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Blood parasite infestation and the leukocyte profiles in adult and immature reed warblers (*Acrocephalus scirpaceus*) and sedge warblers (*Acrocephalus schoenobaenus*) during autumn migration

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Haemoparasites prevalence in birds can vary among species, age and sex groups and ecological conditions. To comprehend the host–parasite assemblages, it is important to investigate physiological mechanisms underlying the interactions in wild bird species. Here, using blood smears we investigated how leukocyte profiles in reed warblers (*Acrocephalus scirpaceus*) and sedge warblers (*Acrocephalus schoenobaenus*) vary in relation to the heamoparasite presence with respect to the host's age and sex. We sampled the birds during their autumn migration in 2008 at a stopover site in northern Poland. We found a significant relationship between the leukocyte profiles and parasite infection. Parasite infection was associated with elevated relative number of heterophils with simultaneous lowered relative number of lymphocytes. These opposite pattern resulted in an elevated H/L ratio in infected birds. Total leukocytes, lymphocytes and heterophils counts were elevated in birds infected with blood parasites as compared with those of non-infected ones. Leukocyte profiles of parasitized birds were similar regardless of individual age. However, non-infected adults had lower total leukocyte counts and values of H/L than non-infected immatures.

Introduction

Haemoparasites prevalence in birds can vary among species, age and sex groups and ecological conditions (e.g. Ots & Hõrak 1998, Valkiūnas 2005). Even closely related species may differ significantly in blood parasites prevalence (e.g. Fokidis *et al*. 2008). Higher prevalence of parasites in juvenile birds than in adults was documented in several studies (e.g. Hudson & Dobson, 1997, van Oers *et al*. 2010), whereas other studies revealed quite an opposite pattern (e.g. Deviche *et al*. 2005). Contradictory results were also found when regarding the sex of birds.

Higher blood parasites prevalence was usually found in females as compared with that in males, but several other studies found the opposite (reviewed in McCurdy *et al*. 1998). Furthermore, within the sampled species, pattern of infection with blood parasites differed among phases of annual cycle, seasons and study sites (Deviche *et al*. 2001, Weatherhead & Bennett 1991, Bennett *et al*. 1995, Bosch *et al*. 1997). Hence, despite many intensive studies on blood pathogens in birds, we still have only a limited knowledge of the pattern and mechanisms behind parasitemia in wild birds.

To fully comprehend the host–parasite assemblages, it is important to investigate physiological mechanisms underlying the interactions in wild bird species. Leukocyte profiles (i.e. the relative numbers of different leukocyte types in the peripheral blood), provide a convenient measure of integrated immune function (Davis 2005, Salvante 2006). Generally, elevated total number of leukocytes indicates an inflammatory processes in response to both microbiological and macroparasite infections (e.g. Dein 1986). Lymphocytes and heterophils make up the majority (nearly 80%) of leukocytes in birds (Campbell 1995, Davis *et al*. 2008). Lymphocytes are responsible for pathogen-specific immune response (Fudge 1989; but *see* Dufva & Allander 1995) and their increased numbers can be found during parasitic infection (Ots & Hõrak 1998, Bonier *et al*. 2007) as well as any immunological challenges (Eeva *et al*. 2005). Heterophils are non-specific phagocytozing cells that enter the tissues during an inflammation, particularly due to microbial challenge (Rose *et al*. 1979, Hawkey *et al*. 1985, Campbell 1995, Maxwell & Robertson 1998). They increase in numbers during stress, trauma and chronic bacterial infections. The heterophil to lymphocyte ratio (H/L) is often used as a stress indicator in birds (Gross & Siegel 1983, Maxwell 1993, Vleck 2000, Bonier *et al*. 2007, Davis *et al*. 2008). This ratio is known to increase during infectious diseases and/or starvation. The remaining 20% of the leukocytes are represented by eosinophils, which are associated with the inflammation process and defense against parasites (Maxwell 1987), monocytes that are phagocytic cells playing a role in a defense against infections and bacteria (Campbell 1995, Davis *et al*. 2004) and basophils, which of function is not fully understood but is thought to involve inflammation (Campbell 1995).

The aim of this study was to compare leukocyte profiles between parasite-infected and parasite-free birds of different age and sex groups, in two small passerines, the reed warbler (*Acrocephalus scirpaceus*) and the sedge warbler (*Acrocephalus schoenobaenus*) during the autumn migration period. The two species are closely related and quite similar in terms of breeding ecology and migration strategy (Cramp 1998). Due to contrasting results of parasitemia in birds reported so far, we were not able to formulate detailed hypotheses on the relationship between parasite prevalence and hematological parameters in the particular studied groups. Nevertheless, since migration period is already a stressful phase in the birds annual cycle, when they experience reduced immune capacity (Owen & Moore 2006), we expected that leukocytes profiles of the birds should give good evidence of blood parasites if they were present. Thus regardless of the species, age and sex, birds infected with blood parasites should differ in leukocyte profiles from parasite-free birds.

Material and methods

Field work

We conducted the study in the southern part of the "Lake Druzno" reserve (54°05´N, 19°27´E) in northern Poland. Lake Druzno is a large, shallow lake, overgrown for much of its area by reedbeds, which makes it an attractive place for breeding and migrating reed and sedge warblers. We captured the birds in 20 nets situated in reedbeds, controlled every 1.0–1.5 hours between 27 July and 26 August 2008. The study time comprised almost the entire autumn migration period of *Acrocephalus* warblers in this area (Jakubas and Wojczulanis-Jakubas 2010). Due to protracted breeding season of both species (Cramp 1998), the birds captured (especially at the end of July) included an unknown proportion of local birds, not yet on migration.

We ringed captured birds and aged them as adults (≥ 2 years old) or immatures, according

to plumage and iris colour (Svensson 1992). A small amount of blood (10–20 μl) was drawn from the brachial vein for preparing a blood smear and molecular sexing. We sampled each individual only once.

Laboratory work

Air dried blood smears were stained using the May-Grünewald-Giemsa method with a Wescor Aerospray Haematology cytocentrifuge. We examined the blood smears for large parasites, such as *Trypanosoma*, microfilaria and *Leukocytozoon* under 400× magnification. Then, we used 100 microscopic views to check for *Haemoproteus*, *Plasmodium*, *Hepatozoon* under 1000× magnification with immersion oil. The birds were considered parasite-infected if at least one parasite was found in the examined fields.

We determined differential leukocyte counts by examining one cell-layer, non-overlapping microscope fields of each smear at 1000× magnification under oil immersion. We counted all types of leukocytes (heterophils, lymphocytes, basophiles, eosinophils and monocytes) until a total of 100 cells were reached. We used a ratio of the relative number of heterophils and lymphocytes (hereafter H/L ratio) as an index of chronic stress (Vleck 2000, Bonier *et al*. 2007, Davis *et al*. 2008). Moreover, we used the number of heterophils, lymphocytes and total leukocytes counts per 10 000 erythrocytes to estimate the overall allocation to the leukocyte production. Blood smears were examined by a single observer, (AC: blood parasites, IK: leukocytes profile) who was oblivious to particular sampled individual characteristics except for the ring number. The repeatabilities of the leukocyte counts obtained from repeated scannings of the same blood smear (Krams *et al*. 2010) were high (r = 0.96, 0.95, 0.95 0.77, 0.65 and 0.63 for relative number of heterophils, lymphocytes, H/L ratio, basophiles, eosinophiles and monocytes respectively, all $p < 0.0001$).

We preserved blood sub-samples for molecular sexing in 70% ethanol. DNA was extracted following evaporation of the alcohol and using the Blood Mini kit (A&A Biotechnology, Gdynia, Poland). We performed sexing by amplification of a 390-bp fragment of the *CHD* gene on the W chromosome (in females only), and a 370-bp fragment on the Z chromosome (in both sexes), using the primer pair P2 and P8 (Griffiths *et al*. 1998). PCR was performed according to the protocol described by Griffiths *et al*. (1998) with 50 °C for annealing the primers. Differences in size of the PCR products were clearly visible on 3% agarose gel.

Data analysis

In total, we considered 116 reed warblers [37 adults (17 females and 20 males); 79 immatures (40 females and 39 males)] and 104 sedge warblers [25 adults (15 females and 10 males); 79 immatures (39 females and 40 males)] in the study.

Prevalence of blood parasites was defined as the percentage of infected individuals among the birds sampled. To test whether haemoparasites prevalence differed among the species, age and sex groups, we used χ^2-test with Yates' correction when sample size for a given category was < 10.

We examined relationships between leukocyte profiles and parasite presence using general linear models (GLMs). Before the analyses, we normalized all leukocyte parameters using an arcsin square-root transformation (Zar 1999). However, we used raw data to present a reference values for the leukocyte parameters in the birds examined (Fig. 1). We performed separate analyses for each leukocyte parameter (dependent variable) with parasites infection as fixed factors. Since species, age and sex might also influence the leukocyte profile, these variables (fixed factors) as well as their interaction with parasite infection were included in the models. We used sums of squares of type III in all analyses. The models were well-adjusted to relative counts of heterophils (r^2 = 0.84) and lymphocytes (r^2 = 0.89), H/L ratio (r^2 = 0.89), as well as counts of heterophils (r^2 = 0.85), lymphocytes (r^2 = 0.41) and leukocytes (r^2 = 0.81) per 10 000 erythrocytes but not to relative counts of basophiles (r^2 = 0.05), eosinophils (r^2 = 0.04) and monocytes (r^2 = 0.06). Therefore, we presented results of the analyses only for well-adjusted models. Since the leukocyte parameters were found to be

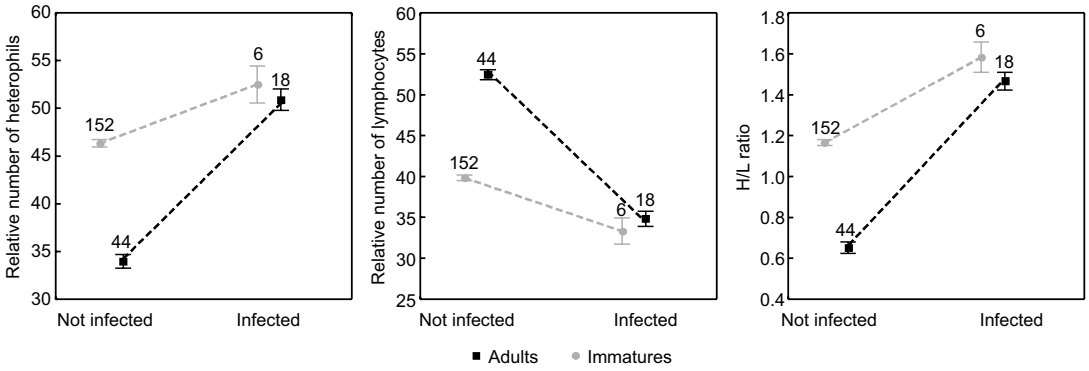

Fig. 1. Relative heterophils and lymphocytes counts and H/L ratios (mean and 95% confidence intervals) of adult and immature reed and sedge warblers (both species combined) in respect to their blood parasite status. Numbers above the bars denote sample size.

related only to parasite infection and age (GLM analyses; Table 1), we pooled data for both species and sexes together when comparing the leukocyte parameters between infected and non-infected birds in both age groups with Student's *t*-test.

Results

In total, we found blood parasites in 24 (11%) of 220 sampled individuals of reed and sedge warblers. We detected three genera of blood parasites, *Haemoproteus* sp. (most frequent: 71% of 24 infected individuals), *Hepatozoon* sp. (42%), and *Leucocytozoon* sp. (least frequent: 8%). In five cases, we recorded mixed infection (one case of *Haemoproteus* sp. × *Leucocytozoon* sp. and four cases of *Haemoproteus* sp. × *Hepatozoon* sp.). Prevalence of blood parasites was similar in reed (12%) and (10%) sedge warblers ($\chi^2_1 = 0.27$, $p = 0.60$). In both species, prevalences of blood parasites were significantly higher in adults (32% and 24% in reed and sedge warblers, respectively) than immatures (3% in reed warblers: $\chi^2_1 = 13.20$, $p < 0.001$; and 5% in sedge warblers: $\chi^2_1 = 4.28$, $p = 0.04$). Females (both age groups combined) were more frequently infected with blood parasites than males both in reed (19% *vs*. 9%, respectively) and sedge warblers (17% *vs*. 4%, respectively), but the sex differences were not significant (reed warblers: $\chi^2_1 = 0.85$, $p = 0.36$; sedge warblers: $\chi^2_1 = 2.36$, $p = 0.12$).

We found that leukocyte profiles were significantly affected by parasite infection, age of birds and an interaction among those two variables (GLM analyses, Table 1). In adults, as compared with non-infected birds, infected individuals had elevated relative numbers of heterophils (*t*-test: $t_{60} = -18.41$, $p < 0.001$) but lowered relative numbers of lymphocytes ($t_{60} = 26.21$, $p < 0.001$; Fig. 1). Infected adults also had a higher H/L ratio ($t_{60} = -25.77$, $p < 0.001$; Fig. 1), as well as total heterophil ($t_{60} = -27.86$, $p < 0.001$), total lymphocyte ($t_{60} = -11.94$, $p < 0.001$) and total leukocyte ($t_{60} = -30.68$, $p < 0.001$) numbers than non-infected ones. Similarly in immatures, as compared with non-infected birds, infected ones had elevated relative heterophils ($t_{156} = -7.26$, $p < 0.001$) but lowered relative lymphocytes ($t_{156} = 8.62$, $p < 0.001$; Fig. 1). In immatures, infected individuals also had a higher H/L ratio ($t_{156} = -10.47$, $p < 0.001$; Fig. 1), as well as total heterophil ($t_{156} = -9.60$, $p < 0.001$) total lymphocyte ($t_{156} = -4.41$, $p < 0.001$) and total leukocyte ($t_{156} = -9.20$, $p < 0.001$) numbers than non-infected individuals.

We found no significant age-related differences in the leukocyte profiles within infected individuals (*t*-test, relative heterophils: $t_{22} = -2.02$, $p = 0.06$; relative lymphocytes: $t_{22} = 1.51$, $p = 0.14$; H/L ratio: $t_{22} = -1.98$, $p = 0.06$; Fig. 1; and total heterophils counts: $t_{22} = -0.66$, $p = 0.52$; total lymphocytes counts: $t_{22} = 0.41$, $p = 0.69$ and total leukocytes counts: $t_{22} = -0.28$, $p = 0.78$). However, in non-infected birds, adults as compared with immatures had significantly lower

values of relative heterophils ($t_{194} = -29.02$, $p < 0.001$) but higher values of relative lymphocytes ($t_{194} = 36.39$, $p < 0.001$; Fig. 1). Moreover, non-infected adults had a lower H/L ratio ($t_{194} = -35.41$, $p < 0.001$; Fig. 1), total heterophils ($t_{194} = -25.93$, $p < 0.001$), total lymphocytes ($t_{194} = -5.91$, $p < 0.001$) and total leukocytes ($t_{194} = -21.03$, $p < 0.001$) than non-infected immatures.

Discussion

Leukocyte parameters are widely used, useful tool in avian ecology and immunoecology (reviewed in Davis *et al*. 2008). However, when considering values of haematological parameters, rarely blood-parasite status of birds is considered and if so, usually lack of significant relationship between the two variables is reported (e.g. Nazifi *et al*. 2008, Krams *et al*. 2010, Shutler *et al*. 2010). Our study clearly demonstrates relationship between leukocytes profile and haemoparasite infection. The reason for the pattern observed in the present study might be the fact that the birds were sampled during their migration period. If immune function is compromised during migration (due to high energy demands), birds may be more susceptible to pathogen or parasite infection during stopover (Owen & Moore 2006).

Increased total lymphocyte and leukocyte numbers revealed in the present study might be a response to infection. Similar effect of parasite infection on such parameters was found in the great tit (*Parus major*) (Ots & Hõrak 1998). However, higher level of heterophils in infected birds is quite unexpected, since these cells are thought to proliferate mainly during bacterial infection (Fudge 1989, Harmon 1998). It is possible that parasite infections are associated with decreased resistance to other forms of infection (e.g. bacterial) that in turn promote heterophilia (Harmon 1998). Such an explanation for elevated heterophils number in birds was also proposed by Fokidins *et al*. (2008). Alternatively, birds with bacterial infection might have been more susceptible to the parasite infection.

Parasite infection was also associated with an increase in relative numbers of heterophils with simultaneous decrease in relative numbers

Table 1. The effects of species (reed and sedge warbler), age (immatures, adults), sex (M, F) and parasite presence on the relative heterophils, relative lymphocytes, and H/L ratio, total heterophils, total lymphocytes and total leukocytes counts per 10 000 erythrocytes (GLM analyses on arcsin-transformed data).

Parameter	df	Relative heterophils		Relative lymphocytes		H/L ratio		Heterophils per 10 000 eryth.		Lymphocytes per 10 000 eryth.		Leukocytes per 10 000 eryth.	
		F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Correct model	7	158.63	< 0.001	236.74	< 0.001	236.43	< 0.001	166.23	< 0.001	21.00	< 0.001	131.57	< 0.001
Intercept	1	50769.49	< 0.001	62468.20	< 0.001	31701.19	< 0.001	15446.72	< 0.001	12386.20	< 0.001	23390.59	< 0.001
Species	1	0.40	0.530	0.12	0.720	< 0.01	0.970	3.51	0.060	< 0.01	0.950	1.56	0.210
Age	1	119.58	< 0.001	179.68	< 0.001	181.97	< 0.001	90.13	< 0.001	3.01	0.080	55.48	< 0.001
Sex	1	0.14	0.710	0.13	0.720	0.16	0.690	0.07	0.800	< 0.01	0.970	0.02	0.890
Parasite presence	1	310.59	< 0.001	500.01	< 0.001	565.76	< 0.001	517.01	< 0.001	87.55	< 0.001	466.75	< 0.001
Species × parasite presence	1	0.26	0.610	1.50	0.220	1.10	0.300	0.58	0.450	0.97	0.330	1.62	0.200
Age × parasite presence	1	71.74	< 0.001	99.78	< 0.001	85.21	< 0.001	56.33	< 0.001	4.42	0.040	38.85	< 0.001
Sex × parasite presence	1	0.01	0.920	0.001	0.970	0.08	0.780	0.29	0.590	0.19	0.660	0.42	0.520
Error	212												

of lymphocytes. These opposite changes resulted in an elevated H/L ratio in the infected birds, which suggests their higher stress level as compared with that of the non-infected individuals. It has recently been reported that parasite infection by itself might be a source of natural stress (Müller *et al*. 2011). Thus, birds infected with parasites might indeed experience an increase of stress level. We cannot also exclude an alternative explanation, that initially more stressed birds could be more susceptible to parasite infection. All birds were captured during migration and so assumed to have elevated stress level (*see* above), but the phase of individuals' migration as well as their body condition might be different, and so might parasite susceptibility.

Most interestingly, we showed that leukocyte profiles of parasitized birds were similarly elevated regardless of age. Non-infected adults had lower values of H/L and total leukocyte counts than non-infected immatures. One possible explanation of the pattern observed between the age groups in parasite-free individuals might be differential costs associated with pathogenic challenge in adults and juveniles. Since a major part of the immunity is acquired, infection might therefore be disproportionately costly for juveniles (e.g. van Oers *et al*. 2010). Indeed, results of the study on the Seychelles warbler (*Acrocephalus sechellensis*) suggest that negative effects of parasitemia are more pronounced in younger birds (van Oers *et al*. 2010). Hence, all numbers of leukocytes may constantly be elevated in immature birds, ready for potential pathogenic challenge. Elevated stress level in juvenile birds during migration might also result from less experience during migration. First-time migrants would be less efficient in habitat selection, foraging, navigating, etc. than experienced birds, and this might lead to higher stress levels during migration. Furthermore, the increased H/L ratio in immatures birds at a stopover sites might be associated with the stress related to competition with more experience and efficiently foraging adults of possibly higher social status (Woodrey & Moore 1997).

Since we captured the birds in nets controlled every 1–1.5 h, we were not able to measure the exact time between the capture and blood sampling. However, it is certain that the time the birds were waiting for their blood to be taken was at the most 1–1.5 h. Davis (2005) showed that although the relative number of each leukocyte type was similar over 1 hour of capture, the total count of leukocytes changed significantly in the same time. Even more rapid changes in the total counts of leukocytes was reported by Cirule *et al*. (2012). That context might raise a concern about the results obtained in the present study. However, as stated earlier the blood samples were taken no longer than 1–1.5 h after the birds were caught. Had that maximum sampling time occured frequently, we should have found a considerable variance in the data set and that was not the case (*see* range of the confidence intervals, Fig. 1). Moreover, had the time affected the leukocyte parameters, we would have observed a blurred pattern of the relationship between the parasites and leukocyte profile, if any pattern at all. Finally, even if the time had affected the blood parameters, it should have worked in the similar manner in the birds of different age, sex and parasite status.

It is worth to mention that both reed and sedge warbler adults were more frequently infected with blood parasites than immature birds. Similar results were reported for the reed warbler from Portugal (Ventim *et al*. 2012). Also, for other avian species such age-related pattern of parasite infection was reported and it is often explained by a longer period of exposure to parasites (e.g. Allander & Bennet 1994, Merilä *et al*. 1995, Dale *et al*. 1996, Pawełczyk *et al*. 2003). On the other hand, Fernandez *et al*. (2010) did not find age differences in prevalence of haemosporidians in reed and sedge warblers caught during migration in Spain. The differences between the two studies are difficult to explain, however, one could speculate that the warm climate of western Spain (Fernandez *et al*. 2010), where parasite vectors are presumably more abundant, leads to rapid infection. In colder climates of northern Poland, with possibly lower abundance of vectors, longer period of exposure could be needed for birds to become infected.

Finally, prevalence of blood parasites in reed and sedge warblers recorded in the present study was relatively low (12% and 10%, respectively), particularly as compared with the values reported for the same two species breeding and migrating

through western Spain (85% and 72%, respectively; Fernandez *et al*. 2010). That, together with some other studies reporting parasitemia in reed [10%–15% in Bulgaria (Shurulinkov & Chakarov 2006); 33% in Portugal (Ventim *et al*. 2012)] and sedge warblers [9% in Bulgaria (Shurulinkow & Golemansky 2003), 19%–37% in the UK (Bench *et al*. 1999), and 62% in Poland (Kruszewicz & Dyrcz 2000)] shows a considerable geographic variation in prevalence of blood parasite in the two warbler species. Variability in prevalence of haemoparasites, both on large and small scales, was reported in many other species (Sol *et al*. 2000, Bensch & Åkesson 2003, Martinsen *et al*. 2008, Fokidis *et al*. 2008). These apparent differences among the studies may arise from differences in habitat conditions, bird communities (presence of alternative host species), as well as vector abundance and activity (Pérez-Tris & Bensch 2005). On the other hand, in contrast to the present study, most of the studies reporting prevalence of heamoparasites in *Acrocephalus* warblers were carried out during the breeding season. There is some evidence that seasonality may affect the parasite prevalence. For example, Deviche *et al*. (2001) showed lower parasitemia in dark-eyed junco (*Junco hyemalis*) during migration as compared with the breeding period.

In conclusion, we found that leukocytes profiles to be significantly related to parasite infection in the two species of passerines. However, it is still not clear which physiological processes are behind those observed differences. That highlights a great need for both comparative and experimental studies investigating a causal mechanism of the pattern observed.

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