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Haemosporidians in birds during spring migration on Ponza island

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CONTENTS

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ABSTRACT

Blood parasites of the genera *Haemoproteus*, *Leucocytozoon* and *Plasmodium* are found in a variety of bird hosts. The effects on the life and behaviour of infected individuals - also regarding bird migration - have already been the subject of numerous studies in the past. In this study, a look was taken at the general occurrence of the three parasite genera in wild birds during their stopover on the island of Ponza (Italy). Birds were caught using nets in spring 2016 and 2017, ringed, and a number of parameters were determined (bird species, age, sex, etc.). Furthermore, blood samples were taken and screened by PCR targeting the standard CytB barcode sequence. Long-distance migrants were more likely to be infested with blood parasites than short-distance migrants. The year of sampling also seems to have influenced the results (more infections in 2017 than in 2016). The age and sex of the host, on the other hand, had no effect on the frequency of blood parasite infections. Overall, the results are in line with previously published studies. However, since there are only few studies on avian haemosporidian infections in migratory birds during their mediterranean stopovers, further studies at other prominent resting places of migratory birds should be considered.

ZUSAMMENFASSUNG

Blutparasiten der Gattungen *Haemoproteus*, *Leucocytozoon* und *Plasmodium* sind in einer Vielzahl unterschiedlicher Wirte anzutreffen, darunter auch in Vögeln. Die Auswirkungen auf das Leben und Verhalten infizierter Individuen - auch im Hinblick auf den Vogelzug - waren bereits in der Vergangenheit Gegenstand zahlreicher Studien. In dieser Studie wurde das allgemeine Vorkommen Parasiten der drei Gattungen bei Wildvögeln während ihres Zwischenstopps auf der Insel Ponza (Italien) untersucht. Die Vögel wurden im Frühjahr 2016 und 2017 mit Netzen gefangen, beringt; weiters wurde eine Reihe von Parametern bestimmt (Vogelart, Alter, Geschlecht, etc.). Es wurden Blutproben entnommen und mittels PCR auf die Standard-CytB-Barcodesequenz untersucht. Bei Langstreckenziehern war die Wahrscheinlichkeit eines Befalls mit Blutparasiten größer als bei Kurzstreckenziehern. Auch das Jahr der Probenahme scheint die Ergebnisse beeinflusst zu haben (mehr Infektionen im Jahr 2017 als im Jahr 2016). Alter und Geschlecht der Wirtsvögel hatten dagegen keinen Einfluss auf die Häufigkeit von Blutparasiteninfektionen. Insgesamt stehen die Ergebnisse im Einklang mit bereits veröffentlichten Studien. Da es jedoch nur wenige Studien über aviäre Hämosporidieninfektionen bei Zugvögeln auf ihren Zwischenhalt im Mittelmeergebiet gibt, sollten weitere Studien an anderen wichtigen Rastplätzen von Zugvögeln in Betracht gezogen werden.

1. INTRODUCTION

Haemosporidian parasites are widespread in birds. So far over 206 species of avian haemosporidians have been described from many different bird species all over the world, except the Antarctic area (Valkiūnas 2005). Besides birds, haemosporidians are also known to parasitize reptiles, amphibians, and mammals. They are transmitted by blood-sucking dipterans such as mosquitoes, biting midges, and louse flies as vectors (Valkiūnas 2005).

The most common avian haemosporidian parasites belong to the genera *Haemoproteus*, *Leucocytozoon*, and *Plasmodium* (Valkiūnas 2005). Since human malaria is also caused by parasites of the genus *Plasmodium*, they are considered of high medical interest. Since “avian malaria” is similar to human malaria, parasites like *Plasmodium relictum* are seen as model systems (Fecchio et al. 2020).

Especially regarding migratory birds, haemosporidian parasites are of interest because of the complex host-parasite relationships. The diversity of haemosporidian parasites is generally higher in migrants (Jenkins et al. 2012). However, *Haemoproteus* and *Leucocytozoon* are more often found in residents (breeding or wintering grounds) whereas *Plasmodium* parasites are more common in migratory birds (Hellgren et al. 2007, Valkiūnas 2005). Furthermore, *Haemoproteus* lineages are often specialized on one host, while *Plasmodium* lineages less often form single-host relationships (Hellgren et al. 2007). In other words, *Plasmodium* parasites can be described as a generalist, and *Haemoproteus* and *Leucocytozoon* parasites as specialists.

As migratory birds travel between continents and are exposed to a range of different environments and vectors, the probability of becoming infected with blood parasites increases (Fecchio et al. 2020). Fecchio et al. (2020) describe possible factors for the spread of the different parasite genera: In addition to the exposure of bird species to different parasite species, the resistance of the birds also plays a role, as do the interactions between the parasites themselves. Not only the spread of the parasites to other areas, but also the transmission to new hosts is apparent due to the migratory behaviour of the animals (Jenkins et al. 2012). The transmission therefore highly depends on the geographical area and the observed bird population. Many European-African migratory birds become infected with the blood parasites in their wintering grounds (Valkiūnas 2005), but transmission does not occur in the breeding grounds in Europe because of the lack of competent vectors (Garcia-Longoria et al. 2015). The occurrence of vectors depends on several factors, but studies have shown that there is a correlation with temperature, rainfall and/or water resources (Atkinson & Samuel 2010, Lachish et al. 2011a, Garamszegi 2011, Wood et al. 2007). Van Rooyen et al. (2014) also show that there are differences in the prevalence of haemosporidian parasites in

European and North African populations in the same bird species and even in populations within Europe different lineages are prevalent. The same study also found that the observed bird population in Algeria featured more infected individuals than the European population.

According to Fecchio et al. (2020), further research is needed to better understand the distribution of vectors, parasites, and hosts as well as the complex transmission patterns. The spread of pathogens due to bird migration is also of relevance to human health. Previous studies on avian influenza and the West Nile virus show how quickly pathogens can spread through the migratory movements of birds (Jourdain et al. 2007). A precise description and overview on the distribution of parasites is necessary to make forecasts for the future, particularly in the wake of climate change (Fecchio et al. 2020). As shown by Garamszegi (2011), an increase in global temperature causes a higher incidence of avian malaria in bird hosts and can put them under pressure, for example by prolonging their breeding season.

Both the possibility of pathogens spreading from birds to humans and the increased risk of the spread of avian malaria parasites to more northern areas through bird migration due to climate change are important issues that require further research (Fuller et al. 2012).

Studying the effects of haemosporidian parasites is difficult because effects of infections on the fitness of wild bird populations are poorly understood (Merino et al. 2000). Infections in wild birds are more difficult to study for several reasons: 1) Wild birds are usually better adapted to their local parasite communities due to long-term co-evolution. 2) Internal and external factors such as food sources differ between wild and captive birds. 3) It is difficult to find heavily infected individuals in nature because these individuals are more prone to dying or being predated (Valkiūnas 2005). For practical reasons, the pathology of avian haemosporidians is better characterized in domestic birds like canaries or poultry. The observations include lethargic behavior, breathing difficulties, paralysis, and even death (Valkiūnas 2005).

Generally, there are prepatent, acute, critical, chronic, and latent stages of infections. Depending on the current state of infection, the birds have different challenges to face (Valkiūnas 2005). Especially during acute infection with high parasitemia, the birds more likely face death than individuals that already passed this critical phase. On the other hand, chronic infections are known to lesser harm the birds' fitness due to the lower quantity of parasites in the organism (Valkiūnas 2005; Atkinson & Samuel, 2010, Bensch et al., 2007). Therefore, most infections in wild bird populations are chronic in appearance (Valkiūnas 2005; Atkinson & Samuel 2010). However, the effect of the chronic phase on the bird should not be underestimated because especially chronic *Plasmodium* infections can lead to reduced chances for survival (Van Oers et al. 2010, Lachish et al. 2011b, Wood et al. 2013, Dawson & Bortolotti 2000). Other studies also point out that the long-term costs of chronic infections include the shortening of telomeres as well as a reduction in survival (Asghar et al. 2015). The

infection status must be considered as a dynamic state, which means that infections can not only be gained but also lost (Knowles et al. 2011). The risk of being exposed to several types of parasites during the life of a bird is very high, as mentioned above, but to a certain extent a problem in terms of the life expectancy of the individual. In a few cases, however, it is possible to overcome the infection as described by Piersma & Van der Velde (2012).

Bird migration is a very stressful period in a bird's annual cycle and, and infections with haemosporidian parasites can negatively affect the birds' fitness. One well observed bottleneck of spring migration, known to be one of the first stopovers for birds after their travel from Africa across the Mediterranean Sea, is the island Ponza (Italy). The island is very popular as a first port of call after several hours of flying over the sea (Maggini et al. 2021) and was for many years the venue for studies on fitness, body condition and hormonal parameters and their effects on bird migration and stop-over ecology (Benedetti et al. 2013, Fusani et al. 2011, Goymann et al. 2017, Lupi et al. 2017). In recent years, the bird ringing station on Ponza has become a place of studies on parasite incidence in migratory birds. Rollins et al. (2021) and Battisti et al. (2020) investigated the ticks of arriving birds. Both studies point out the importance of birds bringing pathogens and parasites to Europe in spring as they travel from the south to the north, therefore studies will also be needed in the future in view of climate change.

When it comes to observations of haemosporidians in wildlife, the focus is often on studying the distribution of the parasites and effects on the bird populations rather than birds migrating from Africa to Europa or vice versa. Infection with haemosporidian parasites indeed can have negative effects on a birds' migratory behavior, including prolonged stopovers (Hegemann et al. 2018), effects on body condition/fat reserves (Cornelius et al. 2014), changes in timing during migration, energy condition, refueling of energy reserves (DeGroote & Rodewald 2010), departure time (Sorensen et al. 2016b), and delay in migration and therefore late arrival at the breeding grounds (López et al. 2013, Santiago-Alarcon et al. 2013). In the context of avian malaria and migratory behaviour, the importance or impact of wintering areas has also been indicated in previous studies. The location of the wintering areas plays a role in the likelihood of being infected with blood parasites as birds wintering in Western Central Africa tend to be infected with more *Plasmodium* lineages compared to birds that settle in southern Europe over the winter (Von Rönn et al. 2015). It has also been shown in waterfowls that longer migratory distances increase the risk of haemosporidian infections (Figuerola & Green 2000).

Other important research questions when dealing with blood parasites are the distribution and effects on age and sex of individuals. The factor "age" does play a role for a bird's life: the older the birds become, the higher the chance of carrying blood parasites (Freeman-Gallant & Taff 2017). Observations in long-lived birds like the mute swan also showed that infections with

Haemoproteus parasites are found more likely in older individuals, which can be interpreted as a correlation of age and infection (Wood et al. 2013). Furthermore, the already mentioned increased mortality in young birds (Atkinson & Samuel 2010, Van Oers et al. 2010) may mean that infections are seen more often in adult birds that have survived the acute phase and are now in the chronic phase. The factor "sex", however, can be looked at from different angles: When studying blood parasites and their influence on the performance of both sexes, studies often focus on the breeding success (Asghar et al. 2011, Norte et al. 2009, Knowles et al. 2010) and sexual dimorphism of the birds (Romano et al. 2019, Bensch et al. 2007). Whether a particular sex is more or less likely to be infected with a blood parasite cannot be answered simply. Norte et al. (2009) did not find any evidence that infections were more frequent in a certain sex or in a certain age group, but this may depend on the host and parasite species studied.

This descriptive study takes a holistic look at the distribution of *Haemoproteus*, *Leucocytozoon*, and *Plasmodium* parasites – in populations of migrating birds on the island of Ponza. The birds were caught in the field via mist nets and blood samples were taken and later investigated in the laboratory. The detection was carried out by light microscopic examination of blood smears as well as PCR and sequencing.

In these studies, we tried to better understand a number of aspects of the host-parasite relationship in migratory birds. These include investigations into the occurrence of blood parasites in the carriers in terms of frequency and species. Furthermore, our studies focus on whether double or triple infections could be detected in the samples in addition to single infections. In addition to the question of whether and how many infections were found in the host birds, we also want to shed light on the relationship between parasite strains and bird species. Another subject of our investigations is whether there were differences in the occurrence of parasites in the years under investigation (2016 and 2017). Finally, we will also try to determine whether factors such as age, sex and migratory behaviour (long-distance versus short-distance migrants) play a role in the likelihood of a bird carrying parasites. As previous studies indicated, we predict that the probability of infection depends on the migration behaviour and thus also on the respective bird species. The age and sex of the sampled bird can also be expected to have an impact, i.e., older individuals should show a higher incidence (carry parasites more often) and the individuals are expected to be higher during the breeding season.

2. MATERIAL AND METHODS

2.1. Study site and species

The sample collection took place on the island of Ponza, Italy (40°55'N, 12°58'E). Ponza is located in the Tyrrhenian Sea, 50 km south of the mainland, along the migratory route between Africa and Central Europe. As shown in previous studies, the island is a well-known stopover for passerine birds during the spring migration (Maggini et al. 2020a, Maggini et al. 2021). The bird ringing station (www.inanellamentoponza.it) is located in the village Le Forna, the narrowest part in the north of the island. The ringing activity took place in the months of March/April till mid of May. The blood samples for this study were collected during the spring season of 2016 (14th of March to 13th of May) and 2017 (29th of March to 25th of May).

Figure 1 Position of net rows at Le Forna, Ponza

The birds were trapped in so-called mist nets. In total, 340 meters of mist nets were installed (Figure 1). They were controlled every 60 minutes from sunrise to sunset – except for bad weather conditions when the nets stayed closed. The captured birds got measured and marked with an individual ring. As far as possible, age and sex of the individual birds were determined using the criteria of the field guides by Svensson (1992) and Jenni & Winkler (1994). The age of the birds was divided into the following three categories: 4 = age not determinable/unknown, 5 = birds hatched in the previous year, 6 = Birds that did not hatch in the previous year but in one of the years before (adults). The sex of the birds was divided into the following three categories: 0 = sex not determinable/unknown, 1 = male, 2 = female.

The standard procedures for measuring birds included the length of the third primary cover with a precision of +/- 0.5 mm and the body weight with a precision of +/- 0.1 g as well as the fat and muscle scores, determined by standardized scales for the pectoral muscles (0-3) and subcutaneous fat (0-8) (Bairlein, 1995). Recaptured birds were not used for the blood sampling.

In 2016, 318 blood samples from eleven different bird species were collected. The subjects of the research were barn swallows (*Hirundo rustica*, Linnaeus 1758), European robins (*Erithacus rubecula*, Linnaeus 1758), black redstarts (*Phoenicurus ochruros*, S.G. Gmelin 1774), common redstarts (*Phoenicurus phoenicurus*, Linnaeus 1758), whinchats (*Saxicola rubetra*, Linnaeus 1758), icterine warblers (*Hippolais icterina*, Vieillot 1817), common whitethroats (*Curruca communis*, Latham 1787), garden warblers (*Sylvia borin*, Boddaert 1783), Eurasian blackcaps (*Sylvia atricapilla*, Linnaeus 1758), spotted flycatchers (*Muscicapa striata*, Pallas 1764) and pied flycatchers (*Ficedula hypoleuca*, Pallas 1764). In 2017, 360 blood samples were collected from the latter eleven bird species, which resulted in a total of 678 blood samples from both years (Table 1).

For this study, the species were divided in (i) “long-distance migrants” and (ii) “short-distance migrants”. Long-distance migrants were defined as species wintering in sub-Saharan Africa (tropical as well as the Sahel zone) and showing a regular distribution in temperate areas like Central and Northern Europe, where they breed. Following birds were assigned in the group of “long-distance migrants”: barn swallows, common redstarts, whinchats, icterine warblers, common whitethroats, garden warblers, spotted flycatchers, and pied flycatchers. European robins, black redstarts and Eurasian blackcaps were defined as “short-distance migrants” because their wintering habitats only extend around the Mediterranean (Maggini et al. 2020b).

Furthermore, the species were divided into three groups (tropical, Sahel region, North Africa), depending on their typical wintering grounds. Bird species from the “tropical” group are defined as species wintering in tropical regions, specifically in sub-Saharan Africa, and which are regularly distributed in temperate areas such as Central and Northern Europe where they breed. Following birds were assigned in this group: garden warblers, whinchats, barn swallows, icterine warblers and pied flycatchers. Bird species from the “Sahel” group are defined as species wintering in the Sahel region of Africa, which includes common redstarts, common whitethroats, and spotted flycatchers. The “North Africa” group includes European robins, black redstarts, and Eurasian blackcaps. Their winter habitats are not based in sub-Saharan Africa but extend around the Mediterranean (Maggini et al. 2020b).

Table 1 Species and number of blood samples of 2016 and 2017

Bird species	Number of samples 2016	Number of Samples 2017	Total number of samples	Wintering grounds
barn swallow (i)	18	20	38	Tropical
European robin (ii)	21	39	60	North Africa
black redstart (ii)	26	3	28	North Africa
common redstart (i)	6	25	31	Sahel
whinchat (i)	30	43	73	Tropical
icterine warbler (i)	17	41	58	Tropical
common whitethroat (i)	74	57	131	Sahel
garden warbler (i)	83	30	112	Tropical
Eurasian blackcap (ii)	1	48	48	North Africa
spotted flycatcher (i)	24	24	48	Sahel
pied flycatcher (i)	18	30	48	Tropical
Total sample size	318	360	678	

Blood samples of 50 µl were taken from the brachial vein by using a single use cannula (Sterican Standard Size 18) and microcapillaries (Minivette POCT 100 µl EDTA violet, Sarstedt). Blood drops were placed on filter paper (Whatman® FTA® Elute FTA Elute Micro Card, Sigma-Aldrich), air dried and stored in paper envelopes. In addition to the blood spots, blood smears (2 glass slides per individual) were prepared and air dried. All the material was stored at room temperature till the transport to the laboratory (Vienna, Austria).

After the procedure, the birds were released unharmed.

2.2. Blood analysis in the laboratory

The laboratory analyses focused on parasites of the genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*.

The blood smears were prepared for examination under the light microscope (x1000; high magnification and immersion oil). After fixing with methanol, the samples were stained with the Giesma staining method (diluted 1:10 for 45−60 mins; Applichem). The examination of the blood smears was used to compare the results of the PCRs and as a basis for possible future morphological studies. To determine the parasite species in the respective blood samples using a microscope, the identification key in Valkiūnas (2005) was used to identify gametocytes in the blood cells of their hosts.

To prepare the samples for later polymerase chain reactions (PCR) a DNA extraction was conducted. A 4 x 4 mm blood stain was cut from the filter paper and transferred to a 1.5 ml tube. The sample was incubated at 4°C during the night with 100 µl of phosphate buffered saline (PBS). Afterwards the sample was centrifuged at 13.000 revolutions

per minute (RPM) for 3 minutes to create a supernatant. The supernatant was pipetted off and the resulting pellet, which remained in the tube, was washed by adding 100 µl of PBS and centrifuging the tube at 13.000 RPM for 2 minutes. Afterwards the supernatant was removed again. In the next step, 60 µl of InstaGene matrix (InstaGene™ Whole Blood Kit; Biorad) were added to the sample before it was incubated for 8 minutes at 70°C. After 15 seconds of vortexing, the sample was incubated for 4 minutes at 95−100°C, followed by another 15 seconds of vortexing and centrifuging (13.000 RPM, 1 minute). The last step of the extraction was the removal of the supernatant and adding 60 µl of InstaGene matrix. The extracted DNA was stored at -20°C for further tests.

In short, the method according to Hellgren et al. (2004) works with a first PCR for the detection of the three parasite genera, followed by two nested PCRs targeting *Plasmodium*/*Haemoproteus* and *Leucocytozoon*. We used the GoTaq G2 Polymerase kit with Green Buffer, (Promega, Fitchburg, USA). An Eppendorf Mastercycler©Pro (Eppendorf AG, Hamburg, Germany) was used for the PCRs. The master mix consisted of nuclease-free water, dNTP's, GoTaq G2 Polymerase, and primer pairs (Table 2). The primers target the mitochondrial *CytB* gene of most *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* parasites in a nested PCR assay (Hellgren et al. 2004, Waldenström et al. 2002).

Table 2 Primer for Haemosporida PCR

Test	**Primer**	**Name**	**Sequence (5'-3')**
Haemosporida	Forward Nest 1	Haem NF1	5´-CATATATTAAGAGAA5TATGGAG-3´
Haemosporida	Reverse Nest 1	Haem NR3	5´-ATAGAAAGATAAGAAATACCATTC-3´
Haemoproteus, Plasmodium	Forward Nest 2	Haem F	5´-ATGGTGCTTTCGATATATGCATG-3´
Haemoproteus, *Plasmodium*	Reverse Nest 2	Haem R2	5´-GCATTATCTGGATGTGATAATGGT-3´
Leucocytozoon	Forward Nest 2	Haem FL	5´-ATGGTGTTTTAGATACTTACATT-3´
Leucocytozoon	Reverse Nest 2	Haem R2L	5´-CATTATCTGGATGAGATAATGG5GC-3´

The composition of the reagents for Haemosporida nest 1 (25,000 µl total) consists of nuclease-free water (15.075 µl), 5 x Green Buffer (5,000 µl), dNTP's (10 mM, 0. 500 µl), Go Taq G2 Polymerase (5 u/ µl, 0.125 µl), Primer Forward (100 pmol/µl, 0.150 µl), Primer Reverse (100 pmol/µl, 0.150 µl) and the DNA (4,000 µl). In comparison, the composition of the reagents for Nest 2 (25,000 µl total) is composed of nuclease-free water (17.075 µl), 5 x Green Buffer

(5,000 µl), dNTP's (10 mM, 0. 500 µl), Go Taq G2 Polymerase (5 u/ µl, 0.125 µl), Primer Forward (100 pmol/µl, 0.150 µl), Primer Reverse (100 pmol/µl, 0.150 µl) and the Nest 1 product (2.000 µl).

Every plate of samples was provided with a positive control and two negative controls (4 µl distilled water). The temperature profile of the PCRs included the following phases: Initialization (95°C, 2:00 min), followed by 30 cycles of alternating phases of denaturation (95°C, 0:45 min), primer annealing (50°C, 0:45 min) and primer extension (72°C, 1:00 min); subsequent final extension phase (72°C, 5:00 min) and the final hold (15°C). In the second round of the PCR (Nest 2), 23 µl of the master mix were merged with 2 µl of the product of the first PCR product. The primer pairs used to detect *Haemoproteus,* and *Plasmodium* (HaemF, HaemR2) differed from the primer pairs used for *Leucocytozoon* (HaemFL, HaemR2L) as shown in Table 2. All other reagents as well as the temperature profile were the same as used for the Nest 1 PCR. The Nest 2 PCRs produced 478 base pair (bp) and 476 bp fragments for *Plasmodium*/*Haemoproteus* and *Leucocytozoon*, respectively.

The PCR products were checked by electrophoresis, running each 5 µl of the nest 2 PCR products on 1% agarose gels. We repeated the PCRs of the negative samples (with positive blood smears) with the primer sets CytB_HPL_intF1 and CytB_HPL_intR1 (Harl et al. 2019). Positive samples were sent to LGC Genomics (Hoddesdon, UK) for purification and sequencing. For the subsequent identification of the parasite species, the sequences were compared with data from NCBI GenBank and the MalAvi database (Bensch et al. 2009) using BLAST searches.

2.3. Statistical analysis

Data elaborations, descriptive statistics and plots were performed using Microsoft Excel (2021) for Microsoft 365 Apps (Microsoft Corporation, https://office.microsoft.com/excel).

3. RESULTS

3.1. Overview infections

In a total of 678 blood samples from eleven different bird species, 342 individuals (50.4%) were uninfected, and 336 individuals (49.6%) were infected with one or more blood parasites. *Haemoproteus* infections were most common with 235 infected birds (34.7% of all samples), followed by *Plasmodium* with 89 (13.1% of all samples) and *Leucocytozoon* with 43 (6.3% of all samples) (Table 3, Figure 2).

The most generalist representatives of each genus were in case of *Plasmodium* the parasites/lineages *P. relictum* GRW11 and *P. relictum* SGS1 (in five bird species of each one), in genus *Haemoproteus* the parasite/lineage *H. attenuatus* ROBIN1 (in four bird species) and in genus *Leucocytozoon* the parasites/lineages *L.* sp. BT2, *L.* sp. SATEC01 and *L.* sp. SFC8 (in three bird species of each one) (Table 4a-c).

In our study, new lineages were also found that differ from already known lineages in just some base pairs. These include AEMO01(477/478), PBPIP1(476/478), SGS1(477/478a), SGS1(477/478b), SGS1(477/478c), SYBOR02(477/478), SYBOR05(472/473a), SYBOR05(472/473b), SYBOR05(472/473c), WW3(477/478) for *Plasmodium*, COLL2(478/479), CWT10(462/463), CWT11(477/478), CWT5(476/478), CWT5(477/478), SFC9(472/473), SYBOR01(477/478a), SYBOR01(477/478b) for *Haemosporidia* and BT1(475/476), BT2(475/476), RS3(475/476), SATEC01(474/476) for *Leucozytozoon* (Table 4a-c).

Table 3 Overview of all Haemosporida infections and their distribution in the 3 individual genera in a total of 678 blood samples

Parasite	**Infection found**	
	N	**%**
Plasmodium	89	13.1%
Haemoproteus	235	34.7%
Leucocytozoon	43	6.3%
total	336	49.6%

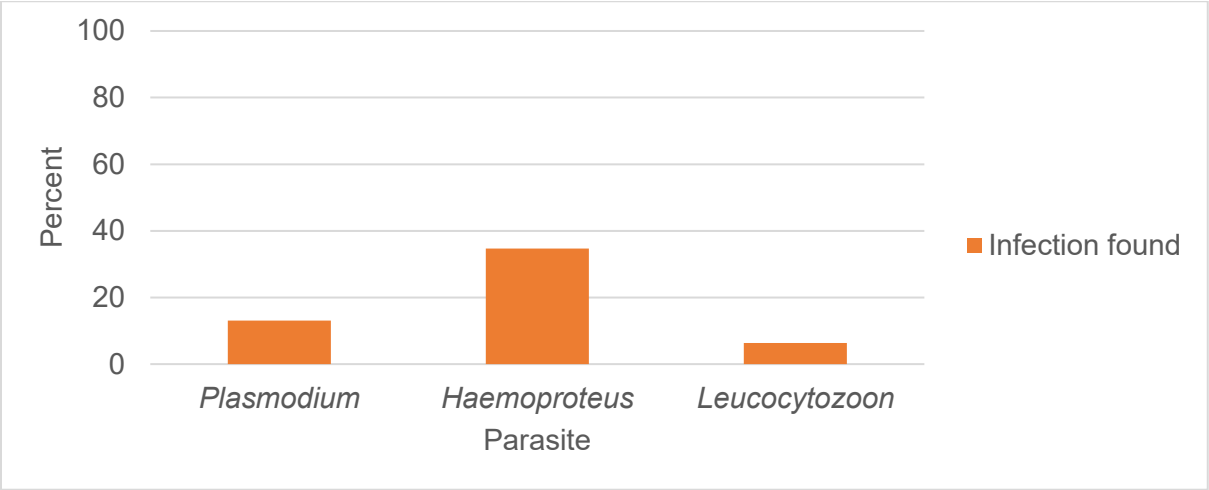

Figure 2 *Plasmodium*, *Haemoproteus* and *Leucocytozoon* infections found in a total of 678 blood samples

Table 4a Parasite species/lineages of Genus *Plasmodium* and bird hosts in 2016 and 2017

Parasite Species/Lineage	**Bird species**
P. ashfordi GRW02	barn swallow (2), garden warbler (1)
P. circumflexum TURDUS1	barn swallow (1), common redstart (4), common whitethroat (1), European robin (2)
P. homonucleophilum SW2	garden warbler (1), whinchat (6)
P. relictum GRW04	common whitethroat (1)
P. relictum GRW11	common whitethroat (2), garden warbler (1), pied flycatcher (1), spotted flycatcher (1), whinchat (1)
P. relictum SGS1	common redstart (3), common whitethroat (18), garden warbler (5), spotted flycatcher (1), whinchat (4)
P. sp. AEMO01	garden warbler (2)
P. sp. AEMO01(477/478)	garden warbler (1)
P. sp. AFR143	whinchat (1)
P. sp. AFR146	whinchat (1)
P. sp. BT7	common redstart (1), pied flycatcher (1)
P. sp. COLL7	common whitethroat (1), spotted flycatcher (1)
P. sp. CUCROC01	barn swallow (1)
P. sp. GRW09	barn swallow (3), spotted flycatcher (1)
P. sp. GRW10	whinchat (1)
P. sp. LK06	black redstart (1), common whitethroat (1)
P. sp. PBPIP1(476/478)	common whitethroat (1)
P. sp. PLOCUC02(477/478)	whinchat (1)
P. sp. PLOPRI01	spotted flycatcher (2)
P. sp. RTSR1	garden warbler (1), whinchat (1)
P. sp. SGS1(477/478a)	spotted flycatcher (1)
P. sp. SGS1(477/478b)	common whitethroat (1)
P. sp. SGS1(477/478c)	common whitethroat (1)
P. sp. SYAT24	whinchat (1)

Table 4b Parasite species/lineages of Genus *Haemoproteus* and bird hosts in 2016 and 2017

Parasite Species/Lineage	**Bird species**
H. attenuatus ROBIN1	European robin (7), spotted flycatcher (1), whinchat (16), common whitethroat (2)
H. balmorali COLL3	pied flycatcher (2)
H. balmorali SFC1	spotted flycatcher (17)
H. belopolskyi HIICT1	pied flycatcher (1), icterine warbler (41), spotted flycatcher (1)
H. belopolskyi HIICT3	icterine warbler (12), pied flycatcher (1)
H. hirundinis DELURB1	barn swallow (1)
H. majoris CWT4	common whitethroat (4)
H. majoris PHSIB1	European robin (1)
H. majoris WW2	Eurasian blackcap (1)
H. pallidus COLL2	pied flycatcher (4), whinchat (1)
H. pallidus PFC1	common redstart (1), pied flycatcher (1)
H. pallidus SFC3	common whitethroat (1), spotted flycatcher (13)
H. parabelopolskyi SYAT01	Eurasian blackcap (16), European robin (1), pied flycatcher (1)
H. parabelopolskyi SYAT02	Eurasian blackcap (4)
H. parabelopolskyi SYBOR01	garden warbler (19)
H. sp. COLL2(478/479)	pied flycatcher (1)
H. sp. CWT10(462/463)	common whitethroat (3)
H. sp. CWT11	common whitethroat (2)
H. sp. CWT11(477/478)	common whitethroat (1)
H. sp. CWT2	common whitethroat (31)
H. sp. CWT2(477/478)	common whitethroat (1)
H. sp. CWT3	common whitethroat (8)
H. sp. CWT5(476/478)	common whitethroat (1)
H. sp. CWT5(477/478)	common whitethroat (1)
H. sp. CWT7	common whitethroat (4)
H. sp. HIICT2	icterine warbler (5)

Table 4c Parasite species/lineages of Genus *Leucocytozoon* and bird hosts in 2016 and 2017

Parasite Species/Lineage	**Bird species**
L. sp. BT1	common redstart (2)
L. sp. BT1(475/476)	European robin (1)
L. sp. BT2	Eurasian blackcap (1), garden warbler (1), spotted flycatcher (5)
L. sp. BT2(475/476)	common redstart (1)
L. sp. BT5	European robin (1)
L. sp. CYAVER03	pied flycatcher (1)
L. sp. HIRUS07	whinchat (1)
L. sp. REB7	whinchat (2)
L. sp. RECOB3	European robin (1)
L. sp. RS2	common redstart (1)
L. sp. RS3(475/476)	common redstart (1)
L. sp. SATEC01	garden warbler (1), icterine warbler (1), spotted flycatcher (3)
L. sp. SATEC01(474/476)	spotted flycatcher (1)
L. sp. SFC8	Eurasian blackcap (1), European robin (1), icterine warbler (1)
L. sp. SYAT22	Eurasian blackcap (1), pied flycatcher (1)
L. sp. SYBOR06	garden warbler (4)

Of the 336 birds infected with haemosporidian parasites (49.6%), 305 (45.0%) featured single infections and 31 (4.6%) double infections. None of the birds featured a triple infection (Figure 3).

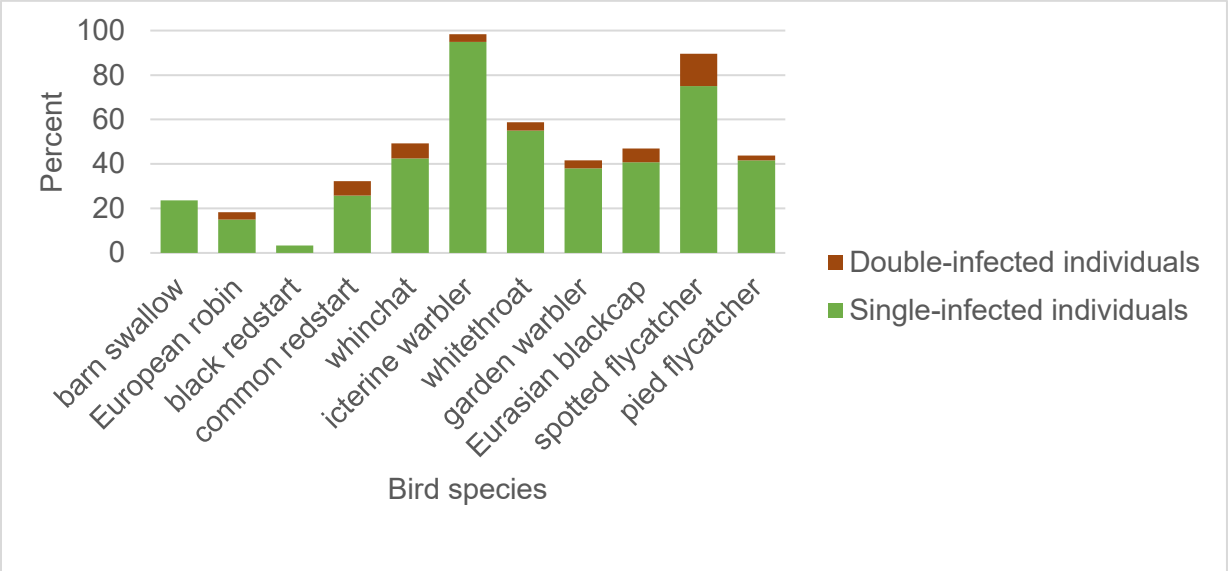

Figure 3 Bird species and their infection status (single-infection and double-infection)

Table 5 Number of birds positive for *Plasmodium*, *Haemoproteus* and *Leucocytozoon* parasites

Bird species	**Number of individuals**	**Positive samples**	***Plasmodium***	***Haemoproteus***	***Leucocytozoon***
barn swallow	38	9	6	2	1
European robin	60	11	2	8	3
black redstart	29	1	1	0	0
common redstart	31	10	8	1	3
whinchat	73	36	22	18	2
icterine warbler	58	57	0	56	3
common whitethroat	131	77	27	54	1
garden warbler	113	47	12	21	18
Eurasian blackcap	49	23	1	22	3
spotted flycatcher	48	43	8	35	7
pied flycatcher	48	21	2	18	2
total	678	336	89	235	43

Icterine warblers (N = 58) featured the highest prevalence of blood parasites with 57/58 (98.3%) positive samples whereas the prevalence was lowest in the black redstarts (N = 29) with 1/29 (3.4%) positives. The spotted flycatchers (N=48) most frequently featured double infections (7 double infections) (Table 5).

3.2. Years

In 2016, 128 of 318 (40.25%) birds were infected with a blood parasite. In 2017, 208 of 360 (57.78%) birds were infected (Table 6).

Table 6 Samples sizes and positive samples of 2016 and 2017

Year	**Sample size**	**Positive samples**	
	N	**N**	**%**
2016	318	128	40.2%
2017	360	208	57.8%
Total	678	336	49.6%

Furthermore, we compared the distribution of *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* infections in the bird species studied between the two years (Table 7).

Table 7 Comparison of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* infections in the bird species from 2016 and 2017

Bird species	Total	*Plasmodium* 2016		*Plasmodium* 2017		*Haemoproteus* 2016		*Haemoproteus* 2017		*Leucocytozoon* 2016		*Leucocytozoon* 2017	
		N	%	N	%	N	%	N	%	N	%	N	%
barn swallow	38	3	16.7%	3	15.0%	0	0.0%	2	10.0%	1	5.6%	0	0.0%
European robin	60	0	0.0%	2	5.1%	0	0.0%	8	20.5%	0	0.0%	3	7.7%
black redstart	28	1	3.8%	0	0.0%	0	0.0%	0	0.0%	0	0.0%	0	0.0%
common redstart	31	3	50.0%	5	20.0%	0	0.0%	1	4.0%	0	0.0%	3	12.0%
whinchat	73	4	13.3%	18	41.9%	9	30.0%	9	20.9%	0	0.0%	2	4.7%
icterine warbler	58	0	0.0%	0	0.0%	17	100%	39	95.1%	0	0.0%	3	7.3%
common whitethroat	131	10	13.5%	17	29.8%	30	40.5%	24	42.1%	0	0.0%	1	1.8%
garden warbler	112	8	9.6%	4	13.3%	9	10.8%	12	40.0%	9	10.8%	9	30.0%
Eurasian blackcap	48	/	/	1	2.1%	/	/	22	45.8%	/	/	3	6.3%
spotted flycatcher	48	4	16.7%	4	16.7%	18	75.0%	17	70.8%	0	0.0%	7	29.2%
pied flycatcher	48	1	5.6%	1	3.3%	5	27.8%	13	43.3%	1	5.6%	1	3.3%

3.3. Age and Sex

The analyses including the variables "age" and "sex" were only conducted with data of those individuals, who could be divided into the categories "yearling" (born in the previous year) and "adult" (born not in the previous year but before) and/or "male" and "female" by means of morphological criteria. The following bird species were used for the investigations with the variable "age" and "sex": pied flycatcher, black redstart, common redstart, whinchat, Eurasian blackcap and common whitethroat. Furthermore, we were able to determine the age of the European robins but not their sex, and the sex of the barn swallows but not their age. In the case of the icterine warblers, garden warblers and spotted flycatchers, neither age nor sex can be determined by morphological characteristics in spring.

The age could be determined in a total of 420 age-able individuals, of which 296 individuals were born in the previous year (70.5%) and 124 individuals were adults (29.5%). Of the 296 individuals born in the previous year (yearling birds), 130 were infected by a blood parasite (43.9%) while 49 of the 124 adults were affected (39.5%). In other words, out of a total of 180 positive samples of this seven bird species, 130 were positive yearling birds (72.2%), 49 adults (27.2%) and one individual of category 4 so not age-able (0.6%) (Table 8).

Table 8 Distribution of the age classes of the individual bird species

Bird species	Yearling birds		Adult birds		Age not determinable	
	Total	Infected	Total	Infected	Total	Infected
European robin	38	7	22	4	0	-
black redstart	15	0	14	1	0	-
common redstart	21	5	9	4	1	1
whinchat	60	31	13	6	0	-
common whitethroat	83	51	48	26	0	-
Eurasian blackcap	45	21	4	2	0	-
pied flycatcher	34	15	14	6	0	-
Total sample size	296	130	124	49	1	1

The sex could be determined in a total of 380 sex-able individuals, of which 203 (53.4%) were males and 177 (46.6%) females. Of the 203 males, 93 (45.8%) were infected while 84 of the 177 (47.5%) females were infected. In other words, out of a total of 178 positive samples of this 7 bird species, 93 were positive males (52.2%), 84 females (47.2%) and 1 individual of category 0 so not sex-able (0.6%) (Table 9).

Table 9 Distribution of the sexes of the individual bird species

Bird species	Males		Females		Sex not determinable	
	Total	Infected	Total	Infected	Total	Infected
barn swallow	14	4	21	5	3	0
black redstart	6	1	8	0	15	0
common redstart	15	5	15	4	1	1
whinchat	42	22	31	15	0	-
common whitethroat	71	37	60	40	0	-
Eurasian blackcap	29	12	20	11	0	-
pied flycatcher	26	12	22	9	0	-
Total sample size	203	93	177	84	19	1

3.4. Migration behaviour

Of the 540 long-distance migrants (barn swallow, common redstart, whinchat, icterine warbler, common whitethroat, garden warbler, spotted flycatcher, pied flycatcher), 301 (55.7%) individuals were infected. Of the 138 short-distance migrants (European robin, black redstart, Eurasian blackcap), 35 (25.4%) individuals were infected (Table 10).

Table 10 Infection status of long- and short-distance migrants

Migration behaviour	Infected individuals		Non-infected individuals		Total
	N	%	N	%	N
Long-distance migrants	301	55.7%	239	44.3%	540
Short-distance migrants	35	25.4%	103	74.6%	138

In terms of migratory behaviour and detected lineages, we observed that short-distance migrants featured exclusively a total of six lineages (*Haemoproteus* with *H. majoris* PHSIB1, *H. majoris* WW2, *H. parabelopolskyi* SYAT02; *Leucocytozoon* with *L.* sp. BT1(475/476), *L.* sp. BT5, *L.* sp. RECOB3). A total of seven lineages were associated with both short- and long-distance migrants (*Plasmodium* with *P. circumflexum* TURDUS1, *P.* sp. LK06; *Haemoproteus* with *H. attenuatus* ROBIN1, *H. parabelopolskyi* SYAT01; *Leucocytozoon* with *L.* sp. BT2, *L.* sp. SFC8, *L.* sp. SYAT22). All remaining lineages were found in long-distance migrants only (Table 4a-c).

4. DISCUSSION

This study centered around haemosporidian parasite prevalence in 678 migratory birds on Ponza Island, looked on migration patterns and host-parasite interactions. Our hypotheses focused on migration distance, age, and sex as factors influencing parasite prevalence. We expected higher infection rates in long-distance migrants and potential differences between age and sex groups. Results showed a high overall infection rate, primarily dominated by *Haemoproteus* (34.7% of samples), followed by *Plasmodium* (13.1% of samples) and *Leucocytozoon* (6.3% of samples), with multiple parasite lineages identified as the most common ones *P. relictum* GRW11 and *P. relictum* SGS1 (in five bird species of each one), *H. attenuatus* ROBIN1 (in four bird species) and *L.* sp. BT2, *L.* sp. SATEC01 and *L.* sp. SFC8 (in three bird species of each one). Single infections (45.0% of all positive samples) were more common than double infections (4.6%). Particularly, differences in parasite prevalence were observed between long-distance (N=540; 55.7% infected) and short-distance migrants (N=138; 25.4% infected), suggesting ecological or behavioral factors involved. Furthermore, variations in infection rates among bird species (highest prevalence in icterine warblers with 57 of 58 samples positive; lowest prevalence in black redstart with 1 of 29 samples positive) indicated potential host-specific differences in distribution or exposure. Compared to age (yearling birds 43.9% infected; adults 39.5% infected) and sex (males 45.8% infected, females 47.5% infected) with no noticeable differences in parasite prevalence, there was a difference in the number of infections between the years (2016 with 40.25% of infected individuals; 2017 57.78% of infected individuals).

Among the bird species investigated, the barn swallow carried parasite lineages such as *Plasmodium* GRW02, GRW09, TURDUS1, and *Haemoproteus* DELURB1, consistent with prior studies conducted in Germany, Switzerland, and Italy (Von Rönn et al. 2015; Romano et al. unpub). Additionally, a new *Plasmodium* lineage, CUCROC01, was identified in our barn swallow sample, previously only found in the Madagascar cuckoo (*Cuculus rochii*) from Madagascar (Musa et al. 2019).

Similarly, the European robin revealed known parasites like *Plasmodium* TURDUS1, *Haemoproteus* PHSIB1, ROBIN1, and *Leucocytozoon* SFC8, identical to findings from Sweden, Lithuania, Russia, Germany, and Nigeria (Ellis et al. 2020; Hellgren et al. 2007). Here as well, some lineages were found the first time in the European robin, such as *Haemoproteus* lineage SYAT01, which was detected in species such as the Eurasian blackcap and garden warbler in Sweden (Ellis et al. 2020), common whitethroat in Slovakia (Šujanová et al. 2021) and other bird species not mentioned in this study; *Leucocytozoon* lineages BT5 which was detected in Eurasian blackcap samples in Germany (Santiago-Alarcon et al. 2011) and other bird species not mentioned in this study; and RECOB3, previous detected in Eurasian blackcap

in Spain (Pérez-Rodríguez et al. 2013), garden warblers in Sweden and Ukraine, common whitethroat in Sweden (Ellis et al. 2020) and other bird species not mentioned in this study.

The only positive blood sample of black redstart showcased a - for the bird species - new *Plasmodium* lineage, LK06, found in so far in common whitethroat in Sweden and Eurasian blackcap in Spain (Ellis et al. 2020; Bodawatta et al. 2020) as well as other bird species.

Furthermore, the common redstart presented previously documented parasites like *Plasmodium* BT7, SGS1, TURDUS1 in countries as Sweden, Armenia and Russia (Hellgren et al. 2007; Drovetski et al. 2014) and *Leucocytozoon* RS2, BT1 and BT2 as recorded in Sweden (Hellgren et al. 2007), alongside the *Haemoproteus* lineage, PFC1, detected in species like the pied flycatcher in Russia and Sweden, collared flycatcher (*Ficedula albicollis*) in Sweden and hawfinch (*Coccothraustes coccothraustes*) in Russia (Hellgren et al. 2007; Fletcher et al. 2019) but never in common redstart ever before.

Our whinchat samples identified once again already known lineages as *Plasmodium* GRW11 and SW2 from Turkey (Ciloglu et al. 2020), RTSR1 and SGS1 from Sweden (Ellis et al. 2020); *Haemoproteus* with ROBIN1 from Sweden and Nigeria (Ellis et al. 2020, Hellgren et al. 2007), and *Leucocytozoon* with REB7 from Nigeria (Hellgren et al. 2007). Furthermore, we found several new *Plasmodium* lineages for whinchat as AFR143, known from trilling cisticola (*Cisticola woosnami*) (Lutz et al. 2015) and collared sunbird (*Hedydipna collaris)* (Lauron et al. 2015); AFR146 in brown-crowned tchagra (*Tchagra australis*) (Lutz et al. 2015) and black-crowned tchagra (*Tchagra senegalus*) (Harvey & Voelker 2017); all of them sampled on the African continent. Other first time found lineages in whinchat are *Plasmodium* GRW10, already confirmed in pied flycatcher in Netherlands and Russia (Jones et al. 2018); SYAT24 in Eurasian blackcap from Spain (Hellgren et al. 2007) and other bird species not further mentioned in this study in both examples. The *Haemoproteus* lineage COLL2 was detected in pied flycatcher samples in Netherlands, Russia, United Kingdom, Sweden, Spain and Finland (Jones et al. 2018) as well as garden warblers in Spain and Sweden (López et al. 2013, Ellis et al. 2020) and other bird species, not discussed in this study. The *Leucocytozoon* lineage HIRUS07 is known from European robin in Sweden (Ellis et al. 2020), barn swallow in Germany (Von Rönn et al. 2015), Eurasian blackcap from Morocco (Bodawatta et al. 2020) and numerous other bird species.

Our study of the icterine warbler is consistent with other studies such as *Haemoproteus* HIICT1, HIICT2 and HIICT3 in Sweden, Nigeria, France and Germany (Ellis et al. 2020, Hellgren et al. 2007, Reullier et al. 2006); while we found for the icterine warbler new *Leucocytozoon* lineages as SATEC01, so far observed in some African bird species (Reullier et al. 2006; Cornuault et al. 2012) and SFC8, found in European robin in Armenia, Portugal and Germany (Drovetski et al. 2014, Strehmann et al. 2023), spotted flycatcher in Nigeria

(Hellgren et al. 2007), black redstart in Sweden (Ellis et al. 2020), Eurasian blackcap in Portugal and Sweden (Drovetski et al. 2014, Ellis et al. 2020), garden warbler in Portugal (Drovetski et al. 2014) and other other bird species not further mentioned in this study

The common whitethroat of our study samples revealed for their species known parasites such as *Plasmodium* GRW11 found in Russia (Križanauskienė et al. 2006), LK06 as well as *Haemoproteus* CWT11, CWT2, CWT3 and CWT4 in Sweden (Ellis et al. 2020); alongside with for common whitethroat newly identified *Plasmodium* lineages as GRW04, detected in barn swallows in Germany (Von Rönn et al. 2015) and other bird species on various continents, COLL7 known from pied flycatcher in Sweden and Spain (Kulma et al. 2013, Jones et al. 2018) and other bird species not mentioned in this study. As for *Haemoproteus*, we found for the first time in whitethroats the lineages ROBIN1, known in European robins, whinchats and Eurasian blackcaps from Sweden and Slovakia (Ellis et al. 2020); and lastly SFC3, known in pied flycatcher in Russia (Valkiūnas et al. 2008) and spotted flycatcher in Sweden (Ellis et al. 2020).

Our garden warbler samples confirmed the presence of previously reported parasites like *Plasmodium* AEMO01, GRW02, GRW11, RTSR1, SGS1 in Nigeria (Hellgren et al. 2013); *Haemoproteus* SYBOR01, ROBIN1 and *Leucocytozoon* BT2, SYBOR06 in Italy (Hellgren et al. 2007, Hellgren et al. 2013). Further we found in garden warblers *Plasmodium* pSW2, observed so far in species such as the whinchat in Turkey (Ciloglu et al. 2020), European robin and icterine warbler from Sweden (Ellis et al. 2020) as well as not further in our study mentioned bird species; and *Leucocytozoon* SATEC01, so far detected in African samples from Réunionand Gabon (Cornault et al. 2012, Loiseau et al. 2017).

The Eurasian blackcap is the only bird species of our samples without any new connections to lineages. The results match with previous studies, as for *Haemoproteus* SYAT01, SYAT02 and WW2 from Belgium, France, Spain and Sweden (Hellgren et al. 2007) as well as *Leucozytozoon* BT2, SFC8, SYAT22 from Sweden (Ellis et al. 2020).

Furthermore, the spotted flycatcher samples match with previous studies, documenting lineages like *Plasmodium* SGS1 in countries as Russia and Morocco (Hellgren et al. 2007, Droveski et al. 2014, Mata et al. 2015), GRW09 in Sweden (Ellis et al. 2020); *Haemoproteus* SFC3 in Sweden and Morocco (Hellgeren et al. 2007B, Mata et al. 2015, Ellis et al. 2020); and *Leucocytozoon* BT2 in Sweden (Hellgren et al. 2007), alongside with spotted flycatcher's new lineages as *Plasmodium* GRW11, detected so far in species as European robin in Portugal and Russia (Drovetski et al. 2014), pied flycatcher in Portugal (Jones et al. 2018), black redstart in Portugal (Drovetski et al. 2014, Mata et al. 2015), whinchat in Turkey (Ciloglu et al. 2020), Eurasian blackcap in Spain (Hellgren et al. 2007), garden warbler in Nigeria, Sweden, Ukraine (Hellgren et al. 2007), common whitethroat in Armenia (Drovetski et al. 2014) and other bird species not mentioned in this study. We found in spotted flycatchers also *Plasmodium* COLL7

and PLOPRI01, both known from pied flycatcher in Czech Republic, Hungary, Poland, Sweden (Jones et al. 2018) and other not further mentioned bird species; *Haemoproteus* ROBIN1, known from European robin in Germany (Strehmann et al. 2023), whinchat in Sweden (Ellis et al. 2020, Hellgren et al. 2007), and other bird species; HIICT1, known from icterine warbler in Sweden (Ellis et al. 2020) as well as whinchat and garden warbler from Nigeria (Hellgren et al. 2007). In our spotted flycatcher samples, we even detected for the first time *Leucocytozoon* SATEC01, till now known in birds from the African continent only (Cornuault et al. 2012, Loiseau et al. 2017).

Finally, our study of the Pied flycatcher aligned with prior research, showing infections with parasites such as *Plasmodium* BT7 in Finland, GRW11 in Spain, and *Haemoproteus* COLL2 in countries as Finland, Netherlands, Russia, Spain, Sweden, United Kingdom COLL3 in Finland, Russia, Spain, United Kingdom and PFC1 in Finland, Netherlands, Russia, Spain, Sweden, United Kingdom (Jones et al. 2018). In addition, we found new lineages in our pied flycatcher samples as for *Haemoproteus* HIICT1 previously found in icterine warbler samples from France, Belgium, Germany, Sweden, Russia (Reullier et al. 2006), garden warbler and whinchat samples from Nigeria (Hellgren et al. 2007), just to name a few. Further we found *Haemoproteus* HIICT3, already known from Swedish samples of icterine warbler and spotted flycatcher (Ellis et al. 2020), hSYAT01 as in Eurasian blackcaps from Sweden, Armenia, Morocco, Portugal, Russia (Ellis et al. 2020, Drovetski et al. 2014), garden warblers from Sweden (Ellis et al. 2020) and more not further in this study mentioned bird species; and as for *Leucocytozoon* CYAVER03 as only known in samples of green-headed sunbird (*Cyanomitra verticalis*) from Gabon (Loiseau et al. 2017), and SYAT22 found in Eurasian blackcaps in Sweden (Ellis et al. 2020) and other bird species not mentioned in this study.

Focusing from the bird species to the area studied - in our case Italy - we also recognise similarities with other studies. In *Plasmodium* we had identical results regarding lineage and host species in GRW02, GRW09, RTS1 and SGS1, in *Haemoproteus* in DELURB1 and SYBOR01 and in *Leucocytozoon* in BT2 and SYBOR06 (Romano et al. unpub, Hellgren et al. 2007, Hellgren et al. 2013). Lineages already detected in Italy, which were detected in our test material but did not correspond to our host species, were GRW11 and SW2 in *Plasmodium* and the lineage WW2 in *Haemoproteus* (Hellgren et al. 2007, Hellgren et al. 2013, Martínez-de la Puente et al. 2015, Marzal et al. 2011, Zehtindjiev et al. 2012).

With regard to the newly detected lineages - i.e. lineages that are similar to known species but differ in a few base pairs - there are similarities to known studies. For example, *Plasmodium* AEMO01(477/478) with a positive detection of garden warbler. As already discussed for AEMO01, there is evidence of this lineage in garden warbler from Nigeria (Hellgren et al. 2013). The situation is similar for *Plasmodium* SGS1(477/478a), SGS1(477/478b) and

SGS1(477/478c): The affected host birds of the three lineages, spotted flycatcher and common whitethroat, were also detected in SGS1 and thus coincide with the studies on spotted flycatcher in countries as Russia and Morocco (Hellgren et al. 2007, Drovetski et al. 2014, Mata et al. 2015) and common whitethroat in Sweden (Ellis et al. 2020). The situation is completely different for *Plasmodium* PBPIP1(476/478), in our case detected in common whitethroat, and known as PBPIP1 in different species from Africa to Asia and Europe (Hellgren et al. 2007, Gupta et al. 2019, Martinsen et al. 2008, Ellis et al. 2020) and whinchat with PLOCUC02(477/478) in several other bird species in the form of PLOCUC02 - all of them sampled on the African continent (Loiseau et al. 2017). In *Haemoproteus* a similar situation arises, both bird species of the new lineage, as well as the originally known lineage - and thus the discourse already carried out before - coincide in the case of pied flycatcher with COLL2(478/479) and common whitethroat with CWT11(477/478) and CWT2(477/478). So far, samples of common whitethroat such as *Haemoproteus* CWT10(462/463) with no record on MalAvi; on the other hand, CWT5(476/478) and CWT5(477/478), similar to the CWT5 samples from Russia (Hellgren et al, 2007B), were already mentioned. In the *Leucocytozoon* samples we found BT1(475/476) in European robin, similar to our samples BT1 from common redstart, and also spotted flycatcher with SATEC01(474/478), similar to the already discussed SATEC01 of the same bird species. In addition, there was a detection of RS3(475/476) in our study material of common redstarts, with RS3 known from samples from Sweden in common redstart as well as icterine warbler (Hellgren et al. 2007, Ellis et al. 2020).

In our investigations, 31 double and not a single triple infection was found. In comparison, depending on the population location, double infections were significantly more frequent in Piersma & Van der Velde (2012) (16% of positive samples from Spain, 28% of positive samples from Denmark). In the study sample of Hellgren et al. (2009), 80 individuals out of 1571 infected birds were infected with 2 or more parasites. Romano et al. (2019) found only 3 individuals with double infections in a total of 209 individuals (98 males and 111 females). The already high numbers of infections in the studies by Granthon & Williams (2017) also show considerable differences in their multiple infections. Especially in vireos (7 single and 55 multiple infections) and waxwings (56 single and 95 multiple infections), significantly more multiple infections were observed. Possible interactions between the different parasite species in the same host (Fecchio et al. 2020) were not part of our evaluations but could also be of interest for the question of parasite infestation and host superiority in future studies.

The results with regard to the individual bird species only coincide to a certain extent. In another study observing barn swallows, significantly fewer infections were found (27 of 575 adult barn swallows) (Von Rönn et al. 2015). Our results of the garden warblers on the other hand are in line with the observations of López et al. (2013), where 66.1% of 183 garden warblers had infections. As seen in our study, garden warblers were indeed more likely to carry

a blood parasite (47 infected individuals out of 113). Surprisingly, there are observations that garden warblers carry double infections with a very high probability (58.5% of the 183 individuals examined were single-infected) (López et al. 2013). This is in line with our research, which also found garden warblers with double infections.

We were able to find differences in our data set between the years 2016 (40.2% positive samples) and 2017 (57.8%). This is consistent with the research of Knowles et al. (2011), who also found fluctuations during their 4-year study on *Plasmodium* prevalence. Piersma & Van der Velde (2012) also found differences in the incidence of blood parasites in the different years of their studies. In most of the years studied, approximately 83% prevalence was found. It should be noted that the occurrence of parasite infections can depend very much on the season and the respective year (Norte et al. 2009). As Norte et al. (2009) noted, different years may well exert the negative effects of infection on, for example, the fitness of birds.

Not only the year but also the season plays a role in the occurrence of blood parasites in the sample material. Norte et al. (2009) found considerable differences (for example 0% prevalence of *Plasmodium* in winter 2004 and 2006 vs. 29.2% prevalence in spring 2005 or prevalence of *Haemoproteus* 0% in winter/autumn 2004 and winter 2006 vs. 27.3% in spring 2006 as well as *Leucocytozoon* prevalence with 4.2% in spring 2005 vs. 38.9% in spring 2003). The emergence of vectors depends on several factors, but studies have shown that there is a correlation with temperature, rainfall and/or water resources or better said the local habitat in general (Atkinson & Samuel 2010, Lachish et al. 2011a, Garamszegi 2011, Wood et al. 2007). It is important to mention that these external factors not only have an influence on the vectors but also on the different blood parasite species, because not every species reacts in the same way to the existing conditions (Lachish et al. 2011a). And even the different lineages of parasites can lead to different levels of prevalence (Wood et al. 2007).

Interpreting the data in relation to the age and sex of the birds was difficult based on the experimental design. As already mentioned, these factors play a role more often in a setting during the breeding period, less during the actual bird migration. While no correlation between parasite infestation and age/sex/fitness was found in our data, other studies suggest that this is the case. For example, a study on House Martins was carried out on 112 juveniles and 358 adults, which resulted in an observation of 0 infected fledglings but 77% infected adult individuals (Piersma & Van der Velde 2012). Another bird species, the great reed warbler, showed to suffer even less from infections during the wintertime, but being surprisingly often of chronic nature (Sorensen et al. 2016a). Juveniles, hatched in the previous summer season of the study, shown a minimally higher (but not significant) prevalence than adults. Granthon & Williams (2017) also failed to find a correlation between parasite infection probability and sex or age in any of the bird species studied. Norte et al. (2009) also came to the conclusion

that no correlation between age/sex and infection can be predicted. Other studies show that a gender difference in infections may well exist. In a study from Germany, it was observed that female carrion crows were more likely to be infected than their male counterparts (Schmid et al. 2017). The results from a Hawaiian study (Atkinson & Samuel 2010) points out that infections with bird malaria can lead to problems with local bird species in form of a mainour decrease of populations. In the resident apapane *Himatione sanguinea* we found a higher mortality in young birds (more than half of them) than in adults (more than 25%). It's conferred by studies of Garcia-Longoria et al. (2015) with house martins *Delichon urbica* that the transmission of haemosporidian parasites can even happen in Europa, before juvenile birds even reach their winter quarter in Africa. The models of Lachish et al. (2011a) show that infections in Eurasian blue tits *Cyanistes caeruleus* can vary in sexes. But even in this case there are differences in the clade of *Plasmodium* causing a more prominent infection-sex-influence (males show greater infection rates) in one (*P. circumflexum*), and an infection-age-influence (adults more likely to be infected than juveniles in a 12-months period) in the other clade (*P. relictum*) (Lachish et al. 2011a). One could have assumed a connection between the sex of the birds and the infection, as the infection can also have an effect on the migratory behaviour of males and females or delay their arrival in the north. However, old studies show that the delays during migration itself are small. The Cornet et al. (2013) studies not only controlled whether mosquitoes target infected birds more frequently than uninfected birds, but also whether this effect could be related to body condition. As in our study, no difference in body mass was found between healthy and infected birds. Similarly, Cornelius et al. (2014) found no evidence of a relationship between infection status and body condition/fat. For example, the comparison of the studies of by Piersma & Van der Velde (2012) and Marzal et al. (2008) show that correlations between infection and body mass as well as survival existed in the population studied in southern Spain but could not be observed in the Danish population. In studies conducted over several years, it is clear that the chance of acquiring a blood parasite infection from one year to the next is 85%, while loss of infection is a rather rare event at 5%. Only 23% of the individuals studied by Piersma & Van der Velde (2012) showed no change in status. Wood et al. (2013) also reported that infections can clear, but again it is the exception. For example, if birds are exposed to predators more frequently, this has a negative impact on their fitness and can thus lead to more frequent parasite infection than, for example, control groups that have not been exposed to predators (Navarro et al. 2004). Another parameter is the fitness of the bird. Although chronic infections are often seen as a non-first-order health problem for birds, studies show that these infections do have a negative impact on fitness (Knowles et al. 2010). As already mentioned, there are also different degrees of effects depending on the parasite species (Lachish et al. 2011b). Several studies suggest that the likelihood of a bird carrying a blood parasite increases over the course of a bird's life and that

adult birds are more often infected than young birds (Marzal et al. 2016, Piersma & Van der Velde 2012, Allander & Bennett 1994). But the opposite, i.e., a more frequent infection of the young birds, can also occur, as the data of Van Oers et al. (2010) prove. However, it should be noted that some study found no connection whatsoever between age and infection (Norte et al. 2009). A cross-studies pattern regarding parasite infestation and age of the host animal can therefore not be identified. In these cases, the occurrence of parasites in adult and juvenile birds certainly depends on several factors - starting with the respective bird species, the parasite species and the region investigated. In the present study, the body condition of infected birds did not differ significantly from that of non-infected individuals, independently of age or sex. It is possible that there is no correlation in our data because bird migration is very stressful and energy-consuming and therefore the weakened individuals have already been eliminated. This applies to individuals of all ages. This could also be a line of reasoning for the lack of correlation between infection and muscle and fat reserves, as only fit individuals start the migration.

Malaria infections can also lead to reduced survival (Van Oers et al. 2010, Lachish et al. 2011b, Wood et al. 2013, Dawson & Bortolotti 2000), especially in areas with increased incidence of malaria (Lachish et al. 2011b). One of the big questions is whether infections in the winter grounds or in stop-go places affect the arrival of migratory birds in Europe. It has been suggested that a serious infection could even prevent the birds from arriving in Europe because they succumb to their weakened health (Garcia-Longoria et al. 2015). We can see that migratory behaviour affects the probability of carrying a blood parasite. This is in line with our expectations. Species coming from sub-Saharan Africa are more likely to carry a parasite than birds that have wintered in the Mediterranean.

The studies of Hellgren et al. (2013) also show that the location as well as the time of year can lead to different parasite occurrences. In this study, garden warblers were examined for parasites in three different areas: sub-Saharan Africa (wintering grounds), Sweden (summering grounds) and the island of Capri in Italy (about 115 km south-east of Ponza), which is another good example of islands frequented during migration. Another example of investigations in the immediate vicinity is the data from Schumm et al. (2021). The island of Ventotene, located 41.8 km east of Ponza, was also able to detect blood parasites in blood samples from European turtle doves.

Furthermore, Maggini et al. (2020a) named the island as a potential sampling site to obtain information not only on bird migration but also on the population status of some species. This shows that migratory birds and especially long-distance migrants need further research to gain an overview of the occurrence of blood parasites. However, so many variables remain to be considered, especially contagion in winter areas or even stop-overs. Observation of migration

routes and weather phenomena of individual species or even populations could be used for forecasting. The fact that many of the studies discussed above deal with either single bird species or even single populations/breeding areas raises the question of whether islands such as Ponza should be considered more often as a study area for parasite infections in the future, mainly for the reasons that a large number of different species pass by and the birds are sampled during migration and not in their reproductive period.

The method according to Hellgren et al. (2004) again proved to be a practical tool because large sample sizes could be examined. Nevertheless, errors can occur. On one hand, it is well advised check blood smears and to run a second PCR. On the other hand, the analyses of blood smears are and remains very time-consuming and tedious. Nevertheless, it was essential for our study to use additional primer pairs and thus the procedure according to Harl et al. (2019). A good example of how difficult it might be to observe infections just by microscope can be the Granthon & Williams (2017) observations. Depending on the bird species the methods was useful, and therefor molecular tests is the tool of choice. If it is not the course of the disease but only the distribution of the parasites that is to be studied, field work will always be the means of choice. However, if the immune response and pathology of the infections need to be studied, laboratory experiments will continue to be important, or experiments on a small scale as with breeding colonies.

5. CONCLUSION

There is a dichotomy elucidated by the data in this investigation, based on the scientific hypothesis. If it were for the course of the disease but the distribution of the parasites that is to be studied, field work would always be the means of choice. However, if the immune response and pathology of the infections need to be studied, laboratory experiments will continue to be important, or experiments on a small scale as with breeding colonies. The topic is quite complex and cannot be discussed in detail with this large amount of data.

Therefore another idea for future projects would be whether migratory behaviour per se, for example migratory restlessness, changes as a result of infection. Projects with this focus are already known from Ponza and combining both topics would be quite exciting. When it comes to climate, climate change - the future spread of blood parasites must be taken into account even more. The weather and climate play a decisive role in the spread of vectors and so bird species or populations that have so far been spared may be endangered.

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LIST OF FIGURES

LIST OF TABLES