplicon bands by running 4 μl of the final PCR product. Positive or negative samples were seen as presence or absence of bands with c. 500 bp length under ultraviolet light (G:BOX - Syngene). Standard sequencing was performed for all amplified fragments from both 5' and 3' ends with the primers HaemF-HaemR2 in Macrogen Inc. There were no birds infected with *Leucocytozoon* spp. and we did not sequence amplicons with the primers HaemFL-HaemR2L. The obtained cyt b sequences of 478 bp were edited and aligned using CodonCode Aligner version 8.0.2 (CodonCode, Dedham, MA, USA). Double nucleotide base peaks on the chromatogram were considered as co-infections of two or more parasite lineages in the host. To identify parasite cyt b lineages we use BLAST tool implemented in MalAvi database (accessed April 2019) [31].

To check for all possible co-infections of the parasites of the genera *Haemoproteus*, *Plasmodium* and *Leucocytozoon*, which are often underestimated by common PCR protocols we applied an additional multiplex PCR protocol [32]. Three pairs of primers were simultaneously used to amplify parasites from genus *Haemoproteus* (HMF – ATTGGATGTCAATTACCACAATC; HMR – GGGAAAGTTTATCCAGGAAGTT) with length c. 530 bp, *Plasmodium* (PMF – CCTCACGAGTCGATCAGG; PMR – GGAAACCGGCGCTAC) with length c. 380 bp and *Leucocytozoon* (LMF – TGGAACAATAATTGSATTATTACAYT; LMR – AACATATCATATTCATCCATTTAGATTA) with length 218 bp. The co-infections of the three parasite genera were visible on the 2% agarose gel stained with GelRed Nucleic Acid Stain as bands of different length if present. The fragments were not sequenced.

2.4 Statistical analyses

One-way ANOVA and Kruskal-Wallis ANOVA by Ranks were used to test for differences in the leukocyte profile (i.e. proportion of each cell type, as well as the H/L ratio) among uninfected, single infected and co-infected birds. Next, we tested for a relationship between parasite intensity and the proportion of each particular type of leukocyte (including the H/L ratio) using Pearson's product moment correlation and Spearman rank order correlation. H/L ratios were log10-transformed prior to analyses. When the assumption of normality and homogeneity was not met we used Box-Cox transformation. Non-parametric statistical tests were used for two variables (proportion of eosinophils and basophils), for whose normality was not achieved via transformation. The intensity of *H. pastoris*

was log-transformed ($x/\text{mean}(x)+k$). Statistical analyses were carried out with JASP Version 0.10.0 (JASP Team 2019).

3 Results

3.1 Prevalence and intensity of haemosporidian parasites

In the 31 rosy starlings examined for haemosporidian parasites we found high overall prevalence (83.9% of the genera *Haemoproteus* and *Plasmodium*). Parasites from the genus *Leucocytozoon* were found neither with microscopic examination nor with molecular PCR diagnosis. The majority of the infections were by *Haemoproteus pastoris* (lineage LAMPUR01) with prevalence of 80.1%. Infections with *Plasmodium* spp. were less common ($n = 9$) with prevalence of 29.0% and the majority of them ($n = 8$) were in co-infection with *H. pastoris*. We managed to sequence and to identify correctly two cyt *b* lineages (DELURB5 and FANTAIL01) of the genus *Plasmodium*. The intensity of *H. pastoris* varied between 0.01% and 1.10%, (0.17 ± 0.26). The intensity of *Plasmodium* parasites signified chronic infections (0.01–0.04%). Therefore, we excluded the intensity of *Plasmodium* infections from the statistical analyses below because of its narrow range and the low prevalence.

3.2 Leukocyte profile and intensity

All birds in this study showed shifts in leukocyte profile consistent with a high degree of stress, presumably from the transport and captivity (Table 1). Heterophils were abundant while lymphocytes were less common, leading to an average H/L ratio for all birds of 3.86 (typical ratios for Passerines are under 0.5, A.K. Davis, *pers. comm.*). Importantly, birds with single or co-infections did not have significantly higher H/L ratios than uninfected

birds ($F_{2, 28} = 3.05$, $p=0.063$). When pooled together, all birds with blood parasites ($n=27$) did have a higher H/L ratio than uninfected birds ($n=4$, Table 1), although this was not significant (Student's *t*-test, $t=-0.256$, $p=0.799$). Further, no differences were found in the proportion of each leukocyte type among uninfected birds and those, infected with single or co-infections: lymphocytes: $F_{2, 27} = 0.1$, $p = 0.9$; heterophils: $F_{2, 27} = 0.05$, $p = 0.95$; $p = 0.9$; monocytes: $F_{2, 27} = 0.5$, $p = 0.6$; eosinophils $H(2, N = 30) = 1.27$ $p = 0.5$; basophils: $H(2, N = 30) = 0.66$, $p = 0.7$. *Haemoproteus pastoris* intensity was not significantly correlated with heterophil, lymphocyte, and basophil proportion ($r = -0.05$, $p = 0.8$; $r = 0.1$, $p = 0.6$; $r_s = 0.08$, $p = 0.7$, respectively), nor it was related to heterophil/lymphocyte (H/L) ratio ($r = 0.05$, $p = 0.8$). There was a weak positive correlation between intensity of *H. pastoris* and eosinophil proportion ($r_s = 0.26$, $p = 0.16$). Lastly, there was a significant negative correlation between proportion of monocytes and intensity of *H. pastoris* ($r = -0.4$, $p = 0.02$, Fig. 3).

Figure 3. Correlation plot of *Haemoproteus pastoris* intensity (calculated as percentage of infected erythrocytes, log-transformed) and proportion of monocytes (per 100 leukocytes) in blood samples of

Table 1. Mean values of leukocyte proportions and heterophil to lymphocyte ratio (H/L) in rosy starlings (*Pastor roseus*) calculated for infected and uninfected individuals. Note that prior to blood sampling, birds were transported to a lab and held overnight in an aviary. As such, these values, especially for heterophils, lymphocytes and the H/L ratio, should not be considered baseline values for this species, but rather they show the effects of these stressors.

	Lymphocytes	Heterophils	Monocytes	Eosinophils	Basophils	H/L ratio
Infected ($n=27$)	31.59	59.33	7.04	2.30	0.22	4.07
Uninfected ($n=4$)	34.25	58.75	5.75	1.75	0.25	2.46

adult rosy starlings.

4 Discussion

The prevalence of blood parasites observed in the rosy starlings we collected was surprisingly high (>80%). Comparatively, certain other bird species from the Sturnidae family, such as common starling (*Sturnus vulgaris*) and spotless starling (*Sturnus unicolor*), have lower reported prevalence (<10%) of haemosporidian parasites [33, 34]. Similarly, three species within the Sturnidae family studied in South Africa [35] also had low prevalence (<10%). And, Greiner et al [20] reported low prevalence (<5%) in *Sturnus vulgaris* in North America. But meanwhile, European blackbirds, *Turdus merula*, were reported with very high prevalence (>80%) when sampled during the breeding season [17]. With such disparity over infection prevalence within this family, it is difficult to draw conclusions from the samples obtained here. Thus, it is unclear if the high prevalence observed is related to the location, time of year, or perhaps something endemic to this species. No prior investigations of haematozoa in this species have been undertaken, though such study would be warranted.

Given the timing of our samples (collected just prior to the migratory flight), such a high haematozoan infection prevalence may have implications for the migratory journey, since so many individuals in this population would be faced with the competing demands of flight and parasite burden. Since these parasites reside within erythrocytes, they presumably compromise oxygen delivery. However, we did note that most infections here appeared to be low-grade, chronic infections, which may not significantly impede migratory performance. Moreover, it is still unclear even if blood parasites hinder migration ability at all; recent work using experimentally-infected birds showed no evidence that mild haematozoan infections impair aerobic capacity [36]. Also, field-based studies have shown minimal effects of blood parasites to migrating passerines [4].

Our examination of leukocyte profiles provided additional, though indirect, evidence for the benign nature of haematozoan parasites. Infection with blood parasites did not appear to lead to additional stress to the birds when they were moved to captivity. Heterophil-lymphocyte ratios in birds invariably rise following stress events [37], usually within 1-2 hours from the start of the event, and importantly, they remain high during the stressor [38]. Indeed, the birds in our study all had exceptionally-high H/L ratios for Passerines (mean = 3.86), which is consistent with the nature of the multiple stressors these

birds underwent (capture, transport, overnight captivity). Typical ratios for Passerines not under stress are closer to 0.2 to 0.5 (e.g. 40, 41-43). We caution though, that our sample size was small (n=31), which greatly reduced the power and reliability of our analyses. In fact, the average raw H/L ratio of infected birds was indeed greater than in uninfected birds (Table 1), which argues that with additional data, this result could change.

One interesting finding of our study was that the intensity of *H. pastoris* infection was negatively correlated with the proportion of monocytes in the blood stream, suggesting that infected rosy starlings with higher intensity of *H. pastoris* exhibited reduced amount of circulating monocytes in the blood. One possible explanation of this finding could be immune suppression due to preparing for migration and resource allocation in fat accumulation and muscles hypertrophy [43]. The investigated colony of rosy starlings left the breeding site a week after we sampled the birds, following a well-known pattern from other breeding sites in Hungary and Greece. We assume that our samples were collected in the period when rosy starlings prepared for long-distance flight, just before leaving Kaliakra cape and the beginning of autumn migration towards India. There is evidence for immune suppression during these periods when migratory birds prepare for migration [44], which is in accordance with this idea. Thus, the increased intensity of *H. pastoris* might be a result of immune suppression and natural relapse of the parasite during post breeding and pre-migratory period in order to infect new juvenile hosts [5]. However, the correlational nature of our data, collected only once, does not allow us to determine whether monocyte proportion decreases as a direct result of the parasite infections or due to resource allocation related to preparation for migration.

5 Conclusions

Rosy starlings appear to have exceptionally high levels of haemosporidian parasites during their pre-migratory phase (>80%). It is unclear if this is endemic to this species, or to this stage of the life cycle. Regardless, the timing of these infections could be important for the birds' migration. There was minimal evidence that blood parasites add further stress to these birds when they are faced with an acute challenge. The finding that monocyte abundance in the blood decreases with increased parasite intensity warrants further study.

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Authors contribution: PZ generated the idea and outlined the main hypothesis of the study. TA, KB and MI participated in the capture of the animals, and collection of the blood samples. DD examined blood smears, evaluated leukocyte proportions and identified parasites morphologically. AB performed molecular analyses of parasite lineages. MM performed the statistical analyses. DD, MI, MM, AB and PZ equally contributed to interpretation and writing of the manuscript.

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