Research Article

Correlates of blood parasitism in a threatened marshland passerine: infection by kinetoplastids of the genus *Trypanosoma* is related to landscape metrics of habitat edge

Justyna Kubacka[1], Alina Gerlée[2], Julien Foucher[3], Judith Korb[4] and Edyta Podmokła[5]

[1]Museum and Institute of Zoology, Polish Academy of Sciences, Wilcza 64, 00-679 Warsaw, Poland; [2]Department of Geoecology, Faculty of Geography and Regional Studies, University of Warsaw, Krakowskie Przedmieście 30, 00-927 Warsaw, Poland; [3]Association pour la Connaissance et la Recherche Ornithologique Loire et Atlantique (ACROLA), 10 rue de la Paix, 44480 Donges, France; [4]Evolutionary Biology and Ecology, Institute of Biology I (Zoology), Hauptstrasse 1, D-79104 Freiburg, Germany and [5]Department of Comparative Anatomy, Institute of Zoology and Biomedical Research, Jagiellonian University, Gronostajowa 9, 30-387 Kraków, Poland

Abstract

In birds, vector-borne parasites invading the bloodstream are important agents of disease, affect fitness and shape population viability, thus being of conservation interest. Here, we molecularly identified protozoan blood parasites in two populations of the threatened Aquatic Warbler *Acrocephalus paludicola*, a migratory passerine nesting in open marsh. We explored whether prevalence and lineage diversity of the parasites vary by population and whether infection status is explained by landscape metrics of habitat edge and individual traits (body mass, fat score, wing length and sex). Aquatic Warblers were infected by genera *Plasmodium*, *Leucocytozoon* and *Trypanosoma*, with seven, one and four lineages, and 29.9, 0.7 and 12.5% prevalence, respectively. No *Haemoproteus* infections were detected. Prevalence did not vary between the populations, but lineage diversity was higher in Polesie than in Biebrza for all the lineages pooled and for *Plasmodium*. Infection by *Trypanosoma* decreased with patch core area and increased with density of habitat edge. Infection status was not predicted by the individual traits. Our study is the first to show an association between edge-related landscape features and blood parasitism in an open habitat bird. This finding will support informed conservation measures for avian species of the globally shrinking marshland and other treeless habitats.

Introduction

The vector-borne hemoparasites that parasitize birds are widespread and diverse (Valkiūnas, 2005; Zídková *et al.*, 2012). The key taxons involve the intra-erythrocytic hemosporidians (Aconoidasida: Haemosporida): *Plasmodium* (the agent of avian malaria), *Haemoproteus* and *Leucocytozoon*, and the extracellular trypanosomatids of the genus *Trypanosoma* (Kinetoplastea: Trypanosomatida). To date, 3173 genetic lineages of the three genera of hemosporidians have been identified (MalAvi database, as of December 2018, Bensch *et al.*, 2009) and at least 80 species of trypanosomes are known (Apanius, 1991). Blood parasites of birds occur worldwide, except for Antarctica, and infect several avian hosts (Kučera, 1982; Sehgal *et al.*, 2001; Valkiūnas, 2005). For instance, in Western Palearctic birds, the most frequently recorded genus is *Haemoproteus* (51% species sampled), followed by *Leucocytozoon* (46%), *Trypanosoma* (36%) and *Plasmodium* (23%), with the mean prevalence of 11, 10, 4 and 1%, respectively (Arriero and Møller, 2008). Vectors of bird blood parasites are dipteran hematophagous insects, such as ceratopogonid and hippoboscid flies, simulids and culicine mosquitoes. Birds become infected through a bite (hemosporidians) or digestion (trypanosomes) of an infected arthropod (Molyneux, 1977; Atkinson *et al.*, 2008; Votýpka *et al.*, 2012; Svobodová *et al.*, 2017). Blood parasites are important agents of disease in birds and affect their fitness. For example, infection by *Plasmodium* causes damage of internal organs and destruction of red blood cells, leading to a reduction in metabolism efficiency and acute anaemia (Marzal, 2012). As shown by medication experiments, malaria parasites decrease clutch sizes, offspring hatching success, parental provisioning rates and offspring fledging success (Merino *et al.*, 2000; Marzal *et al.*, 2005; Knowles *et al.*, 2010). In birds infected by trypanosomes, males arrive later to the breeding grounds in spring [which is known to decrease their breeding success (Verhulst and Nilsson, 2008)], sire fewer young, and show compromised nest defence against a predator, relative to uninfected males (Rätti *et al.*, 1993; Hakkarainen *et al.*, 1998; Dyrcz *et al.*, 2005). Blood parasites are therefore expected to limit bird population growth and viability (Loye and Carroll, 1995; Holmes, 1996; Marzal, 2012). Especially in species of conservation concern, it is important to gain further knowledge of their distribution, diversity and prevalence.

The diversity and prevalence of blood parasites within an avian host species are explained by a range of factors, of which potent ones are landscape features (Sehgal, 2015; Ferraguti *et al.*, 2018;

Pérez-Rodríguez et al., 2018). Prevalence is related to, for instance, the altitude and distance to water course or water body, as well as habitat degradation (van Riper et al., 1986; Wood et al., 2007; Bonneaud et al., 2009; Loiseau et al., 2010; Belo et al., 2011; Knowles et al., 2011; Gonzalez-Quevedo et al., 2014; Krama et al., 2015; Reinoso-Pérez et al., 2016). Also, habitat fragmentation and heterogeneity are expected to affect parasite infection in birds, through increased edge effects and the introduction of new parasite lineages by species occupying ecotones and areas between fragments (Loye and Carroll, 1995; Holmes, 1996). However, studies that addressed the relationship between habitat fragmentation/edge and blood parasite infection in birds are scarce, focused solely on forest habitats and only two of them involved intraspecific comparisons (Sebaio et al., 2010; Laurance et al., 2013; Gudex-Cross et al., 2015; Pérez-Rodríguez et al., 2018). For example, in a forest passerine, the Antillean Bullfinch (Loxigilla noctis), prevalence of two hemosporidian lineages is positively related to variables describing habitat fragmentation and amount of edge (Pérez-Rodríguez et al., 2018). To our knowledge, treeless habitats have not been studied in this respect, while a similar pattern could be expected due to negative edge effects on parasitism in some animal taxa (Ries et al., 2004). It appears that in particular habitat specialist birds inhabiting peatlands need to be addressed in this context, as these ecosystems have been globally degrading and shrinking due to habitat loss and fragmentation. Blood parasite prevalence in birds is also explained by individual traits. For example, within species, blood parasite prevalence is female-biased (McCurdy et al., 1998), higher in older than in younger individuals (Wood et al., 2007) and higher in individuals of poorer body condition (Shutler et al., 1999; Dawson and Bortolotti, 2000; Hatchwell et al., 2001; Dyrcz et al., 2005; Shurulinkov et al., 2012). Negative effects of blood parasite infection on body mass in birds were demonstrated experimentally, through inoculation and medication treatments (Atkinson et al., 1988; Merino et al., 2000; Valkiūnas et al., 2006). To gain a broader understanding of the link between individual features and parasitism, more bird species of diverse ecologies should be studied.

The Aquatic Warbler Acrocephalus paludicola is a globally-threatened passerine bird with the Vulnerable IUCN status. It breeds in temperate regions, in extensive, permanently water-logged fen mires with low vegetation, and winters in sub-Saharan Africa. Following massive habitat loss, the species saw a steep decline. Today, the Aquatic Warbler is fluctuating around approximately 12 200 singing males and breeds in the highly fragmented and scarce remaining fens of East-Central Europe (Aquatic Warbler Conservation Team, 1999; Briedis and Keišs, 2016; BirdLife International, 2017; Flade et al., 2018). Blood parasites have been studied in the Aquatic Warbler during migration and wintering (Neto et al., 2015) but only to a limited extent on the breeding grounds (Dyrcz et al., 2005). Here, we identified blood parasites in breeding Aquatic Warblers from two core populations using molecular techniques and tested for differences in prevalence and diversity between the populations. We further evaluated whether infection status is related to habitat edge and individual traits. Specifically, we predicted that (1) the amount, proximity and/or complexity of edges is positively related to infection probability, (2) individuals of lower body condition are more likely to be infected than individuals in higher body condition, and (3) females are more likely to be infected than males.

Methods

Study area and sampling

Aquatic Warblers were sampled from two core breeding populations, in the Biebrza Valley (NE Poland; four sites sampled) and

the western Polesie region (SE Poland; seven sites sampled, Fig. 1). The distance between the two regions is c. 250 km and they differ by habitat fragmentation, vegetation, patch size, as well as density and numbers of Aquatic Warblers. The Biebrza Valley comprises relatively large, continuous and extensive fen mires, while the Polesie is more fragmented and consists of smaller habitat patches (Fig. 1). The Biebrza Valley holds 2000–2600 singing males, with the density in the sampled areas of about 10 males per 10 ha. The Polesie population is 400–700 singing males and the density is about half that of the Biebrza (Kubacka et al., 2014; Polish Society for the Protection of Birds, 2014; Kubacka unpubl.).

We captured 144 Aquatic Warblers with mist-nets during the breeding season, between May and August of 2014. Locations of captures were saved with a GPS device. We sampled 54 individuals in the Biebrza Valley (17 adult females, 33 adult males and four first-year individuals of unknown sex) and 90 individuals in Polesie (18 adult females, 71 adult males and one first-year individual of unknown sex). Sex was identified by the presence of cloacal protuberance (adult males) or brood-patch (adult females), and age was determined by examination of plumage: partially or completely worn in adults and completely new for first-year birds (Svensson, 1992). Wing length was measured with a ruler to the nearest millimetre, according to the method described by Svensson (1992). Fat score was assessed on the scale of 0–8 according to Kaiser (1993). Body mass was measured with a Pesola spring balance to the nearest 0.1 g. Approximately 5–100 µL of blood was sampled from each individual through brachial venepuncture. The blood samples were immediately transferred to tubes with 1 mL of 96% ethanol and stored at room temperature until laboratory analysis.

Identification and diversity of blood parasites

DNA was isolated using a phenol-chloroform protocol (Sambrook and Russell, 2001). With molecular techniques, the blood samples were screened for the presence of blood parasites by amplifying:

1) A 478 bp fragment of the mitochondrial cyt b gene in the case of Leucocytozoon, Haemoproteus and Plasmodium, with the thermal profile according to Hellgren et al. (2004). The polymerase chain reaction (PCR) was performed in volumes of 25 µL, which included 2 µL of genomic DNA, 0.125 mM of each dNTP, 3 mM $MgCl_2$, 0.6 µM of each primer (first-round primers: HaemNFI and HaemNR3; second-round primers: HaemF and HaemR2 in the case of Plasmodium/Haemoproteus, HaemFL and HaemR2L in the case of Leucocytozoon), and 0.5 units of Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) with the accompanying PCR buffer at 1 × final concentration.
2) A 326 bp fragment of the small subunit ribosomal RNA (SSU rRNA) gene in the case of Trypanosoma, with the PCR thermal profile according to Sehgal et al. (2001). Each 25 µL reaction mixture contained 2 µL of genomic DNA, 0.125 mM of each dNTP, 3 mM $MgCl_2$, 1.25 µM of each primer (first-round primers: S-762 and S-763; second-round primers: S-755 and S-823) and 0.5 units of Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) with the accompanying PCR buffer at 1 × final concentration.

Four µL of PCR products from the second round were run on 2% agarose gels stained with GelRed (Biotium, Hayward, CA, USA) and visualised under ultraviolet light. Each plate contained the positive (DNA from birds with confirmed infection based on blood smear screening) and negative (ddH_2O) control. Products of positive samples were purified with FastAP (Fermentas) and sequenced directly with an automated ABI 3130 DNA analyzer
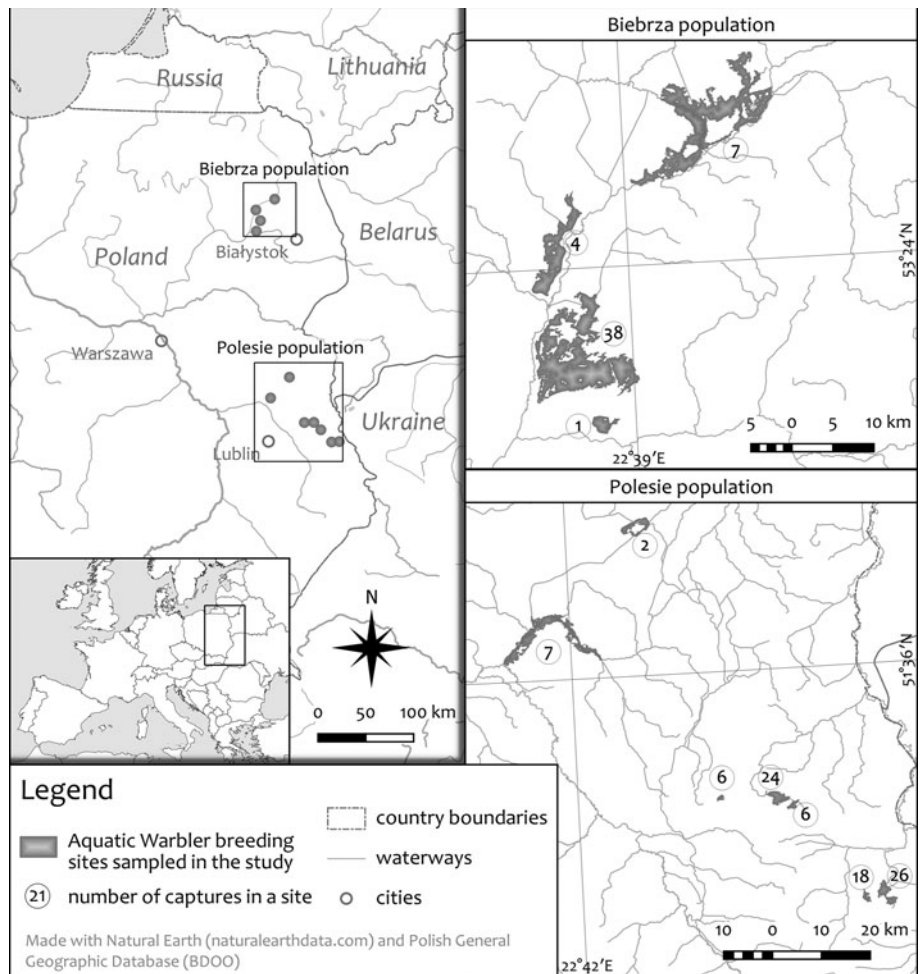
Fig. 1. Distribution of the 11 sampling locations including the numbers of Aquatic Warblers. The map was created using QGIS (QGIS Development Team, 2018).

(Applied Biosystems), using BigDye terminator v3.1 (Applied Biosystems). The obtained sequences were visually inspected and aligned using the BioEdit software (Hall, 1999), which offers a local BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1990). They were then compared against the MalAvi database (Bensch et al., 2009) in the case of Leucocytozoon, Haemoproteus and Plasmodium, and GenBank (Benson et al., 2013) in the case of Trypanosoma, using nucleotide BLAST and selecting the best hit match. Leucocytozoon, Haemoproteus and Plasmodium were identified at the lineage level, and Trypanosoma was identified at the species/genus level, due to the short length of the sequenced fragment. In the case of double infections (indicated by double peaks in the chromatogram), parasite lineages were assigned visually by comparing DNA sequences with the pool of lineages known to occur in our study (in the pool of other analysed samples). This method was confirmed to be reliable in a previous study, by cloning PCR products and showing that the sets of lineages identified by cloning and by visual comparison of lineages were in complete accordance (Podmokła et al., 2014).

To describe the diversity of the identified blood parasites, two ecological diversity indices were used: the species richness (S; the total number of detected lineages) and the Shannon index (H; combines information on species richness and relative abundance).

Patch and landscape metrics

Each site was a patch of continuous breeding habitat, separated from another site by a stretch of non-breeding habitat (other than fen mire or peat meadow) of at least a few kilometres. Using QGIS software (QGIS Development Team, 2018) and

ellipsoidal (GRS 1980) measurement, for each patch and/or capture point, we calculated biologically informative metrics characterizing the proximity, amount and complexity of edge in the breeding habitat (Table 1). These metrics are commonly applied in landscape ecology, also in the context of blood parasitism and vector occurrence (McGarigal et al., 2012; Li et al., 2016; Pérez-Rodríguez et al., 2018). To ease interpretation of the effects of the metrics on infection status, we provide their correlation matrix, alongside with the total length of edge and the area of a patch (Table S1).

Statistical analysis

Data were analysed in the R environment (R Core Team, 2018). We first computed population prevalence, Shannon diversity and species richness, for all the lineages pooled and for each of the identified genera separately. The two diversity indices were calculated in the 'vegan' package (Oksanen et al., 2019). The prevalences were compared between the two populations with the test for equal proportions (command 'prop.test' from the 'stats' package) and asymptotic 95% confidence intervals (CIs) were calculated with a custom function. The 95% CIs of the Shannon indices and their differences between the populations were calculated in the 'boot' package (Canty and Ripley, 2017) according to the recommendations of Gardener (2014). Asymptotic CIs were estimated based on 1000 bootstrap resamples [custom R command 'H_boot' from Gardener (2014)]. The statistical significance of the difference in the diversity index between populations was estimated using a randomisation method based on 2000 bootstrap resamples [custom R command 'H_bss' from Gardener (2014)].

Table 1. Patch and landscape metrics of habitat edge used in the study

Metric	Description	Units	Range
Core area index (CAI)	Percentage of the core area within a patch. The core area was delimited with a 100-m-wide buffer drawn parallel to patch edge. We chose this buffer width based on our field observations (Kubacka unpubl.) indicating that the density and survival of nests of Aquatic Warblers, as well as their activity, is lower close to edges of the breeding habitat, approximately within 100 m. Therefore, we assumed that the 100-m-buffer contains areas with the most pronounced edge effects in this species, and the core area beyond this buffer is less affected by edge effects. In general, bird responses to edges have been reported within 50–200 m (Ries *et al.*, 2004). Edge included both the patch boundary and islands of non-breeding habitat (searched for manually using QGIS and satellite imagery), such as wood, shrubs, water or road, of minimum dimensions 20×5 m^2. This cut-off allowed finding a vast majority of non-breeding habitat that could be of biological significance (for instance through harbouring vectors or hosts) without a gross drop in work efficiency. CAI is affected both by edge length and patch area.	percent	0–100
Non-breeding habitat in a patch (NB)	Percentage area of non-breeding habitat islands (see above) within the total patch area. This metric indicates the heterogeneity of a patch due to the occurrence of non-breeding habitat.	percent	0–100
Distance to nearest edge (DISTANCE)	Distance between a capture point and the edge of the nearest island of non-breeding habitat (see above) or patch boundary.	m	>0
Edge density (ED)	Length of all edges in an area unit, calculated per a grid square of 400×400 m^2 and averaged over a patch.	m/ha	>0
Shape index (SHAPE)	Patch edge length divided by the square root of patch area (m^2) and normalised by a circle of the same area. SHAPE is metric of patch edge complexity that is unaffected by patch area. A shape index of one corresponds to a shape of the lowest edge complexity and larger values indicate more edges given the patch size.	no unit	⩾1
Non-breeding habitat around a capture point (BUFFER NB)	Percent area of non-breeding habitat in a 450-m-radius buffer around a capture point. We chose this radius because it corresponds to the home range of Aquatic Warblers, averaged between males and females during the pre-nesting period, when they are the most mobile (Schaefer *et al.*, 2000). This metric quantifies landscape heterogeneity within the activity area of the birds.	percent	0–100
Edge density around a capture point (BUFFER ED)	Percent edge in a 450-m-radius buffer around a capture point. This metric quantifies ED in the activity area of the birds.	m/ha	⩾0

To evaluate the effect of the patch and landscape metrics, and individual variables on infection status, we used generalized linear mixed models with binomial error structure and logit link, fitted with the package 'lme4' and the 'glmer' command (Bates *et al.*, 2015). The information theoretic (IT) approach was used for inference and the Akaike information criterion corrected for small sample size (AICc) was applied for model selection (Burnham and Anderson, 2002). The IT approach has been increasingly used in ecology, especially in non-experimental studies. In this approach, unlike in null hypothesis testing, an *a priori* set of biologically plausible candidate models is constructed and each model within the set is evaluated in terms of how well it approximates the relationship between the predictors and the dependent variable relative to the other models, given the data. Then, the models are ranked by AICc and the best-ranking model (or a subset of best models) is selected. Models that are within 2 AICc units of the top model (which has the lowest AICc) are considered to be highly supported (Burnham and Anderson, 2004). Including a null model in the candidate set, i.e. a model assuming that the response variable is constant, enables evaluation of 'absolute' support for each model. The strength of evidence of each model is provided by computing model likelihood (the relative likelihood of a model in the candidate set, given the data), the Akaike weight (ωAICc) and the evidence ratio. ωAICc is the probability of a model for a given set of candidate models. For a given model, the evidence ratio is the ratio of its probability to the top model probability, which has an evidence ratio equal to one.

We ran two separate analyses, each with four candidate model sets, with the response being infection status (coded as a binary variable: 1 – infected, 0 – uninfected) by (1) any of the blood parasite lineages pooled, (2) *Plasmodium*, (3) lineage SW2 (the most frequent, Table 3) and (4) *Trypanosoma*. Each model included the random effect (random intercept) of site, to account for possible non-independence between individuals mist-netted within a given patch (Bolker *et al.*, 2009). Because *Leucocytozoon* was found only in one individual and no *Haemoproteus* infections were detected (see Results), we did not run separate models for these genera. The two lineages that are not known to be transmitted in Europe (PAS106 and PAS112, detected in 8 individuals, Table 3) are suggested to have a broad geographical distribution (Šlapeta *et al.*, 2016), and hence we included all the lineages in both analyses.

In the first analysis, we tested whether the infection status was explained by any of the patch and landscape variables. Each of the four candidate model sets consisted of eight models, with seven models each containing one patch or landscape metric (Table 1) as the fixed variable, and a null model assuming that infection status was constant across individuals. This enabled us to see which of the patch and landscape variables was the best predictor of infection status (by all the parasites pooled, *Plasmodium*, *Trypanosoma* and the SW2 lineage) and whether the most parsimonious model was more informative than the null model. In the second analysis, we assessed whether infection status was explained by variables related to the individual: sex (binary categorical variable), fat score, wing length and body mass (all continuous variables). This analysis was performed on a dataset that excluded missing values for the biometrical parameters (hence the retained sample size for the second analysis was $N = 111$). Each of the four candidate model sets consisted of five models, with four models allowing the infection status to vary with (1) sex, (2) fat score, (3) wing length, (4) body mass and (5) a null model, which assumed a constant infection status. We reported support for the models with the evidence ratio and Akaike weight (ωAICc).

Table 2. Blood parasites identified in breeding Aquatic Warblers. For *Plasmodium* and *Leucocytozoon*, information on the species, host range and transmission was obtained from the MalAvi database (Bensch *et al*., 2009), as of December 2018, with the transmission of SYAT24, SW5 and SYBOR02 additionally checked in other available literature. A lineage was considered to be transmitted in Europe if it has previously been found in juvenile birds of a migratory species or adult/juvenile birds of a resident species. For *Trypanosoma*, data were obtained from GenBank (features of deposited sequences) and through screening of published literature

Lineage and morphological species	Prevalence (%)			Host range (number of orders)	Transmission in Europe	GenBank accession no.
	overall (N = 144)	Biebrza Valley (N = 54)	Polesie (N = 90)			
Plasmodium	43 (29.9%)	17 (31.5%)	26 (28.9%)			
SW2 (*Plasmodium homonucleophilum*)	30 (20.8%)	15 (27.8%)	15 (16.7%)	6	known	AF495572
GRW04 (*Plasmodium relictum*)	5 (3.5%)	0	5 (5.6%)	4	known	MG018687
SW5 (*Plasmodium circumflexum*)	4 (2.8%)	0	4 (4.4%)	7	known[a]	AB741486
TURDUS1 (*Plasmodium circumflexum*)	2 (1.4%)	1 (1.9%)	1 (1.1%)	4	known	AF495576
GRW06 (*Plasmodium elongatum*)	1 (0.7%)	1 (1.9%)	0	12	known	KJ488566
SYAT24 (*Plasmodium* sp.)	1 (0.7%)	0	1 (1.1%)	1	known[b]	JN164732
SYBOR02 (*Plasmodium* sp.)	1 (0.7%)	0	1 (1.1%)	2	known[c]	DQ368392
Trypanosoma	18 (12.5%)	3 (5.6%)	15 (16.7%)			
Trypanosoma culicavium	8 (5.6%)	1 (1.9%)	7 (7.8%)	1	known	HQ107967
PAS112 (*Trypanosoma thomasbancrofti*)[d]	6 (4.2%)	2 (3.7%)	4 (4.4%)	1	unknown	JN006832
Trypanosoma avium	2 (1.4%)	0	2 (2.2%)	5	known	AY099320
PAS106 (*Trypanosoma thomasbancrofti*)[d]	2 (1.4%)	0	2 (2.2%)	1	unknown	JN006833
Leucocytozoon	1 (0.7%)	1 (1.9%)	0			
SYBOR23 (*Leucocytozoon* sp.)	1 (0.7%)	1 (1.9%)	0	1	known	KJ488654

[a]Ventim *et al*., 2012.
[b]Shurulinkov *et al*., 2018.
[c]Hellgren *et al*., 2013.
[d]Šlapeta *et al*., 2016.

Results

Description of blood parasites

The blood parasites found in breeding Aquatic Warblers belonged to three genera (*Plasmodium*, *Trypanosoma* and *Leucocytozoon*; no *Haemoproteus* infections were detected) and 12 lineages (Table 2). One out of the five first-year individuals was infected (with *Trypanosoma culicavium*). Six lineages were found in the Biebrza and ten lineages were found in the Polesie population. In both populations, the most prevalent lineage was SW2 (*Plasmodium homonucleophilum*). One individual was co-infected by two different *Plasmodium* lineages (SW2 and SW5) and eight individuals were infected by both *Plasmodium* and *Trypanosoma*. None of the identified lineages were specific to the *Acrocephalus* genus. Prevalence did not differ between the populations, however, lineage diversity was higher in Polesie than in the Biebrza Valley, in the case of all the parasites pooled and *Plasmodium*, but not *Trypanosoma* (Table 3).

Association of patch and landscape metrics with infection status

There was no clear support for the correlation of a patch or landscape metric with infection by any parasite or by *Plasmodium*, as none of the models ranked better than the null model and all the odds ratio (OR) estimates spanned 1 (Table 4a). The probability of infection by the most frequent *Plasmodium* lineage, SW2, appeared to increase with the relative amount of non-breeding habitat within a patch, however, the confidence interval of its OR reached 1 (Table 4a). In the case of *Trypanosoma*, we found support for two patch metrics, core area index (CAI) and edge density (ED), to affect infection status. The ORs of CAI and ED were both different from one, the models with these variables

were the only ones within $\Delta AICc \leqslant 2$ and they were about three times more probable than the null model. The probability of infection by trypanosomes decreased 0.94 times with one unit change in CAI and increased 1.07 times with one unit change in ED (Table 4a, Fig. 2). These two metrics, CAI and ED were correlated (Table S1), which explains their similar support and exactly opposite mutual effects.

Effects of sex, wing length, fat deposits and body mass

Infection status was not clearly related to sex or biometrics variables, whether for all the parasites pooled, *Plasmodium*, SW2 lineage, or *Trypanosoma*. Constant infection models always ranked best and for each of the explanatory variables the confidence intervals of its estimated odds ratio spanned one (Table 4b).

Discussion

Our study is the first account of blood parasites in the Aquatic Warbler from a range of its breeding locations. We showed population differences in lineage diversity but not in prevalence. While we did not find support for infection status to vary with body mass, fat reserves, wing length and sex, we demonstrated, apparently for the first time in an open habitat bird, that infection probability by a blood parasite is affected by metrics of habitat edge.

Diversity and prevalence of blood parasites

The prevalence of blood parasites in the Aquatic Warbler in our study is difficult to compare with that of other birds due to high variation in prevalence attributable to location, habitat, year, age or sample size. Overall, considering studies carried out during the breeding season that inspected more than 20 individuals,

Table 3. Diversity and prevalence of blood parasites in the two studied breeding populations of the Aquatic Warbler. Differences significant at the 0.05 level are marked in bold

Blood parasites	Species richness (number of lineages)		Shannon index (95% CIs)		Difference in Shannon indices between populations		Prevalence (95% CIs)		Difference in prevalence between populations	
	Biebrza Valley	Polesie	Biebrza Valley	Polesie	Z	P	Biebrza Valley	Polesie	χ^2 (df = 1)	P
All parasites	6	10	1.044 (0.38–1.34)	1.925 (1.51–2.05)	2.91	**0.011**	0.389 (0.259–0.519)	0.456 (0.353–0.558)	0.008	0.929
Plasmodium	3	6	0.444 (0.00–0.75)	1.288 (0.83–1.50)	2.39	**0.048**	0.315 (0.191–0.439)	0.289 (0.195–0.383)	0.108	0.742
Trypanosoma	2	4	0.637 (0.00–0.64)	1.245 (0.73–1.36)	0.35	0.740	0.056 (−0.006–0.117)	0.167 (0.090–0.244)	2.861[a]	0.091

[a]Yates correction applied.

Plasmodium prevalence in the Aquatic Warbler appears to be higher than in at least four out of seven other *Acrocephalus* species breeding in Europe, and is the closest to that found in the Great Reed Warbler (*A. arundinaceus*), 24–27% (Bensch *et al.*, 2000, 2007; Zehtindjiev *et al.*, 2008, 2009; Dimitrov *et al.*, 2010, 2018; Biedrzycka *et al.*, 2015). Although absent in Aquatic Warblers, also during migration and wintering (Neto *et al.*, 2015), *Haemoproteus* was detected in other acrocephalids breeding in Europe, with a high variation in prevalence within and across species. Studies that examined at least 20 individuals during the breeding season reported prevalences of 10–96% in the Great Reed Warbler, 9% in the Common Reed Warbler (*A. scirpaceus*), 4–54% in the Sedge Warbler (*A. schoenobaenus*) and 21–24% in the Paddyfield Warbler (*A. agricola*) (Bensch *et al.*, 2000, 2007; Zehtindjiev *et al.*, 2008, 2009; Dimitrov *et al.*, 2010, 2018; Fernández *et al.*, 2010; Biedrzycka *et al.*, 2015). The prevalence of *Leucocytozoon* in our study was remarkably low, and so it is in other European *Acrocephalus* species, ranging between 0 and 1% during the breeding season (Shurulinkov and Chakarov, 2006; Svoboda *et al.*, 2009; Fernández *et al.*, 2010; Biedrzycka *et al.*, 2015; Dimitrov *et al.*, 2018). The lack of *Haemoproteus* and the low prevalence of *Leucocytozoon* in our study can be due to lack of vectors in the breeding habitat of Aquatic Warblers. *Trypanosoma* was found in Aquatic Warblers previously in one of the locations that we studied, the Biebrza Valley (Dyrcz *et al.*, 2005), with 48.5% breeding males being infected. This is a high prevalence compared to the one that we found in that population (12.5%), which could stem from the change in the occurrence of vectors, for example, due to different weather and humidity conditions in the year of sampling, relative to Dyrcz *et al.* (2005).

The three genera of hemosporidians that we targeted in our molecular analysis were previously studied in Aquatic Warblers during migration and wintering (Neto *et al.*, 2015). In that study, only three *Plasmodium* lineages were found, with a prevalence of 26.9% on the migration stop-over site in Portugal and 5.5% on a wintering site in Senegal. In our study, we identified seven lineages of *Plasmodium*, with the 29.9% prevalence being similar to the one during migration but clearly higher than in wintering quarters. Aquatic Warblers could have higher infection rates during the breeding period due to the spring relapse of blood parasites, associated with higher vector activity (Beaudoin *et al.*, 1971; Valkiūnas, 2005; Hellgren *et al.*, 2013), but also because infected birds die before reaching their wintering sites. In addition, Neto *et al.* (2015) established that two threatened acrocephalids, the Seychelles Warbler (*A. seychellensis*) and the Aquatic Warbler, had the lowest number of *Plasmodium* and *Haemoproteus* lineages found in relation to host samples screened, compared to common acrocephalids. They hypothesized that this is due to their rarity and habitat specialisation. Our results refute this hypothesis and demonstrate that the Aquatic Warbler shows an average number of lineages per number of analysed samples, relative to other acrocephalids listed in Neto *et al.* (2015), which is likely attributable to our study being conducted on breeding birds.

As parasites affect survival and breeding success of birds, studying population differences is important to inform conservation management of threatened species. The comparable prevalence rates in both populations indicate that blood parasitaemia is of similar importance in terms of population viability in both locations. The higher diversity of lineages in Polesie than in the Biebrza Valley is likely a result of different sample sizes (90 and 54, respectively), as the number of lineages identified grows asymptotically with the number of screened individuals (e.g. Neto *et al.*, 2015). Another possibility is the variation in vector diversity between the two regions, especially that Polesie

Table 4. Model selection results and estimates of the fixed variables converted to odds ratios (OR) from the analysis relating a) landscape metrics and b) individual variables to infection status. Models within ΔAICc⩾2 (i.e. with strong relative support) and ORs different from 1, based on their 95% confidence intervals (CIs), are marked in bold. K – number of estimable parameters. AICc – Akaike information criterion corrected for small sample size, ΔAICc – difference of AICc relative to the top model, ωAICc – Akaike weight (probability of a model)

a)							
Model	K	AICc	ΔAICc	Evidence ratio	ωAICc	Cumulative AICc	Estimated OR (95% CIs)
All lineages pooled							
null	2	194.62	0.00	1.00	0.27	0.27	
DISTANCE	3	195.99	1.37	0.50	0.14	0.41	0.47 (0.08–2.75)
SHAPE	3	196.48	1.87	0.39	0.11	0.51	0.98 (0.90–1.07)
NB	3	196.63	2.02	0.36	0.10	0.61	1.01 (0.95–1.06)
CAI	3	196.65	2.03	0.36	0.10	0.71	1.00 (0.96–1.03)
ED	3	196.67	2.06	0.36	0.10	0.81	1.00 (0.94–1.05)
BUFFER NB	3	196.69	2.08	0.35	0.10	0.90	1.00 (0.97–1.03)
BUFFER ED	3	196.70	2.09	0.35	0.10	1.00	1.00 (0.98–1.02)
Plasmodium							
null	2	179.67	0.00	1.00	0.27	0.27	
BUFFER NB	3	181.39	1.71	0.42	0.12	0.39	0.99 (0.95–1.02)
DISTANCE	3	181.48	1.80	0.41	0.11	0.50	0.61 (0.09–3.90)
NB	3	181.49	1.82	0.40	0.11	0.61	1.02 (0.96–1.07)
ED	3	181.62	1.94	0.38	0.10	0.71	0.99 (0.93–1.05)
CAI	3	181.70	2.02	0.36	0.10	0.81	0.99 (0.96–1.03)
SHAPE	3	181.74	2.07	0.36	0.10	0.90	0.99 (0.90–1.08)
BUFFER ED	3	181.76	2.09	0.35	0.10	1.00	1.00 (0.98–1.02)
Lineage SW2							
NB	3	150.14	0.00	1.00	0.30	0.30	1.06 (1.00–1.13)
null	2	150.94	0.80	0.67	0.20	0.50	
SHAPE	3	152.18	2.03	0.36	0.11	0.61	1.06 (0.96–1.17)
CAI	3	152.74	2.60	0.27	0.08	0.70	0.98 (0.92–1.05)
BUFFER NB	3	152.74	2.60	0.27	0.08	0.78	0.99 (0.94–1.03)
BUFFER ED	3	152.84	2.70	0.26	0.08	0.86	0.99 (0.96–1.02)
DISTANCE	3	153.03	2.88	0.24	0.07	0.93	0.95 (0.08–11.13)
ED	3	153.03	2.88	0.24	0.07	1.00	1.00 (0.90–1.11)
Trypanosoma							
CAI	3	109.80	0.00	1.00	0.37	0.37	**0.94 (0.90–0.99)**
ED	3	110.23	0.43	0.81	0.30	0.66	**1.07 (1.01–1.15)**
null	2	112.07	2.28	0.32	0.12	0.78	
NB	3	113.81	4.01	0.13	0.05	0.83	0.96 (0.88–1.05)
DISTANCE	3	114.06	4.27	0.12	0.04	0.87	0.59 (0.02–15.33)
SHAPE	3	114.10	4.30	0.12	0.04	0.92	0.98 (0.83–1.15)
BUFFER ED	3	114.10	4.30	0.12	0.04	0.96	1.00 (0.96–1.03)
BUFFER OT	3	114.15	4.35	0.11	0.04	1.00	1.00 (0.95–1.05)

(Continued)

consists of several patches, scattered over a larger area, which are more diverse than the fairly uniform habitat in the Biebrza Valley. The higher heterogeneity of the Polesie sites also likely translates to a more diverse bird community, which could be another factor affecting parasite lineage diversity, due to cross-species infections (Waldenström *et al.*, 2002; Reullier *et al.*, 2006; Sehgal, 2015). Host community and species are known to be powerful predictors of parasite diversity, including *Plasmodium* (Scordato and Kardish, 2014; Fecchio *et al.*, 2018; Ferraguti *et al.*, 2018).

Effects of patch and landscape metrics on infection

Aquatic Warblers breeding in patches with a relatively larger core area (CAI) and lower amount of habitat edge per area unit (ED)

Table 4. (*Continued.*)

b)

Model	K	AICc	ΔAICc	Evidence ratio	ωAICc	Cumulative AICc	Estimated OR (95% CIs)
All lineages pooled							
wing	3	153.03	0.00	1.00	0.34	0.34	0.79 (0.59–1.07)
null	2	153.19	0.16	0.93	0.31	0.65	
fat	3	155.03	2.00	0.37	0.13	0.78	0.90 (0.59–1.36)
sex	3	155.24	2.21	0.33	0.11	0.89	0.89 (0.32–2.41)[a]
mass	3	155.30	2.27	0.32	0.11	1.00	0.99 (0.63–1.57)
Plasmodium							
null	2	145.42	0.00	1.00	0.39	0.39	
wing	3	146.64	1.22	0.54	0.21	0.59	0.86 (0.64–1.17)
sex	3	147.50	2.08	0.35	0.14	0.73	1.11 (0.38–3.19)[a]
mass	3	147.52	2.10	0.35	0.13	0.87	0.97 (0.61–1.57)
fat	3	147.52	2.11	0.35	0.13	1.00	0.98 (0.63–1.52)
Lineage SW2							
null	2	127.20	0.00	1.00	0.39	0.39	
mass	3	128.85	1.65	0.44	0.17	0.56	0.83 (0.49–1.38)
sex	3	129.07	1.86	0.39	0.15	0.71	1.38 (0.38–5.00)[a]
fat	3	129.17	1.97	0.37	0.15	0.86	0.90 (0.55–1.43)
wing	3	129.25	2.05	0.36	0.14	1.00	1.04 (0.74–1.48)
Trypanosoma							
null	2	87.85	0.00	1.00	0.33	0.33	
mass	3	87.97	0.12	0.94	0.31	0.65	1.75 (0.81–3.78)
fat	3	89.91	2.06	0.36	0.12	0.77	1.12 (0.47–2.64)
sex	3	89.95	2.09	0.35	0.12	0.88	1.12 (0.21–5.87)[a]
wing[b]	3	89.95	2.10	0.35	0.12	1.00	0.97 (0.63–1.51)

[a]Males relative to females.
[b]Model did not converge.

were less likely to be infected by trypanosomes. The CAI is a function of patch size and total edge length, while ED is affected by edge length (Table 1). These two metrics are correlated (Table S1) and it is difficult to conclude which landscape feature specifically determines the probability of infection. Nevertheless, our results show that the known factors of habitat change and loss in peatlands, such as succession, fragmentation, drainage or eutrophication, could exacerbate the risk of infection by trypanosomes in the Aquatic Warbler, through reduction of the size of a patch and/or increase in habitat heterogeneity and edge length. This could translate into a lower breeding success, survival and population growth. We are not aware of other studies linking landscape features associated with edge to blood parasite infection in birds of open habitats. Core area of habitat (positively) and edge density (negatively) were found to be related to bird abundance in a grassland passerine (Herse *et al.*, 2018). Within-species research on blood parasite infection in relation to habitat edge and fragmentation all concerned forest habitats and showed negative (Pérez-Rodríguez *et al.*, 2018), positive (Laurance *et al.*, 2013) or no associations (Sebaio *et al.*, 2010; Gudex-Cross *et al.*, 2015). This area clearly requires more research, especially in birds of open habitats.

Our results should be given some reservation since the study covered a limited number of patches (11) and because we did not know whether all of the identified parasite lineages are transmitted specifically in the studied area. For instance, although the blood parasites identified in our study areas are all most likely transmitted in Europe (Table 2), some of them could be transmitted to Aquatic Warblers only during migration or on African wintering grounds, e.g. due to the absence of lineage-specific vectors in fen mires. In our study, removing the unexplained variance caused by individuals infected only by lineages not transmitted in Central-European fens could have revealed additional relationships with the landscape and patch metrics. For a more robust result, the association that we found requires further study in more breeding sites and fine-scale determination of transmission.

A range of mechanisms can underlie the relationship between trypanosome infection, CAI and ED. There is some evidence for increased parasitism near habitat edges for birds, mammals and amphibians (Ries *et al.*, 2004). In the case of arthropod-borne blood parasites, this effect could be due to exposure of birds to more (and different) vectors and/or presence of different parasite lineages in the adjacent habitat, infecting the birds utilising this habitat. Hematozoan vectors are more abundant in the forest than in treeless habitats (Bennett *et al.*, 1992) and forest bird species have higher prevalences of blood parasites than species from open habitats (Tella *et al.*, 1999). One reason for the low vector abundance in open habitats could be greater wind speeds, which are known to reduce host-seeking activity of vectors such as biting midges and black flies (Lillie *et al.*, 1988; Fredeen and Mason, 1991; Martin *et al.*, 1994; Martínez-De La Puente *et al.*, 2009). In addition, increased
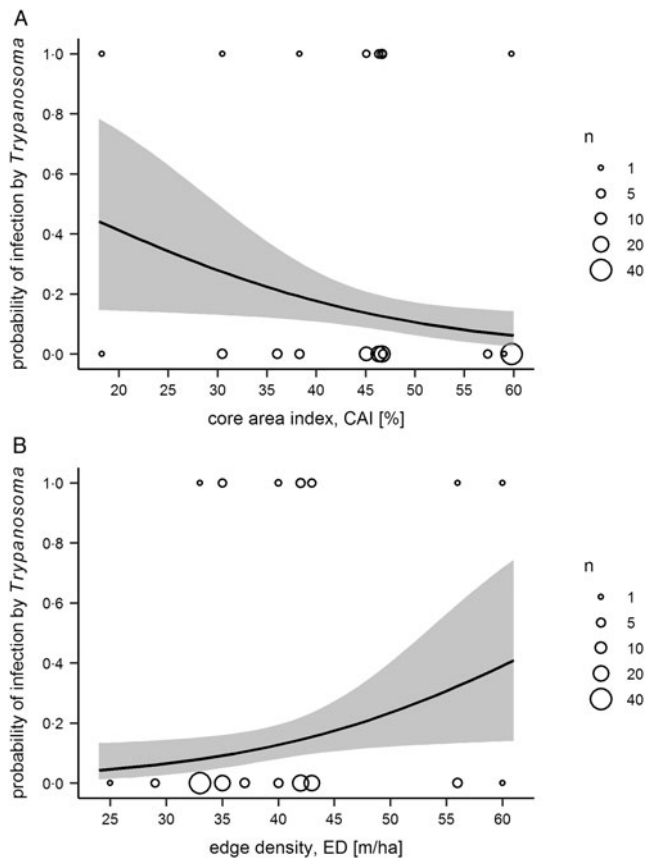
Fig. 2. Probability of infection of Aquatic Warblers by genus Trypanosoma in relation to (a) core area index and (b) edge density of breeding habitat patches. The lines were fitted based on the estimates from the respective top models in Table 4a and the bands show confidence intervals. Circles show infected ($y = 1$) and uninfected ($y = 0$) individuals, with the circle area proportional to the number of individuals (n) for a given value of the metric.

heterogeneity of habitat reduces its quality for open-marshland specialist birds and might attract avian predators occupying overgrown or fragmented areas (Thomson *et al.*, 2010). This can lead to social and physiological stress, which is known to compromise immune function (Lyles and Dobson, 1993; Oppliger *et al.*, 1998). On the other hand, if social or physiological stress was elevated and affected immunity of individuals in the patches with more edge in our study, we would have observed this effect also in *Plasmodium*.

Individual traits and infection status

In our study, sex, wing length, fat score or body mass had no discernible effect on the probability of infection of Aquatic Warblers by blood parasites. This was true both for overall parasite infection and when the analysis was performed separately for *Plasmodium*, the SW2 lineage and *Trypanosoma*. In an earlier study, breeding Aquatic Warbler males infected by trypanosomes were lighter than uninfected males but their wing length and fat deposits did not differ (Dyrcz *et al.*, 2005). In other bird species, experimental studies demonstrated negative effects of blood parasite infection on body mass (Atkinson *et al.*, 1988; Merino *et al.*, 2000; Valkiūnas *et al.*, 2006) and females of lower body condition had higher parasitaemia (Shutler *et al.*, 1999; Dawson and Bortolotti, 2001; Hatchwell *et al.*, 2001). Sex effects have also been observed, for example, breeding females are more often infected by *Haemoproteus* than breeding males (McCurdy *et al.*, 1998). It is possible that in our study variation arising due to differences in capture date or age confounded potential associations between blood parasite infection and body mass, fat score, wing length and sex.

Concluding remarks

In this study, we describe the diversity and prevalence of blood parasites in the threatened Aquatic Warbler, a breeder of fen mires, and demonstrate that infection risk by the genus *Trypanosoma* increases with habitat edge density and decreases with the amount of core area in a patch. This relationship indicates that habitat changes causing reduction of breeding area or increase in edge length could negatively affect the fitness of Aquatic Warblers through higher parasitism by trypanosomes. This could also be true for other avian habitat specialists breeding in open habitats, such as marshland and grassland, but to date research in this area is lacking. Our result contributes to informed conservation management of the Aquatic Warbler, as well as other bird species of the globally shrinking marshland ecosystems.

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