Genetic analyses of Haemosporidian parasites infecting captive and free populations of wild birds

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

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This thesis is the product of my work and the assistance and advice from others is fully acknowledged

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Merit González Olvera

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Abstract

Genetic analyses of Haemosporidian parasites infecting captive and free populations of wild birds.

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Haemosporidian parasites are a diverse group of protozoans whose definitive host and vectors are dipteran insects, and their intermediate hosts are vertebrates. The most common haemosporidian parasites found infecting birds are *Plasmodium* spp., *Haemoproteus* spp. and *Leucocytozoon* spp., from which the first one is the most pathogenic. *Plasmodium* spp. produces a disease known as avian malaria, which has been one of the main causes of mortality for penguins kept in captivity. The genetic diversity of *Plasmodium* parasites was evaluated from mosquitoes, captive-wild birds and free-wild birds during 2017 in Chester Zoo. In this period, the Humboldt penguin colony of Chester Zoo experienced an avian malaria outbreak. It was observed that three main species were circulating in the area, *P. relictum*, *P. vaughani* and *P. matutinum*. They were found in the mosquitoes and wild birds; however, only *P. matutinum* infected penguins and was associated with their mortality. The distribution of *Plasmodium* parasites is cosmopolitan; however, for some regions of the world available information is limited. One of these understudied regions is the Peruvian Amazonia. With the aid of Yagua hunters from the Nueva Esperanza community, located in the Yavarí riverbank, blood samples were collected in FTA® cards or filter paper. DNA was extracted and PCR was performed with a nested protocol for haemosporidian parasites. Statistical analyses were done to look for significant differences in parasites prevalence by bird species and year. Cluster analysis and phylogenies were made to determine parasite species and lineages. *Leucocytozoon* spp. was not present in the samples and *Plasmodium* spp. and *Haemoproteus* spp. were found in 5.2% and 64.5% of the birds, respectively. Significative differences in parasite prevalence were found by bird species but not by collection year. New lineages and possibly new haemosporidian species were documented. Diagnosis of haemosporidian parasites is challenging due to the characteristics of their life cycles and may be avoided to prevent additional stress to sick birds. An alternative source to blood for detecting haemosporidian parasites is required. Feathers have been used in to diagnose viral diseases that directly affect the feather, and it has been proven that DNA can be extracted from them. Feathers from multiple sources were collected; DNA from feathers was extracted, quantified, and tested for haemosporidians. The three most common haemosporidian parasites were detected from the feathers of multiple bird species. Factors affecting the detection of haemosporidian parasites were the storage temperature and time from collection to analysis of the sample, the amount of DNA extracted and the bird´s body weight, as it directly affects DNA yield. Haemosporidian parasites represent bird conservation threats, losses to poultry industry and emerging diseases. Continuous surveillance of these parasites is needed to better understand their behavior and further improve prevention and treatment protocols.

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CHAPTER 1

GENERAL INTRODUCTION

1.1. Common haemosporidians: *Haemoproteus* spp., *Plasmodium* spp. and *Leucocytozoon* spp.

Haemosporidians (*Phylum Apicomplexa*, Order Haemosporida) (Borner, 2016) are a diverse group of vector-transmitted blood parasites (Ishtiaq, 2017) with an intermediate life stage infecting all major vertebrate taxa, except fish (Levin, 2009; Huang, 2018). The haemosporidian genera more frequently found infecting birds are *Haemoproteus*, *Leucocytozoon* and *Plasmodium* (Atkinson C, 1991, Valkiunas G, 2005); they are globally distributed in domestic and wild species (Ishtiaq, 2017) and to date, they have been reported in 2,043 bird species (MalAvi, 2021). Haemosporidian infections range from asymptomatic to severe and potentially fatal depending on the parasite species and lineage, and bird species involved. In general, juveniles and immunologically naïve birds are highly susceptible to infection, with the following disease resulting in mortality at their first exposure. Birds that survive the acute phase become chronically infected and may act as reservoirs of infection to vectors and susceptible birds (Chaisi, 2019).

The extent of damage produced by haemosporidians on their bird hosts is variable (Knowles, 2009; LaPointe, 2012). The diseases produced by *Plasmodium* spp., *Haemoproteus* spp., and *Leucocytozoon* spp. are known as avian malaria, haemoproteosis and leucocytozonosis respectively. Some of these parasites are a main concern for the poultry industry (Valkiunas G. 2005; Lee, 2016), some have affected the health of captive bird populations at zoos and rescue centers (LaPointe, 2012), some are considered as conservation threats to endangered bird species

(Bunbury, 2007; Chaisi, 2019), and some have even been declared as emerging infectious diseases (Derraik 2008; Sijbranda 2017). The most devastating effects of these parasites were observed when they caused the extinction and decline of multiple endemic Hawaiian bird populations (Atkinson, 2008; Foster, 2007; Ortego, 2008; Rock, 2012; Alley, 2008).

Haemosporidian parasites are transmitted by dipteran vectors of different families; *Plasmodium* is vectored by mosquitoes (Culicidae), *Haemoproteus* is transmitted by flies of the Ceratopogonidea and Hippoboscidae families, and *Leucocytozoon* vectors belong to the Simuliidae family (Evans, 1998). Life cycles of haemosporidians are composed of two phases, the merogony that occurs in birds and the sporogony, which happens in the dipteran vector (Njabo, 2011). The merogony comprises three stages. In the first stage, the exoerythrocytic merogony, sporozoites are inoculated into the birds´ blood stream when the vector feeds, the parasites undergo a first cycle of schizogony in either hepatocytes or endothelial cells of capillaries producing meronts. These meronts undergo multiple cycles of schizogony forming mononuclear cells called merozoites, following generations of merozoites turn into metacryptozoites and phanerozoites which infect reticuloendothelial cells of various tissues (Borner, 2016; Grilo, 2016). At the second stage, the erythrocytic merogony, merozoites can either undergo multiple cycles of exoerythrocytic merogony, or develop into gametocytes in erythrocytes or leucocytes (Borner, 2016). At the third stage, the secondary exoerythrocytic merogony, gametocytes reproduce in the vector's gut producing an ookinete which matures into an oocyst, as the oocyst develop sporogony takes place and sporozoites are produced; the sporozoites migrate to the salivary glands to be inoculated in the mosquito´s next blood meal (Borner, 2016; Grilo; 2016) (Figure 1.1).

Key aspects to differentiate between haemosporidians are as follows. *Plasmodium* spp. is the only one that forms erythrocytic meronts (multinucleated cells) (Grilo, 2016). Haemosporidians infect amongst other tissues peripheral red blood cells of their vertebrate hosts; nevertheless, only *Leucocytozoon* spp. infects leucocytes

(Fallon, 2003). Lastly, with the exception of *Leucocytozoon*, haemosporidians form a characteristic pigment in the red blood cells called hemozoin, which is a metabolite from hemoglobin digestion (Borner, 2016).

Infection in birds by haemosporidians consists of different periods. A brief acute period is characterized by the brief appearance of parasites in the blood, with a distinctive sharp increase of parasitaemia; this is a critic stage because mortalities are often observed at this time (Asghar, 2011) and it can occur either on a new infection or in a relapse of a previous infection. A crisis period is when parasitaemia reaches its peak. After the development of the initial blood stages, a prepatent period presents. If individuals survive the acute phase, a long-term chronic period develops, in which parasites persist at low density controlled by the host´s immune system. Lastly, the latent period is observed when the parasitaemia has a sharp decrease and is subsequently removed by the host´s immune system (Valkiūnas, 2005).

1.2. Haemosporidian species and lineages

Currently there are more than 250 named species of avian haemosporidians from the genera *Haemoproteus*, *Leucocytozoon*, and *Plasmodium* (Fecchio, 2018b). Originally, the first descriptions of haemosporidian species at the end of the 19th century were based on morphological characters of blood stages (Valkiūnas, 2014; Ishtiaq, 2017) and under a strict host specificity assumption, the host species was used to distinguish between similar morphological parasites (Galen, 2018b). However, now it is known that avian haemosporidians have a highly variable host range (Huang, 2018) making that distinction obsolete.

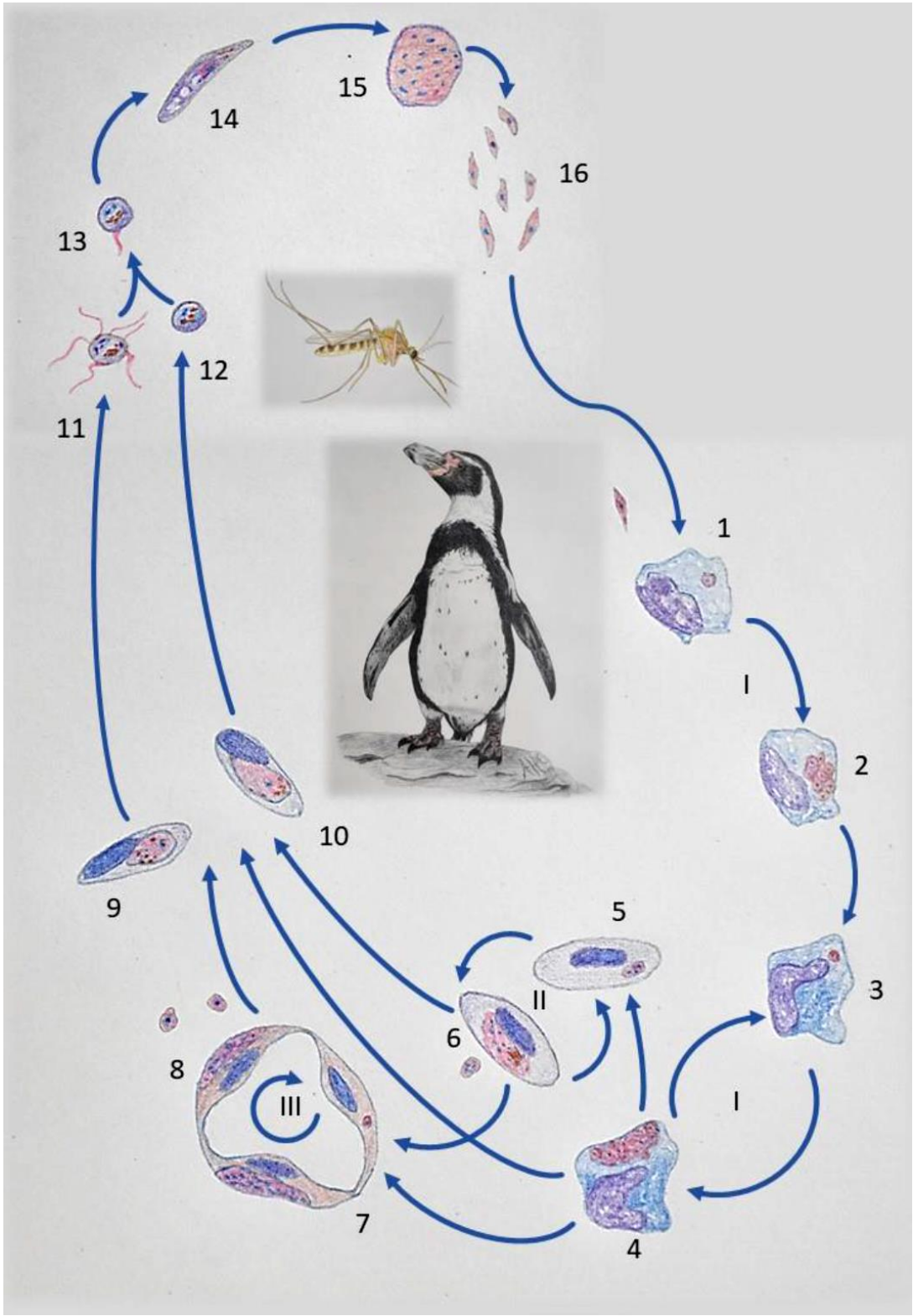

Figure 1.1 Life cycle of avian haemosporidians. Stages: I – primary exoerythrocytic merogony; II – erythrocytic merogony; III – secondary exoerythrocytic merogony; 1 – sporozoite invading a reticuloendothelial cell; 2 – cryptozoite; 3 – merozoite invading a reticuloendothelial cell; 4 – metacryptozoite; 5 – merozoite invading an erythrocyte; 6 – erythrocytic meront; 7 – merozoite invading an endothelial cell; 8 – phanerozoite; 9 – microgametocyte; 10 – macrogametocyte; 11 – exoflagellation of microgametes; 12 – macrogamete; 13 – zygote; 14 – ookinete; 15 – oocyst; 16 – sporozoites. Taken from Hernández-Colina (2019).

The recent molecular approaches to study haemosporidians had led to the establishment of lineages, although there has been no universal guidelines for this purpose; for instance, some authors define a lineage based on a difference of one nucleotide, while others define it by differences of three or more nucleotides (Chaisi, 2019). Current molecular approaches use the cytochrome b (*cytb*) as a species identifier (Galen, 2018b) and as a lineage barcode. On this basis, over 2,000 unique haemosporidian lineages have been described around the world (Ishtiaq, 2017). Numerous phylogenetic studies have been carried out in an effort to determine haemosporidian lineages and their relationships. Most of which, are based on a single gene (*cytb*) (Field, 2018) and therefore, lack the necessary depth to solve this query. Multi-gene approaches have also been done, resulting in well-resolved phylogenies, but with unresolved relationships for some species and even genus (Field, 2018). The vast majority of recent studies focus solely on molecular data, due to its relative ease of use, leaving morphological characteristics aside, which has produced a mismatch between species and lineages (Lotta, 2019). Furthermore, this situation has also generated the challenge of the validity of the morphospecies concept under the hypothesis that several of them represent cases of cryptic speciation, in which species with the same phenotype have different genotypes, thus making them different species (Ortego, 2008).

The genus *Plasmodium* is divided into five subgenera, *Bennettinia, Novyella, Haemamoeba, Giovannolaia* and *Huffia* (Hernández-Lara, 2018). A unique feature in this genus is the presence of schizogony in erythrocytes (Borner, 2016). Currently, there are over 60 species and 900 recognized lineages of avian *Plasmodium* (Zélé, 2014b; Ilgunas, 2016). Nonetheless, DNA sequencing of some avian malaria morphospecies has found them to be composed of a number of independent evolutionary units, suggesting that this group is formed by a larger number of species than previously recognized (Ortego, 2008).

The genus *Haemoproteus* is currently formed by two subgenera: *Parahaemoproteus* and *Haemoproteus*; these subgenera share the production of hemozoin, undertaking of merogony in endothelial tissue cells and development of

gametocytes after invading erythrocytes (Field, 2018). However, they differ in the range of birds they infect and in the insects they are vectored by; while parasites in the subgenus *Parahaemoproteus* are transmitted by biting midges of the Ceratopogonidae family and infect birds of various species, parasites in the *Haemoproteus* subgenus are transmitted by hippoboscid flies of the Hippoboscidae family and mainly infect birds of the order Columbiformes (doves and pigeons) (Field, 2018; Hernández-Lara, 2018). In spite of this, and the occasional recovery of these subgenera in separate clades within phylogenies (Galen, 2018), it has been suggested to consider them as different genus (Hernández-Lara, 2018). To date over 150 species of the genus *Haemoproteus* are recognized (Ilgunas, 2019) and about 1148 *cytb* lineages have been reported in the MalAvi database, from these lineages 97.4% are found within the *Parahaemoproteus* subgenus and only 30 in the *Haemoproteus* subgenus; interestingly, 16.6% of those 30 lineages belong to one species (*H. columbae*) (Field, 2018).

Leucocytozoon is the least studied haemosporidian; it comprises two subgenera, *Leucocytozoon* and *Akiba* (Hernández-Lara, 2018). To date 50 species of the genus *Leucocytozoon* are known along with 749 lineages (Fecchio, 2018b); however, that number is possibly underestimated due to the low sensitivity of PCR detection methods towards certain *Leucocytozoon* species (Lotta, 2019). Additionally, not much molecular research has been done on this genus, to the point that no genome or transcriptome are available yet (Field, 2018). Major biological traits present only in this genus, and that are used for classification are that its gametocytes undergo schizogony in white blood cells and that is vectored by black flies (Simuliidae) (Murdock, 2015); with one known exception, the species *L. caulleryi* belonging to subgenus *Akiba* is transmitted by biting midges of the Ceratopogonidae family (Walther, 2016).

1.3. Haemosporidians geographic distribution

The distribution of avian haemosporidians differs by genus and among geographical regions (Holarctic, Ethiopian, Oriental, Australian, Neotropical and Antarctic). The spread of these taxa is influenced by a dynamic interplay among hosts (intermediate and definitive) and their environment. The abundance, richness and distribution of birds and vectors regulate the transmission rates of haemoparasites and can promote their diversification (Doussang, 2019). For many taxa, high levels of diversity typically occur in biogeographic 'hotspots', which are generally centered on tropical regions in low latitudes (Chaisi, 2019). In addition to climate and latitude, landmass also regulates species diversity with island communities often exhibiting reduced diversity compared to continental regions (Clark, 2014).

Lineage diversity of *Haemoproteus* (n=1788), *Plasmodium* (n=1381) and *Leucocytozoon* (n=1314) found worldwide by molecular methods is remarkable (Moens, 2016b) with 4483 unique lineages recorded so far in the MalAvi database (MalAvi, 2021), a public database specialized in avian haemosporidian parasites of these three genus (Bensch, 2009). Biogeographic patterns of haemosporidian parasites may reflect the evolutionary and ecological host-parasite interactions and how they can influence parasite diversity (Clark, 2014); nevertheless, there are still many areas where not enough sampling has been carried out (Bell, 2015), and most of the work has been done on one genus, *Plasmodium*.

Parasites of the genus *Plasmodium* are of global distribution, they can be found on all continents, except Antarctica (Ilgunas, 2016), with varying prevalence across geographical regions (Svensson-Coelho, 2013). Their diversity is greater in tropics, but some *Plasmodium* species are actively transmitted in temperate climates and it has recently been reported in the high arctic tundra (Valkiunas, 2017). *Plasmodium* parasites are rarely found in cold areas probably because of the absence of suitable vectors and an air temperature too low for completing their sporogonic development (Valkiunas, 2017; Fecchio, 2017b). It has been demonstrated experimentally that *Plasmodium* spp. development halts below 13°C (Svensson-

Coelho, 2013) which should particularly affect its development in its ectothermic vector. *Plasmodium* spp. prevalence in the arctic tundra is considerably low in comparison with other haemosporidians. Additionally, in the arctic tundra *Plasmodium* spp. was found almost exclusively in migrant birds and it was found only in one resident bird by PCR, and given that this test does not indicate the life stages of the parasite, this bird was not proven to be a competent host (Oakgrove, 2014).

Plasmodium spp. is probably the most widely dispersed haemosporidian parasite, finding it even on island ecosystems such as Hawaii, French Polynesia and New Zealand; however, its presence in these sites is believed to be the consequence of anthropogenic introductions (Clark, 2014)**.** Despite its cosmopolitan distribution, some *Plasmodium* species seem to be restricted to small areas, whereas others are widely spread. The latter is the case for *P. relictum* (Figure 1.2); moreover, not only this species but some of its lineages (SGS1, GRW11, and GRW4) are widespread globally (Hellgreen, 2015). The most dispersed *P. relictum* lineage, GRW4, is frequently recorded in migratory bird species (Hellgreen, 2015), which might help in the spread of this lineage (Doussang, 2019).

The genus *Haemoproteus* is distributed worldwide in temperate and tropical habitats (Valkiunas 2005) and its prevalence varies across different regions (Svensson-Coelho, 2013). For the majority of the zoogeographical regions, *Haemoproteus* species richness is higher in comparison to other haemosporidians (Clark, 2014). This genus also reports species and lineages apparently restricted to small areas, and species and lineages with broad distributions, like *H.syrnii* (Figure 1.3). Interestingly, in contrast to *Plasmodium*, this genus has little presence on islands; one possible explanation is that this genus has not been as widely introduced by human activities as *Plasmodium*, and their vectors have not been able to colonize ecosystems isolated from mainland (Clark, 2014).

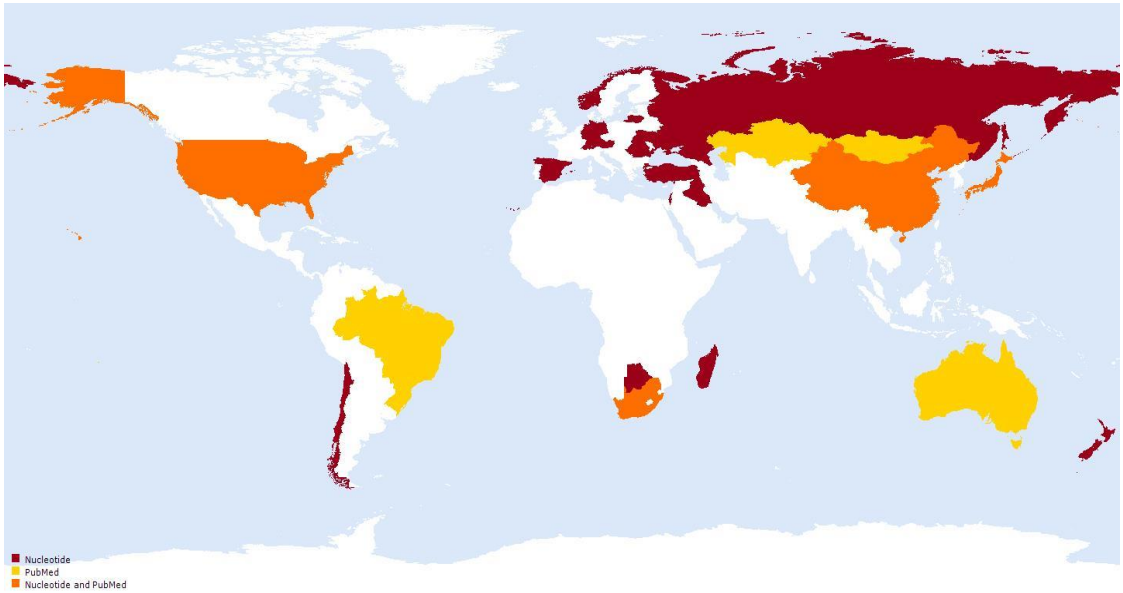

Figure 1.2. *Plasmodium relictum* distribution according to the EID2 database (Wardeh, 2015). Based on genetic data.

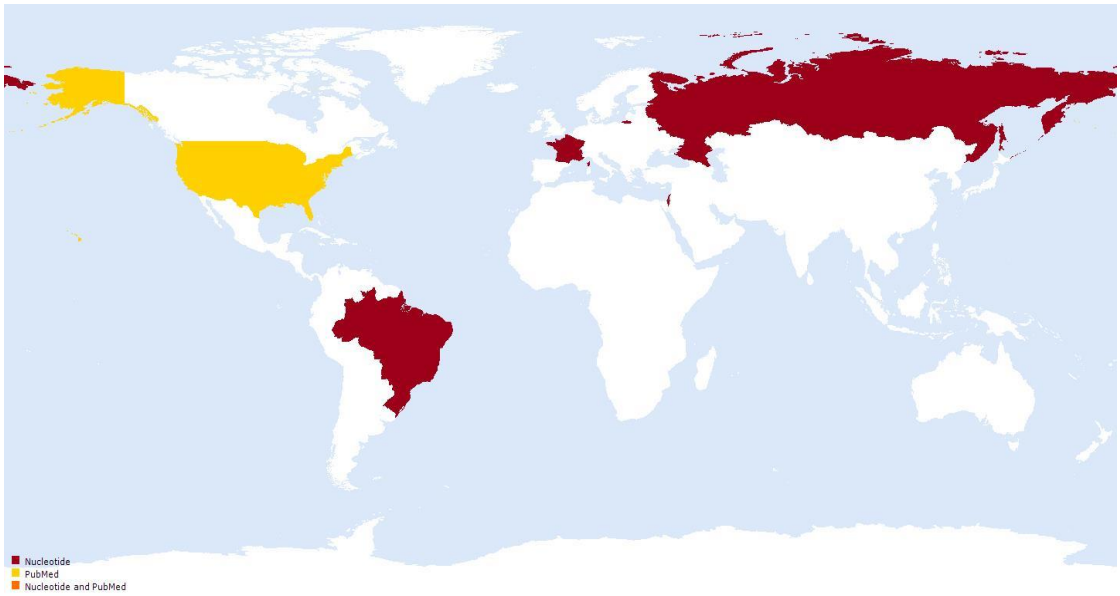

Figure 1.3. *Haemoproteus syrnii* distribution according to the EID2 database (Wardeh, 2015). Based on genetic data.

Leucocytozoon spp. have been reported worldwide (Murdock, 2015) with the exception of Antarctica in a wide variety of birds (Walther, 2016). As observed with other related parasites, some common species and lineages of *Leucocytozoon* have

wide geographic ranges (Murdock, 2015), such as *L. lovati* (Figure 1.4), whereas others seem restricted to specific areas. It has been suggested that high temperature represents a constraint on the development of these parasites, since their prevalence and diversity in tropical climates is scarce (Matta, 2014; Fecchio, 2018b; Fecchio, 2020), but is the most prevalent and genetically diverse haemoparasite in the arctic tundra (Oakgrove, 2014). For instance, in the Neotropics its prevalence ranges from 0.06 to 4.6% (Lotta, 2019) and it has only been reported in sites with low annual average temperatures (Fecchio, 2018b). Moreover, a latitudinal gradient for its prevalence in South America has been proposed (Matta, 2014), implying higher diversity and higher probabilities of infection at higher latitudes where temperature drops (Fecchio 2020). Similarly; it has been reported that *L. quynzae* is transmitted at a 3,155 masl altitude, where temperatures range from 0 to 14°C (Matta, 2014).

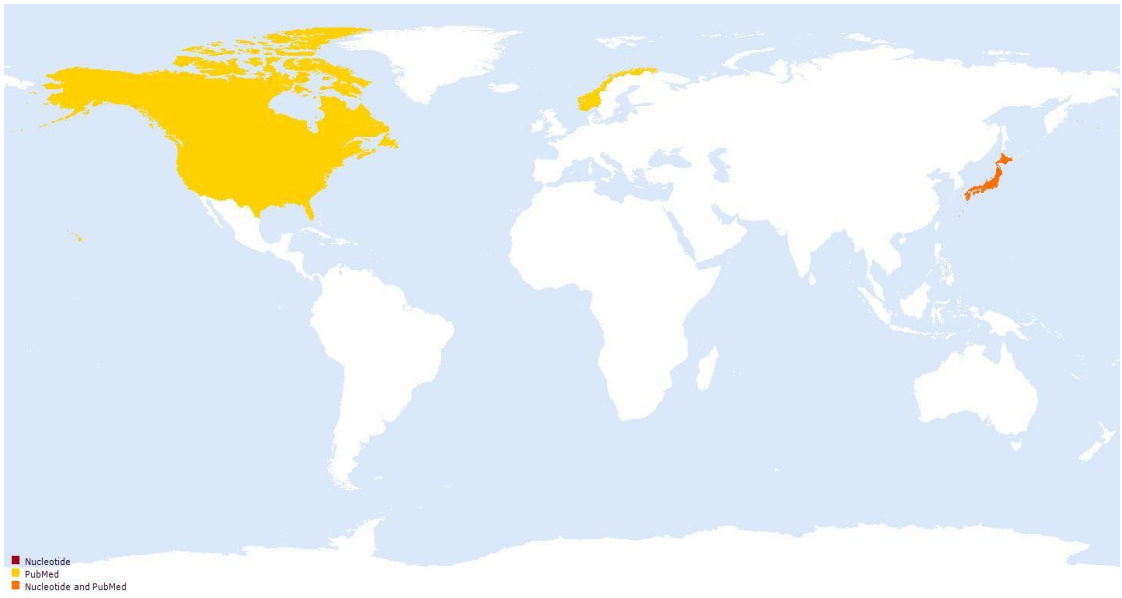

Figure 1.4. *Leucocytozoon lovati* distribution according to the EID2 database (Wardeh, 2015). Based on genetic data.

1.4. Haemosporidians host specificity

Host specificity is the extent to which a parasite colonizes a single or a broad range of host species, and may be reflected in the prevalence and infection intensities in each of its available host species (Huang, 2018). Usually, multiple species of haemosporidian parasites coexist locally and exhibit varying levels of host specificity, ranging from infecting a single host to several distantly related hosts (Fecchio, 2017b). Parasites capable of infecting diverse host species are considered to be generalists, whereas specialist parasites infect a single or a narrow range of hosts. A generalist parasite may become more specialized on a subset of their host species resulting in these hosts displaying higher infection intensities and prevalence than the others (Huang, 2018). This has been observed in different lineages of *H. majoris* (PARUS1, WW2, PHSIB1) which were found infecting birds from 9-19 different families; however, their prevalence was significantly higher in one family or a couple of bird species (Huang, 2018). Over time, host-parasite relationships can co-evolve towards a higher tolerance and moderated immune response of the host against the microorganism, avoiding inappropriate inflammatory reactions and allowing hosts to carry parasites without severe pathological signs. (Sijbranda 2017)

Host specificity of avian malaria parasites differs greatly both among and within parasite genera (Moens, 2016b). Comparison of avian host and parasite phylogenies indicates that closely related haemosporidian parasites may be found in distantly related host species and distantly related parasites can share a single host species (Njabo, 2011). Overall avian malaria parasites are considered as generalists (Huang, 2018); hence it is suspected that they have diversified mainly by host-switching (Moens, 2016b, Doussang, 2019). Several lineages of *Plasmodium* show extreme generalist host-parasitism (Hellgreen, 2015, Doussang, 2019), while others appear to be restricted to particular host families over recent evolutionary history (Zélé, 2014b; Doussang, 2019). The best example of low host specificity in this genus is found in the lineage GRW4 of *P. relictum*, which has been recorded across different geographic regions (Clark, 2014) in 59 bird species in 18 families (Hellgreen, 2015).

In contrast to what has been observed for *Plasmodium* parasites, high host specificity has been recorded in *Haemoproteus* lineages due to their vector specialization on ceratopogonid and hippoboscid flies (Doussang, 2019). Nevertheless, recent research by Moens (2016b) established *H. witti*, a species that was previously only known in hummingbirds, as the greatest generalist of the genus after recording this parasite in 36 species of 11 bird families. However, these results should be taken with caution, since they are based on PCR results from which the presence of the parasite´s life stage transmissible to the vector cannot be confirmed; thus, it cannot be used to determine host viability. Moreover, the highest prevalence of this parasite was observed in hummingbirds (Trochilidae), and gametocytes and high infection intensities were only observed in these birds.

The case for *Leucocytozoon* spp. is similar, with different studies describing varying infection rates in different bird families (Lotta, 2019). The same pattern is observed in *Leucocytozoon cytb*-based lineages, which reflect varying degrees of host specificity; some lineages are found consistently only in one bird species, family or order (Walther, 2016), whereas other lineages develop successfully in a wide range of taxonomically varied hosts (Murdock, 2015).

1.5. Host susceptibility

Hosts present different levels of susceptibility to haemosporidian infections which translates into pathogenicity variation (Sorci, 2010). Mainly, host susceptibility is determined by factors that influence the immune response (Calero-Riestra, 2016), such as age (Ishtiaq, 2017), species, sex (Lachich, 2011), physiology, body condition and reproductive effort (Ortego, 2008).

The immune system matures as birds age (Sorci, 2010), causing a decline in infection probability with host age (Knowles 2011). Concerning sex, the immune response triggered on males and females is of a different type as a result of the endocrine-immune interactions; while testosterone favours cell-mediated

immunity, oestrogen boosts humoral immunity (Calero-Riestra, 2016). Likewise, it has been observed that behavioural differences between sexes (i.e. parental investment) affect the trade-off of energy used for the immune response (Ortego, 2008).

The onset of avian malaria infections on susceptible hosts is known to be driven by stress (Alley, 2008) and possibly it is benefitted by the synchronized breeding season, which generates periodic recruitment of immunologically naive juveniles (Lachish, 2011). Numerous bird species are known to be highly susceptible to these parasites such as Passeriformes and captive Sphenisciformes; whereas Psittacines, Charadriiformes, Pelecaniformes, and Procellariiformes seem to be rarely infected. A number of possible explanations for the latter have been suggested, such as a limited number of vectors in the bird´s habitat, a highly specific host-parasite association, and the intrinsic immune response of each bird group (Quillfeldt, 2011). Although, this can simply be related to the sampling effort done on the different taxonomic groups; for instance, Passeriformes is the most abundant taxonomic group and most of the studies on haemosporidian prevalence are usually dominated by this order. Reports of deaths by *Plasmodium* spp. have been done on puffins (*Fratercula arctica*) (Meinster, 2021), mohuas (*Mohoua ochrocephala*) (*Alley, 2008)* and kiwis (*Apterix* spp.) (Banda, 2013); additionally, these parasites have caused the decline and extinction of Hawaiian honeycreepers (Drepanidinae) and they have threatened the survival of the Hawaiian crow (*Corvus hawaiiensis*) (Alley, 2008).

Likewise, host susceptibility to *Haemoproteus* parasites is variable, with the effects of each species on its host varying widely; while some species cause severe anaemia and death, others persist in barely detectable chronic infections (Svensson-Coelho, 2013; Field, 2018). A well-established association is that of *H. minutus* and parrots (Psittaciformes), which is often lethal (Ilgunas, 2019). More recently lethality reports caused by *H. velans* in woodpeckers (*Dryobates albolarvatus*) have been described (Groff, 2019).

Leucocytozoon parasites have been commonly found on diurnal raptors (*Falco sparverius sparverius*) with high prevalence and parasitaemia (Walther, 2016). Cases of mortality in owlets (*Bubo virginianus*) infected with *L. danilewskiy* have been observed (Niedringhaus, 2018), and survival of the Pink pigeon (*Columba mayeri*) has been reported to be affected by infections with *L. marchouxi* (Bunbury, 2007). This genus is the most understudied within the haemosporidian group; hence, data on host ranges and susceptibility is lacking, though recent efforts are being made to collect this information (Walther, 2016).

1.6. Haemosporidians pathogenicity

The pathogenic effects of haemosporidians have been recorded on domestic and captive avian populations (Bunbury, 2007); nevertheless, they affect the health of their hosts differently (Knowles SCL. 2009, LaPointe DA. 2012). Previously, it was believed that long-term haemosporidian chronic infections produced little to mild effects on fitness; however, it has been seen lately that these infections can produce important hidden costs for hosts. Additionally, the frequency of reports detailing the adverse effects caused by these parasites is increasing (Calero-Riestra; 2016). In general, the pathogenic effects from haemosporidians are mainly caused by two life stages, merozoites and phanerozoites, and partly as collateral damage from the host´s immune response. Merozoites reproduce in erythrocytes producing a regenerative haemolytic anaemia due to the replacement of infected erythrocytes with immature erythrocytes, and as a result of the metabolization of haemoglobin, they produce an increase of biliverdine, which is excreted in faeces. The phanerozoites invade capillary vessels of various organs (heart, lung, liver, spleen, brain and kidneys) blocking them partially or totally, producing fugue of plasma, proteins and consequently oedema and haemorrhages. The immune response triggered by these parasites can generate a severe inflammation turning in hepatomegaly, splenomegaly, cardiomegaly and nephromegaly (Valkiūnas, 2004).

It used to be thought that most birds infected with *Plasmodium* spp. were not significantly affected by it (Jeffrey, 2007); although this assumption was made from netting birds with apparent good health that are generally at the chronic (relatively benign) stage of infection with low levels of parasitaemia (Ishtiaq, 2017). Likewise, as deaths from wild birds were not observed regularly, the disease was not considered to be severe (Valkiūnas, 2005). Nevertheless, finding damage from a disease in wildlife is challenging because there are limited opportunities to observe the course of the disease and it is difficult to detect sick birds in the wild and similarly, carcasses are easily removed by scavengers reducing the chances to record them (Valkiūnas, 2005); hence, rates of mortality caused by malaria infections in wild birds is unknown (Ilgunas, 2016).

On individual bases, detrimental effects observed from *Plasmodium* infections include reduction on packed cell volume, reduction on host body condition (Podmokla, 2014), reduced lifespan, and in chronically infected birds reduced number and quality of offspring (Ortego, 2008; Knowles, 2009; LaPointe, 2012; Sijbranda, 2017). The majority of these effects are possibly associated with an energetic resource reallocation to fight off infection (Ortego, 2008). On a population scale, *Plasmodium* parasites have produced important avian malaria outbreaks in zoos, rescue centres (LaPointe, 2012) and susceptible wild populations. They have induced mortality in common European birds (Ilgunas, 2016), limited the population distribution and even caused the extinction of certain avian species (Jeffrey, 2007). Some species are known for their high mortality rates (i.e. *P. relictum*) (Grilo, 2016). Pathogenic species have been associated with the decline and death of native and endangered avian species in Hawaii, New Zealand, Australia and the Galapagos Islands and are therefore a conservation threat, especially in captivity where animal densities are high (Alley, 2008; Chaisi, 2019). In New Zealand, the recent introduction of *Plasmodium* parasites, have resulted in morbidity and mortality in several native bird species, and avian malaria has been declared as an emerging disease (Sijbranda 2017) (diseases whose geographic range, host range, or prevalence have increased in recent years) (Derraik 2008). Although, the most devastating effect was observed on naïve populations of

Hawaiian wild birds, (Atkinson, 2008; Foster, 2007; Ortego, 2008; Rock, 2012), from which numerous endemic Hawaiian honeycreepers species went extinct and others declined drastically (*Alley, 2008*).

The disease produced by *Haemoproteus* infection is known as haemoproteosis (Ishtiaq, 2017). Compared to avian malaria, this disease has been little studied; however, fitness reduction (Levin, 2009), negative effects on reproductive activities (Tomas, 2007) and reduced survival of wild birds (Martínez-de la Puente, 2010; Marzal, 2008) have been associated to it. Additionally, numerous well-documented cases of severe haemoproteosis in non-adapted avian host have been reported lately (Ilgunas, 2019). For instance, infections in parrots with *H. minutus* have been stated to be lethal (Ilgunas, 2019). Another well-documented case is that of the relatively common *H. columbae*, which can cause anaemia, pulmonary, myocardial and hepatic obstructions, and enlargement of spleen and liver during heavy parasitaemia often producing mortality in its hosts (Field, 2018).

Leucocytozonosis (the disease produce by *Leucocytozoon* spp.), has been endemic to south-eastern-Asian countries affecting the poultry industry. This disease causes severe health disease in geese, duck, chicken and turkey, as well as considerable economic losses to the farms (Valkiunas G. 2005; Lee, 2016). Known effects from this disease are internal haemorrhages and anaemia, splenomegaly, degenerated ovaries and oviducts, hepatomegaly and ovarian follicular atrophy, resulting in increased mortality and decreased egg production (Lee, 2016). Mortality caused by leucocytozonosis has also been reported in Great horn owlets (*B. virginianus*) (Niedringhaus, 2018), and on diurnal raptors (*F. sparverius sparverius*). The parasites´ pathogenicity ranges from impaired flight, lack of coordination and weight loss to mortality (Walther, 2016). Furthermore, survival decrease has been reported in young Pink pigeon (*C. mayeri*) infected with *L. marchouxi*.; the Pink pigeon, formerly endangered, now vulnerable, was affected by *L. marchouxi*, which made this parasite to be considered as a conservation threat for that species (Bunbury, 2007).

1.6.1 Abortive infections

Development of haemosporidian parasites is often partial or abortive both in birds and vectors. When this happens, the initial parasite development occurs (i.e. tissue, or initial sporogonic stages develop), but the parasites cannot complete their life cycles, resulting in absence of gametocytes in birds or sporozoites in vectors, which are the infective stages necessary to be transmitted to a new host. Such abortive development often causes severe disease and sometimes even mortality both in avian hosts and vectors (Valkiūnas, 2014). The abortive parasite stages may persist in partly resistant hosts for several weeks, being difficult to detect and identify by microscopy (Valkiūnas, 2014). In contrast, PCR is highly effective for the detection of abortive infections, but as life stages of the parasites are not examined by PCR, host competence cannot be confirmed using this method; hence, positive PCR results do not necessarily indicate coevolved host-parasite associations. For instance, numerous *Plasmodium* lineages have been recorded by molecular techniques in blood samples from penguins, in which the parasites abort development at the tissue stage without producing gametocytes (Valkiūnas, 2014). Abortive development of haemosporidian parasites has been described in captive bird populations, when a host species is brought to a new location where it is exposed to local parasites and vectors (Levin, 2013) which it has not encountered before in its evolutionary history.

For *Plasmodium* parasites, the generalist strategy, that allows them to switch to new hosts and consequently evolve into new lineages (Huang, 2018), leads to the onset of abortive infections. For instance, encounters of new lineages in non-adapted hosts are often described in captive birds (Chaisi, 2019; Huang, 2018).

Haemoproteus spp. infections used to be considered as relatively benign; however, the recent discovery of numerous well-documented cases of severe haemoproteosis in non-adapted avian hosts is changing this view. Severe disease and even mortality have been reported, particularly when *Haemoproteus* infection was established in non-adapted avian hosts, resulting in abortive infections.

Interestingly, sporogonic stages (ookinetes) of avian *Haemoproteus* parasites can markedly damage the midgut of blood-sucking insects (both vectors and non-vectors) and even kill them after blood meals with heavy gametocytaemia (Ilgunas, 2019).

Regarding *Leucocytozoon* parasites, little is known about abortive infections. To the best of my knowledge, no reports of abortive infections produced by *Leucocytozoon* spp. exist; however, since it has been pointed out that these parasites might also diversify by host switching (Walther, 2016), a strategy where the parasite changes constantly to new bird species, there is a possibility that one of those encounters happen with a non-adapted host species and abortive infections may occur.

1.7 Haemosporidians diagnostic methods

Microscopy and Polymerase Chain Reaction (PCR) are the two main ways to diagnose haemosporidians in live birds (Valkiūnas, 2005). A less usual method is the Enzyme-Linked Immunosorbent Assay (ELISAS) (Grilo, 2016). For dead birds, diagnosis is mainly done by histopathology, although molecular methods such as Chromogenic *In Situ* Hybridization (CISH) have been developed recently to aid in the post-mortem diagnosis (Ilgunas, 2019).

Microscopy is highly effective for hematozoa diagnosis (Moens, 2016) and it can also be used to estimate parasitaemia (Hasselquist, 2007; Stjernman, 2008); however, it heavily relies on the quality of slide preparation, the number of microscope fields analysed, observer´s expertise (Ishtiaq, 2017) and parasite load (Zélé, 2014b). It has been pointed out that microscopy usually fails to diagnose low intensity parasitaemia infections, (Biedrzycka, 2015; Ishtiaq, 2017, Ciloglu, 2019) misidentifying birds as uninfected. Nevertheless, the screening effort is overlooked; it should be considered that a smear test has the potential to examine 40,000,000

cells on a typical slide (Feldman, 1995). Hence, by increasing the area of the blood film screen, the probability of detecting parasites increases (Bell, 2015).

Numerous PCR techniques have been developed for haemosporidian diagnosis (Table 1.1). Although the detection rates are relatively high with these methods, low intensity infections can pass as false negatives, particularly if they are mixed infections (Jarvi, 2002). This is due to the fact that the host DNA is much more concentrated than the parasite DNA, which affects the ability to detect haemosporidian DNA or to amplify larger fragments of parasite DNA (Bell, 2015). If the DNA is degraded or copy number of parasite DNA is low, false negatives could be obtained due to a weak template (Ishtiaq, 2017). In addition, varying primer affinity has been observed, resulting in the amplification of only some species and lineages (Martínez, 2009). Additionally, most of the molecular approaches cannot distinguish between parasite species and successive methods are needed for identification. For example, Thymine adenine (TA) cloning and DNA sequencing, laser microdissection of single blood cells or vector stages, and next-generation sequencing (NGS) assays. It should be noted that all these methods are expensive and labour-intensive (Ciloglu, 2019).

For lethal cases, diagnosis is mainly achieved by histopathological examination, supported by necropsy findings and PCR. Parasites can be observed in stained histological sections; however, conclusive determination of the parasites is not always achieved, even on severe cases. Hereby, CISH protocols were recently developed to aid in the identification of haemosporidian parasites. Noteworthy is that one key aspect for this method is the tissue fixation, poorly and over fixated samples will induce autolytic degradation; hence, this method requires tissue samples to be fixed immediately after necropsy (Himmel, 2019).

Table 1.1. PCR based methods to diagnose haemosporidian parasites

Reference	PCR type	Notes	Genera	Gene targeted
Biedrzycka (2015)	qPCR	Previous nested PCR needed	*Plasmodium* *Haemoproteus*	Cyt b
Hellgreen (2004)	Nested	Needs to asses total DNA quality	*Haemoproteus* *Leucocytozoon* *Plasmodium*	Cyt b
Fallon (2003)	Touchdown	6 times more conservative than *cytb*	*Haemoproteus* *Plasmodium*	LSU rRNA SSU rRNA
Martinsen (2008)	Nested	Low *Haemoproteus* detection	*Haemoproteus* *Plasmodium*	Cyt b Co1 Clpc Asl
Bell (2015)	Real time	More conserved than *cytb*	*Haemoproteus* *Leucocytozoon* *Plasmodium*	Mitochondrial rDNA
Hellgreen (2013)	Nested	Previous *cytb* to confirm species	Plasmodium	MSP1
Ciloglu (2019)	Multiplex	A single reaction	*Haemoproteus* *Leucocytozoon* *Plasmodium*	Cyt b (*Haemoproteus*) COX1 (*Leucocytozoon*) mtDNA (*Plasmodium*)

PCR= Polymerase chain reaction; qPCR= Quantitative polymerase chain reaction; *cytb*= cytochrome b; LSU= Large subunit; SSU= Small subunit rRNA= Ribosomal RNA CO1= cytochrome c oxidase subunit; Clpc= Caseinolytic protease; Asl= Adenylosuccinate lyase; MSP= Merozoite surface protein; COX1= cytochrome oxidase 1

In addition to the individual challenges of haemosporidian diagnostic methods mentioned early, life cycle characters of these parasites complicate their detection further. The majority of natural haemosporidian infections are of low intensity, this and the presence of abortive infections, where gametocytes are not developed (Valkiūnas, 2014), makes it difficult to either diagnose or detect haemosporidians. Moreover, the acute stage of infection, where the parasitaemia increases considerably, occurs in such a short period of time (Asghar, 2011) that they can be easily missed.

Diagnosis of avian malaria is usually done by microscopy and molecular methods. Microscopy is not only useful for diagnosis, it is also required for the identification of *Plasmodium* species, in which different cell types of the parasite within host cells are recorded (Ishtiaq, 2017); likewise, this technique is necessary to establish the competence of a host by the observation of gametocytes (life stages required to infect vectors) (Valkiūnas, 2014). Many PCR protocols targeting different genes for the detection of *Plasmodium* spp. exist (Table 1.1); but despite their high sensitivity they usually fail to diagnose mixed infections as well as some parasite lineages, because the primers present preferences for certain strains (Bernotiene, 2016). Additional methods for the diagnosis of avian malaria include ELISA (Palmer, 2013), CISH and histopathology. The ELISA test was developed to detect anti-*P*. *relictum* and anti-*P*. *elongatum* antibodies from infected birds using *P*. *falciparum* antigens; this technique is sensitive, rapid and relatively inexpensive, although some authors have suggested that this method may have limited specificity (Grilo, 2016). For dead birds, histopathology is a classic method to diagnose avian malaria through the visualization of tissue meronts within macrophages and endothelial cells, and along with lesions, this method also aids to establish *Plasmodium* parasites as the cause of death (COD). Post-mortem diagnosis is performed by examining for *Plasmodium* infection associated lesions, such as hepatomegaly, splenomegaly, hydropericardium and lung congestion (Grilo, 2016); nevertheless, visualization of exoerythrocytic meronts may be difficult in some cases. To overcome this diagnostic difficulty, CISH protocol was recently developed for detection of avian *Plasmodium* spp. (Ilgunas, 2016). The coloured binding probes used in this method facilitate the observation of the parasites even in light infections (Dinhopl, 2015). None of the methods described is absolutely effective in the diagnosis of avian malaria and each one has its own limitations and sources of variation. The variation in diagnostic sensitivity among screening methods represents a potential source of bias that may lead to erroneous inference in comparisons of prevalence across studies (Ishtiaq, 2017).

Similarly to avian malaria, haemoproteosis is mainly diagnosed by microscopy and molecular methods (Table 1.1). For lethal infections, histopathology is commonly used, and it is fundamental to establish the parasites as the COD. In addition to this techniques, CISH using specific probes can be used for distinguishing *Haemoproteus* (Ilgunas, 2019); however, this method aims to detect parasites belonging to the subgenera *Parahaemoproteus* (*Haemoproteus)* as it contains most of the species within the genus (Himmel, 2019).

Leucocytozonosis are also detected mainly by microscopy and molecular methods (Table 1.1). Regarding the molecular methods, it has been observed that some PCR protocols fail to detect some *Leucocytozoon* species (Lotta, 2019). As it is done with other haemosporidians, histopathology is frequently used to diagnose *Leucocytozoon* spp. infections in dead birds and more recently, a CISH protocol targeting the *18S* rRNA has been developed to detect parasites within the *Leucocytozoon* subgenus (*Leucocytozoon*), since the species in this genus are the most prevalent (Himmel, 2019).

1.8 Aims

The aim of this thesis is to describe the prevalence, diversity, phylogeny and genetic assemblage of *Plasmodium* spp. and other related haemosporidians in wild birds. This was explored in a captive penguin population and mosquito vectors in a UK zoo and in a wild bird community from a little studied area of the Peruvian Amazonia. Additionally a novel, non-invasive method for detecting haemosporidians was explored using feathers. The purpose of Chapter two is to quantify the temporal and spatial variation in prevalence, diversity and the phylogeny of *Plasmodium* parasites in captive penguins and mosquito vectors within a zoological garden from the UK to suggest preventive measures for the disease. The third chapter targets the prevalence and diversity of haemosporidia infecting a wild bird community in

the Peruvian Amazonia, which is virtually an unexplored area and an unexplored group of hosts; terrestrial game birds. The fourth chapter aims to explore the recovery of haemosporidian DNA from feathers of infected individuals and to develop a new method for detecting avian blood parasites to facilitate the diagnosis of infection using a non-invasive and readily available source of genetic material. Finally, the purpose of the fifth chapter is to integrate the information obtained here, highlighting relevant considerations, to draw general conclusions and recommendations for the study, prevention and control of haemosporidians in wild birds.

CHAPTER 2

Molecular Ecology of Avian Malaria at Chester Zoo

2.1 Introduction

2.1.1 Avian malaria in penguins

One of the most susceptible species to avian malaria are penguins (Order Sphenisciformes, Family Spheniscidae) and their high susceptibility (Vanstreels, 2014) is possibly due to a lack of exposure to the mosquito vector and parasite in their natural habitats. Hence, as a group, they have developed little adaptation to *Plasmodium* parasites and therefore, their immune response is limited (Grilo, 2016). Naïve individuals, such as chicks, juvenile birds and adults without previous exposure to mosquitoes, show the highest susceptibility. The most severe presentation of disease occurs after primary exposure, and subsequent exposures to the parasite are usually not fatal with surviving birds developing what appears to be protective immunity against endothelial parasite stages along with a low-level parasitaemia without clinical signs. Infected birds are not capable of clearing the infection and when re-infected with homologous strains of *Plasmodium* spp., they will only have short and low intensity parasitaemia, without mortality (Grilo, 2016).

To date, there are reports of seven *Plasmodium* species infecting penguins (*P. relictum, P. elongatum, P. juxtanucleare, P. tejerai, P. cathemerium, P. unalis,* and *P. nucleophilum*) (Bier, 1980), with the first two causing the majority of infections (Vanstreels, 2016). Additionally there are reports of unique lineages infecting

penguins in the Galapagos Islands (Levin, 2009). From the eighteen species of penguins, thirteen have been reported to be infected with *Plasmodium* parasites in captivity or in the wild (Vanstreels, 2016), and from these, the highest mortality rates have been reported in the Magellanic (*Spheniscus magellanicus*) (Bueno, 2010), Humboldt (*Spheniscus humboldti*) (Sallaberry-Pincheira, 2015), and African penguins (*Spheniscus dermesus* (Sallaberry-Pincheira, 2015; Beier, 1980). This observation should be taken with caution, since it has to be considered that Humboldt and African penguins are usually amongst the most common penguin species kept at zoological collections (Hernández-Colina, 2021b) and large numbers of the Magellanic penguins are often taken into rehabilitation centres (Taunde, 2019); hence the number of studied individuals from this species is larger. Additionally, these species are not Antarctic, for which they are mostly kept in outdoors exhibits (Hernández-Colina, 2021b), increasing their exposure to mosquito bites.

2.1.2 Avian malaria in captive penguins

Plasmodium infections in wild penguin populations have been reported previously in Galapagos penguins (*Spheniscus mendiculus*) (Levin, 2009), Yellow-eyed penguins (*Megadyptes antipodes*) (Palmer, 2013), African penguins (*Spheniscus demersus*) and Magellanic penguins (*Spheniscus magellanicus*) (Vanstreels, 2015). In most of these cases high mortality was observed; however, the penguins were wild-caught and reallocated to outdoor facilities (Palmer, 2013). Avian malaria is a key concern in captive penguin colonies, as it represents one of the major causes of mortality worldwide in penguin colonies exhibited outdoors (Sallaberry-Pincheira, 2015; Bueno, 2010). Several outbreaks have affected penguins in zoos and rehabilitation centers internationally with rapid mortalities varying from 10 to 83% (Vanstreels, 2016; Vanstreels, 2014; Gracyzk, 1995). When penguins are imported into temperate climate zoos, they may suffer high mortality from avian malaria (Beier, 1980). The first report of avian malaria in penguins was in 1926, from a King Penguin (*Aptenodytes patagonicus*) kept at London Zoo (Scott, 1927). Since then,

infections have been described in many zoos around the world, especially in African (*S. demersus*) and Humboldt penguins (*S. humboldti*) (Bueno, 2010). In the Northern Hemisphere, 12.5% of 40 zoos that participated in a survey have had, at some point, diagnosed cases of avian malaria in their penguin collections (Grilo, 2016).

Avian malaria outbreaks present a highly marked seasonality, occurring from early summer to late autumn for the Northern hemisphere; because of the seasonal occurrence of their mosquito vector species (Vanstreels, 2015; Fix, 1988). Malaria outbreaks have a rapid onset and they have been commonly associated with stress induction (Levi, 2009). Aspergillosis, a disease frequently attributed to immunosuppression states (Xavier, 2011), has been often observed concurrently in avian malaria outbreaks (Vanstreels, 2015; Levin, 2009; Fix, 1988), and because it produces unspecific signs and lesions, just like avian malaria, the diagnosis and treatment of both diseases is challenging (Hernandez-Colina, 2021b).

Fix (1988) details an outbreak of avian malaria presented from April to December of 1986, in a zoo in Michigan, USA, where 38 out of 46 Magellanic penguins died. The penguins showed inactivity, weakness, anemia, regurgitation and dyspnea; at postmortem examination, 22 birds showed avian malaria gross lesions, with the most common ones being splenomegaly, hepatomegaly and severe pulmonary oedema. Of the 22 birds presenting avian malaria lesions, histopathology demonstrated schizogony in 13, occurring mainly in the spleen, lung and liver. Other avian malaria related findings included splenic and pulmonic haemosiderosis. Nevertheless, other diseases were also found; aspergillosis was diagnosed in 23 penguins of which 11 cases were also associated with avian malaria. Likewise, heliminthiasis and bacterial enteritis were also seen in 10 penguins. Vanstreels (2014) examined an avian malaria outbreak in a wildlife rehabilitation center in Brazil from the summer of 2008 till early 2009, in which 9 casualties out of 28 Magellanic penguins were recorded. Prior to death, the affected penguins had good body condition, normal appetite and behaviour. *Plasmodium* species found were *P. tejerai*, *P. elongatum* and possibly *P. cathemerium*. Gross lesions included pneumonia, pulmonary oedema and congestion, splenitis, hepatitis, and nephritis.

Histopathology of the penguins infected with *P. tejerai* revealed meronts frequently identified in cardiac and renal tissues. As with Fix (1988), additional avian malaria findings were splenic, pulmonic and hepatic haemosiderosis (Vanstreels, 2014).

2.1.3 Avian malaria outbreaks in the UK

Throughout the UK, there are at least 41 zoological gardens and aquaria exhibiting penguins (Hernandez-Colina, 2021), some of which have reported cases of avian malaria; but complete epidemiological data about them is scarce. Quintavalle (2015) briefly mentions one outbreak and some others are eventually published as press releases. There are few public records about the death of some or even all of the penguins from colonies at different UK zoos in the past 20 years associated to avian malaria. Outbreaks were reported at Marwell Zoo in 1999, Bristol Zoo in 1999, Edinburgh Zoo in 1999, London Zoo in 2005, London Zoo in 2012, Exmoor Zoo in 2016, Longleat Safari in 2016 and Flamingo Land in 2016 (Table 1). These events mainly affected Humboldt penguins (*S. humboldti*), but also African penguins (*S. demersus*), King Penguins (*A. patagonicus*) and Macaroni Penguins (*Eudyptes chrysolophus*). As these were press releases, there is no information about the malaria prevalence in these colonies, epidemiology of the disease, methods used for the diagnosis and parasite species or lineages involved.

Table 2.1. Avian malaria outbreaks in the UK reported by the press from 1999 to 2016

Year	Zoo	Penguin species	Source
1999	Marwell Zoo	African penguins (*Spheniscus demersus*) Macaroni penguins (*Eudyptes chrysolophus*)	BBC News, 1999
1999	Bristol Zoo	King penguins (*Aptenodytes patagonicus*)	Wales Online, 2003
1999	Edinburgh Zoo	Not mentioned	BBC News, 1999
2005	London Zoo	African penguins (*Spheniscus demersus*)	Quintavalle, 2015
2012	London Zoo	Humboldt penguins (*Spheniscus humboldti*)	The Guardian, 2012
2016	Exmoor Zoo	Humboldt penguins (*Spheniscus humboldti*)	The Guardian, 2016
2016	Longleat Safari	Humboldt penguins (*Spheniscus humboldti*)	BBC News, 2016
2016	Flamingo Land	Humboldt penguins (*Spheniscus humboldti*)	The Mirror, 2016

2.1.4 Penguin mortality event at Chester Zoo

Chester Zoo is the single largest zoo in the UK, it was established in 1934, and currently it houses over 35,000 animals belonging to more than 500 species. A fifth of Chester Zoo´s animals are birds, comprising 139 species and 1760 individuals (TNEZS, 2019). Chester Zoo has held Humboldt penguins since 1964, housed in an outdoor exhibit. Their penguin colony has maintained stable numbers with sporadic isolated deaths each year since then and no avian malaria cases or outbreaks have

been observed previously. Chester Zoo's veterinary team were aware of avian malaria outbreaks occurring in UK zoological collections prior to 2017, and were concerned about the possibility of their penguin colony becoming infected, which prompted this research project. By early April 2017, mosquito traps were in place around the site and trapping was underway. After a few weeks, the first mosquitoes infected with *Plasmodium* spp. were identified, confirming that the parasite was circulating in the area. Around that time, Chester Zoo began a program to refurbish the penguin exhibit. In early September 2017, the penguin colony was temporarily relocated to a former orangutan exhibit, which was closed except for window openings and it also had a pool. Humidity was a concern for the zoo´s staff, so they installed fans to improve ventilation. To control for mosquitoes in the facility, CDC-miniature light traps were set inside (n=2) and around (n=4) the enclosure to catch mosquitoes, collecting nets every week. After mosquitoes were caught from the inner traps, the decision was taken to install fine plastic mesh around the windows and to apply spray foam on any gaps where mosquitoes could get through. No more mosquitoes were found inside the enclosure after those modifications and the outer traps only caught seven mosquitoes throughout the time they were set (Hernández-Colina, 2019).

When penguins were moved to their temporary accommodation, Chester Zoo veterinarians did a health check and took blood smears and blood samples from 21 penguins, which were screened for avian malaria. None of these samples were positive for *Plasmodium* spp. (Table 2.9). On September 21st 2017, the penguins started to show signs of disease (Table 2.2); therefore, the zoo staff examined them for diagnosis and treatment providing further blood samples or smears, which were also negative for *Plasmodium* spp. (Table 2.9). The first signs observed in the penguins were mild and unspecific, such as anorexia, depression, weight loss, lethargy and regurgitation, which is consistent with reports from other outbreaks (Fix, 1988; Grilo, 2016). As days passed, the signs progressed to ataxia, dyspnea and opistotonus, until the condition of the penguins deteriorated so much that they were euthanized or died. Sudden deaths without clinical signs were also observed. Sick penguins were initially treated with antibiotics and anti-fungals because these

aetiologies were suspected. However, *Plasmodium* spp. was eventually discovered post-mortem in penguin organs, and thereafter, the colony was treated with antimalarial drugs (primaquine and chloroquine).

In total, 22 of the 44 penguins in the Chester Zoo colony died between September 29th 2017 and January 21st 2018, with the majority of deaths occurring in October and November. Penguin carcasses were examined to establish the causes of death (COD) by Dr Julian Chantrey, chief veterinary pathologist at the University of Liverpool, Leahurst campus. It was established that seven penguins died from aspergillosis, four due to various inflammatory processes, two from foreign body stomach perforations, one from avian malaria and four most likely from avian malaria. Additionally, there were four penguins whose COD could not be determined (Table 2.2). Contributing to the establishment of CODs, organs from the 22 dead penguins were tested for *Plasmodium* spp. using PCR and its presence was confirmed in five of them (Table 2.2). Furthermore, the organs of the 22 penguins were sent for *in situ* hybridization testing for *Plasmodium* spp. to the University of Veterinary Medicine, Vienna. The results from this test agreed with the histopathology findings that attributed one death to avian malaria.

The penguins were moved back to their refurbished exhibit on February 7th 2018; after this date no more penguin deaths were recorded. Soon after, the zoo acquired 25 penguins to re-populate their colony and a preventive malaria treatment was applied throughout the mosquito season (April – October 2018). More strategies for malaria prevention were established, such as regular cleaning of organic matter from nearby ponds, elimination of water containers, and removal of plants that provided shelter for mosquito larvae (Hernández-Colina, 2019).

Table 2.2. Signs, lesions and tests contributing to the establishment of a cause of death for the penguins in Chester Zoo.

ID	Death date	Main signs observed	Main histopathology findings	PCR	CISH	COD
CO587	29/Sep/17	Anorexia Lethargy Ataxia	Splenitis, hepatitis, pneumonia	P	N	Suspected AM
C13178	05/Oct/17	Anorexia Respiratory distress Vomiting	*Aspergillus* spp airsaculitis Hepatitis Pneumonia	P	N	Aspergillosis Suspected AM
C06094	08/0ct/17	Sudden death Sternal recumbancy	Intersitial pneumonia Liver haemosiderosis	P	N	Suspected AM
C12185	10/Oct/17	Respiratory distress	Airsacculitis, pneumonia, trachetis, pericarditis and capsular nephritis	N	N	Aspergillosis
C07093	14/Oct/17	Weight loss Vomiting	Necrogranulomatous pneumonia	N	N	Aspergillosis
950128	15/Oct/17	Depression	Liver IMH Haemosiderosis Chronic neprhitis	P	P	AM
C14138	15/Oct/17	Depression	Pulmonary oedema Splenitis Hepatic haemosiderosis IMH	N	N	Multisystemic bacterial infection
C07080	17/Oct/17	Weight loss Anorexia Vomit	Necrotising hepatitis Haemosiderosis	N	N	Suspected AM
C12181	23/ Oct/17	Lethargy Anorexia Weight loss	Cerebellar hemorrhage Necrotising cloacitis Hepatic haemosiderosis	N	N	Suspected AM
C16135	24/Oct/17	Lethargy Weight loss Opistotonus Ataxia	Necrotising hepatits Intersticial nephritis Airsaculitits High iron levels	N	N	Undetermined
CZ1636	30/Oct/17	Weight loss	Hepatic haemosiderosis Pulmonary oedema	N	N	Undetermined
C06131	06/Nov/17	Weight loss	High iron levels Hepatic, splenic and pulmonary haemochromatosis	N	N	Undetermined
C14129	08/Nov/17	Weight loss	Tracheal *Aspergillus* spp granuloma Hepatic haemosiderosis Late stage necrotic enteritis	N	N	Aspergillosis Necrotic enteritis
CZ1379	10/Nov/17	Lethargy Dyspnea Weight loss	Chronic aspergilosis	N	N	Foreign body perforation of GIT
BO1319	16/Nov/17	Weight loss	Hepatic haemosiderosis	N	N	Undetermined

			Atherosclerosis			
C13190	17/Nov/17	Weight loss	Necrogranulomatous airsaculitits Hepatic haemosierosis		N	Aspergillosis
C12214	17/Nov/17		Ulcerative gastritis Hepatic haemosiderosis	P	N	Foreign body perforation
C13176	21/Nov/17	Neurological signs	Myocarditis Hepatitis Pneumonia Nephritis Splenitis	N	N	Myocarditis
C14175	14/Dec/17	Anorexia	Necrotising oesophagitis Necrogranulomatous airsaculitis		N	Oesophagitis
C08131	02/Jan/18	Anorexia Lethargy Respiratory distress Vomiting	Necro-ulcerative enteritis Splenitis	N	N	Necro-ulcerative enteritis
C07108	13/Jan/18	Anorexia Weight loss Lethargy	Necrogranulomatous airsaculitis Hepatitis Pericarditis Splenomegaly	N	N	Aspergillosis
C13398	21/Jan/18	Anorexia Weakness	Abscesation around the hearth base		N	Aspergillosis

ID= Penguin identification; PCR= Polymerase Chain Reaction; ISH= *in situ* hybridazation; COD= Cause of death; P= Positive for *Plasmodium* spp.; N= Negative for *Plasmodium* spp.; AM= avian malaria; IMH= Increased medullary haematopoiesis

2.1.5 Objective

The Flamingo Land avian malaria outbreak in 2016 (The Mirror, 2016) raised concern for the Chester Zoo penguin colony. Aiming to prevent an avian malaria outbreak and investigate the situation of the disease in Chester Zoo, a protocol was developed and started in April 2017 (prior to the outbreak described before). This chapter presents the research to uncover the situation of *Plasmodium* spp. in the zoo premises by quantifying its presence and its genetic diversity within vectors, captive wild birds and free-living wild bird reservoirs over an 8-month period (April to November 2017).

It is expected that a better understanding of the temporal or spatial variations in parasite prevalence and of parasite diversity will help to explain the 2017 avian malaria outbreak and provide guidance on avoiding similar events in the future. Analysis of the parasite species present in the vectors and wild birds will contribute to understanding the source of the infection for the penguins as two explanatory possibilities exist. The first one is that the penguins became infected from parasites circulating in the area, in which case their parasite species would match to the one found in mosquitoes and birds in the surroundings as well as species reported in the country. The second possibility is that they got infected by a foreign parasite species introduced by overseas imported birds (a relatively common practice in a zoo); these birds could have developed a chronic stage of the disease making it undetectable at quarantine and for routine examinations and resurging under specific conditions. In the latter case, the parasite species found on the penguins may or may not match the ones found on mosquitoes and birds in the area; but, would be different from typical British species.

2.1.6 Hypothesis

Chester Zoo has suitable breeding habitats for mosquitoes and it is located within the distribution area of the main European vector for avian *Plasmodium* parasites, *Culex pipiens*. Therefore, the vector is thought to be present in the area as well as the parasite, and it is hypothesized that multiple *Plasmodium* species are on site and their diversity should be similar to other European sites. *Plasmodium´*s vector is affected by weather conditions; therefore, parasite prevalence is expected to differ at each site, depending on the particular microclimate.

2.2. Materials and Methods

2.2.1. Sampling

During 2017, the author collaborated with Chester Zoo in a project for the surveillance of avian malaria on their premises. Mosquitoes, blood samples and blood smears of captive-wild birds and carcasses of captive and free-living wild birds were analysed. All procedures carried out in this project were approved by the University of Liverpool Veterinary Ethics Committee (reference VREC532a) and the Chester Zoo scientific committee. The Approved Research Proposal Form can be found in Appendix (2.6.1).

2.2.2. Mosquito sampling

From April to November of 2017, mosquitoes were collected at Chester Zoo, UK (Upton-by-Chester, Chester, CH2 1EU). Two types of traps were deployed BG-Mosquitaire traps, which are highly effective at catching *Culex* spp. mosquitoes (Hernández-Colina, 2019), and the CDC Gravid traps, which are built specifically to catch the mosquitoes of interest, *Culex* spp. The BG-Mosquitaire® trap has a plastic funnel located at the centre of the trap on which a capture net is attached. It has a fan on one of its inner sides and a compartment for the attractant opposite to it (Figure 2.1). This trap is intended to capture mosquitoes looking for a blood-meal and it must be plugged into power to operate. The attractant needed is the BG-Sweetscent®, a pouch with a lactic acid based formulation that imitates the scent of mammalian sweat. The attractant was replaced every six weeks, two less than suggested by the manufacturer to prevent efficacy reduction. The BG-Mosquitaire traps operated continuously and the nets were collected twice a week, first, after six days, and then, on the following day replacing them with empty nets on both occassions. This allowed us to capture mosquitoes on a weekly and on a daily basis.

CDC-Gravid traps consist of a plastic cylinder with a fan in the middle. The cylinder is placed vertically over a tray supported by metal bars and screws on each side, and the upper end of the tube is covered with a net (Figure 2.2). To operate, the trap must be plugged in to a 6 V battery and the bottom of the tray must be filled with oviposition medium (John W. Hock Company, 2013). The oviposition medium attracts female mosquitoes looking for a place to lay their eggs; as they fly over the water and close to the tube, they get sucked into the net. Laying eggs requires a great amount of nutrients, which mosquitoes acquire from blood meals; therefore, detection of pathogens in gravid females ensures that they have had at least one blood-meal. The infusion media was prepared with tap water (40L), hay (200g), brewer's yeast (2g) and milk powder (2g) and it was rested for at least one week before use, as suggested by Reiter (1983). Four litres of infusion media were poured into the tray of each trap and the traps were operated for one day per week, collecting mosquitoes after 24 hours.

Figure 2.1. BG-Mosquitaire trap.

Figure 2.2. CDC-Gravid trap.

2.2.3. Mosquito sampling locations

Ten sampling areas were established in or near the bird enclosures at the zoo. These areas were 30 m in diameter and contained one trap of each kind, which were separated by at least 10 m to avoid interference between them and pseudo-replicates. Traps were protected from direct sunlight, artificial lighting, wind, and rain, but near vegetation and, whenever possible, near to water bodies. Before setting the traps, Chester Zoo staff were consulted to confirm that each site complied with the following requirements: access to electricity (for the BG-mosquitaire traps either to plug in directly or using extension cords); ease of access, avoiding sites that would require a staff member to grant us access; safety for animals, ensuring that traps and all its elements would not be dangerous or stressful for animals; safety for visitors, ensuring that the traps were not in plain sight of visitors. Ultimately, the sites that satisfied these criteria were as follows: A1 (Flamingos´ pond), A2 (Penguins snack shed), A3 (Off-show aviaries), A4 (Greenhouses), A5 (Red panda), A6 (Dragons), A7 (Vultures), A10 (Wetlands), A11 (Conservation-golf) and A12 (Penguin's kitchen) (Figure 2.3).

Figure 2.3. Chester Zoo map indicating the location of the sampling sites. (Made by A. Hernández-Colina) Red line: perimeter of the zoo; Orange circles: sampling areas, containing each one a BG-Mosquitaire and a CDC-Gravid trap.

2.2.4. Mosquito screening

Mosquito nets from each trap were stored at -20°C for one hour to kill the mosquitoes. All mosquitoes were identified using a morphological key (Cranston 1987) and counted by Dr Arturo Hernández-Colina. Once the mosquito species was determined, total DNA was extracted individually from females. DNA extractions were carried out in 96 deep well plates (1.5ml), placing mosquitoes individually in each well. For the first 2072 mosquitoes this was done using OMEGA Bio-Tek E.Z.N.A ® Tissue DNA kits; for the remaining flies, the Livak extraction method (Livak, 1984) was used due to budgetary reasons. When the OMEGA kits were employed, proteinase K and the first lysis buffer were added to each well and the mosquitoes were macerated using autoclavable pestles inserted in a homogenizer; then, the plate was sealed with a mat and left for incubation to complete the lysis. Lysed samples were transferred to individual columns of the kit and from there the

extraction was done as instructed by the manufacturer. For the Livak method, half of the lysis buffer volume was added to macerate the mosquitoes with autoclavable pestles inserted in an homogenizer, then the remainder of the lysis buffer was added, the plate was sealed with a mat and incubated as required. The rest of the extraction was done using 96 deep well plates and the centrifugation steps were carried on in an eppendorf 5810R plate centrifuge. For the elution step, 200 μl of nuclease free water was added to each well and the elute was deposited individually in 1.5 ml tubes. To ensure that the change of extraction method would not affect the results, a pairwise trial was conducted using both extraction methods on a 239 mosquito set collected on the same day. For the trial, the first extraction steps of the Livak method were carried out on each mosquito, adding the buffer and heating up the sample to complete the lysis process. Afterwards, the sample was split in half, and DNA extraction was completed on each half using the Livak method and OMEGA columns respectively. The difference in DNA yields was analysed using a Fisher´s Exact Test of independence using R. The level of agreement between the results of the two methods was calculated with Cohen´s Kappa coefficient (Landis, 1977) at 95% CI.

2.2.5. Bird screening: blood samples

Surplus blood samples from routine or diagnostic sampling were provided by the zoo's staff throughout 2017 and 2018. The blood was collected into an EDTA-coated microtainer and if enough blood remained, three thin blood smears were carried out for each bird. The smears were air dried for three minutes, fixed with absolute methanol and then stored at 4°C until they could be processed. In the laboratory, the blood smears were stained with Giemsa's solution and screened with a light microscope for parasite detection. Each blood smear was examined for 30 min; given that diverse haemosporidia (including *Haemoproteus* spp.) may be present, a parasite was only recorded as *Plasmodium* spp. if merozoites were observed (Valkiunas G. 2005). Intensity of infection was reported as percentage counting the number of parasites found in 2000 erythrocytes as suggested by Godfrey (1987).

The intensity of infection was classified depending on the percentage of infected erythrocytes as Benedikt (2006) proposed: low (<0.1%), medium (0.1-0.5%) and high (>0.5%). DNA was extracted from blood samples using QIAGEN DNeasy kits according to the manufacturer´s instructions and eluted in 50 μl of elution buffer. DNA extracts were tested by PCR for haemosporidia following Hellgreen´s protocol (2004).

2.2.6. Bird screening: organ samples

Traditionally, *Plasmodium* spp. prevalence is assessed by analysing fresh blood samples from netted birds. However, it was not possible to catch free-living wild birds at the zoo; hence, *Plasmodium* spp. prevalence was evaluated in organ samples from free-living wild birds found dead in the zoo grounds. During 2017 the zoo staff picked up bird carcasses each morning and stored them at -20°C degrees until they could be transported to the laboratory. Due to the avian malaria outbreak, collection of dead free-living wild birds continued in 2018. In the laboratory, post-mortem examinations (PME) were carried out assessing body condition, conservation state, weight, sex and cause of death. Due to risk of infection with zoonotic pathogens, all PMEs were carried out inside a class 2 microbiological safety cabinet. After PME, brain and liver samples were taken from each bird. In addition, brain and liver samples were taken from zoo captive wild birds that died during 2017 whenever possible. Occasionally, these organs were removed by the zoo staff, and in those cases different organs than those typically affected by *Plasmodium* spp., (e.g. lung, heart, spleen and kidney) were sampled. In the case of the penguins, an effort to screen all organs typically affected by *Plasmodium* spp. (brain, hearth, liver, lung, kidneys and spleen) was done. In all cases, a small piece (1 cm^3) of each organ was taken and stored in 1.5 ml microcentrifuge tubes at -20°C. For DNA extraction, a 0.5 cm^3 piece of each organ was taken and dissected into smaller pieces with disposable scalpels in a petri dish. For each organ, a different scalpel and clean sections on the petri dish were used to avoid contamination. Organ dissections and the first steps of DNA extraction were

done inside a biosecurity hood. After adding the first extraction buffer and proteinase K, DNA extraction was completed outside the hood with a QIAGEN DNeasy kit, following the manufacturer´s instructions.

2.2.7. Additional penguin samples from Chester Zoo and other UK collections

Organs stored at -20°C from Chester Zoo penguins that died in previous years (2011-2016) were tested for *Plasmodium* spp. Similarly, for comparison, organs from penguins suspected to have died of avian malaria at London Zoo (n=2), Blackpool Zoo (n=3), Cotswold Wildlife Park (n=1) and Paignton Zoo (n=13) were tested using the same methodology for organ samples as described in section 2.2.6.

2.2.8. PCR Protocol

DNA extracted from female mosquitoes and bird blood and organ samples was tested by nested PCR using the protocol described by Hellgreen (2004). PCR is the gold standard method for identification of *Plasmodium* to species level. Hellgreen primers amplify a conserved 479-bp fragment of the cytochrome b (*cytb*) gene located in the mitochondrial genome of avian haemosporidians, including *Plasmodium* spp., *Leucocytozoon* spp. and *Haemoproteus* spp. The nested PCR consisted of two amplification parts. For the first part, each reaction included 1 μl of DNA template, 1 μl of forward primer HaemNF1 (5'-CATATATTAAGAGAAITATGGAG-3'), 1 μl of reverse primer HaemNR3 (5'-ATAGAAAGATAAGAAATACCATTC-3'), 10 μl of Bioline mix, 1 μl of BSA (Bovine Serum Albumin) and 6 μl of nuclease free water, to reach a final volume of 20 μl. The PCR profile was 22 cycles at 94°C for 3 min, 94°C for 30 s, 50°C for 30 s, 72°C for 45 s, followed by an extension of at 72°C for 10 min. The second part, intended to detect *Plasmodium* spp. and *Haemoproteus* spp., was performed using 2 μl of PCR product from the previous reaction, 1 μl of forward primer HaemF (5'-ATGGTGCTTTCGATATATGCATG-3'), 1 μl of reverse

primer HaemR2 (5'-GCATTATCTGGATGTGATAATGGT-3'), 10 μl of Bioline mix, 1 μl of BSA and 5 μl of nuclease free water, to reach a final volume of 20 μl. The profile for the second part was 36 cycles at 94°C for 3 min, 94°C for 30 s, 50°C for 30 s, 72°C for 45 s, followed by an extension at 72°C for 10 min. To detect *Leucocytozoon* spp. the exact same procedure was followed; but, using the primers HaemFL (5'-ATGGTGTTTTAGATACTT ACATT-3') and HaemR2L (5'-CATTATCTGGATGAGATAATGGIG C-3') in the second stage. The number of cycles in the first reaction was increased from 20, in the original protocol, to 22. Molecular grade water was used as a negative control; for *Plasmodium* spp. and *Haemoproteus* spp. positive control, Dr Blandine Franke-Fayard of Leiden University provided genomic DNA from *Plasmodium bergei* ANKA (rodent malaria parasite); for *Leucocytozoon* spp., genomic DNA of *Leucocytozoon* spp. isolated from a Mallard (*Anas platyrhynchos*) was used. A positive and a negative control were used on every occasion. Amplicons were visualised on a 1.5% agarose gel stained with SYBR Safe DNA gel satin (Thermo Fisher Scientific). Amplification of *cytb* from mosquito DNA was done using the same protocol but in 96-well plates in a Techne TC-412 Thermal Cycler. Mosquito DNA samples (2-4) were pooled per well, plus 8 negative controls and 4 positive controls per plate. In the event of a PCR-positive well, all individual samples from the well were then tested individually. For the bird blood and organ DNA, a T3 Thermocycler (Biometra®) was used, adding a negative and a positive control every 10 samples.

2.2.9. Statistical analysis

The proportion of *Plasmodium*-positive mosquitoes was compared by mosquito species, sampling areas, trap type and months, using Chi-square tests of independence, and by analysing the residuals of the test to find the values with the highest contributions to significance. For the species comparison, only completely identified females of *Culex pipiens* and *Culiseta annulata* were included because they were the only positive species. In the area and month comparisons, the December collection was excluded due to low catches. When analysing the

proportions by trap type, only mosquitoes from one-day collections in the BG-mosquitaire trap were used as they were equivalent to the collections of the CDC-gravid traps. To calculate the 95% confidence intervals a binomial test was used. All analyses were done using R.

2.2.10. Gene sequencing

Positive PCR products were sent for sequencing in forward direction (primer HAEMF) with the Sanger method. Sequence reads were compared to previously published avian haemosporidia in the GenBank nucleotide database (http://www.ncbi.nlm.nih.gov/genbank/) using BLASTn to identify the genus of the parasite. On this basis, proven *cytb* gene sequences were deposited in GenBank (accession numbers MW813993 - MW814510). Mixed sequences, as indicated by electropherograms with double peaks, were considered as mixed infections (Lutz, 2015) and were excluded from further analysis.

2.2.11. Phylogenetic analysis

No common naming of haemosporidian lineages exists yet; this sometimes leads to species misidentifications (Vanstreels, 2014) and publishing of identical sequences with different names on available databases (Bensch, 2009). Therefore, to identify each *Plasmodium* sequence obtained here most accurately, sequence clustering and phylogenetic analysis were used to relate each sequence to all previously published sequences. To ensure the most thorough comparison of new sequences with those recorded by previous studies, one *Plasmodium cytb* sequence, randomly chosen, was used to search GenBank. The best 10,000 matches in the database were obtained; when ranked in descending sequence identity, matches to *Plasmodium* spp. were listed first, followed by *Haemoproteus* spp. and *Leucocytozoon* spp sequences. Therefore, having recovered non-*Plasmodium* sequences in the search, we may be confident that the list of *Plasmodium cytb*

sequences recovered is exhaustive. After removing duplicates, partial sequences, and sequences containing ambiguous bases, the reference sequences (n = 5,292) were aligned in Bioedit (Hall, 1999) using ClustalW (Chenna, 2003) with all new *Plasmodium* sequences identified in this study (n = 518) to produce a 378 bp multiple sequence alignment (n = 5,810). The length of the *cytb* sequence used for genetic barcoding is 480 bp; nevertheless, shorter sequences with up to 50% of missing data can still provide a precise identification, as demonstrated by simulation studies (Fecchio, 2017b). Mammalian *Plasmodium* sequences were removed because they have an extreme AT bias in base composition that is problematic in phylogenetic analysis (Fallon, 2003). This has no detrimental effect on the current analysis since mammalian *Plasmodium* is monophyletic and distinct from all avian *Plasmodium* (Borner, 2016).

This multiple sequence alignment, while comprehensive, was too large and contained too many near-identical sequences to resolve a phylogeny. Therefore, the program CD-HIT was used to cluster sequences into common lineages, defined by a sequence divergence threshold. The precise threshold in divergence that constitutes a 'species', or other taxon of biological relevance, is a matter of debate. So sequences were clustered multiple times at different divergence thresholds (0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0% and 3.5%). After analysing the clustering behaviour at each threshold, the percentage value that best retained morphospecies of the reference lineages (i.e. where all sequences pertaining to a given morphospecies is found mainly or exclusively in a single cluster) was chosen; this value was 1.0%. Single sequences with a different label found on clusters composed mainly by sequences with the same morphospecies label were thought to be mislabelled and considered to be of the same morphospecies as the cluster they were found in.

Leucocytozoon sequences were retained for use as an outgroup in phylogenetic analysis. *Haemoproteus* sequences were included to confirm the identity of all "*Plasmodium*" labelled sequences; for example, if a *Plasmodium* sequence clustered in an otherwise all *Haemoproteus* cluster, it was considered to be mislabelled and

was not used in further analysis. Having checked this, *Haemoproteus* sequences were not included in phylogenetic analyses. Sequences clusters containing sequences derived from a named morphospecies were assigned to that morphospecies.

After sequence clustering, the multiple sequence alignment contained 322 sequences, including one representative of each cluster defined by CD-HIT as well as Chester Zoo penguin sequences isolated from the 2017 outbreak, Chester Zoo penguin sequences from other years (Table 2.8) and penguin sequences from other UK zoos (Table 2.9). A Neighbour Joining (NJ) phylogenetic tree including the 322 sequences mentioned above was estimated using Jukes-Cantor genetic distances in MEGA X (Sudhir, 2018). A uniform molecular rate was applied to all sites. The tree topology was rooted with *Leucocytozoon* sequences. Node robustness was evaluated with 100 non-parametric bootstraps. Given that *Plasmodium* phylogeny is known to be adversely affected by base composition bias at the third codon position, a Maximum Likelihood (ML) tree was estimated for comparison using MEGA X (Sudhir, 2018) with a Tamura-Nei nucleotide substitution model after automatic model estimation. The data set was partitioned to allow independent modelling of base substitution rates at each codon position, a measure that will reduce the effect of homoplasy at the third position. Node robustness was evaluated with 100 non-parametric bootstraps. The software FigTree v1.3.1 was used to visualize the phylogenetic trees.

2.3. Results

2.3.1. Mosquito sampling

In total, 7,888 mosquitoes were collected at Chester Zoo during eight months in 2017. These belong to eight different species: *Culex pipiens*, *Culex torrentium*, *Culiseta annulata*, *Culiseta morsitans*, *Anopheles plumbeus*, *Anopheles maculipennis*, *Anopheles claviger* and *Cochilletidia richiardii* (Hernández-Colina, 2019). *Culex pipiens* (63.65%) was the most common mosquito species, followed by *Culiseta anulata* (3.49%). From all captured mosquitoes, 6,814 (87.1%) individuals were females; these mosquitoes were used for avian malaria testing since only females feed on blood and these parasites are not vertically transmitted in the vector. It should be mentioned that overall some mosquitoes were damaged (17.4%) and could not be identified to species level; however, they were employed in subsequent testing.

When comparing the efficiency of BG-Mosquitaire traps versus CDC-Gravid traps, it was found that CDC-Gravid traps are more efficient for trapping mosquitoes, particularly *Culex* spp. (Hernández-Colina, 2019). With regards to the different sampling areas, A1 (22.20%), A3 (19.50%) and A4 (9.50%) collected more mosquitoes than the other sites. A1 caught significantly more mosquitoes; however A1 is not statistically different from A3, which in turn is not statistically different from A4 and A7 (Table 2.3).

Table 2.3. Total and proportion of mosquitoes caught per sampling area in Chester Zoo during 2017.

Area*	Total	Percentage
A1[a]	1752	22.20%
A2[c]	426	5.40%
A3[ab]	1535	19.50%
A4[b]	752	9.50%
A5[c]	398	5.00%
A6[c]	475	6.00%
A7[b]	534	6.80%
A10[c]	593	7.50%
A11[c]	743	9.40%
A12[c]	411	5.20%

*: Areas with the same superscript letters were not significantly different from each other.

Catches in July and August were significantly greater than in the other months followed by June and September, then by October and finally by May and November (Hernández-Colina, 2019) (Table 2.4).

Table 2.4. Total and proportion of mosquitoes caught per month in Chester Zoo during 2017.

Month*	Total	Porcentage
May[c]	269	3.4%
June[b]	2552	32.4%
July[a]	2678	34.0%
August[a]	1216	15.4%
September[b]	497	6.3%
October[c]	377	4.8%
November[c]	272	3.4%
December	27	0.3%
Total	7888	100.0%

*: Months with the same superscript letters were not significantly different amongst themselves.
Note: December was excluded from the analysis because it only contained one observation.

2.3.2. Mosquito DNA extraction method

Regarding the comparison of two different extraction methods on the mosquitoes, 16 samples extracted with columns were positive for *Plasmodium* spp., but negative when extracted by Livak´s method. Conversely, 18 of the positives extracted by Livak´s method were negative when extracted with columns. Thirty samples were positive by both methods and 175 were negative by both methods. The difference in the detection of avian malaria by the two assays was not statistically significant (p=1). The strength of agreement between the two assays was considered to be good according to Cohen´s Kappa coefficient (k = 0.55; CI 95%) (Table 2.5).

Table 2.5. Comparison of Livak and column extraction methods for detecting *Plasmodium* spp. infection by PCR.

	Column (+)	**Column (-)**
Livak (+)	30	18
Livak(-)	16	175

2.3.3. *Plasmodium* infection and prevalence in mosquitoes

In 2017 (May-December), overall *Plasmodium* spp. prevalence of mosquitoes was 10%; it started at 2% in May with a peak of 46.8% in the summer (August) and ending at 5% in November. Looking at the number of infected mosquitoes, one infected mosquito was caught on the last day of May and the highest number of infected mosquitoes occurred in July with 162, 147 and 108 on three different weeks, followed by August with 77 and 34. Mosquitoes collected on the 19th of July were crossed contaminated while processing them in the lab; therefore, they were not used for prevalence calculations, nor included in subsequent analyses. (Figure 2.4)

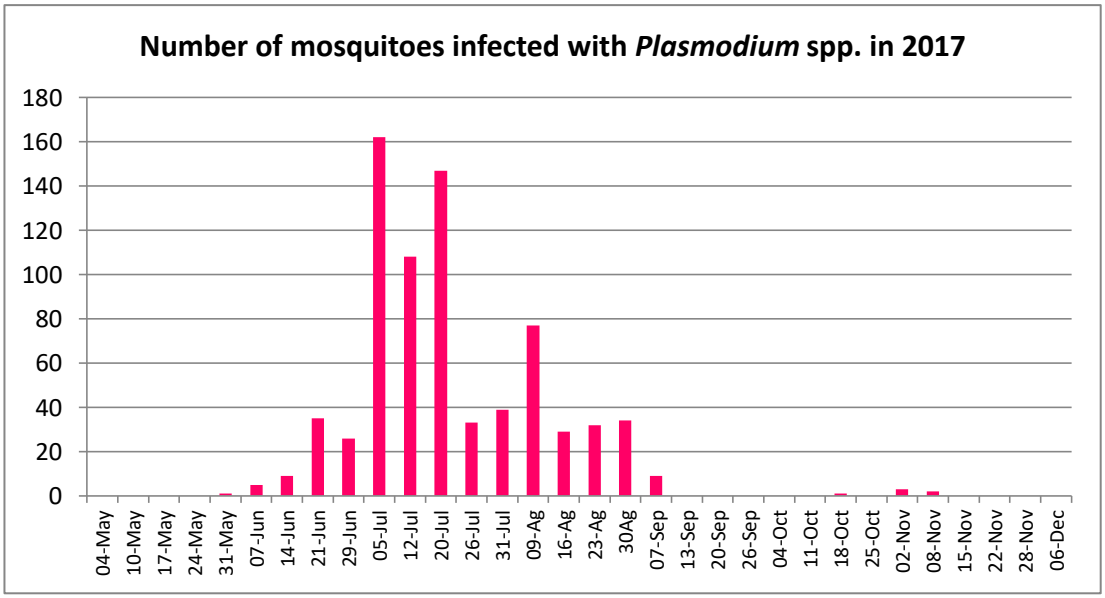

Figure 2.4. Number of *Plasmodium* spp. infected mosquitoes collected in Chester zoo during 2017 per week. Samples from 19th of July were excluded due to contamination.

From the eight species of mosquitoes captured at Chester Zoo during 2017, only two were infected with *Plasmodium* spp., *Culex pipiens* and *Culiseta annulata*, which were also the most abundant mosquito species caught. A total of 753 mosquitoes were infected with *Plasmodium* spp.; from these, 106 could not be identified to species level due to damage and they are not included in this analysis. The total number of captured female mosquitoes by species was 5,469 for *Cx. pipiens* and 244 for *Cs*. *annulata*; of these, 11.4% (626) of *Cx. pipiens* and 10.3% (25) of *Cs. annulata* mosquitoes were infected (Table 2.4). Statistical analysis revealed no difference in the infection prevalence by species (Chi-square test of independence, X^2= 0.18, df=1, p= 0.69) (Figure 2.5).

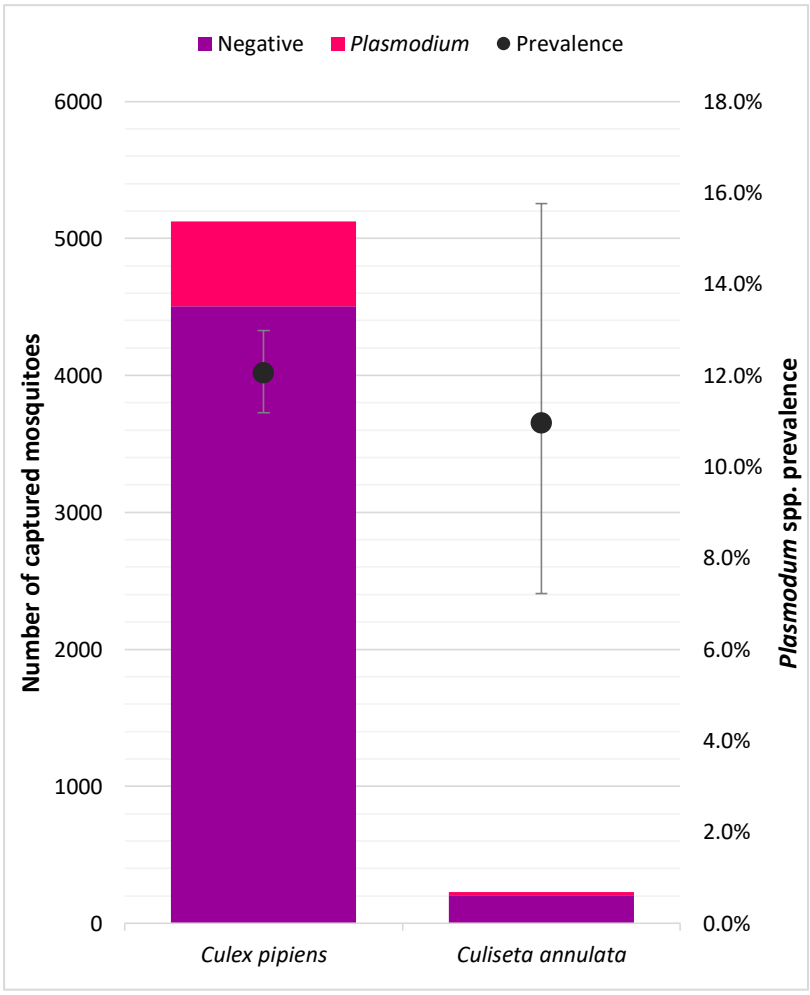

Figure 2.5. Number of captured mosquitoes and Plasmodium spp. prevalence. On the left axis captured mosquitoes and *Plasmodium* spp. prevalence by mosquitoe species on the right axis. Error bars are 95% confidence intervals.

There was a significant difference in *Plasmodium* spp. prevalence by sampling area (Chi-square test of independence, X^2= 71.2, df= 9, p< 0.001). The areas where more infected mosquitoes were caught were A1 (Flamingos´ pond) (n= 234), A3 (Off-show aviaries) (n= 139), A2 (Penguins´ snacks shed) (n= 73) and A4 (Greenhouses) (n= 71); by analysing its residuals, the greatest contributions to the significance of the test came from areas A1 (Flamingos´ pond), A2 (Penguins´ snack shed) and A5 (Red Pandas); and negative contributions from A7 (Vultures), A10 (Wetlands), A11 (Conservation Golf) and A12 (Penguins´ kitchen) (Figure 2.6).

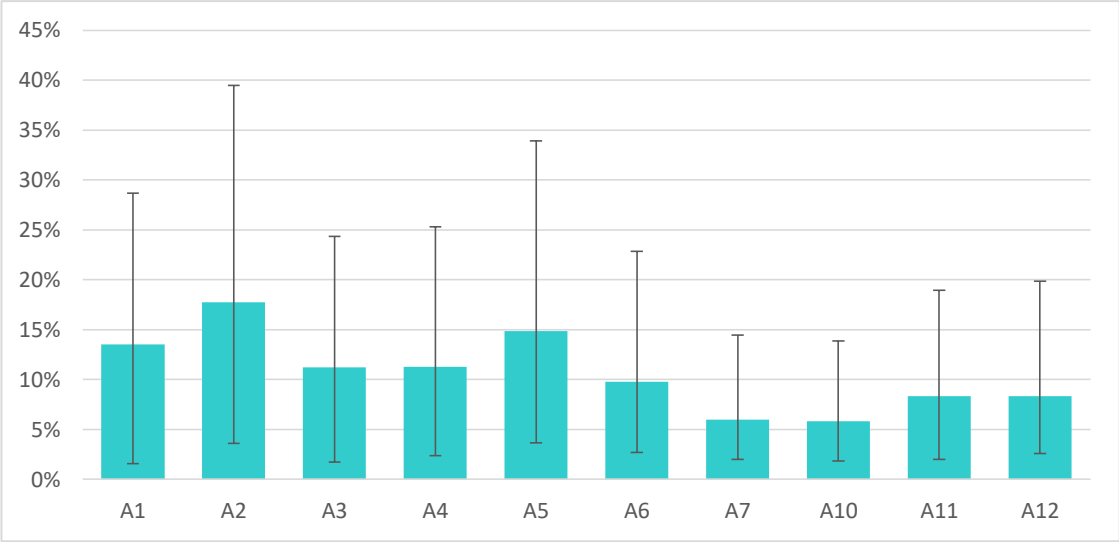

Figure 2.6. Proportion of mosquitoes infected with *Plasmodium* spp. by sampling area. Error bars are 95% confidence intervals.

The trap type analysis showed a difference in the number of infected mosquitoes captured (Chi-square test of independence X^2= 5.43, df=1, p= 0.02), where the BG-mosquitaire traps caught significantly more mosquitoes than the gravid traps. For this analysis the gravid catches and the BG-mosquitaire catches of one day were compared. In that way, 15.2% of the mosquitoes trapped by the BG- mosquitaires were infected with *Plasmodium* spp. and 11.0% of the mosquitoes trapped by the CDC-gravid traps were infected.

Prevalence by month also showed a difference in mosquito infection (Chi-square test of independence, X^2= 447.27, df=6, p<0.001). The months with the highest number of mosquitoes infected were July (495), August (172) and June (74); with July and August having significantly more infected mosquitoes (Figure 2.7).

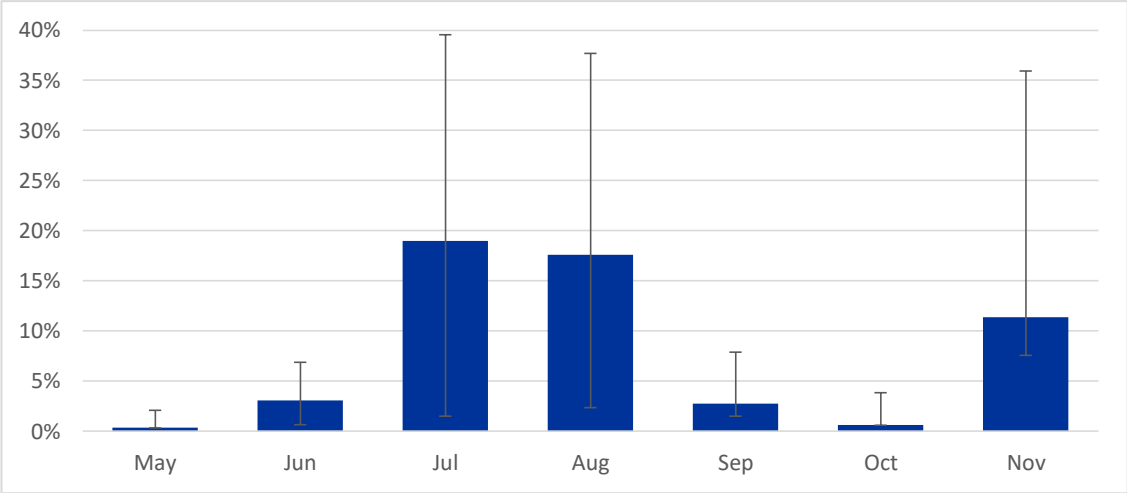

Figure 2.7. Prevalence of mosquitoes caught in Chester Zoo during 2017 infected with *Plasmodium* spp. per month. Error bars are 95% confidence intervals.

2.3.4. Birds screening

A total of 81 free-living wild birds belonging to 27 species were recovered from Chester Zoo grounds. Eurasian blackbirds (*Turdus merula*) (n=10) and Common moorhens (*Gallinula chloropus*) (n=10) were the most common dead birds found on site followed by Mallards (*Anas plathyrhynchos*) (n=9) and Common starlings (*Sturnus vulgaris*) (n=9). Post-mortem examinations did not reveal lesions associated with haemosporidia infection and the most common causes of death for these birds were window crashes and head trauma; details are shown in Appendix (2.6.2). Only two Eurasian blackbirds (*Turdus merula*) were infected with *Plasmodium* spp. Overall, *Plasmodium* spp. prevalence was 2.5% and by species, *Plasmodium* spp. prevalence in Eurasian blackbirds was 20% (Table 2.6). *Haemoproteus* spp. and *Leucocytozoon* spp. were also found on these birds as shown in Appendix (2.6.3).

Table 2.6. *Plasmodium* spp. prevalence in dead free-living wild birds found in Chester zoo during 2017

Order	Family	Species	Common name	P (n)	n	%
Accipitriformes	*Accipitridae*	*Buteo buteo*	Eurasian buzzard		2	0
Anserifirme	*Anatidae*	*Anas platyrhynchos*	Mallard		9	0
Charadiformes	Laridae	Larus argentatus	European herring gull		2	0
		Larus ridibundus	Black-headed gull		5	0
Columbiformes	*Columbidae*	*Columba livia*	Rock dove		5	0
		Columba palumbus	Common woodpigeon		1	0
		Streptopelia decaocto	Eurasian collared-dove		5	0
Gruiformes	*Rallidae*	*Gallinula chloropus*	Common moorhen		10	0
Passeriformes	*Corvidae*	*Corvus corone*	Carrion crow		1	0
		Corvus monedula	Eurasian jackdaw		1	0
		Pica pica	Eurasian magpie		1	0
	Fringilidae	*Chloris chloris*	European greenfinch		1	0
		Pyrrhula pyrrhula	Eurasian bullfinch		1	0
	Hirundinidae	*Hirundo rustica*	Barn swallow		1	0
	Muscicapidae	*Erithacus rubecula*	European robin		1	0
	Paridae	*Periparus ater*	Coal tit		1	0
		Parus major	Great tit		1	0
		Cyanistes caeruleus	Eurasian blue tit		1	0
	Passeridae	*Passer domesticus*	House sparrow		2	0
	Phylloscopidae	*Phylloscopus trochilus*	Willow warbler		3	0
	Regulidae	*Regulus regulus*	Goldcrest		2	0
		Regulus ignicapilla	Common firecrest		3	0
	Sturnidae	*Sturnus vulgaris*	Common starling		9	0
	Troglodytidae	*Troglodytes troglodytes*	Northern wren		1	0
	Turdidae	*Turdus merula*	Eurasian blackbird	2	10	20
		Turdus philomelos	Song thrush		1	0
Pelecaniformes	*Ardeidae*	*Ardea cinerea*	Grey heron		1	0
		Total		2	81	2.5

P= *Plasmodium* spp.

Throughout 2017, fresh blood samples or blood smears were obtained from captive wild birds from 21 different individuals belonging to four orders, five families and five species. Most of the samples (n=13) were from Sumatran laughingthrushes (*Garrafax bicolor*), followed by Chilean flamingo (*Phoenicopterus chilensis*) (n=4),

Waldrapp ibis (*Geronticus eremita*) (n=2) and a single observation for Black vulture (*Coragyps atratus*) and Asian glossy starling (*Aplonis panayensis*). All of these birds were negative for *Plasmodium* spp. by blood smear and PCR; however, two Sumatran Laughingthrushes were found to be infected by PCR with *Haemoproteus* spp. and *Leucocytozoon* spp. respectively (Table 2.7).

Table 2.7. *Plasmodium* spp. screening from fresh blood samples of captive wild birds at Chester zoo during 2017, excluding penguins.

Order	Family	Species	Common name	N	Blood smear	PCR
Cathartiformes	*Cathartidae*	*Coragyps atratus*	Black vulture	1	N	N
Passeriformes	*Leiotrichidae*	*Garrafax bicolor*	Sumatran Laughingthrush	13	N	N*
	Sturnidae	*Aplonis panayensis*	Asian glossy starling	1	N	N
Pelecaniformes	*Threskiornithidae*	*Geronticus eremita*	Waldrapp ibis	2	N	N
Phoenicopteriformes	*Phoenicopteridae*	*Phoenicopterus chilensis*	Chilean flamingo	4	N	N

N= Negative; *= PCR results found one bird infected with *Haemoproteus* spp and one bird infected with *Leucocytozoon* spp.

A total of 88 dead captive-wild birds, were tested for avian malaria in 2017. The birds belonged to seven orders, 11 families and 26 species. Only one species was found to be infected with *Plasmodium* spp., the Humboldt penguin (*Spheniscus humboldti*), with 25% prevalence; these infections were associated with the penguin mortality event. Most observed birds were *Streptopelia risoria* (n=22), followed by the *Spheniscus humboldti* (n=20) and *Lonchura orizivora* (n=13) (Table 2.8). Although *Plasmodium* spp. was absent from these birds, other haemosporidia were identified, as described in Appendix (2.6.4).

Table 2.8. *Plasmodium* spp. prevalence in dead captive wild birds found in Chester Zoo during 2017

Order	Family	Species	Plasmodium	n	%
Anserifirme	*Anatidae*	*Dendrocygna viduata*		1	0
Columbiformes	*Columbidae*	*Ptilinopus superbus*		1	0
		Otidiphaps aurensis		1	0
		Streptopelia risoria		22	0
Galliformes	*Phasianidae*	*Tragopan temminckii*		1	0
Musophagiformes	*Musophagidae*	*Tauraco fischeri*		2	0
Passeriformes	*Corvidae*	*Cissa thalassina*		1	0
	Estrildidae	*Lonchura oryzivora*		10	0
		Taeniopygia guttata		1	0
	Leiotrichidae	*Garrulax bicolor*		1	0
		Leiothrix lutea		1	0
	Ploceidae	*Ploceus cucullatus*		1	0
	Sturnidae	*Aplonis panayensis*		2	0
Piciformes	*Lybiidae*	*Pogonornis melanopterus*		1	0
Psittaciformes	*Psittacidae*	*Pyrrhura griseipectus*		1	0
Sphenisciformes	*Spheniscidae*	*Spheniscus humboldti*	5	20	25
		Total	5	67	7.5

2.3.5. Chester Zoo penguin screening

Humboldt penguins (*S. humboldti*) were always a priority during this study; therefore, this species accounts for most of the samples and analysis. The first samples analysed from penguins were received on the 1st of September of 2017, when the penguins were being moved (see section 2.1), on that date six blood samples and 11 blood smears were found to be negative for *Plasmodium* spp. From the 12 penguins whose samples were submitted on the 1st of September, three never developed symptoms, six died and three were tested on a later occasion and died sometime afterwards. Dates of death and organs tested for the single-time tested penguins are as follows. Three negative penguin on the 10th, 14th and 23rd October and 17th of November; one positive died on the 15th of October.

A second set of blood samples or smears from three penguins that were tested on the 1st of September arrived on the 29th of September, 6th and 10th of October; the results were once more negative and the penguins died within 3, 20 and 45 days respectively after the last sample was taken. The first re-tested penguin died on the 2nd of October, after which *Plasmodium* spp. was discovered in its lung and liver. A second re-tested penguin died on the 10th of November with no sign of *Plasmodium* spp., although its liver tested positive for *Leucocytozoon* spp. Lastly, a third penguin died on the 30th of October and its organs were negative for *Plasmodium* spp. On the 2nd of September, another batch of samples collected after the exhibit move was taken; six penguins were sampled. All of them were negative for *Plasmodium* spp. either by blood sample (n= 5) or blood smear (n= 2). Five of those penguins died on the 5th 15th, 17th and 24th of October and 17th of November. The first one had a *Plasmodium* spp. infection in its lung, the last one was positive for *Plasmodium* spp. in all organs and the other three showed no *Plasmodium* spp. infections. A final batch of samples from the reallocation arrived on the 4th of September; it consisted of blood samples and smears from five penguins, all of which tested negative for avian malaria and survived.

When the reallocation was completed, samples were only received from penguins exhibiting signs of disease. From the 26th of September to the 10th of October a second sample from five different penguins was received, consisting either of a blood sample or a blood smear; all of these penguins tested negative for avian malaria on their first and second screening, but then died in a period of 1-35 days after the second sample was taken. On the 6th of October a blood sample was received from a penguin that had not been tested previously; the results were negative but the penguin died a month later on the 8th of November. All organs tested post-mortem were negative. The last blood sample was received on the 27th of October from a penguin not tested before and it tested negative. From that date onwards, only organs were received from penguins that had not previously been sampled. After testing these organs, it was found that a penguin that died on the 7th of October was positive and that the penguins that died on the 21st of November and the 13th of January were negative.

In summary, 32 different penguins were tested for *Plasmodium* spp. either from blood samples, blood smears or organs. *Plasmodium* spp. was not detected in blood smears or blood samples; and from the 22 dead penguins (n=44) *Plasmodium* spp. were detected in the organs of five (Table 2.9). Causes of death of the 22 penguins were established by pathologist Julian Chantrey, the details can be seen in the introduction (Table 2.2). Although *Plasmodium* spp. was found in the organs of five penguins, avian malaria was determined as the cause of death in only one, and suspected in other four because of the observed signs and lesions.

Table 2.9. *Plasmodium* spp. screening from Chester Zoo penguin's colony during 2017. Blood smears and fresh blood and organs PCR results.

ID	Sampling Date	Blood sample	Smear	Date of death	Brain	CB	Liver	Kidney	Spleen	Lung
C08159	01/Sept	N								
C12187	01/Sept	N	N							
940015	01/Sept		N							
C12185	01/Sept	N	N	10/Oct	N	N	N		N	N
C07093	01/Sept		N	14/Oct	N	N	N			
950128	01/Sept			15/Oct	N	**P**[L]	**P**			
C12181	01/Sept		N	23/ Oct	N	N	N	N	N	
CO587	01/Sept			02/Oct		**P**	N			**P**
	29/Sept		N							
CZ1636	01/Sept	N	N	30/Oct	N	N	N	N		
	10/Oct	N								
CZ1379	01/Sept	N	N	10/Nov	N	N	N[L]	N	N	N
	06/Oct	N*								
BO1319	01/Sept	N	N	17/Nov	N		N	N	N	
C08131	01/Sept		N	02/Jan[+]						
C13178	02/Sept			05/Oct		N	N	N	N	**P**
	04/Oct	N								
C14138	02/Sept	N	N	15/Oct	N	N	N	N	N	N
C07080	02/Sept	N		17/Oct	N	N	N	N	N	N
C16135	02/Sept	N		24/Oct	N	N	N	N	N	N
C12214	02/Sept	N		17/Nov	**P**	**P**	**P**		**P**	
C06077	02/Sept		N							
C16145	04/Sept	N								
C16125	04/Sept	N								
C14175	04/Sept									
C13171	04/Sept	N	N							
C12213	04/Sept	N	N							
C14129	26/Sept	N		08/Nov	N		N	N	N	N
	02/Oct	N	N							
C06131	06/0ct	N		08/Nov	N	N	N	N	N	N
CZ33	27/Oct	N								
C06094				07/0ct	**P**	N	**P**			**P**
C13190				17/Nov						
C13176				21/Nov	N				N	N
C14175				14/Dec						
C07108				13/Jan[+]		N	N	N		
C13398				21/Jan[+]						

CB= Clotted blood; PME= Post-mortem exam; N= negative for *Plasmodium* spp; [L] = Samples were positive for *Leucocytozoon* spp; + = This death occurred in 2018

2.3.6. Bird screening in 2018

Concern was raised after the mortality event suffered by the penguins; therefore, testing of zoo birds continued throughout 2018. Blood samples from 10 Sumatran laughingthrushes (*G. bicolor*) and 19 Humboldt penguins (*S. humboldti*) were analyzed. PCR results showed that only one penguin was infected (Table 2.10); this penguin showed signs of disease and its blood smear revealed infection with *Plasmodium* spp. (Figure 2.8) of high intensity (1.5%); nevertheless the penguin survived and to date it is still at Chester Zoo.

Table 2.10. *Plasmodium* spp. screening from fresh blood samples of captive wild birds at Chester zoo during 2018.

Order	Family	Species	Common name	*Plasmodium*	n
Passeriformes	*Leiotrichidae*	*Garrulax bicolor*	Sumatran laughingthrush	0	10
Sphenisciformes	*Spheniscidae*	*Spheniscus humboldti*	Humboldt penguin	1	19

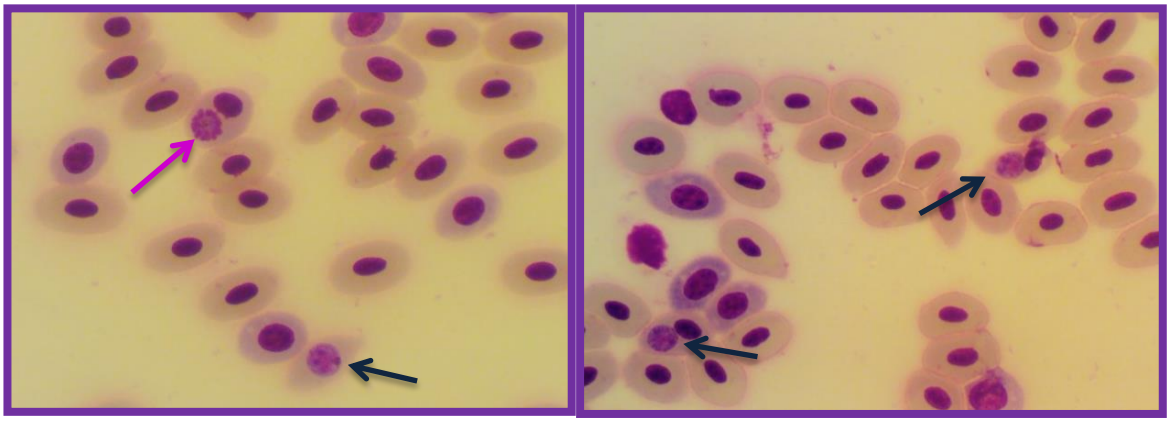

Figure 2.8. Blood smear from a Chester Zoo penguin infected with *Plasmodium* spp. in 2018. The purple arrow indicates a *Plasmodium* merozoite; black arrows indicate gametocytes.

Testing of dead captive wild birds in 2018 showed three birds infected with *Plasmodium* spp. a Red-breasted goose (*Branta ruficollis*), a Grey-winged blackbird (*Turdus boulboul*) and a Humboldt penguin (*Spheniscus humboldti*) (Table 2.11). The infected Red-breasted goose was euthanized for medical reasons and a blood smear was taken prior to its death, which showed a high intensity infection (4.5%) with *Plasmodium* spp. as seen on Figure 2.9. The Humboldt penguin infected died on the 2nd of August of 2018 and gross post-mortem lesions were highly suggestive of multisystemic avian malaria, although no parasites were seen by histopathology. Results from testing of dead free- living wild birds in 2018, can be seen in Appendix2.6. 6.

Table 2.11. *Plasmodium* spp. screening of dead captive wild birds from Chester Zoo in 2018.

Order	Family	Species	*Plasmodium* spp.	n	%
Anserifirme	*Anatidae*	*Branta ruficollis*	1	2	50
Passeriformes	*Estrildidae*	*Lonchura oryzivora*	0	1	0
	Turdidae	*Turdus boulboul*	1	1	100
Sphenisciformes	*Spheniscidae*	*Spheniscus humboldti*	1	1	100
		Total	3	5	60

Note: No other haemosporidian infections were observed

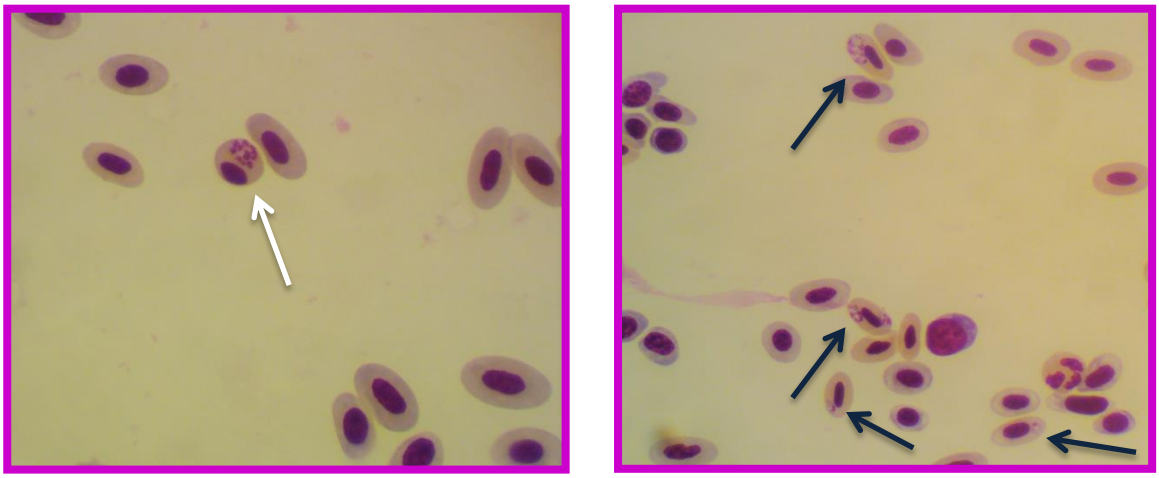

Figure 2.9. Blood smear from a Chester Zoo Read-breasted goose infected with *Plasmodium* spp. in 2018. White arrow points a *Plasmodium* spp. merozoite, black arrows point at different life stages of the parasite.

2.3.7. Additional penguin screening from Chester Zoo and other UK collections

The mass mortality observed in the penguins involving avian malaria raised another question: Was the parasite present in the area before 2017 or has it recently settled there? Therefore, stored organs of penguins that died in previous years at Chester Zoo were sampled. Organ samples from 11 penguins were tested, two from 2011, two from 2012, one from 2013, one from 2014, two from 2015 and three from 2016; of these, one penguin from 2013 was infected with *Plasmodium* spp. and the rest were negative. To compare parasite species within UK penguins, organs from 19 penguins suspected or confirmed to have died of avian malaria in different years and zoos in the UK were tested. This sampling included different penguin species; Magellanic penguins (*Sphenicus magellanicus*) (n=2), Macaroni penguins (*Eudyptes chrysolophus*) (n=5) and Humboldt penguins (*Spheniscus humboldti*) (n=12). Samples were submitted from five zoos at different locations; Lancashire (Blackpool Zoo), Pembrokeshire (Folly Farm), Devon (Paignton Zoo), Oxfordshire (Cotswold Wildlife Park) and Middlesex (London Zoo). Out of the 19 analyzed, 14 samples were positive; of those, nine were from Paignton Zoo (n=13) two from Blackpool (n=2), two from London Zoo (n=2), and one from Cotswold Wildlife Park (n=1) (Table 2.12).

Table 2.12. PCR result of tested penguin samples suspected to die of avian malaria from different years and zoos in UK.

ID	Zoo	Year	Species	PCR
Pai8100	Paignton	ND	*Spheniscus humboldti*	N
Pai1525	Paignton	2016	*Spheniscus humboldti*	N
Pai1688	Paignton	ND	*Spheniscus humboldti*	N
Pai0909	Paignton	ND	*Spheniscus humboldti*	P
Pai6840	Paignton	ND	*Spheniscus humboldti*	P
Pai6518	Paignton	2014	*Eudyptes chrysolophus*	P
Pai9190	Paignton	2014	*Eudyptes chrysolophus*	N
Pai9032	Paignton	2014	*Eudyptes chrysolophus*	P
Pai6509	Paignton	2014	*Eudyptes chrysolophus*	P
Pai9690	Paignton	2014	*Eudyptes chrysolophus*	P
Pai6515	Paignton	ND	*Spheniscus humboldti*	P
Pai9626	Paignton	ND	*Spheniscus humboldti*	P
Pai12317	Paignton	ND	*Spheniscus humboldti*	P
Bpool1	Blackpool	2018	*Spheniscus magellanicus*	P
Bpool2	Blackpool	2017	*Spheniscus magellanicus*	P
Lon823	London Zoo	ND	*Spheniscus humboldti*	P
Lon828	London Zoo	ND	*Spheniscus humboldti*	P
Ffarm	Folly Farm	2017	*Spheniscus humboldti*	N
Cots	Cotswold Wildlife Park	ND	*Spheniscus humboldti*	P

ND= Not determined; P= Positive for *Plasmodium* spp.; N= Negative for *Plasmodium* spp.

2.3.8. Genetic analysis

A total of 774 *Plasmodium cytb* sequences were obtained in this study. All sequences showed a sequence identity of between 96 and 100% with *Plasmodium* spp. *cytb* reference sequences from the NCBI database. From Chester Zoo, 750 *cytb* sequences were recovered from mosquitoes in 2017, two from wild birds (*T. merula*) and five from Humboldt penguins (*S. humboldti*). Three additional Humboldt penguin sequences from Chester Zoo were obtained, one from 2013 (deceased) and two from 2018 (one alive, one deceased) (Table 2.10, Table 2.11). Lastly 14 penguin sequences were obtained from different Zoos in the UK; two from Magellanic penguins (*S. magellanicus*), four from Macaroni penguins (*Eudyptes chrysolophus*) and eight from Humboldt penguins (*S. humboldti*) (Table 2.12).

2.3.9. Phylogenetic analysis

A portion of mosquito *cytb* sequences (n=258) represented either mixed infections or partial sequences and could not be analysed further. The remaining sequences retrieved from this study were clustered at different sequence divergence thresholds (0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0% and 3.5%) with the objective of choosing the threshold level that best retains morphospecies. Each divergence threshold produces a different number of clusters; this number diminishes as the sequence divergence percentage increases (Table 2.13). Ideally, all reference lineages of one morphospecies should group in a single cluster, but when applying different divergence percentages, two situations occurred. First, sequences labelled as the same morphospecies appeared in two or more clusters, which suggest that one morphospecies is composed of more than one distinctive lineage; second, and most important, sequences of two or more morphospecies were combined into a single cluster, which implies that different morphospecies are actually not different. Within the range of divergence thresholds applied, there was a tendency to observe morphospecies splitting into multiple clusters as the divergence percentage decreased, whilst the number of morphospecies mixing in one cluster increased along with the sequence divergence threshold (Table 2.13). Hence, the sequence divergence percentage that best represents the concept of morphospecies would be the one with the lowest number of morphospecies combining in the same clusters at the same time as having the lowest number of morphospecies splitting onto different clusters; such percentage was found to be 1.0% (Table 2.13).

Table 2.13. Sequence divergence thresholds analysis.

Divergence porcentaje (%)	Clusters (n)	Splitted morphospecies (n)	Mixed morphospecies (n)	Scoring
0.5	2096	14	4	18
1.0	**1275**	**9**	**6**	**15**
1.5	903	4	13	17
2.0	670	3	23	26
2.5	523	5	23	28
3.0	406	4	30	34
3.5	319	2	32	34

For a better description of the lineage clustering behaviour, we should note the distribution of the clusters; as the sequence divergence threshold increases the number or clusters mixing morphospecies and the number of morphospecies per cluster do too. Conversely, the number of clusters splitting morphospecies and the number of clusters on which a morphospecies is spread decreases as the divergence threshold increases (Table 2.14).

Table 2.14. Sequence divergence clustering behavior.

Divergence percentage (%)	Clusters (n)	Clusters mixing morphospecies (n)	Maximum number of mixed morphospecies in a cluster (n)	Clusters splitting morphospecies (n)	Maximum number of clusters were the same morphospecies is found (n)
0.5	2096	2	2	33	6
1.0	1275	3	2	14	5
1.5	903	6	3	11	4
2.0	670	9	5	10	4
2.5	523	7	5	12	3
3.0	406	11	8	7	3
3.5	319	12	9	2	2

CD-HIT analysis revealed that the majority of the samples clustered within four reference lineages: LINN1 (n=284), AH0824 (n=127), GRW11 (n=16) and SGS1 (n=10) with the following GenBank accession numbers; MK443241, MK652243, JN164731 and LN835311 respectively. Each of the lineages mentioned before are linked to a morphospecies, which are *P*. *matutinum*, *P*. *vaughani*, *P*. *relictum* and *P*. *relictum* respectively. Hence, from here onwards the first two letters of the associated morphospecies was added to the lineage name of the sequence, thus: LINN1Pm, AH0824Pv, SGS1Pr, and GRW11Pr. The remaining sequences formed one small cluster referred to as C20123 (n=7) and multiple individual clusters (n=71); the latter did not cluster with any reference sequence at a 1% sequence divergence threshold, meaning that they represent new lineages (Table 2.15).

From the 492 mosquito-derived *cytb* sequences obtained from Chester Zoo in the phylogenetic analysis, 269 (54.7%) sequences cluster with LINN1Pm lineage, 128 (26.0%) sequences match the AH0824Pv lineage, 13 (2.6%) sequences match the GRW11Pr lineage, 9 (1.8%) sequences match the SGS1Pr lineage, 7 (1.4%) sequences form the new cluster C20123 and 68 (13.8%) are new lineages. Both of the sequences from Chester Zoo wild birds from 2017 (*Turdus merula*) matched the LINN1Pm lineage. Similarly, four of the five sequences from the Humboldt penguins (*S. humboldti*) that died in 2017 cluster with the LINN1Pm lineage, and the fifth one was recorded as a new lineage. The sequence obtained from a Chester Zoo penguin deceased in 2013 also matches the LINN1Pm lineage; whilst one of the two sequences from Chester Zoo penguins from 2018 clusters with the GRW11Pr lineage, and the second one clusters with the LINN1Pm lineage.

Regarding the penguin sequences retrieved from different zoos, from Paignton Zoo, six sequences cluster with the LINN1Pm lineage, two represent new lineages and one clusters with the GRW11Pr lineage. From Blackpool Zoo, one sequence clusters with the LINN1Pm lineage, and another sequence clusters with the SGS1Pr lineage. From London Zoo, one sequence matches the SGS1Pr lineage and the other one clusters with the LINN1Pm lineage; the sequence from Cotswold matched the AH0824Pv lineage (Table 2.15). Interestingly, the LINN1Pm lineage was found in all

the zoos but Cotswold Wildlife Park, coincidentally this was the only zoo where the AH0824Pv lineage was observed in birds. Also the lineage SGS1Pr was only seen in birds at Blackpool Zoo. Unique sequences in birds only occurred at Chester Zoo and Paignton Zoo (Table 2.15).

Table 2.15. *Plasmodium* lineages observed in samples from the different zoos analyzed in this study.

Source	LINN1Pm (n)	AH0824Pv (n)	SGS1Pr (n)	GRW11Pr (n)	C20123 (n)	Unique (n)
Chester Zoo mosquitoes 2017	269	128	9	13	7	68
Chester Zoo wild birds 2017	2					
Chester Zoo penguins 2017	4					1
Chester Zoo penguins 2013	1					
Chester Zoo penguins 2018	1			1		
Paignton Zoo	6			1		2
Blackpool Zoo	1		1			
London Zoo	1			1		
Cotswold		1				
Total	284	129	10	16	7	71

To determine to which *Plasmodium* species each *cytb* sequence belonged, phylogenetic trees were constructed following two different methodologies. The trees were in agreement with regard to the cladistics placement of all sequences. In agreement with the sequence clustering under a 1% divergence threshold, sequences that were previously matched with LINN1Pm, AH0824Pv, SGS1Pr and GRW11Pr reference lineages were also found to fall into three clades corresponding to the same species, *P. matutinum*, *P. vaughani* and *P. relictum*. The remaining sequences, which are proposed to represent new lineages also grouped within

those three species clades. The clades containing sequences isolated in this study had only one labelled morphospecies reference sequence, except for *P. relictum* clade, which includes three named lineages; nevertheless, they were all of the same species (Figures 2.10, 2.11). Hence, the sequences obtained from this study belong to three different morphospecies, *P. matutinum*, *P. vaughani* and *P. relictum,* each of which comprises multiple lineages. In this study, four new lineages were recorded for *P. relictum*, 16 for *P. vaughani* and 43 for *P. matutinum*.

In the case of Chester Zoo, four of the five penguins that died in 2017 were infected with a lineage identical to the LINN1Pm reference lineage, and the fifth penguin was infected with a unique lineage included in the *P. matutinum* clade. Therefore all avian *Plasmodium* infections recorded at Chester Zoo in 2017, including wild and captive birds, were from a single species: *P. matutinum*. The Chester Zoo penguin sequence from 2013 was also identical to the LINN1Pm reference lineage, while one of the Chester Zoo penguin sequences from 2018 falls within the *P. relictum* clade and the other one is on the *P*. *matutinum* clade. Sequences originating from penguins at other zoos included more than one species; most of the Paignton Zoo sequences fall within the *P. matutinum* clade (n=7) while the rest are *P. relictum* (n=2). Similarly, one Blackpool Zoo sequence falls within the *P. relictum* clade and the other within the *P. matutinum* clade. One London Zoo sequence is within the *P. matutinum* clade and the other is within the *P. relictum* clade. Finally, the Cotswold Wildlife Park penguin sequence falls within the *P. vaughani* clade.

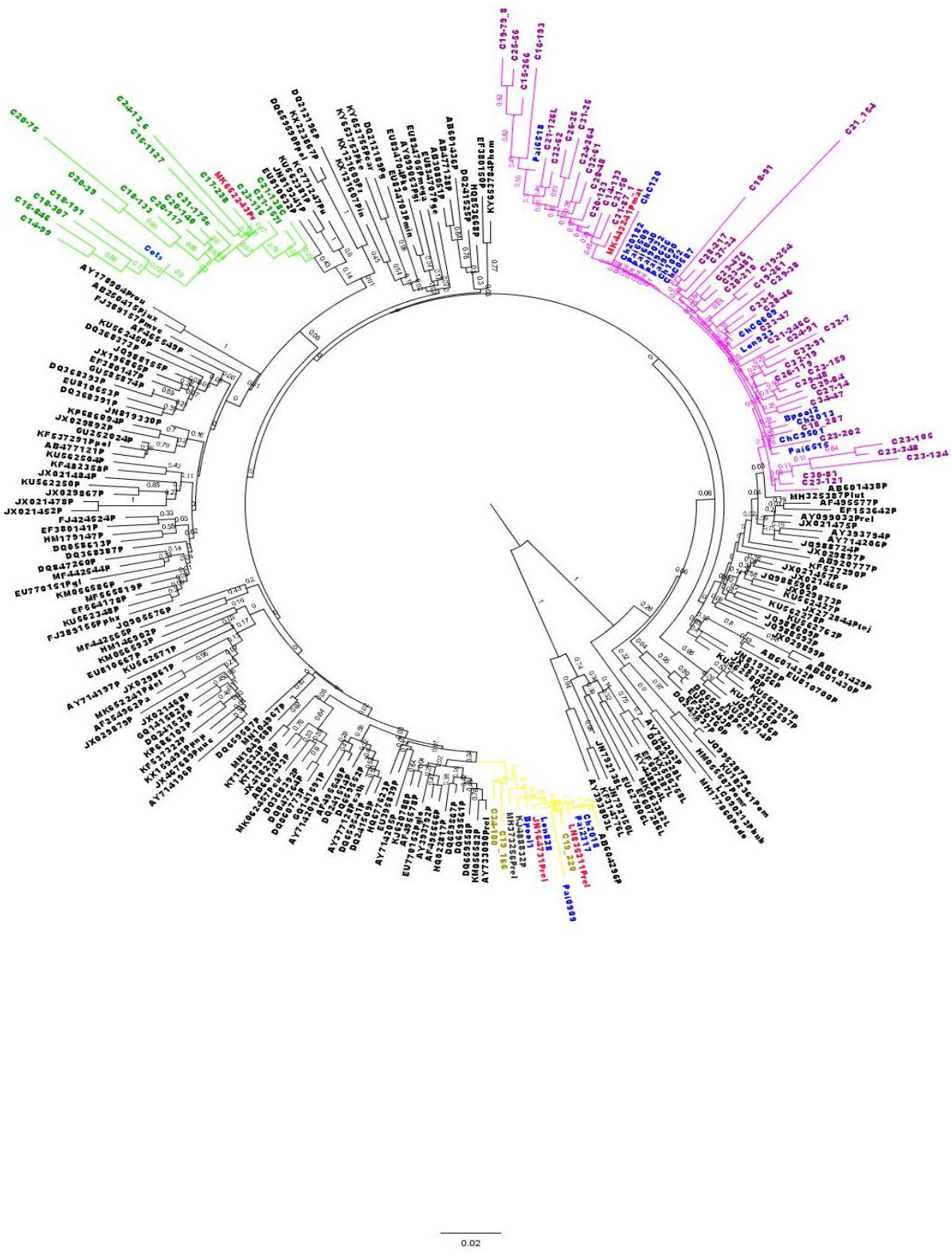

Figure 2.10. Neighbor joining phylogeny of *cytb* sequences representative of sequence clusters obtained with a 1% sequence divergence threshold. The tree was estimated with 100 bootstrap replicates and using a Tamura-Nei nucleotide substitution model. Clades containing sequences obtained in this study are highlighted in purple, green and yellow; penguin sequences are shaded blue and reference sequences that clustered most closely to sequences collected in this study are shaded in red. Sequences are identified by their accession numbers on NCBI database.

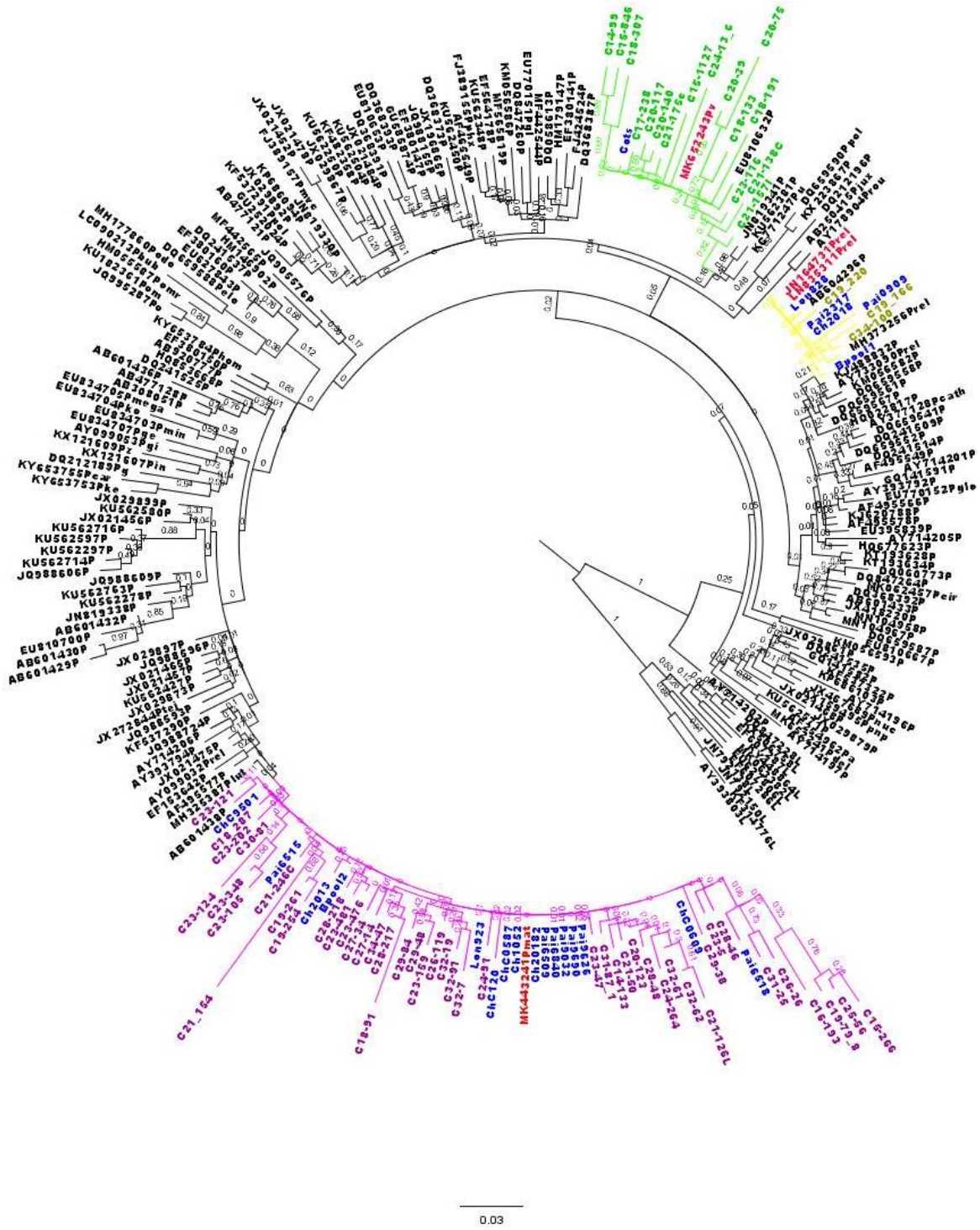

Figure 2.11. Maximum likelihood phylogeny of *cytb* sequences representative of sequence clusters at a 1% sequence divergence threshold. The tree was estimated with 100 bootstrap replicates and using a Tamura-Nei nucleotide substitution model. Clades containing sequences obtained in this study are highlighted in purple, green and yellow. Penguin sequences are shaded blue and reference sequences most closely related to sequences obtained in this study are shaded in red. Sequences are identified by their accession numbers on NCBI database.

2.3.10. *Plasmodium* temporal and spatial distribution

Mosquitoes from Chester Zoo were infected with three *Plasmodium* species, predominantly by *P. matutinum*, followed by *P. vaughani* and *P. relictum* least of all. On a temporal scale, *P. matutinum* was present on every week and it was the only species found in the first and in the last week (May 31st, Oct 18th). *P. vaughani* was first detected on June 7th and was last detected on August 30th; it was relatively more abundant than *P. matutinum* on three consecutive weeks, June 7th, 14th and 21st, and it reached its peak on July 5th. *P. relictum* appeared for a shorter time, it was first detected on June 21st and last detected on August 30th. *P. relictum* abundance was always below *P. matutinum* and *P. vaughani*; except on August 30th when it overtook *P. vaughani*. The three species reached their peak in July; first *P. relictum* and *P. vaughani* in the week of July 5th and afterwards *P. matutinum* on July 20th (Figure 2.12).

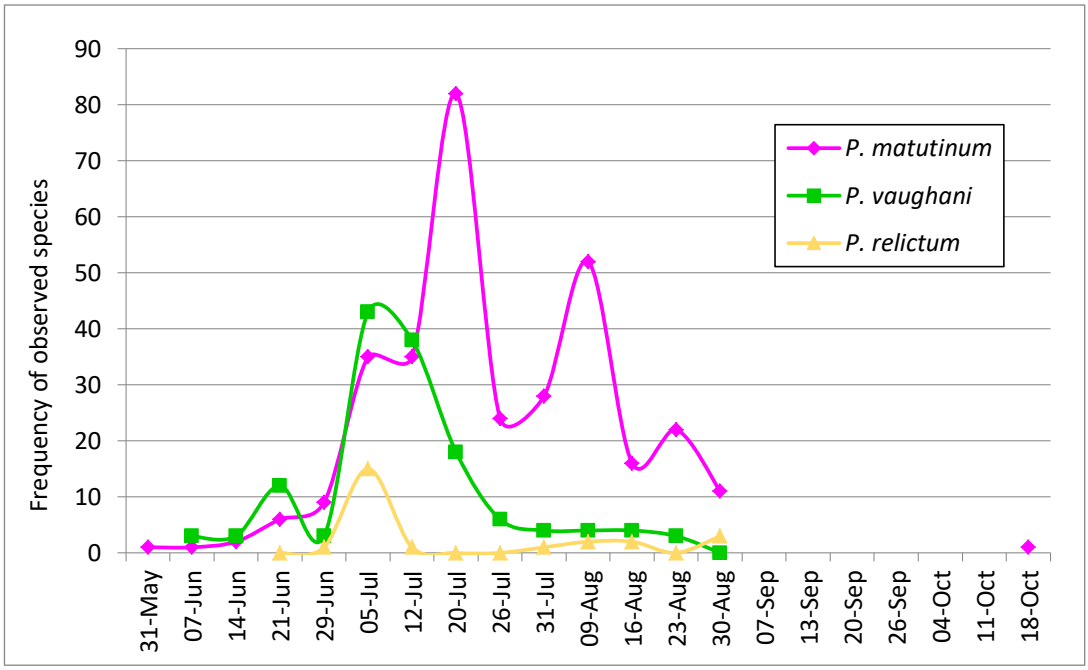

Figure 2.12. Weekly distribution of *Plasmodium* species found in mosquitoes collected at Chester Zoo in 2017.

Cytb lineages from Chester Zoo mosquitos in 2017 divide in six groups: LINN1Pm, AH0824Pv, SGS1Pr, GRW11Pr, C20123 and Unique; this latter one includes 68 new lineages. LINN1Pm is dominant overall; it occurs from May 31st - Oct 18th and its peak occurs on July 20th. AH0824Pv occurs from June 7th - August 23rd, reaching its peak on July 5th. The unique lineages appear later on June 21st and are not seen after August 30th. GRW11Pr occurs from June 29th until July 12th, then reappears between July 31st and August 9th. SGS1Pr was observed in four weeks: July 5th, August 9th, 16th and 30th. Lastly C20123 is seen in three weeks: July 12th, July 26th and 31st (Figure 2.13).

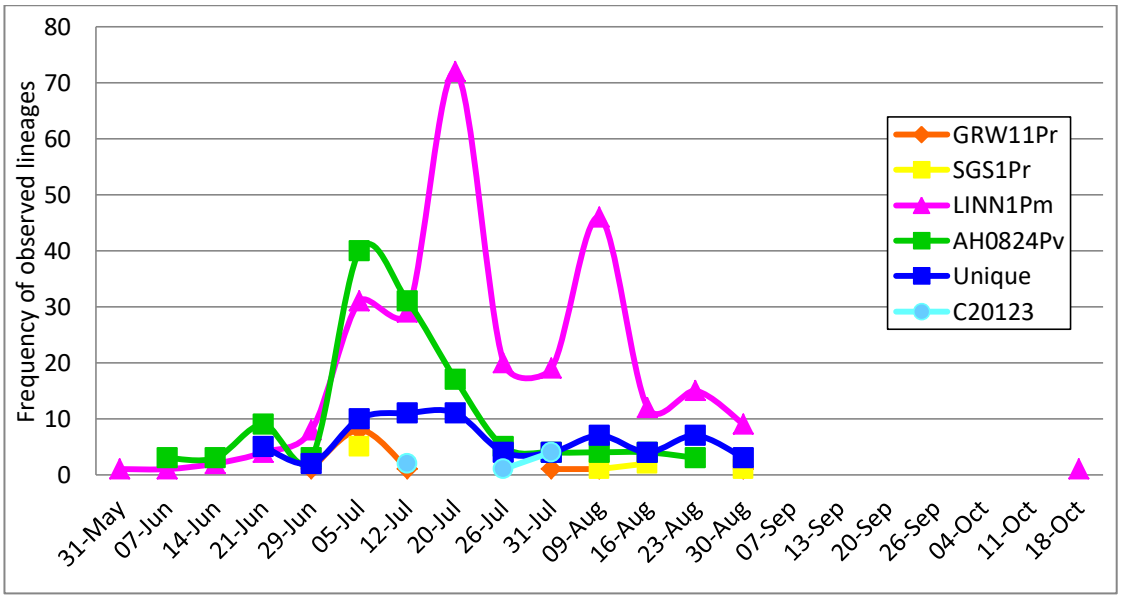

Figure 2.13. Weekly distribution of *Plasmodium cytb* lineages found in mosquitoes collected at Chester Zoo between May and October 2017.

When analyzing *Plasmodium* species distribution by area, we observe that *P. matutinum* and *P. vaughani* are present in all areas; whilst *P. relictum* is present in all but A4 and is also less frequently seen in A5. Proportions of the species are more or less the same across areas; *P. matutinum* is the most abundant followed by *P. vaughani* and *P. relictum*; although in A12 the three species proportions are more similar and in A7 *P. relictum* is observed more frequently than *P. vaughani* (Figure 2.14).

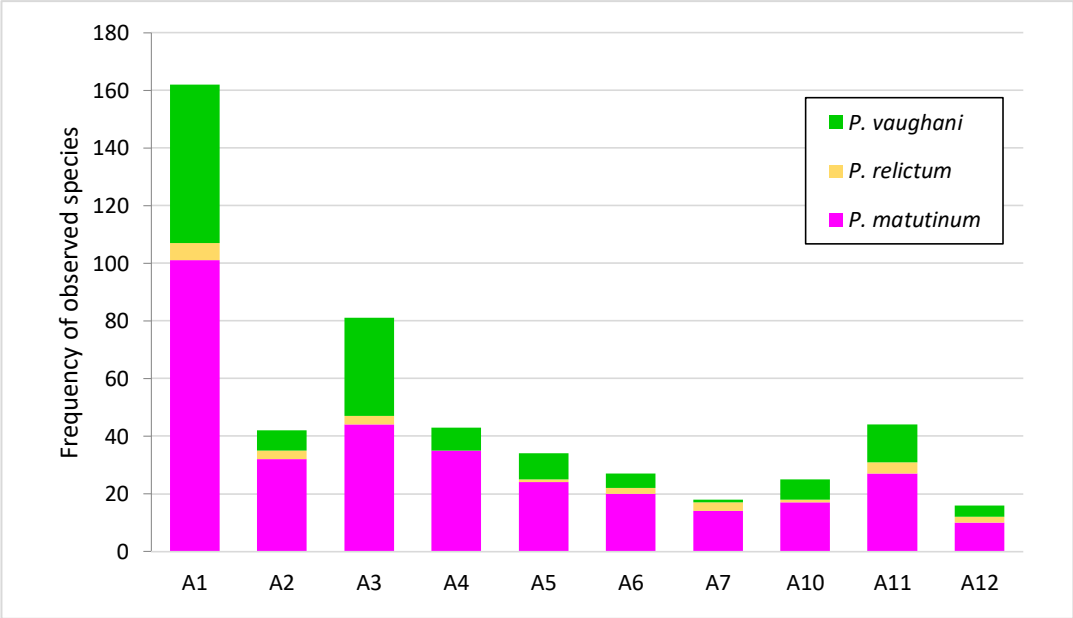

Figure 2.14. Distribution by area of *Plasmodium* species found in mosquitoes collected at Chester Zoo in 2017.

In terms of *Plasmodium* lineages distribution by area, it can be seen that LINN1Pm is predominant in all areas followed by AH0824Pv. In all areas there are only three lineages present; LINN1Pm, AH0824Pv and Unique and the rest of the lineages are seen as follows: lineage C20123 is present in A1, A4, A5 and A12; lineage SGS1Pr is observed in the areas A1, A2, A3, A7 and A12 and GRW11Pr lineage is seen in A1, A2, A3, A5, A7, A10 and A11. There is only one area where it is possible to find all lineages: A1; meanwhile A2, A3 and A7, include all lineages except Unique; A12 also includes all lineages except GRW11Pr. Similarly, A5 contains all lineages except SGS1Pr (Figure 2.15).

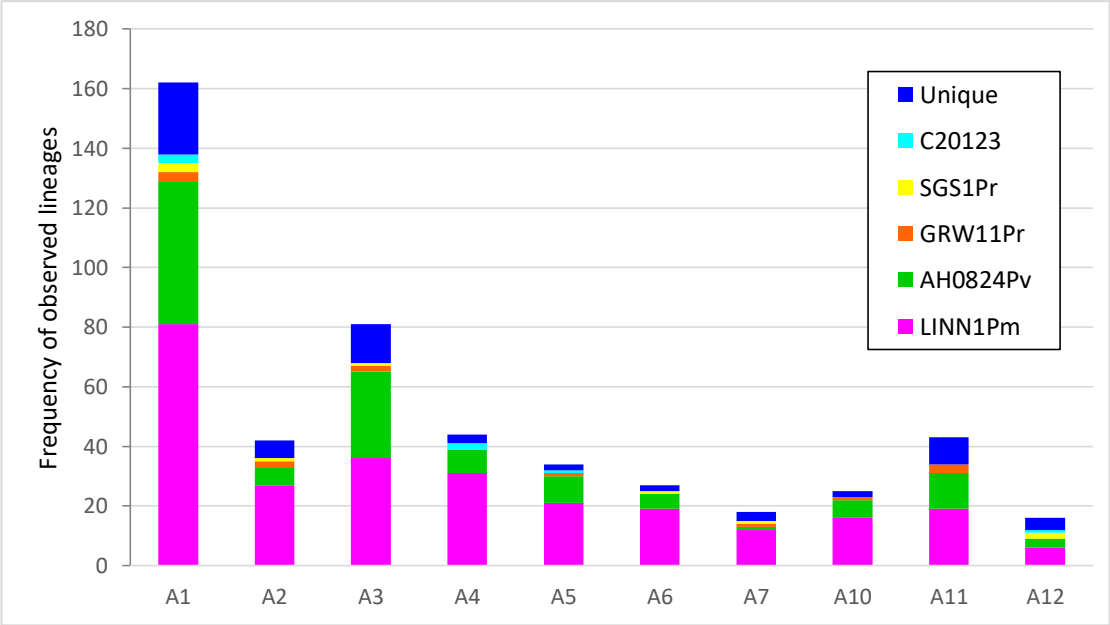

Figure 2.15. Distribution by area of *Plasmodium cytb* lineages found in mosquitoes collected at Chester Zoo in 2017.

Plasmodium infection in wild birds at Chester in 2017 was observed only in two individuals of the same species, *T. merula* (Eurasian blackbird); these infections took place at the beginning and at the end of the year, specifically in March and November. The infection of captive birds at Chester Zoo in 2017 was also restricted to one species, *S. humboldti* (Humboldt penguin), where five individuals were positive, one in September and the remaining four in October. In the *Plasmodium* infections of birds in Chester Zoo in 2017 two lineages were detected; the Unique lineage in one penguin and the lineage LINN1Pm in two blackbirds and four penguins. Ultimately, all avian *Plasmodium* infections in 2017 were from a single species, *P. matutinum* (Figure 2.16).

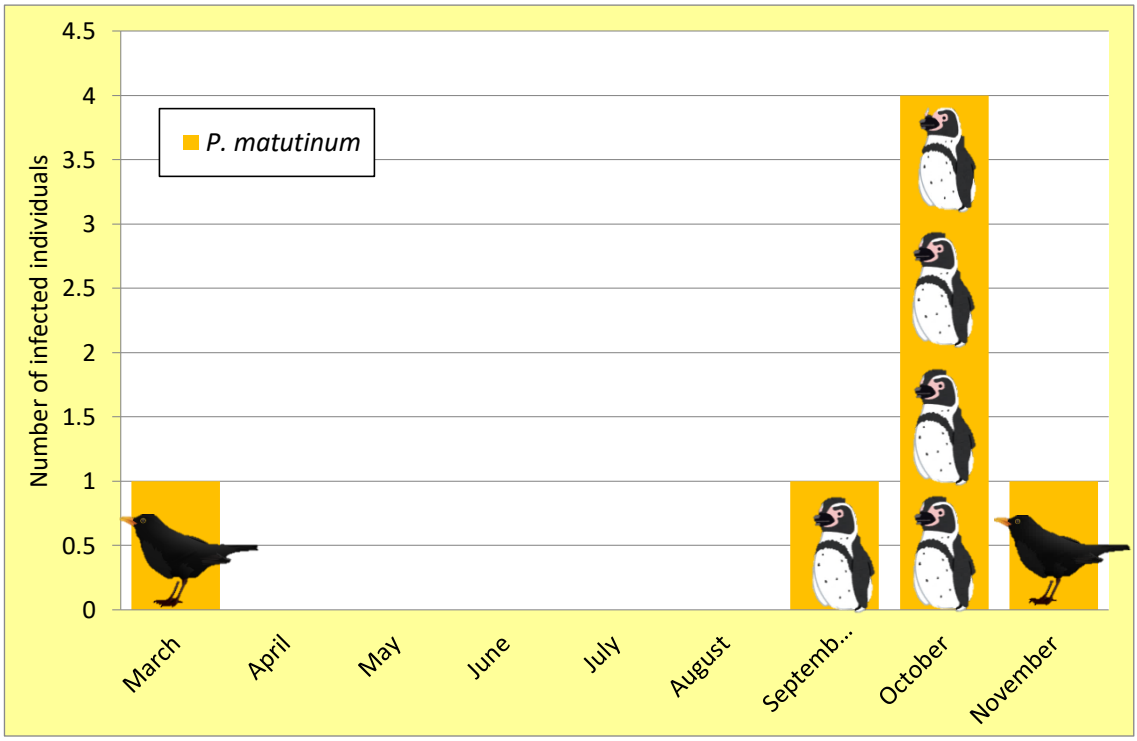

Figure 2.16. *Plasmodium* species temporal distribution on Chester Zoo captive and free-living wild birds in 2017.

2.4 DISCUSSION

The prevalence of *Plasmodium* spp. in *Cx. pipiens* (10%), as well as the marked seasonal variation in *Plasmodium* spp. prevalence, observed in this study is similar to that observed in other European countries such as France (9.52%) (Zélé, 2014b) and Switzerland (11.4%) (Lalubin, 2013). The temporal pattern in prevalence is mostly consistent with patterns observed elsewhere; infected mosquitoes occurred from May to November, whereas in other studies infections were recorded from April to November (Zélé, 2014b). These findings agree with the behaviour of European populations of *Cx. pipiens*, which cease blood feeding from October to March while in diapause. The delay in mosquitoes becoming infected in the spring, and their prolonged infection into November observed here, might reflect the

particular climatic conditions during the 2017 sampling period. Considerable rain was recorded in 2017, raising the average humidity (79%) and lowering the average temperature on site (13.6°C) (Hernández-Colina, 2019).

From the 515 mosquitoes infected with *Plasmodium* spp. on which genetic analysis was done, three different species were identified, *P. matutinum* (66.2%), *P. vaughani* (28.7%) and *P. relictum* (5.1%), all of which have been previously described in birds and mosquitoes in Europe. With respect to *Plasmodium* lineages, 72 new lineages (based on a 1% sequence divergence threshold) were recorded in addition to the four familiar lineages, LINN1Pm (n=284), AH0824Pv (n=127), SGS1Pr (n=10) and GRW11Pr (n=16). One of the novel lineages, C20123 (n=7), was observed repeatedly, while the other 71 were observed only once in the sample. The number of new lineages recorded indicates a high diversity of lineages in the area; however, the sequences were not entirely free of errors, represented by undetermined base pairs on the sequence reads, and these errors could be increasing the apparent diversity of lineages. Merging of forward and reverse sequences, plus manual correction of the resulting sequence may reveal more accurate lineage diversity. Research suggests that *Plasmodium* lineages infecting penguins in a given collection should reflect those that are present in local birds (Grilo, 2016). During this study, this was supported; free-living and captive wild birds were infected with the same parasite species, which was also the predominant parasite species in the mosquitoes. However, *P. vaughani* and *P. relictum* were substantial proportions of the parasites carried by mosquitoes, but they were not observed in the birds, perhaps because of the relatively small bird sample size in this study, as well as its opportunistic nature.

An interesting finding was that the Flamingo´s pond location had the highest abundance of immature mosquitoes, compared with other areas; it had the highest catch of adult mosquitoes and also the highest *Plasmodium* prevalence. This would suggest a high risk of infection for the flamingos (the closest captive birds), nevertheless, no flamingo samples were positive for *Plasmodium* spp. and the zoo has no records of flamingos becoming infected with avian malaria or experiencing

mortality events. A complementary study on mosquito feeding behavior carried out at Chester Zoo from 2017 to 2019 recorded mosquitoes feeding on flamingos just once, indicating that mosquitoes have little preference for feeding on flamingos (Hernández-Colina, 2021a). The free-living wild bird species on which mosquitoes most commonly feed were Eurasian jackdaw (*Corvus monedula*), Eurasian magpie (*Pica pica*) and Eurasian blackbird (*Turdus merula*). From the captive wild birds, the Schalow's turaco (*Tauraco schalowi*) and Humboldt penguin (*Spheniscus humboldti*) were the most common (Hernández-Colina, 2021a). Unfortunately, few samples of those species were available for this study, except for the Eurasian blackbirds and Humboldt penguins, which coincidentally were the only species found to be infected with *Plasmodium* spp. A further study on this regard would benefit from testing large numbers of the bird species that mosquitoes most commonly feed on, looking to establish a relationship between bird infection and mosquito feeding preferences.

Plasmodium spp. was observed in 20% of the sampled Eurasian blackbirds (*Turdus merula*), this value should be taken with caution given that the sample size and the species richness of our sampling are not representative of the community due to its opportunistic design. The species tested were only the ones found dead on the zoo premises and the species abundance and habits influence the sampling, creating a bias towards the more abundant species or those which are more likely to collide with windows (this was the most common cause of death in these birds). Hence, a systematic sampling (i.e. bird netting or cage trapping) of the bird community is needed to account for *Plasmodium* spp. prevalence in free-living wild birds in the area as well as for establishing the main host and reservoir of haemosporidians in Chester Zoo.

Another limitation of this study for prevalence estimation is that it is based on observations of dead birds, whereas most studies take samples from live birds. These are in most cases indicative of an acute infection, while samples from dead birds mainly indicate chronic infections. This difference in methodology leads to the measurement of different parameters, which precludes us from making

comparisons. For instance *Plasmodium* spp. prevalence found around Oxford on a blue tit (*Cyanistes caeruleus*) population was 42%; however, the authors tested up to 900 live birds per year occasionally re-testing individuals (Knowles, 2011). If the sampling at Chester would have been on live birds and with a similar sampling size to the mentioned above, possibly the observed prevalence would have been higher.

Wild birds are suspected to be the natural reservoirs of the disease. All birds tested in this study were resident species and the only one positive, the Eurasian blackbird, was infected with *P. matutinum*, (lineage LINN1Pm). The lineage LINN1 (*P. matutinum*) has been found in dead Eurasian blackbirds before (*T. merula*) (Dinhopl, 2015); although its competent avian hosts remain unknown as the production of gametocytes was not proven (Valkiunas, 2017). It is possible that Eurasian blackbirds act as reservoirs for *Plasmodium* spp. at Chester Zoo; especially because we found the same parasite species (*P. matutinum*) in all infected penguins and the same lineage LINN1Pm in four of the five infected penguins; nevertheless, there could be more species acting as reservoirs and further sampling and testing is required to draw conclusions.

Plasmodium spp. was recorded in 7.5% of captive wild birds and it was restricted to one species, the Humboldt penguin, in which, 25% of the individuals were infected. Detection of the infection was not possible prior to the penguins' deaths despite having tested blood samples and examined blood smears from samples collected prior and during the outbreak. In a similar situation during an avian malaria outbreak in Michigan, Fix (1988) describes difficulties diagnosing avian malaria from blood smears despite extensive effort, and detecting parasitaemia in only one of 46 penguins. The difficulty for detecting avian *Plasmodium* parasites from blood could be related to the life cycle of the parasite, which only appears in blood from 2 – 4 weeks, and as we did not sample periodically, we could have missed this parasitaemic period. Alternatively, the Humboldt penguins may not be a competent host for *P. matutinum*; hence, producing an abortive infection where gametocytes are not produced. Detection of *Plasmodium* spp. in the penguins was successful by PCR using organ samples, although the parasite was only observed in one out of five

infected penguin´s organs by histopathology. This is not an uncommon result, Spottiswoode (2020) reports detection of *Plasmodium* spp. infections by PCR, but in some cases parasites were not observed on their corresponding organs impressions. In the present study, a possible explanation for this observation is that treatment with anti-malarial drugs may have caused the degradation of the parasites precluding us from observing them, as suggested by Vanstreels (2014).

Avian malaria outbreaks are reported to occur from June to November (Beier, 1980; Grilo, 2016), which is consistent with the occurrence of the penguin deaths at Chester Zoo. Avian malaria outbreaks have also been reported to occur concurrently with aspergillosis, West Nile Virus infection, poxviruses, helminthiasis, *Chlamydophila psittaci* infection, bacterial gastroenteritis (Grilo, 2016) and cholestasis (Vanstreels, 2015); from these, aspergillosis is the most common. Similarly, at Chester Zoo, avian malaria was observed simultaneously with aspergillosis, and inflammatory processes of unknown aetiology. Another factor associated to avian malaria onset is stress; in this study, the onset of the disease happened after the penguins were moved to a temporary facility; possibly the move out and reallocation process could have stressed the penguins inducing an immunosuppressive state related to the production of corticosteroids (Fix, 1988). An avian malaria outbreak under very similar circumstances was recorded by Fix (1988), where a group of wild-caught Magellanic penguins sickened after reallocation into a zoo facility. Observed signs in Chester Zoo penguins were unspecific and consistent with the avian malaria outbreak described by Fix (1988), although they differ from what was observed on an outbreak in Brazil where no signs were observed prior to the penguin deaths (Vanstreels, 2014); this could be attributed to the parasite species which were different at each location. The fundamental difference among reports of malaria outbreaks elsewhere and at Chester Zoo is the proportion of individuals diagnosed with avian malaria; in Michigan it was 23 of 38 deaths (Fix, 1988), at Chester five of 22 and at Brazil nine of 28 (Vanstreels, 2014). In summary, all avian malaria outbreaks present high mortality in a short period of time and different diseases besides avian malaria are found, with aspergillosis being quite commonly associated.

P. matutinum was the species involved with the penguin deaths at Chester Zoo, this species was recently characterized by Valkiunas (2017), and currently it only has one entry in the Malavi sequence database documenting the association of *P. matutinum* with Humboldt penguins (although, there is no information available about the circumstances of this observation). Spottiswoode (2020) also recorded an association of *P. matutinum* with penguins, either African or Magellanic (not specified), where this species was established as the cause of death for one penguin. Hence, this is the first time that *P. matutinum* has been associated with an avian malaria outbreak in penguins and the first time that the outcome of this host-parasite association is described. *P. matutinum* lineage LINN1 has been recorded in North America, Europe, Asia and New Zealand in the following bird species: *Apteryx haastii*, *Apteryx mantelli*, *Fulica cristata*, *Philesturnus carunculatus*, *Carduelis cannabina*, *Corvus cornix cornix*, *Anthornis melanura*, *Erithacus rubecula*, *Luscinia luscinia*, *L. svecica*, *Baelophus bicolor*, *Cyanistes caeruleus*, *Passer montanus*, *Petroica longipes*, *Turdus merula*, *T. philomelos*, *Zosterops lateralis*, *Spheniscus humboldti*, *Eudyptyula minor*, and *Athene noctua*. *Culex pipiens* is suspected to be its vector, but no sporozoites have been isolated from salivary glands. Parasitaemia developed by this lineage is low, even in its peak (0-0.1%); however, the pathogenesis was described from an experimental infection on canaries, a species that probably has coevolved with the parasite and therefore is less susceptible to the infection. The pathogenesis of this lineage in other hosts is not known; hence, the infection in more susceptible hosts could behave differently (Valkiunas, 2017), as observed in Chester Zoo penguins.

Sampling and testing for avian malaria at Chester Zoo continued for one year after the outbreak; analysis of blood samples from the remaining penguins revealed one of them was infected with *P. relictum* with high infection intensity. In April 2018, the penguin showed breathing difficulties, a relapsed aspergillosis infection was suspected but analysis showed a *Plasmodium* spp. infection. Coincidentally, the penguin was moulting at that time and moulting is known to exert stress on birds and therefore is listed as one of the triggers for avian malaria. The penguin was treated with antifungals and antimalarial drugs and survived. It is worth noting that

this is the only case in which evidence of *Plasmodium* spp. was observed in blood samples and smears. Observing one penguin that survived an avian malaria infection of *P. relictum*, while the other five died of an infection with *P. matutinum* could suggest that specific parasite species or even lineages, impact hosts differently (Vanstreels, 2015). This idea is supported by the findings of Spottiswoode (2020), who observed that over the course of 20 years the parasite lineages obtained from dead birds that died of avian malaria (*P. cathemerium* SEIAUR01, *P. matutinum* LINN1) were different from parasite lineages found on the blood of live birds sampled in the last three years of the study period (*P. relictum* SGS1). Furthermore, it was documented that while an Italian strain of *P. matutinum* was lethal, an American strain was less virulent (Valkiunas 2017).

P. *relictum* has been recorded as one of the most pathogenic species (Grilo, 2016) and one with a cosmopolitan distribution, although this may not be true for all its lineages. It has been found that each *P*. *relictum* lineage has a very distinctive geographic profile (Hellgreen, 2015) and that certain *P*. *relictum* lineages seemed to benefit host reproductive output (Lachish, 2011). *P. relictum* is also known to be a generalist parasite able to infect a wide variety of avian species (Vanstreels, 2015; Spottiswoode, 2020); this attribute would cause it to interact with many non-competent hosts leading to abortive infections with high mortality. Nevertheless, its pathogenesis has been described on a general basis, and details on different lineages or particular host interactions are lacking. Therefore, it is important to understand the dynamics of avian malaria infections locally, and for this, the study of the mosquito species in the area that transmit the parasites and their breeding sites is needed, as well as investigating the parasite species and lineages circulating and their abundance. That information could be used to install specific preventive measures, like reducing breeding sites for mosquito species carrying the parasites, or choosing a treatment for the organ or blood stages of the parasite depending on their described pathogenesis. Continuous surveillance on zoological gardens could also alert of the presence of the parasite with more accuracy, which could start preventive measures such as keeping the penguins indoors during risk periods.

2.5. CONCLUSION

This is the first time that *P. matutinum* has been reported associated with an avian malaria outbreak in penguins and the first time that the effects of *P. matutinum* on Humboldt penguins have been described. The species and lineages of *Plasmodium* and the host they infect are key determinants of the disease severity; therefore, to preserve the health of birds in captivity, it is necessary to maintain an epidemiological surveillance of the diversity of avian malaria species and lineages within an area, from both birds and mosquitoes. Special attention should be given to the biology and ecology of vectors in the area, particularly identifying potential vectors in order to target their populations with control strategies. Diagnosis is often difficult to achieve in live birds, particularly in acute presentations of the disease and in abortive infections. Complementary to the surveillance, an individual-based post-mortem examination practice should be established to detect all possible parasite species and lineages involved in an outbreak. This practice would give us more information about the epidemiology of the disease and of any possible outcomes of different host-parasite associations. Additionally, in face of the lack of information available about the occurrence of outbreaks and cases of infected individuals, publishing of data detailing the epidemiology of this disease should be promoted. The shared information should include at least observed signs, diagnosis methodology, parasite species and lineage if possible, gross and microscopic lesions, established causes of death and treatment protocols. This data will contribute to the characterization of the onset, pathology and outcomes of the infection produced by different parasite species in association with different hosts. Overall, this study highlights the importance of knowing the ecology of parasites in a particular area in addition to the identification of *Plasmodium* species and lineages for each individual. It also points out the need of standardizing diagnosis to determine causes of death, as well as the need to make all infection-related data available to provide better insight into the disease ecology.

2.6. Appendixes

Appendix 2.6.1: Approved Research Proposal Form of Chester Zoo

Research Proposal Form

CHESTER ZOO

FOR OFFICE USE ONLY

Received:
Logged □
Rejected □ Approved □

Section 1 Project Summary

Project Title			
Study of avian malaria epidemiology and genetics in Chester Zoo.			
Brief project summary *(Max. 100 words; please include justification for study, project aims, methods and expected project outcome)*			
The concern about avian malaria has increased after recent reports in zoo birds. Until now, Chester Zoo has not had cases of the disease; therefore, the aim of this proposal is to evaluate the avian malaria risk in Chester Zoo. For this, we will study the mosquito community to identify the main vector, evaluate blood and organs samples of zoo birds and wild birds, and perform genetic analysis of the parasite. This will allow us to assess the epidemiology and genetics of avian malaria and propose explanations for the current situation in the zoo and recommendations for preventing the infection of zoo birds.			
Proposed start date of data collection with Chester Zoo (dd/mm/yyyy)	13/03/2017	**Proposed end date of data collection with Chester Zoo** (dd/mm/yyyy)	13/11/17

Section 2 Researchers involved in the study

Please add as many rows as is required.			
Name and Institution Please include all people involved in the project	**Role(s) in the study** e.g. Main Researcher or Principal Investigator	**Is this contributing to your academic qualification?** If so, please include qualification level (e.g. M.Sc.)and course subject	**Institutional contact details**
Arturo Hernandez-Colina, University of Liverpool	Main researcher	PhD, not related to any course.	**Address :** Leahurst campus, Chester High Road, Neston CH64 7TE **Email :** arturoh@liverpool.ac.uk **Tel :** 07401496983
Merit Gonzalez-Olvera, University of Liverpool	Main researcher	PhD, not related to any course.	**Address :** Liverpool Science Park IC2, 146 Brownlow Hill Liverpool L3 5RF **Email :** meritmx@liverpool.ac.uk **Tel :** 07463006383
Matthew Baylis, University of Liverpool	Supervisor		**Address :** Leahurst campus, Chester High Road, Neston CH64 7TE **Email :** matthew.baylis@liverpool.ac.uk **Tel :** 0151 794 6084
Andrew Jackson, University of Liverpool	Supervisor		**Address :** Liverpool Science Park IC2, 146 Brownlow Hill Liverpool L3 5RF **Email :** A.P.Jackson@liverpool.ac.uk **Tel :** 0151 795 0225

Section 3 Project Outline

Section 3a Introduction

Background introduction:

Avian malaria is a world-wide distributed disease caused by the infection of blood parasites from the genus *Plasmodium* [1]. From the more than 40 *Plasmodium* morphological species, *P. relictum* is the most relevant since it has been reported in more than 400 bird species and has affected wild birds and caused outbreaks in zoos worldwide [1, 2].

This parasite requires a vector for its transmission and although there is scarce information about the species that it infects, the most recognised one is *Culex* spp. mosquitoes [2]. Only few *Culex* species have been proven to be natural vectors but other mosquito genera in which it can complete its life cycle are *Aedes*, *Anopheles, Coquillettidia* and *Culiceta* [1-4].

The impacts of avian malaria in wild bird populations are poorly understood, varying from subclinical fitness effects to population decline and extinction. It can produce significant long-term effects like reduced reproduction fitness of the avian host [1, 5], but perhaps the most evident case is the population declines, distribution restriction and extinction of several Hawaiian birds [1].

In captive birds the morbidity can be severe and often provokes death, predominantly in penguin populations and young birds [2]. There have been reports of zoo birds affected by avian malaria in Europe [2] and in UK zoos, the disease has affected African black-footed penguins (*Spheniscus demersus*) [3].

Climate change is expected to increase the distribution and intensity of vector-borne diseases like avian malaria [5] and despite there are no reports of penguin mortality due this disease in wild populations, the spread of mosquito species can represent a risk for them [1]; therefore, understanding how this disease affects penguins in zoos can also provide valuable information for the conservation of these birds in their natural habitats.

The study of *Plasmodium* species in the field is limited and the differences of host susceptibility, vector competence and pathogenicity has not been clarified; nevertheless, the analysis of its genetic structure, especially mitochondrial genes, can provide relevant information [1].Lately, molecular methods are revealing that the prevalence of the disease is higher in non-native birds and that the genetic lineages structure of this parasite is more complex than previously though [1]. Likewise, the highest mortalities have been reported in cases where the hosts present mixed infections of two or more malaria species, with the exception of the Hawaiian birds epidemic caused by a single lineage [5]. The genetic analysis of the infected hosts is key to disclose these epidemiological features.

Understanding the vector ecology and the inherent epidemiology of mosquito born-diseases can help to optimize the preventive measures and improve considerably the

animal health management [3]. So far, there have not been avian malaria cases in Chester Zoo, despite the sudden and serious outbreaks that have occurred in other zoos under similar conditions in the UK. Therefore, investigating the local epidemiology of the disease encompassing mosquitoes, birds, and genetics of the parasite, can provide insights of the reasons for the current situation and potential risks in Chester Zoo and provide information to suggest measures for preventing the infection of the zoo birds and propose strategies for the surveillance of this disease.
Research aim(s):
Investigate the presence and distribution of avian malaria and avian malaria vectors at Chester Zoo in order to establish baseline data for the recommendation and assessment of preventative and management measures and to inform the disease potential risk. If avian malaria is identified, to analyse the parasite epidemiologic and genetic properties and to compare its genetic lineage with other lineages reported in the UK and Europe.
List individual hypotheses:
- The vector of avian malaria is not present in the area. - The vector is present but it is not infected with the parasite. - The vector is present and infected with the parasite but transmission to the wild or zoo birds has not been detected. - The avian malaria parasite has infected wild birds or zoo birds. - The genetic lineage of the parasite belongs to one or more of the previously reported ones.

Section 3b Methods

Does this study involve human subjects?*	☐ Yes ☒ No
Does this study involve non-human animal subjects (hereafter known as 'animals')?	☐ Yes ☒ No
Will the researcher need to use a camera or video camera as part of the study to record animal or human subjects?	☐ Yes ☒ No

If yes please refer to section 2.5 of the Chester Zoo Research Policy	
Will the animals or their environment be manipulated for research purposes during the course of the study? *For example altering feeding practices, adaptation of enclosure or use of contraception, wild animal trapping.**	☒ Yes ☐ No ☐ N/A

***N.B.** Projects which involve human subjects and projects which involve manipulation of the animal or its environment may be reviewed by Chester Zoo's Ethical Review Committee*

For projects involving animals, please fill in the following information. Alternatively, if the project involves several species please attach a species list on a separate sheet including the information required below. *If you require a Taxon Report in order to fill in this section, please contact research@chesterzoo.org*	
Species:	Total N:
Control animals (if applicable): N (infants): N (juveniles): N (adolescents): N (adults):	Subjects (non-control): N (infants): N (juveniles): N (adolescents): N (adults):
Research design:	
Vector screening **Larvae sampling:** The method consists on submerging a standard dipper in the potential breeding sites for mosquitoes to capture larvae which then are filtered and preserved in a plastic container. We will choose ten sampling sites randomly in water bodies in the zoo birds' enclosures and in the zoo facilities from which we will take no more than 1 L of water; these sites will be of different depth, sizes and kinds (artificial, natural, with or without vegetation, with or without shade) and separated at least 50 m from each other. The larvae collected will be identified morphologically. **Adult mosquito sampling:** 10 BG-Mosquitaire traps and 10 CDC Gravid traps Model 1712 will be used to capture adult mosquitoes. The traps will be distributed in the zoo birds' enclosures, prioritising the penguins, and near potential breeding or resting sites for the	

mosquitoes in the zoo facilities. The traps will be placed where they can remain without interfering with the activities in the zoo, considering staff and visitors, and do not represent a risk for any person or the zoo animals.

Both types of traps will be placed near vegetation, but not covered by it, or water bodies; away from busy areas, protected from direct sunlight, artificial lighting, wind and rain, and at a distance of at least 50 m form each other to avoid interference. Neither the BG-Mosquitaire nor the CDC Gravid traps release any dangerous substance to people or zoo birds. The mosquitoes will be inactivated by refrigerating at 4 °C and preserved until their analysis at -20 °C [4].

BG-Mosquitaire: the BG-Mosquitaire is a 40 cm trap that works by releasing an odour (BG-Sweetscent) to attract mosquitoes that are looking for a blood meal and sucking them with a fan into a capture funnel net; it needs to be plugged to electricity with a cable and it does not require gases to operate like propane or CO^2 [6].

CDC Gravid Trap: the CDC Gravid trap was designed to capture *Culex* mosquitoes attracting females that are looking for a place to lay their eggs. It has a tray that contains an oviposition medium, which is an infusion of hay in tap water (4 L per trap), and a collection bag in which mosquitoes are blown by a fan. It operates using a 6 volt, 12 amp DC battery [7].

Sampling Protocol: The larvae sampling will be performed once a week and will be used for the mosquito community assessment.

The BG Mosquitaire traps will be operating continuously all weekdays and we will collect two sets of samples. The first one will gather the mosquitoes captured during a week, which is consistent with the MOSI project methodology [3] and will be useful to assess the mosquito community. The second one represents the mosquitoes captured during a 24 hours period, minimizing their mortality, which is ideal for the molecular analysis.

The CDC Gravid traps will capture mosquitoes during one day per week, which are going to be used for the molecular analysis.

We will need to visit Chester Zoo every week in two consecutive days at 9:00 hrs during the sampling period, from March to November 2017; we suggest that these days can be Tuesday and Wednesday.

In the first day we will:

- Do the larvae sampling.
- Collect the adult mosquitoes from the BG Mosquitaire traps and turn them on.
- Prepare the CDC Gravid traps with the oviposition medium and turn them on.

In the second day we will:

- Collect the adult mosquitoes from the BG Mosquitaire traps and turn them on.

- Collect the adult mosquitoes from the CDC Gravid traps and turn them off.
- Pick up the bird samples processed during the week.

Zoo staff assistance will be required where it is necessary to access animal areas.

Mosquito identification: the collected mosquitoes will be counted and identified morphologically using the key from Cranston P. S. (1987) [11]. Mosquitoes from the Culex complex will be identified to the species level by extracting the DNA with ammonium acetate [5] and performing a PCR following the procedure used by Hesson J. C. (2015) [12]. We will look for differences and similarities in the mosquito community and trap locations across the sampling period.

Avian malaria screening

Samples required: Surplus blood from routine or diagnostic blood sampling of zoo birds, ideally 200 µl but could be less depending on the bird size and condition. The blood sample should be collected into an EDTA-coated microtainer for capillary collection for immediate dispatch to our laboratory for DNA extraction. At the moment of collection, we also request that three thin blood smears are carried out per bird, air-dried for 3 minutes, and fixed with 100% methanol. [8, 9]. This may be done by Zoo staff or by ourselves as convenient. We recommend that the zoo staff inform us if they decide to take samples for the diagnostic tests if any bird of the collection presents avian malaria symptoms (anaemia, lethargy, anorexia and ruffled feathers [1]).

Tissue samples (liver, spleen, lungs, brain, heart and kidneys) from dead zoo birds and tissues or whole wild birds found dead within the zoo, stored at -20 °C.

Plasmodium diagnosis: blood smears will be stained with Giemsa's solution and screened with a light microscope for parasite detection according to Valkiunas G. (2005) [9]. From mosquitoes, DNA extraction will be performed by standardized methods (phenol extraction and ethanol precipitation) [2] and from zoo birds and wild birds' blood, with ammonium acetate standard protocols [8]. The diagnosis of *Plasmodium* spp. and the characterization of its lineage will be done by nested PCR using specific designed primers for mitochondrial cytochrome B. The positive samples will be precipitated and sequenced to compare the present *Plasmodium* lineage to others using the MalAvi Database [4, 10]

Data Analysis

All samples will be analysed in University of Liverpool after its collection.

Section 3c Additional information required

Is this project being carried out at more than one site?
☐ Yes ☒ No *If yes, please provide details of collaborating institutions including, if applicable, the fate of the animals at the end of the research project:*
Is this project endorsed by the TAG/EEP or BIAZA approved? *For more information about BIAZA research support please click here.*
☐ Yes ☒ No *If yes, please provide details:*
Does any of the work in this project require a Risk Assessment to be carried out? *For example manual handling, hazardous substances, use of equipment or access to behind-the-scenes.*
☒ Yes ☐ No *If yes, please attach completed risk assessments from your institution. Please note that for some research activities, Risk Assessments need to been drawn up by Chester Zoo's Science Team.*
Irrespective of the location of the actual study, would it require a Home Office licence if it was being conducted in the UK? *For guidance, please click here to see The Animals (Scientific Procedures) Act, 1986 and the Performance of Procedures by Veterinary Surgeons. We use the same criteria for projects conducted outside the UK.*
☐ Yes ☒ No *If yes, please provide details:*
Are other licenses or permits required in this project (e.g. for capture and handling of wild animals)?
☐ Yes ☒ No *If yes, please provide details:*
Are there any costs for project activities outside of standard husbandry and/or zoo operational procedures?

Costs	**Description**	**How are these costs being met?**

Section 4 Project Plan and Timetable

Please list the specific project activities that will allow you to achieve your research aims. *Add as many rows as necessary.*			
List of project activities	**Where will these activities take place?**	**Proposed start date for each activity** (dd/mm/yyyy)	**Proposed end date for each activity** (dd/mm/yyyy)
Literature review	University of Liverpool	01/02/2017	28/02/2017
Data collection at Chester Zoo or other study site	Chester Zoo	13/03/2017	13/11/2017
Data analysis	University of Liverpool	13/03/2017	15/01/2018
Report writing	University of Liverpool	13/11/2017	19/02/2018
Submission of written report to Chester Zoo's Science Team	University of Liverpool	12/03/2018	26/03/2018
Expected details of any publications arising from this work, if applicable	University of Liverpool	02/04/2018	28/05/2018

Section 5 Project Ethics, Benefits and Output

N.B. All applicants must complete Section 5a. Please also ensure that you complete each section that applies to your project (5b and/or 5c).

Section 5a Complete for all projects:

Will this project be or has it been submitted to another ethical review committee for approval?
☐ Yes ☒ No *If yes, please provide evidence if approval has already been obtained or please give details of the status of the application:*

How will this project contribute to science, education, welfare, husbandry and/or conservation?
This project will help us to understand the particular epidemiology and genetics of avian malaria in Chester Zoo, providing information about the transmission, prevalence, genetic features and epidemiologic risks. In this way, recommendations for the further disease survey and prevention of the infection could be made, in order to protect the health and welfare of the zoo birds, including those species which conservation is threatened.
How will the results of this project be disseminated?
The outcomes of the project will be presented in a written report to Chester Zoo's Science Team, may be published in a peer-reviewed journal and will constitute part of the degree thesis of the participating Ph.D. students, Merit Gonzalez-Olvera and Arturo Hernandez-Colina.

Section 5b Complete for projects involving animals:

What is the scientific basis for using the number of animals you have stipulated? *Please show sample size calculations or give justification why this is not needed.*
Summarise the potential negative effects to the animals used in this project. *For example, due to animal handling, presence of observer, disease transmission, etc.*
.
If, during the course of the research, the negative effects to the animal(s) rose above that expected, please describe the point at which you would remove the animal from the research. *Please also include any systems in place you have to monitor any deleterious effect and what system you have to adapt the experimental design if required.*

Section 5c Complete for projects involving human participants (e.g. visitors and zoo employees):

Please outline the number of human participants involved in the project and the scientific basis for using the sample size you have stipulated. *Please show sample size calculations or give justification why this is not needed.*
How will potential participants be identified and recruited to take part in this project? How will informed consent be obtained? *Please attach any additional document, e.g. project information sheet for participants, consent form, if applicable.*
Will any of the participants be vulnerable people? *For example, children under 16, adults and children with learning disabilities or mental illnesses.*
☐ Yes ☐ No *If yes, please justify their inclusion:*
Will participants be able to withdraw from the research at anytime?
☐ Yes ☐ No *If no, please explain why:*
Please outline any potential stress, anxiety or other negative consequences, which may be caused by the research and how this will be addressed. *For example, does the research involve discussion of sensitive topics, exposure to upsetting imagery, etc.?*
What steps will be taken to ensure confidentiality and anonymity of participants during data collection, data storage, dissemination or sharing, and publication of the results?

Please include who will have access to the data, and how long will the data be stored.

Section 6 Bibliography

Please ensure all cited references are listed
1. Lapointe, D.A., C.T. Atkinson, and M.D. Samuel, *Ecology and conservation biology of avian malaria.* Ann N Y Acad Sci, 2012. 1249: p. 211-26. 2. Huijben, S., et al., *Avian malaria in Europe: an emerging infectious disease?*, in *Emerging Pests and Vector-Borne Diseases in Europe*, W. Takken and B.G.J. Knols, Editors. 2007. p. 59-74. 3. Quintavalle Pastorino, G., et al., *Project MOSI: rationale and pilot-study results of an initiative to help protect zoo animals from mosquito-transmitted pathogens and contribute data on mosquito spatio-temporal distribution change.* International Zoo Yearbook, 2015. 49(1): p. 172-188. 4. Ventim, R., et al., *Avian malaria infections in western European mosquitoes.* Parasitol Res, 2012. 111(2): p. 637-45. 5. Marzal, A., *Recent Advances in Studies on Avian Malaria Parasites*, ed. O. Okwa. 2012: InTech. 6. AG, B. *BG-Mosquitaire*. 2016 [cited 2017 03/01/2017]; Available from: http://www.biogents.com/bg-mosquitaire/. 7. Company, J.W.H. *CDC Gravid Trap*. 2017 [cited 2017 03/01/2017]; Available from: http://johnwhock.com/products/mosquito-sandfly-traps/cdc-gravid-trap/. 8. Ventim, R., et al., *Characterization of haemosporidian infections in warblers and sparrows at south-western European reed beds.* Journal of Ornithology, 2012. 153(2): p. 505-512. 9. Valkiunas, G., *Avian malaria parasites and other haemosporidia*. 2005, United States of America: CRC Press.

10. Egerhill, M., B. Canbäck, and S. Bensch, *MalAvi, Version 2.2.9*. 2016: Lund University, Department of Biology.

Section 7 Acceptance of terms and conditions

All people named in Section 2 must sign this form *By signing this form, applicants/supervisors accept the terms and conditions of Chester Zoo's Research Policy and agree to provide the Science Team with an electronic and hardcopy of the project report.*		
Name (please print)	**Signature**	**Date**
Arturo Hernandez-Colina		26-01-2017
Matthew Baylis		26-01-2017
Merit Gonzalez-Olvera		26-01-2017
Andrew Jackson		26-01-2017

Section 8 Submission Status - For office use only

Review by Chester Zoo staff	
Reviewed by member of Science Team	*Approved (Y/N):* *Enter name and signature below*:

If the species' role in the collection plan is purely research, what is the fate of those animals at the end of the research project?	
Reviewed by Curator/ Head of Relevant Department	*Approved (Y/N):* *Enter name and signature below*:

Review by Chester Zoo's Ethical Review Committee (if required)			
Date sent: dd/mm/yyyy	**Date due back:** dd/mm/yyyy	**Date returned:** dd/mm/yyyy	**Approved (Y/N):**

Appendix 2.6.2 Post-mortem examinations on dead free-living wild birds

ID	Species	Date found	PM Date	Weight (g)	Sex	Age	BC	Degradation	Abnormalities
M1	*Sturnus vulgaris*	15/05/17	25/05/17	63	F	J	3	2	Craneal trauma
M2	*Sturnus vulgaris*		25/05/17	72	F	J	3	2	
M3	*Sturnus vulgaris*	11/05/17	25/05/17	72			3	2	
M4	*Anas platyrhynchos*	17/05/17	25/05/17	1006	M	A	2	2	
M5	*Columba palumbus*		25/05/17	461		A	2	2	Craneal trauma
M6	*Anas platyrhynchos*	01/05/17	25/05/17	904	F	A	2	2	Trauma lower neck
M7	*Anas platyrhynchos*	24/05/17	25/05/17	1081	M	A	3	2	
M8	*Sturnus vulgaris*	11/05/17	25/05/17	73	M	J	3	2	
M9	*Anas platyrhynchos*	24/05/17	25/05/17	74	F	J	2	2	
M11	*Anas platyrhynchos*	24/05/17	25/05/17	32		C	2	4	
M15	*Erithacus rubecula*	22/05/17	25/05/17	19	M	J	2	3	Hemorrage on right side of the liver
M18	*Troglodytes troglodytes*		25/05/17	6	M	A	3	3	
M20	*Ardea cinerea*	13/05/17	01/06/17	1849	F	A	3	2	Cranial trauma Left humerus broken
M21	*Anas platyrhynchos*	02/05/17	01/06/17	1073	M	A	2	2	Abdominal cut
M22	*Larus argentatus*	19/03/17	01/06/17	725	M	A	3	3	Abdominal hemorrhage
M23	*Gallinula chloropus*	10/02/17	01/06/17	457	F	A	1	4	Pectoral subcutaneous trauma
M25	*Periparus ater*	02/02/17	01/06/17	16	F	A	3	3	Cranial trauma
M26	*Turdus merula*	13/03/17	01/06/17	96	M	A	3	3	Celomic cavity hemorrhage

M27	*Turdus philomelos*	03/02/17	01/06/17	90		J	3	3	Cranial trauma
M28	*Streptopelia decaocto*	17/03/17	01/06/17	106	F	J	3	2	Cranial apteric area
M29	*Sturnus vulgaris*	11/03/17	01/06/17	70	M	A	2	2	
M30	*Passer domesticus*	23/01/17	01/06/17	37	M	A	3	3	
M35	*Regulus regulus*	26/03/17	01/06/17	5	F	A	3	3	
M36	*Regulus ignicapilla*	26/03/17	01/06/17	5		A	2	3	
M37	*Regulus ignicapilla*	08/03/17	01/06/17	3	M	A	2	3	
M38	*Chloris chloris*	23/03/17	01/06/17	27	M	A	3	3	
M62	*Cyanistes caeruleus*	08/06/17	15/06/17	9	M	A	2	2	
M63	*Anas platyrhynchos*	12/06/17	15/06/17	319	M	J	1	4	Keel haemorrage
M64	*Gallinula chloropus*	06/06/17	15/06/17	311	M	A	2	4	Beheaded
M65	*Turdus merula*	27/05/17	15/06/17				2	3	
M66	*Corvus monedula*	05/06/17	15/06/17	164	F	A	1	4	Predated
M67	*Larus ridibundus*	10/06/17	15/06/17	231	M	A	1	4	
M68	*Sturnus vulgaris*	19/05/17	15/06/17	69	M		2	3	
M69	*Sturnus vulgaris*	19/05/17	15/06/17	67	M		1	2	
M70	*Sturnus vulgaris*	19/05/17	15/06/17	80			2	2	Hepatomegaly
M71	*Anas platyrhynchos*	20/05/17	15/06/17	868	F	A	3	3	Internal bleeding
M73	*Buteo buteo*	17/05/17	15/06/17	970			3	5	
M74	*Turdus merula*	11/06/17	15/06/17	49	F	J	2	3	
M76	*Hirundo rustica*	02/06/17	15/06/17	16	F	A	3	2	
M77	*Sturnus vulgaris*	22/05/17	15/06/17	72	M		2	3	
M78	*Parus major*	29/05/17	15/06/17	19			3	2	Cranial trauma
M81	*Phylloscopus trochilus*	26/06/17	30/06/17	6			3	3	
M82	*Larus ridibundus*		30/06/17	380	M	A	3	2	

M83	*Anas platyrhynchos*	25/06/18	30/06/17	600	F	J	1	2	Cranial trauma
M84	*Corvus corone*	29/08/17	30/06/17	134	M	A	2	2	Hepatomegaly Pericardial effusion
M85	*Columba livia*	20/06/17	30/06/17	224		J	3	3	Cranial trauma
M86	*Turdus merula*	14/06/17	30/06/17	97	F	J	1	3	
M88	*Passer domesticus*	05/07/17	13/07/17	24	F	J	3	3	
M89	*Turdus merula*	11/07/17	13/07/17	88	F	J	3	2	Beheaded
M90	*Turdus merula*		13/07/17	94		J	3	3	Cranial trauma
M91	*Gallinula chloropus*		13/07/17	96	M	A	1	3	
M92	*Turdus merula*	20/07/17	27/07/17	83	M	J	3	4	
M93	*Turdus merula*	17/07/17	27/07/17	84	F	J	3	3	
M94	*Buteo buteo*		27/07/17	791	M	A	3	2	Cranial trauma
M95	*Gallinula chloropus*	27/07/17	27/07/17	289	M	J	2	2	Cranial trauma
M96	*Gallinula chloropus*		27/07/17	248	F	J	2	2	
M97	*Columba livia*		27/07/17	285	F	J	2	4	Beheaded
M98	*Gallinula chloropus*	21/07/17	27/07/17	310		J	3	3	Cranial trauma
M100	*Larus ridibundus*		27/09/17	1260	M	A	2	3	
M101	*Gallinula chloropus*		27/09/17	280	M	J	2	2	
M102	*Gallinula chloropus*		27/09/17	258	F	J	2	2	
M103	*Phylloscopus trochilus*		27/09/17	18	F	A	1	3	Cranial haemorrage
M105	*Columba livia*	08/08/17	27/09/17	343	M	A	1	3	
M106	*Columba livia*	17/10/17	09/11/17	377	M	A	3	2	
M107	*Larus argentatus*		09/11/17	293	F	A	3	2	
M108	*Gallinula chloropus*		09/11/17	248	M	J	2	3	Cranial and abdominal haemorrage

M110	*Streptopelia decaocto*	31/10/17	09/11/17	107	F	J	2	3	
M111	*Columba livia*	21/10/17	09/11/17	362	F	A	2	4	
M112	*Turdus merula*		09/11/17	105	M	A	3	3	Cranial trauma
M113	*Turdus merula*		09/11/17	97		A	3	3	Cranial trauma
M114	*Phylloscopus trochilus*	31/08/17	09/11/17	8	M	A	3	2	Epidural haemorrage
M115	*Regulus ignicapilla*	06/10/17	09/11/17	5	M	A	3	2	Hepatomegaly
M116	*Regulus regulus*		09/11/17	5	M	A	3	2	White nodules of 1mm on the liver
M118	*Streptopelia decaocto*	01/10/17	09/11/17	220	F	A	3	2	
M120	*Larus ridibundus*		19/12/17	279	F	A	3	4	
M121	*Streptopelia decaocto*	12/11/17	19/12/17	132	M	A	3	3	
M122	*Larus ridibundus*		19/12/17	225	M	A	2	3	
M124	*Pyrrhula pyrrhula*		19/12/17	14	M	A	3	3	
M125	*Pica pica*	28/11/17	19/12/17	176	M	J	2	3	
M126	*Gallinula chloropus*	06/12/17	19/12/17	250	F	J	2	4	Abdominal haemorrage
M127	*Streptopelia decaocto*	18/12/17	19/12/17	485		A	3	2	Multiple skin wounds

BC= Boby condition. The scale is 1-5 with 1 being emaciated and 5 over weighted; The degradation scale is 1-5 with 1 being just died and 5 highly autolysed; Age is measured as J (juvenile), A (adult) and C (chick); Sex is represented as M (male) and F (female)

Appendix 2.6.3. Haemosporidia identified in dead free-living wild birds in 2017.

Order	Family	Species	H (%)	P (%)	L (%)	n	%
Accipitriformes	***Accipitridae***	***Buteo buteo***	**1 (50)**			**2**	**50**
Anserifirme	***Anatidae***	***Anas platyrhynchos***			**1 (11.1)**	**9**	**11.1**
Charadiformes	***Laridae***	***Larus argentatus***				**2**	**0**
		Larus ridibundus				**5**	**0**
Columbiformes	***Columbidae***	***Columba livia***			**1 (20)**	**5**	**20**
		Columba palumbus			**1 (100)**	**1**	**100**
		Streptopelia decaocto	**1(20)**		**1 (20)**	**5**	**40**
Gruiformes	***Rallidae***	***Gallinula chloropus***			**3 (33.3)**	**10**	**33.3**
Passeriformes	***Corvidae***	***Corvus corone***			**1 (100)**	**1**	**100**
		Corvus monedula				**1**	**0**
		Pica pica			**1 (100)**	**1**	**100**
	Fringilidae	***Chloris chloris***			**1 (100)**	**1**	**100**
		Pyrrhula pyrrhula				**1**	**0**
	Hirundinidae	***Hirundo rustica***				**1**	**0**
	Muscicapidae	***Erithacus rubecula***				**1**	**0**
	Paridae	***Periparus ater***			**1 (100)**	**1**	**100**
		Parus major				**1**	**0**
		Cyanistes caeruleus				**1**	**0**
	Passeridae	***Passer domesticus***				**2**	**0**
	Phylloscopidae	***Phylloscopus trochilus***	**1 (33.3)**			**3**	**33.3**
	Regulidae	***Regulus regulus***	**1(50)**			**2**	**50**
		Regulus ignicapilla	**1 (33.3)**			**3**	**33.3**
	Sturnidae	***Sturnus vulgaris***			**1 (11.1)**	**9**	**11.1**
	Troglodytidae	***Troglodytes troglodytes***				**1**	**0**
	Turdidae	***Turdus merula***	**4*(40)**	**2 (20)**	**3*(30)**	**10**	**70**
		Turdus philomelos				**1**	**0**
Pelecaniformes	***Ardeidae***	***Ardea cinerea***				**1**	**0**
		Total	**10 (12.3)**	**2 (2.5)**	**15 (18.5)**	**81**	**30.9**

H = *Haemoproteus* spp; L = *Leucocytozoon* spp; P = *Plasmodium* spp.

*Two of the *Turdus merula* infected with *Haemoproteus* were also infected with *Leucocytozoon*

Appendix 2.6.4. Post-mortem examinations on dead captive wild birds from 2017 excluding penguins

ID	Species	Death date	PM Date	Weight (g)	Sex	Age	BC	Degradation	Abnormalities
M10	*Dendrocygna viduata*	28/04/17	25/05/17	690		A	3	2	Cranial trauma PPM
M12	*Lonchura oryzivora*	08/05/17	25/05/17	19		J	3	2	Broken wing PPM
M13	*Lonchura oryzivora*	12/04/17	25/05/17	23		A	3	2	PPM
M14	*Lonchura oryzivora*	12/04/17	25/05/17	24			3	2	PPM
M16	*Aplonis panayensis*	27/04/17	25/05/17	35		A	2	5	PPM
M17	*Pogonornis melanopterus*	01/05/17	25/05/17	39		A	2	3	PPM
M19	*Leiothrix lutea*	07/05/17	25/05/17	19		A	3	2	Knee injure PPM
M24	*Ptilinopus superbus*	23/02/17	01/06/17	94		A	2	4	PPM
M31	*Lonchura oryzivora*	20/02/17	01/06/17	22	F	A	3	2	PPM
M32	*Lonchura oryzivora*	20/02/17	01/06/17	24	M	J	2	3	PPM
M33	*Lonchura oryzivora*	02/04/17	01/06/17	23	M	A	1	3	PPM
M34	*Lonchura oryzivora*	03/05/17	01/06/17	20		J	1	3	PPM
M40	*Streptopelia risoria*	04/04/17	08/06/17	193		A	3	2	PPM
M41	*Streptopelia risoria*	04/04/17	08/06/17	150		J	4	3	PPM
M42	*Streptopelia risoria*	04/04/17	08/06/17	155	M	A	3	2	PPM
M43	*Streptopelia risoria*	04/04/17	08/06/17	179	M		3	3	PPM
M44	*Streptopelia risoria*	05/04/17	08/06/17	154	F	A	3	2	PPM
M45	*Streptopelia risoria*	05/04/17	08/06/17	162		A	3	3	PPM
M46	*Streptopelia risoria*	05/04/17	08/06/17	170	M		3	2	PPM

M47	*Streptopelia risoria*	05/05/17	08/06/17	169	F		3	2	PPM
M48	*Streptopelia risoria*	06/05/17	08/06/17	155	F		3	2	PPM
M49	*Streptopelia risoria*	06/05/17	08/06/17	157	F	A	3	2	PPM
M50	*Streptopelia risoria*	06/05/17	08/06/17	146	F		2	3	PPM
M51	*Streptopelia risoria*	06/05/17	08/06/17	174		J	4	3	PPM
M52	*Streptopelia risoria*	06/05/17	08/06/17	158	M	A	2	2	PPM
M53	*Streptopelia risoria*	06/05/17	08/06/17	198	M		4	3	PPM
M54	*Streptopelia risoria*	06/05/17	08/06/17	150	F		3	3	PPM
M55	*Streptopelia risoria*	06/05/17	08/06/17	6	F		3	3	PPM
M56	*Streptopelia risoria*	06/05/17	08/06/17	176	M		3	3	PPM
M57	*Streptopelia risoria*	06/05/17	08/06/17	154	F		3	2	PPM
M58	*Streptopelia risoria*	06/05/17	08/06/17	185			3	3	PPM
M59	*Streptopelia risoria*	06/05/17	08/06/17	164	F		3	3	PPM
M60	*Streptopelia risoria*	06/05/17	08/06/17	149			4	3	PPM
M61	*Streptopelia risoria*	06/05/17	08/06/17	162	F	A	4	3	PPM
M75	*Pyrrhula griseipectus*	01/06/17	08/06/17	86			3	4	PPM
M80	*Lonchura oryzivora*	09/06/17	15/06/17	18	M	J	2	2	PPM
M87	*Lonchura oryzivora*	20/06/17	30/06/17	83	F	J	2	3	PPM
M99	*Tragopan temminckii*	24/08/17	27/09/17	84		A	3	3	PPM
M104	*Cissa thalassina*	12/09/17	27/09/17				3	2	PPM
M109	*Garrulax bicolor*	14/10/17	09/11/17	75			3	2	PPM
M117	*Aplonis panayensis*	09/10/17	09/11/17	53	M	A	3	3	PPM
M119	*Ploceus cucullatus*	08/11/17	09/11/17	27	M	J	2	3	PPM
M123	*Lonchura oryzivora*	22/10/17	09/11/17			J	3	3	PPM
M128	*Taeniopygia guttata*	21/10/17	09/11/17		F	A	2	3	PPM

M129	*Otidiphaps aurensis*	23/10/17	09/11/17	J	2	2	PPM
M130	*Tauraco fischeri*	30/10/17	09/11/17		3	2	PPM
M131	*Tauraco fischeri*	06/11/17	09/11/17		3	3	PPM

BC= Boby condition. The scale is 1-5 with 1 being emaciated and 5 over weighted; The degradation scale is 1-5 with 1 being just died and 5 highly autolysed; Age is measured as J (juvenile), A (adult) and C (chick); Sex is represented as M (male) and F (female); PPM= Previously Post-Mortem examined

Appendix 2.6.5. Haemosporidia identified in dead captive wild birds from Chester Zoo in 2017

Order	Family	Species	H	P	L	n	%
Anserifirme	***Anatidae***	***Dendrocygna viduata***				**1**	**0**
Columbiformes	***Columbidae***	***Otidiphaps aurensis***				**1**	**0**
		Ptilinopus superbus				**1**	**0**
		Streptopelia risoria				**22**	**0**
Galliformes	***Phasianidae***	***Tragopan temminckii***				**1**	**0**
Musophagiformes	***Musophagidae***	***Tauraco fischeri***				**2**	**0**
Passeriformes	***Corvidae***	***Cissa thalassina***				**1**	**0**
	Estrildidae	***Lonchura oryzivora***			**1 (10)**	**10**	**10**
		Taeniopygia guttata				**1**	**0**
	Leiotrichidae	***Garrulax bicolor***	**1**			**1**	**100**
		Leiothrix lutea				**1**	**0**
	Ploceidae	***Ploceus cucullatus***				**1**	**0**
	Sturnidae	***Aplonis panayensis***				**2**	**0**
Piciformes	***Lybiidae***	***Pogonornis melanopterus***				**1**	**0**
Psittaciformes	***Psittacidae***	***Pyrrhura griseipectus***				**1**	**0**
Sphenisciformes	***Spheniscidae***	***Spheniscus humboldti***		**5* (25)**	**2* (10)**	**20**	**25**
		Total	**1 (1.5)**	**5 (7.5)**	**3 (4.5)**	**67**	**10.5**

H = *Haemoproteus* spp.; L = *Leucocytozoon* spp.; P = *Plasmodium* spp.

*Two of the penguins infected with *Plasmodium* were also infected with *Leucocytozoon*

Appendix 2.6.6. Haemosporidia identified in dead free-living wild birds in 2018.

Order	Family	Species	H	P	L	n	%
Anserifirme	***Anatidae***	***Anas platyrhynchos***				**1**	**0**
Charadiformes	***Laridae***	***Larus argentatus***				**1**	**0**
		Larus ridibundus	**1 (33.3)**			**3**	**33.3**
Columbiformes	***Columbidae***	***Columba livia***	**1 (25)**		**2 (50)**	**4**	**75**
Gruiformes	***Rallidae***	***Gallinula chloropus***			**1**	**1**	**100**
Passeriformes	***Corvidae***	***Corvus monedula***			**7 (58.3)**	**12**	**58.3**
		Pica pica	**2* (50)**		**4* (100)**	**4**	**100**
	Passeridae	***Passer domesticus***				**1**	**0**
	Phylloscopidae	***Phylloscopus trochilus***	**1 (50)**			**2**	**50**
	Troglodytidae	***Troglodytes troglodytes***				**1**	**0**
	Turdidae	***Turdus merula***	**1 (14.2)**	**4 (57.1)**	**1 (14.2)**	**7**	**85.7**
Piciformes	***Picidae***	***Dendrocopos major***				**1**	**0**
		Total	**6 (15.7)**	**4 (10.5)**	**15 (39.4)**	**38**	**60.5**

H = *Haemoproteus* spp.; L = *Leucocytozoon* spp.; P = *Plasmodium* spp.

*Two of the *Pica pica* infected with *Haemoproteus* were also infected with *Leucocytozoon.*

CHAPTER 3

Haemosporidian diversity in birds of the Peruvian Amazonia

3.1 Introduction

The Amazon region has been long known for its high biodiversity and endemism (Fecchio, 2018a). Species diversification in Amazonia is mainly thought to have occurred through allopatric evolutionary processes; that is, that the separation of populations of the same species by geographic barriers leading to genetic differentiation. The two main hypotheses to explain the formation of physical barriers are 1) The Pleistocene refuge hypothesis, which assumes the formation over time of isolated forest refugia due to retraction and fragmentation of the rainforest, and 2) The riverine barrier hypothesis, which postulates that large Amazonian rivers functioned as boundaries for population movements (Fecchio, 2018a). Amazonian biodiversity should extend to haemosporidian parasites as well, but these parasites have been studied little within the area; particularly, information regarding blood parasites is lacking in the Peruvian Amazonia. Indigenous human settlements can be found in the Peruvian Amazonia, these communities subsist by hunting local wildlife, which they are allowed to do legally. On previous occasions, researchers have found an opportunity to study diseases and physiological traits of the local wildlife with the collaboration of the indigenous settlers in the area (Aston (2014); Aysanoa (2017); Morales (2017)), where the latter provide researchers with a variety of samples and data from their hunts (Bowler 2015). As this has been a successful strategy to provide information about areas of difficult access, it has been applied here for the study of bird haemosporidia.

3.1.1 Amazonian haemosporidians

The most common avian haemosporidian parasites (*Plasmodium* spp., *Haemoproteus* spp. and *Leucocytozoon* spp.) have been recorded in Amazonia before. However, a marked difference in their prevalence and diversity has been noted. For instance, the species and lineages of *Plasmodium* and *Haemoproteus* were found to be highly diverse and dispersed across major rivers (Fecchio, 2017b; Fecchio, 2018a). By contrast, a single lineage of *Leucocytozoon* associated with one bird species has been recorded in Amazonia in a low proportion (n= 5/90) (Fecchio, 2018c). These parasites have different temperature requirements for their development (Fecchio, 2018c) and the climatic conditions (i.e. temperature and moisture) of the Amazonia are constantly in a range that facilitates transmission of *Plasmodium* and *Haemoproteus* parasites (Fecchio, 2017b). This is reflected in the lack of seasonal variation in the prevalence of these parasites described in birds from the area (Fecchio, 2017a). A possible explanation is that vector abundance might be sufficiently stable over seasons to maintain haemosporidian transmission year-round. Alternatively, the parasites are maintained in the bird´s blood for longer periods allowing infections to be detectable even under low vector abundance (Fecchio, 2017a).

Based on the physical barriers present in Amazonia, the area has been divided into different endemism areas. Fecchio (2018a) conducted a biogeographic analysis on the diversity and distribution of haemosporidian lineages in these areas and showed that haemoparasites were capable of dispersing among avian communities within or between areas of endemism. Dispersal across endemism areas can occur either when parasites move within infected hosts, by host switching, by a combination of these two routes, or carried by their vectors (Fecchio, 2018a). Interestingly, it was found that the dispersion of haemosporidian parasites occurred with increasing frequency during the last five million years, after the modern riverine pattern was established. This finding suggests that these parasites are not constrained by major Amazonian rivers (Fecchio, 2018a). Amazonia haemoparasite

studies by Svenson-Coelho (2013) and Fecchio (2017a, 2017b, 2018a) demonstrate that multiple haemosporidian lineages coexist in the same bird communities with varying levels of host prevalence and specialization, and host exploitation ranging from infecting one to several distantly related avian host species (Fecchio, 2018a). Finding shared lineages among highly diverse bird communities implies that parasites in the area have gone through host switching processes. This agrees with the historic placement of host switching as the principal strategy in the evolutionary diversification of this group of parasites (Fecchio, 2018a).

Evolutionary strategies of haemosporidian parasites in Amazonia have also been analysed. Fecchio (2018a) performed an event cost analysis of four speciation strategies: codivergence (co-speciation), sorting (extinction), duplication (within-host speciation), and host switching. His results indicate that host switching is the most frequent strategy employed by *Plasmodium* and *Parahaemoproteus*. Alternatively, if host switching was very costly, other evolutionary events increased in frequency. In the case of *Plasmodium*, host switching was followed by duplication, whereas in *Haemoproteus*, it was followed by codivergence (Fecchio, 2018a).

3.1.2 Geographical distribution of haemosporidian parasites

Current parasite distributions include many examples of closely related haemosporidian lineages restricted to one biogeographic region and, at the same time, multiple lineages with apparently global distributions; these distributions provide strong evidence for long-distance dispersal (Ellis, 2019). Recent phylogenies place haemosporidian lineages with a marked African and Eurasian distribution nested within a clade of American lineages, implying continental dispersal in the evolutionary history of those parasites (Ellis, 2019). The broad haemosporidians geographic pattern could be partially due to dispersal by migratory birds. Studies of avian haemosporidia distribution in migratory birds have found that these birds

share lineages with birds inhabiting their breeding and wintering sites. This suggests that migratory birds can introduce haemoparasite lineages from wintering sites to their breeding sites and vice versa, transmitting them to the local bird communities (Ellis, 2019). Additionally, it has been proven that haemosporidian parasites have moved from mainland to islands by migratory birds (Ellis, 2019). For instance, two *Plasmodium* lineages detected on the Galapagos Islands matched lineages isolated from North American breeding bobolinks (*Dolichonyx oryzivorus*), the only passerine bird species that regularly migrates through Galapagos (Levin, 2016). Nevertheless, it is unlikely that migrant birds are entirely responsible for global haemosporidian distribution. Evidence suggests that the avian malaria lineage GRW04 was most likely introduced to the Hawaiian Islands by anthropogenic activities; similarly, the presence of haemosporidian parasites in New Zealand is likely to be the result of the introduction of infected birds from Europe. However, other haemosporidians may have dispersed large distances without human intervention and outside of typical bird migration routes (Ellis, 2019).

It is important to consider that haemosporidian dispersal in continental ecosystems is not as constrained as in insular ecosystems. For example, sharing of parasites lineages is commonly observed within bird communities in mainland, but the number of shared parasites decreases as the geographic range of the birds increase. This situation has been observed in malaria parasites from bird communities across island archipelagos (Fecchio, 2017b) and it is, perhaps, associated with the lack of overlap in the distributions of competent vector species (Fecchio, 2017a). On a global scale, the dispersal of avian haemosporidian parasites appears to have been relatively unimpeded by geographic barriers. It is expected then, to find little evidence of populations evolving as a result of changes in their area that physically separated them, and indeed, allopatric speciation is thought to account for only 10-15% of parasite lineages distributed across multiple continents (Ellis, 2019).

3.1.3 Objective

This chapter aims to characterise haemosporidian infections in birds hunted for consumption by the local Yagua indigenous people in a slightly disturbed area of the Peruvian Amazonia, which has been little studied. The objectives were to explore the haemosporidian diversity in the region, describe host-parasite associations, estimate the infection prevalence by species and year, and analyse the phylogenetic relationships of the species and lineages found.

3.1.4 Hypothesis

Due to the cosmopolitan presence of haemosporidians in birds, environmental influences in their transmission, and genetic relationships observed before, these parasites will be found in Amazonian birds with a prevalence variation amongst bird species and year. In addition, it is expected that the species and lineages isolated from the area will be highly related. Likewise, due to the limited research done on haemosporidians in the area, it is expected that the number of lineages and species is underrepresented, and therefore, non-reported ones will be found here.

3.2 Materials and Methods

For this study, bird samples were collected from the Peruvian Amazonia by local Yagua hunters, DNA was extracted from the samples by Stephanie Montero and Winnie Contreras, and I tested the DNA extracts for haemosporidian parasites and performed all statistical and genetic analyses.

3.2.1 Sampling location

Blood samples from game birds were collected by local indigenous Yagua hunters. The study area is located around the Nueva Esperanza community (04°19’53’’S; 71°57’33’’W; UT5:00), which is established on the Yavarí-Mirín River bank, within the Loreto state in Peru (Figure 3.1). The area is composed of 322,500 ha of continuous, predominantly non-flooding *terra firme* forest. The climate is typically equatorial with an annual temperature of 22–36°C, relative humidity of 80% to 100%, and annual rainfall of 1500 to 3000 mm (Bowler, 2015). The Nueva Esperanza village, which is the only one still occupied, is comprised of 281 people. The area they inhabit is mostly forest-covered although, the activities carried out by the settlers, such as small-scale agriculture, fishing, logging and subsistence hunting have altered the landscape. The area utilized for hunting by the locals occupies approximately 422km^2 (Mayor, 2015).

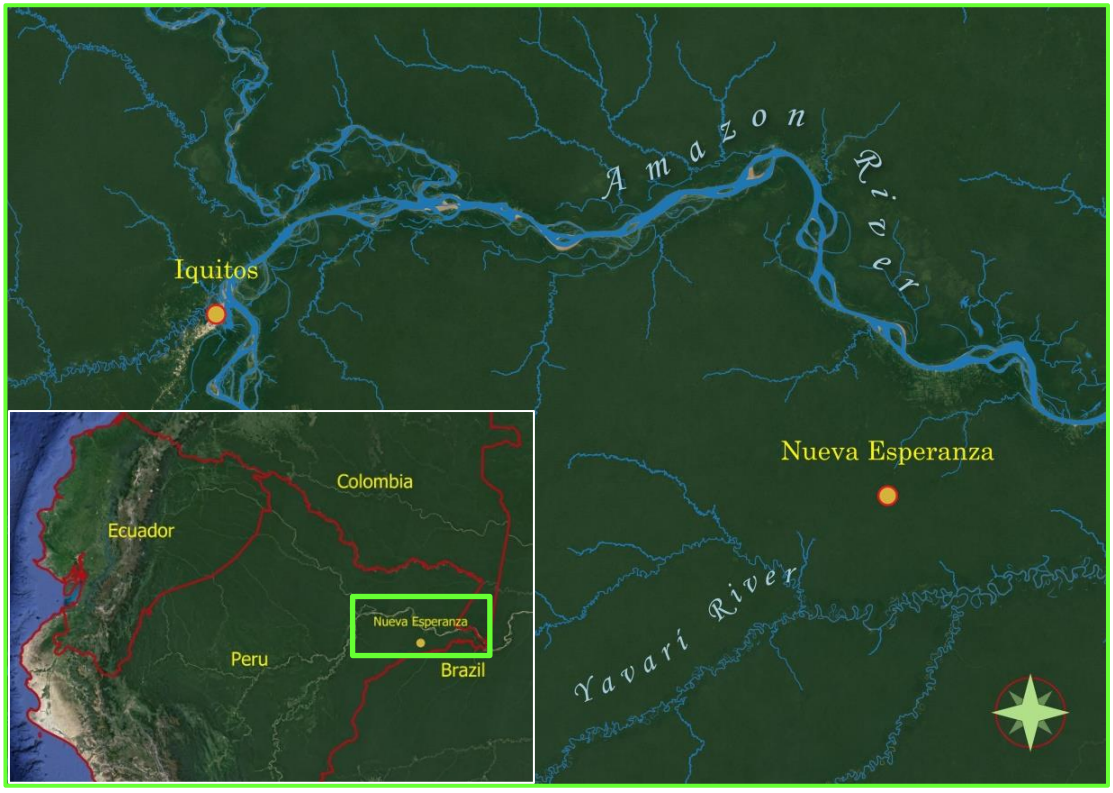

Figure 3.1. Sampling area. Birds used in this study were hunted in an unspecified area around Nueva Esperanza settlement.

3.2.2 Sample collection

From 2007 to 2015, blood samples were collected from 172 wild game birds by subsistence hunters as part of a wildlife conservation program. The indigenous Yagua hunters recorded the hunting date, site, birds´ species, and birds´ sex if sexual dimorphism was present on the species. Access to the area is complex, it implies a three-day boat journey from the nearest settlement, and there is no electricity on-site, which makes it difficult to preserve samples taken by traditional methods (i.e., blood and tissue samples). Therefore, other studies in the area have successfully used filter paper or FTA® cards to collect samples (Aston (2014); Aysanoa (2017); Morales (2017)). In this study, the Yagua hunters impregnated blood from the caudal cava vein of the hunted birds on either Whatman filter paper No. 3 or FTA® cards. Dried blood samples on these cards were placed individually in tagged paper envelopes and stored at environmental temperature protected from light and humidity. Posteriorly, the samples were sent to the Emerge-Lab, LID 412, at the Cayetano Heredia Peruvian University, where they were stored at -20°C.

3.2.3 DNA extraction

DNA extractions were done by MSc Stephanie Montero and Winnie Contreras at the Emerge-Lab LID 412 at the Cayetano Heredia Peruvian University. Whatman filter paper No. 3 or FTA® cards were placed in a petri dish, and a 6 mm^2 piece was cut with a scalpel. The square was cut into smaller pieces and transferred to a 1.5 ml reaction tube. The extraction was done using a QIAamp® DNA Mini Kit according to the manufacturer´s instructions except for the incubation period, which elapsed overnight instead of one hour to increase the amount of DNA extracted.

3.2.4 Polymerase chain reaction

DNA extracted from the filter papers and FTA® cards were tested for the most common haemosporidians (*Haemoproteus* spp., *Plasmodium* spp. and *Leucocytozoon* spp.) following the protocol suggested by Hellgreen (2004). The protocol consists of a nested PCR that employs the primers HaemNF1 and HaemNR3 in the first part to detect a conserved region shared amongst the three parasites. In the second part, two different sets of primers were employed, HaemF and HaemR2, to detect *Plasmodium* spp. and *Haemoproteus* spp., and HaemFL and HaemR2L to detect *Leucocytozoon* spp. Samples were run on a Techne TC-412 Thermal Cycler adding a negative and a positive control every ten samples (see section 2.2.8 for details). Molecular grade water was used as a negative control, and genomic DNA from *Plasmodium bergei* ANKA or genomic DNA from *Leucocytozoon* spp. as a positive control. PCR products were visualised on a 1.5% agarose gel.

3.2.5 Statistical analysis

Generalized linear models (GLM) were constructed to analyse the influence of bird species and collection years in the prevalence of haemosporidians. The presence of *Haemoproteus* spp., *Plasmodium* spp., and both parasites together was taken as a binary response with bird species, year and the interaction of species and year as explanatory variables. A binomial family and a logit link were used for all models (a complementary log-log link was tried, but it produced higher deviance values). Model simplification was done backwards comparing models with an ANOVA test until a significant difference between models was observed. Additionally, a Fisher´s exact test was done to compare the number of lineages to the number of positives by bird species and to the number of birds trapped by species. All analyses were done in the software R version 3.6.0.

3.2.6 Gene sequencing

Positive PCR products were sent for sequencing by the Sanger method in the forward and reverse directions employing the primers HAEMF and HAEMR. Forward and reverse sequences were visualized, assembled and manually corrected using BioEdit. Sequence reads were compared to previously published avian haemosporidia in the GenBank nucleotide database (http://www.ncbi.nlm.nih.gov/genbank/) using BLASTn to identify the genus of the parasite. Mixed sequences, as indicated by electropherograms with double peaks, were considered as mixed infections (Lutz, 2015) and were excluded from further analysis.

3.2.7 Phylogenetic analysis

Currently, there are no standard criteria to establish haemosporidian lineages, which leads to species misidentifications (Vanstreels, 2014) and publishing of identical sequences with different names on available databases (Bensch, 2009). Hence, to identify each haemosporidian sequence derived from this study, sequence clustering and phylogenetic analysis were used to relate each sequence to all previously published sequences. To guarantee the most thorough comparison of new sequences with those recorded previously, one *Haemoproteus cytb* sequence, randomly chosen, was used to search GenBank. The best 10,000 matches in the database were obtained; when ranked in descending sequence identity, matches to *Haemoproteus* spp. and *Plasmodium* spp. were listed first, and thereafter sequences belonging to *Leucocytozoon* spp. Additionally, 224 voucher sequences from MalAvi (MalAvi, 2021) were included in the database. After removing duplicates, partial sequences, and sequences containing ambiguous bases, the reference sequences (n = 4,620) were aligned in Bioedit (Hall, 1999) using ClustalW (Chenna, 2003) with all new *Plasmodium* sequences identified in this study (n = 106) to produce a 475 bp multiple sequence alignment (n = 5,810). Mammalian

Plasmodium sequences were removed because they have an extreme AT bias in base composition that is problematic in phylogenetic analysis (Fallon, 2003). This has no detrimental effect on the current analysis since mammalian *Plasmodium* are monophyletic and distinct from all avian *Plasmodium spp.* (Borner, 2016). This multiple sequence alignment, while comprehensive, was too large and contained too many near-identical sequences to resolve a phylogeny. Therefore, CD-HIT was used to cluster sequences into common lineages, defined by a 1% sequence divergence threshold, as this value retained the best morphospecies of the reference lineages used (See section 2.3.7 for full details on the analysis). On this basis, new lineage sequences were deposited in GenBank under the accession numbers MZ614915 – MZ614938. *Leucocytozoon* sequences were retained for use as an outgroup in the phylogenetic analysis. Sequence clusters containing sequences derived from a named morphospecies were assigned to that morphospecies. After sequence clustering, the multiple sequence alignment contained 403 sequences, including one representative of each cluster defined by CD-HIT as well as the Peruvian sequences isolated. A Neighbour Joining (NJ) phylogenetic tree including the 403 sequences mentioned above was estimated using the Tamura-Nei substitution model in MEGA X (Sudhir, 2018). The tree topology was rooted with *Leucocytozoon* sequences. Node robustness was evaluated with 100 non-parametric bootstraps. Given that *Plasmodium* phylogeny is known to be adversely affected by base composition bias at the third codon position, a Maximum Likelihood (ML) tree was estimated for comparison using MEGA X (Sudhir, 2018) with a General time reversible nucleotide substitution model after automatic model estimation with additional parameters for α, the gamma distribution of among-site rate heterogeneity and for the proportion of invariant sites (GTR + G + I). The data set was partitioned to allow independent modelling of base substitution rates at each codon position, a measure that will reduce the effect of homoplasy at the third position. Node robustness was evaluated with 100 non-parametric bootstraps. Finally, a Maximum Parsimony tree was estimated for comparison in MEGA. The software FigTree v1.3.1 was used to visualize the phylogenetic trees.

3.3 Results

3.3.1 Bird sampling

A total of 174 game birds belonging to three orders (*Galliformes*, *Gruiformes*, *Sthrutiniformes*), three families (*Cracidae*, *Psophiidae*, *Tinamidae*) and five different species were collected by the Yagua hunters from 2007 to 2015. The catches per bird in decreasing order were as follows: Spix´s guan (*Penelope jacquacu*; n=70), Razor-billed curassow (*Mitu tuberosum*; n=46), Great tinamou (*Tinamus major*; n=20), Blue-throated piping-guan (*Pipile cumanensis*; n=17), and White-winged trumpeter (*Psophia leucoptera*; n=16) (Table 3.1). For four of the birds the capture date was not recorded.

Table 3.1. Number of birds collected by species and year around Nueva Esperanza settlement in the Peruvian Amazonia.

Species	2007 (n)	2008 (n)	2009 (n)	2010 (n)	2011 (n)	2012 (n)	2013 (n)	2014 (n)	2015 (n)	Total (n)
Spix´s guan (*Penelope jacquacu*)	1	11	8	8	3	15	16	1	8	71
Great tinamou (*Tinamus major*)		8	2	7	1		1		1	20
Razor-billed curassow (*Mitu tuberosum*)	3	3	7	13	3	4	7	1	5	46
White-winged trumpeter (*Psophia leucoptera*)			3	2		7	1		3	16
Blue-throated piping-guan (*Pipile cumanensis*)	2		7	4	1		1	2		17

3.3.2 Haemosporidian prevalence

Diagnostics from PCR revealed infections with *Haemoproteus* spp. and *Plasmodium* spp.; however, *Leucocytozoon* spp. was not found. The overall prevalence of *Haemoproteus* spp. was 64.4% (n= 111/174) and for *Plasmodium* spp. was 5.2% (n= 9/174). Haemosporidian prevalence varied amongst bird species. In the Spix´s guan, the most represented bird in the sampling, an 86.3% *Haemoproteus* spp. prevalence was found, although no *Plasmodium* spp. infections were observed. In the Great tinamou, a similar prevalence for both parasites was recorded, 10% for *Haemoproteus* spp. and 15% for *Plasmodium* spp. In the Razor-billed curassow a contrasting prevalence was observed; 73% for *Haemoproteus* spp. and 6.3% for *Plasmodium* spp. For the White-winged trumpeter a 6.7% *Haemoproteus* spp. prevalence was observed, whereas *Plasmodium* spp. reached a 13.3% prevalence. Lastly, 62.5% of the Blue-throated piping-guan were infected with *Haemoproteus* spp. and 6.3% were infected with *Plasmodium* spp. (Figure 3.2a, 3.2b). Combined haemosporidian prevalence and *Haemoproteus* spp. prevalence was higher in the Spix´s guan and the Razor-billed curassow. By contrast, the highest *Plasmodium* spp. prevalence was observed in the Great tinamou and the White-winged trumpeter.

Regarding sampling years, *Plasmodium* spp. infections were not observed during 2007, 2008, and 2014. The highest *Plasmodium* spp. prevalence was observed in 2011 (12.5%) and 2010 (11.8%), and the lowest in 2007 (0%), 2009 (3.7%), 2012 (3.8%), 2013 (3.8%) and 2015 (5.9%). *Haemoproteus* spp. infections were observed in all years, except 2007. The lowest prevalence of *Haemoproteus* spp. was observed in 2008 (40.9%), whereas its highest occurrence was in 2013 (88.5%) (Figure 3.3a, 3.3b). For eight samples, the year was not recorded.

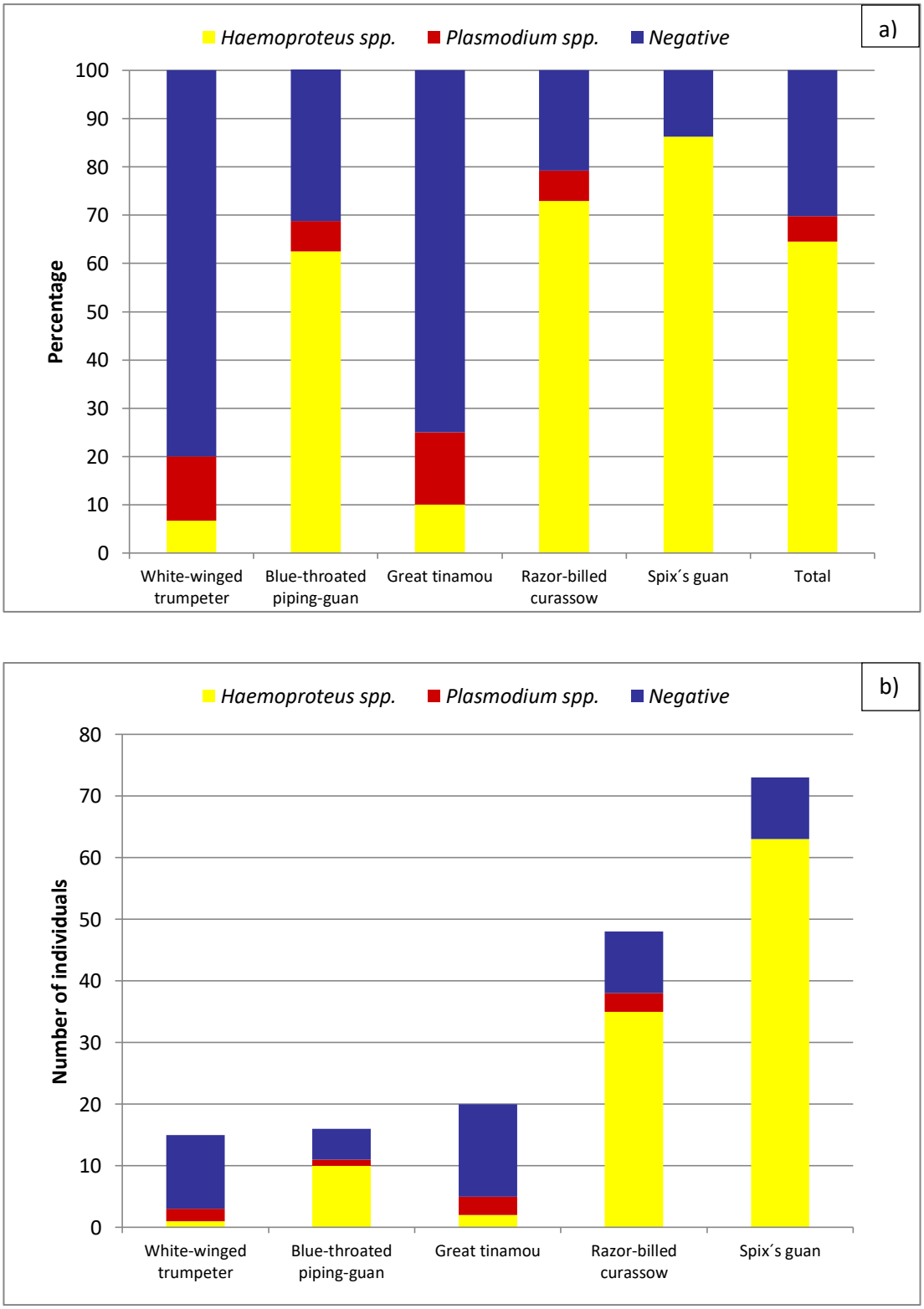

Figure 3.2. *Plasmodium* spp. and *Haemoproteus* spp. prevalence by bird species. a) Percentage of birds infected. b) Number of birds infected.

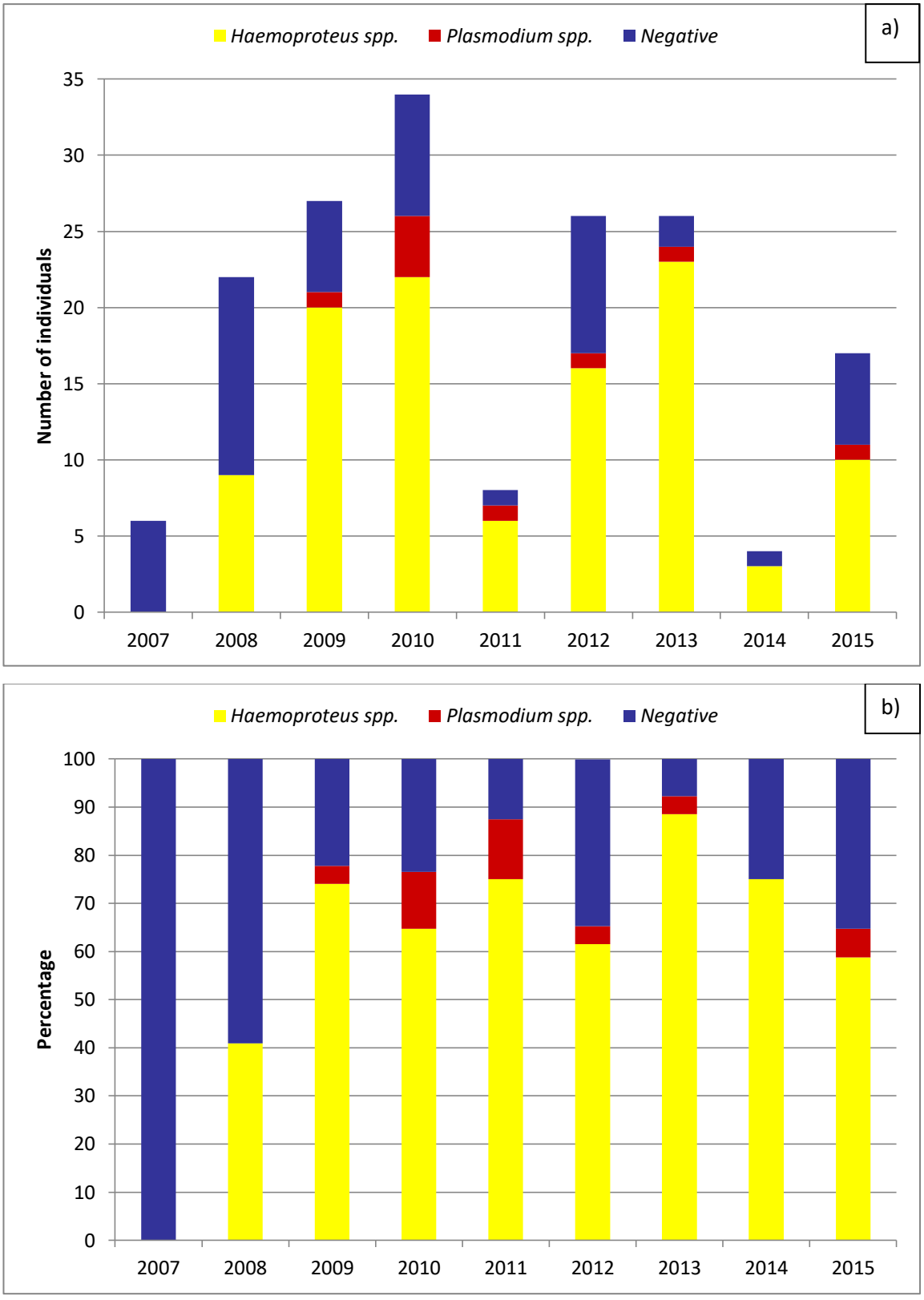

Figure 3.3. *Plasmodium* spp. and *Haemoproteus* spp. prevalence by year. a) Number of birds infected. b) Percentage of birds infected.

3.3.3 Statistical analysis

The GLMs showed that bird species had a significant effect on *Haemoproteus* infection, in particular the White-winged trumpeter and the Great tinamou were highly significant, with a negative effect, and Spix´s guan was close to significance (P = 0.06) but with a positive effect (Table 3.2). In the case of *Plasmodium* spp., no significant influences were found (Table 3.3), and for both parasites combined, the minimal adequate model was very similar to the one for *Haemoproteus* spp. (Table 3.4).

Table 3.2. Parameters of the GLM for the presence of *Haemoproteus* spp**.**

Variable	Estimate	Standard Error	z value	P value
Intercept	-17.624	1564.116	-0.011	0.991
Penelope	1.037	0.57	1.819	0.069
Pipile	-0.737	0.782	-0.943	0.346
Psophia	-4.039	1.134	-3.561	<0.001
Tinamous	-3.375	0.878	-3.845	<0.001
2008	17.349	1564.116	0.011	0.991
2009	19.622	1564.116	0.013	0.99
2010	19.16	1564.116	0.012	0.99
2011	19.076	1564.116	0.012	0.99
2012	18.437	1564.116	0.012	0.991
2013	19.896	1564.116	0.013	0.99
2014	18.964	1564.116	0.012	0.99
2015	18.192	1564.116	0.012	0.991
NA	18.272	1564.116	0.012	0.991
Null deviance: 226.64 on 173 degrees of freedom				
Residual deviance: 128.59 on 160 degrees of freedom. AIC: 156.59				

NA: not applicable, the year for some observations was not recorded.

Table 3.3. Parameters of the GLM for the presence of *Plasmodium* spp.

Variable	Estimate	Standard Error	z value	P value
Intercept	-2.708	0.596	-4.542	<0.001
Penelope	-17.858	2075.183	-0.009	0.993
Pipile	-0.065	1.191	-0.054	0.957
Psophia	0.762	0.963	0.792	0.429
Tinamous	0.973	0.865	1.126	0.26
Null deviance: 70.839 on 173 degrees of freedom.				
Residual deviance: 59.015 on 169 degrees of freedom. AIC: 69.015				

Table 3.4. Parameters of the GLM for the combined presence of *Haemoproteus* spp. and *Plasmodium* spp.

Variable	Estimate	Standard Error	z value	P value
Intercept	-17.501	1583.699	-0.011	0.991
Penelope	0.649	0.607	1.069	0.285
Pipile	-0.76	0.879	-0.866	0.387
Psophia	-3.19	0.817	-3.903	<0.001
Tinamous	-2.769	0.756	-3.663	<0.001
2008	17.448	1583.699	0.011	0.991
2009	19.683	1583.699	0.012	0.99
2010	19.805	1583.699	0.013	0.99
2011	20.1	1583.7	0.013	0.99
2012	18.74	1583.699	0.012	0.991
2013	20.37	1583.699	0.013	0.99
2014	18.905	1583.7	0.012	0.99
2015	18.578	1583.699	0.012	0.991
NA	18.302	1583.7	0.012	0.991
Null deviance: 213.92 on 173 degrees of freedom.				
Residual deviance: 129.96 on 160 degrees of freedom. AIC: 157.96.				

NA: not applicable, the year for some observations was not recorded.

3.3.4 Cytb haemosporidian genetic analysis

From the 120 *cytb* sequences obtained in this study, 14 were either mixed, partial or poor-quality sequences; hence, they could not be further analysed. The remaining sequences were clustered at 1% divergence thresholds since it was observed that it best retains morphospecies (See section 2.3.7 for details). A total of 106 *cytb* sequences were examined in this study, seven within the genus *Plasmodium* and 99 within the genus *Haemoproteus*. Clustering with CD-HIT defined five different *Plasmodium* lineages, of which four were reported for the first time. For *Haemoproteus*, 23 lineages were observed, of which 20 were new records (Table 3.5).

Previously reported parasite lineages that matched sequences from this study were: KU562606.1 (n=1), HPENOBS01 (n=6), HTOFLA03 (n=50) and KF482346 (n=6). KU562606.1 and KF482346 belong to undetermined species of *Plasmodium* and *Haemoproteus,* respectively, whereas HPENOBS01 corresponds to the morphospecies *H*. *ortalidum* and HTOFLA03 to *H. paraortalidum*. The new lineages observed here were named combining the first two letters of the Latin species name on which they were seen (Pi, Ps, Pe, Mi, Ti), followed by the first letter of the genus they belong to (h or p) and at last a distinguishing code composed of two digits (Table 3.5). All of the parasite lineages were found in a single bird species, except for HTOFLA03, which was found on four of the bird species captured in this study (Spix´s guan, Razor-billed curassow, Blue-throated piping-guan and Great tinamou). Most of the lineages were observed in a single year with the following exceptions: Tip01, (2010, 2013), Mih09 (2010, 2013), Mih10 (2010, 2013), Mih11 (2010, 2012, 2015), Mih12 (2009 - 2013), HPENOBS01 (2008 – 2010, 2013, 2015), KF482346 (2008, 2012 - 2015) and HTOFLA03 (2008 - 2015) (Table 3.5).

The parasite lineages were distributed differently across bird species. A small number of lineages were seen in the Great tinamou (n=2), White-winged trumpeter (n=3) and Blue-throated piping-guan (n=5). Similarly, in the Spix´s guan, only seven lineages were recorded; however, the lineages in this species are the most

frequently observed; particularly, the lineage HTOFLA03, which was the most prevalent in this study, was mostly recovered from the Spix´s guan. Interestingly, 14 lineages were registered in the Razor-billed curassow, although this species was not the most abundant, nor the one with the highest parasite prevalence (Table 3.6). Comparison of the number of parasite lineages indicated that the Spix´s guan and Razor-billed curassow had significantly more lineages compared to the other species ($P< 0.001$). However, with regards to the number of parasite positive samples, the Spix´s guan had a significantly lower number of lineages ($P= 0.03$). In relation to the number of birds sampled by species, the Razor-billed curassow, the Blue-throated piping-guan and the White-winged trumpeter had significantly more lineages ($P= 0.12$).

Table 3.5. *Plasmodium* and *Haemoproteus* lineages isolated from birds captured from 2007 to 2015 in the Nueva Esperanza settlement of the Peruvian Amazonia.

Lineages	Genus	Species	(n)	Host species	Year
KU562606.1	P	ND	1	White-winged trumpeter	2012
*Pip01	P	ND	1	Blue-throated piping-guan	2010
*Mip01	P	ND	1	Razor-billed curassow	2011
*Psp01	P	ND	1	White-winged trumpeter	2015
*Tip01	P	ND	3	Great tinamou	2010, 2013
H PENOBS01	H	*H. ortalidum*	6	Spix´s guan	2008, 2009, 2010, 2013, 2015
H TOFLA03	H	*H. paraortalidum*	50	Spix´s guan Razor-billed curassow Blue-throated piping-guan Great tinamou	2008 - 2015
KF482346	H	ND	6	Spix´s guan	2008, 2012, 2013, 2014, 2015
*Peh01	H	ND	1	Spix´s guan	2011
*Peh02	H	ND	1	Spix´s guan	2012
*Peh03	H	ND	1	Spix´s guan	2012
*Peh04	H	ND	3	Spix´s guan	2008
*Mih01	H	ND	1	Razor-billed curassow	ND
*Mih02	H	ND	1	Razor-billed curassow	2010

*Mih03	H	ND	1	Razor-billed curassow	2009
*Mih04	H	ND	1	Razor-billed curassow	2010
*Mih05	H	ND	1	Razor-billed curassow	2010
*Mih06	H	ND	1	Razor-billed curassow	2011
*Mih07	H	ND	1	Razor-billed curassow	2010
*Mih08	H	ND	1	Razor-billed curassow	2013
*Mih09	H	ND	2	Razor-billed curassow	2010, 2013
*Mih10	H	ND	4	Razor-billed curassow	2010, 2013
*Mih11	H	ND	5	Razor-billed curassow	2010, 2012, 2015
*Mih12	H	ND	8	Razor-billed curassow	2009 – 2013
*Pih01	H	ND	1	Blue-throated piping-guan	2010
*Pih02	H	ND	1	Blue-throated piping-guan	2009
*Pih03	H	ND	1	Blue-throated piping-guan	2010
*Psh01	H	ND	1	White-winged trumpeter	2012

n= Total number of observations; P= *Plasmodium*; H= *Haemoproteus*; ND= Not determined; *= New lineages found in this study

Table 3.6. *Plasmodium* and *Haemoproteus* lineages distribution by bird species.

Lineages	Great tinamou	Blue-throated piping-guan	Spix's guan	White-winged trumpeter	Razor-billed curassow
H PENOBS01			6		
H TOFLA03	1	6	39		3
KF482346			6		
KU562606.1				1	
Mih01					1
Mih02					1
Mih03					1
Mih04					1
Mih05					1
Mih06					1
Mih07					1
Mih08					1
Mih09					2
Mih10					4
Mih11					5
Mih12					8
Mip01					1
Peh01			1		
Peh02			1		
Peh03			1		
Peh04			3		
Pih01		1			
Pih02		1			
Pih03		1			
Pip01		1			
Psh01				1	
Psp01				1	
Tip01	3				

To determine the systematics of the parasite sequences, phylogenetic trees were estimated using three different methodologies. In most instances, the trees agreed regarding the topological position of each sequence. In accordance with the 1% divergence threshold, in all the trees it was observed that the sequences 11, 70 and 150 (shaded in red) formed a clade; the sequence 138 (shaded in light blue) and its

matching reference sequence KU562606.1 originated from the same branch; and the sequences 24, 88 and 251 (shaded in black) were located on individual branches. In addition, all these sequences were positioned in the *Plasmodium* clade (Figures 3.4, 3.5, 3.6). The remaining sequences were positioned within the *Haemoproteus* clade in all trees. However, on a few occasions, they formed clades that correspond with the clusters formed at the 1% divergence threshold. For instance, in all the trees the sequences that clustered with the reference sequence KF482346.1 (shaded in orange), as well as the sequences identified as Mih09 (shaded in brown), formed clades. Nevertheless, sequences in the HTOFLA03 cluster (shaded in purple) were mixed with branches containing the KF482346.1 and the Peh04 clusters (shaded in light green). Additionally, one sequence (197) from the HTOFLA03 cluster was positioned in a secondary branch apart from the rest of the sequences in this cluster. The Mih11 (shaded in pink) and Mih12 (shaded in yellow) clusters were mixed with each other and with some unique clusters. Meanwhile, the clusters Mih09, Mih10 (shaded in turquoise) and HPENOBS01 (shaded in dark green) were always situated next to each other forming sister branches and in some trees, single clades (Figures 3.4, 3.5, 3.6).

No *Plasmodium* species could be identified in this study since only one matched a reference sequence (KU562606.1) belonged to a *Plasmodium* parasite whose species has not been determined. The remaining *Plasmodium* sequences (n=6) retrieved here could represent four new species, particularly the clade containing the sequences 11, 70 and 150 (shaded in red and highlighted in blue), as it is always placed in a separate branch. For *Haemoproteus*, two species were identified in this study: *H. ortalidum* (PENOBS01) (shaded in dark green) and *H. paraortalidum* (TOFLA03) (shaded in purple). There was another match to a *Haemoproteus* reference sequence (KF482346) (shaded in orange); but, as occurred with *Plasmodium*, the species was not determined. For the rest of the sequences, it is likely that new *Haemoproteus* species were found; however, the number of new species cannot be estimated due to the unresolved placing of these sequences in the trees (Figures 3.4, 3.5, 3.6).

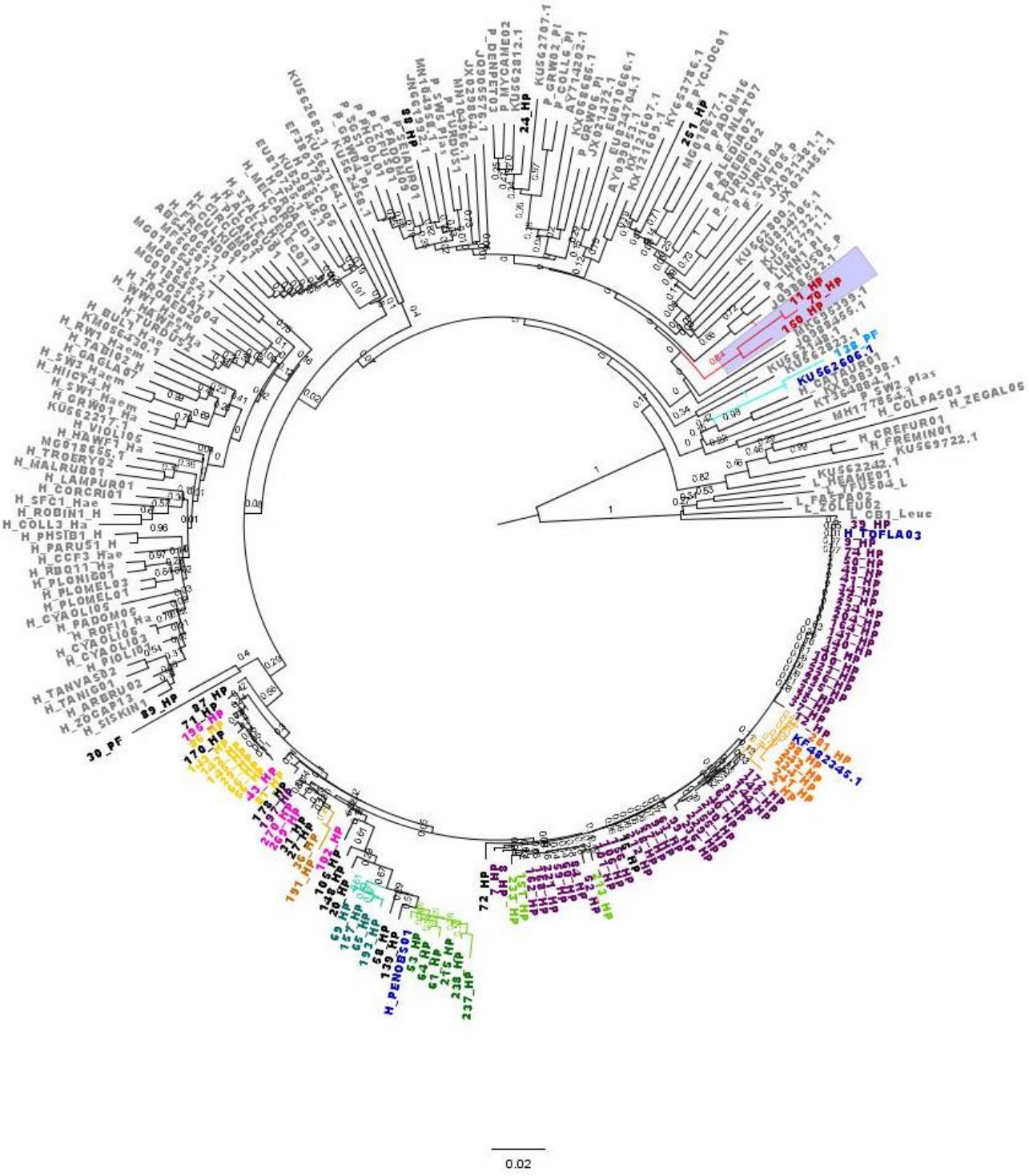

Figure 3.4. Neighbor joining phylogeny of *cytb* sequences representative of sequence clusters obtained with a 1% sequence divergence threshold. The tree was estimated with 100 bootstrap replicates and using a Tamura-Nei nucleotide substitution model with a Gamma distribution. Reference sequences matching sequences obtained in this study are shaded in blue, the rest of them are shaded in grey. Sequences obtained in this study are shaded in different colours (red, blue purple, pink, yellow, orange, light green, dark green, brown, aquamarine); sequences grouped in the same cluster share the same colour. Sequences in black represent unique lineages (no other lineage was found in the same cluster). The highlighted cluster represents a possible new species of *Plasmodium*.

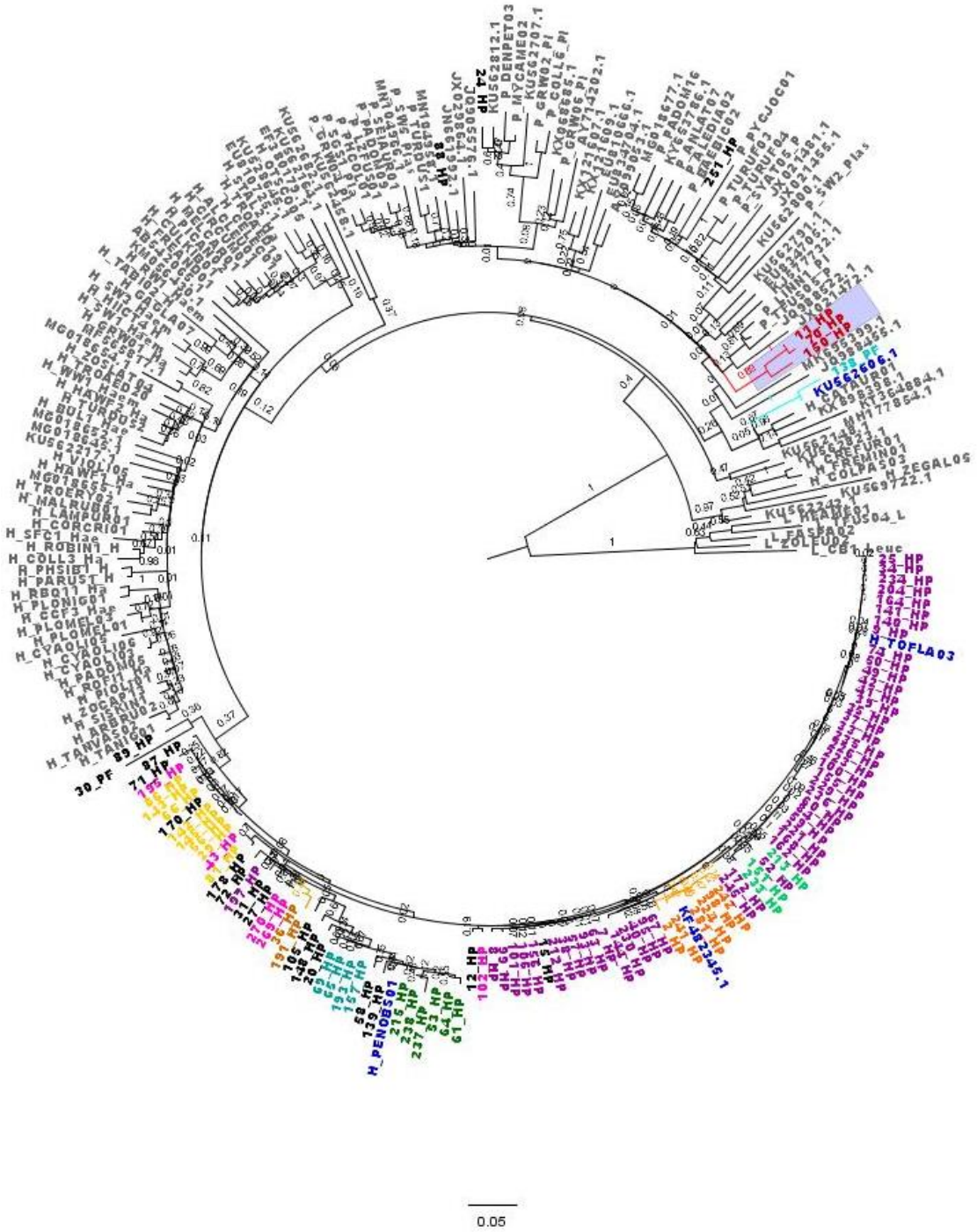

Figure 3.5. Maximum likelihood phylogeny of *cytb* sequences representative of sequence clusters obtained with a 1% sequence divergence threshold. The tree was estimated with 100 bootstrap replicates and using a General time reversible model with a Gamma distribution and invariable evolutionarily. Reference sequences matching sequences obtained in this study are shaded in blue, the rest of them are shaded in grey. Sequences obtained in this study are shaded in different colours (red, blue purple, pink, yellow, orange, light green, dark green, brown, aquamarine); sequences grouped in the same cluster share the same colour. Sequences in black represent unique lineages (no other lineage was found in the same cluster). The highlighted cluster represents a possible new species of *Plasmodium*.

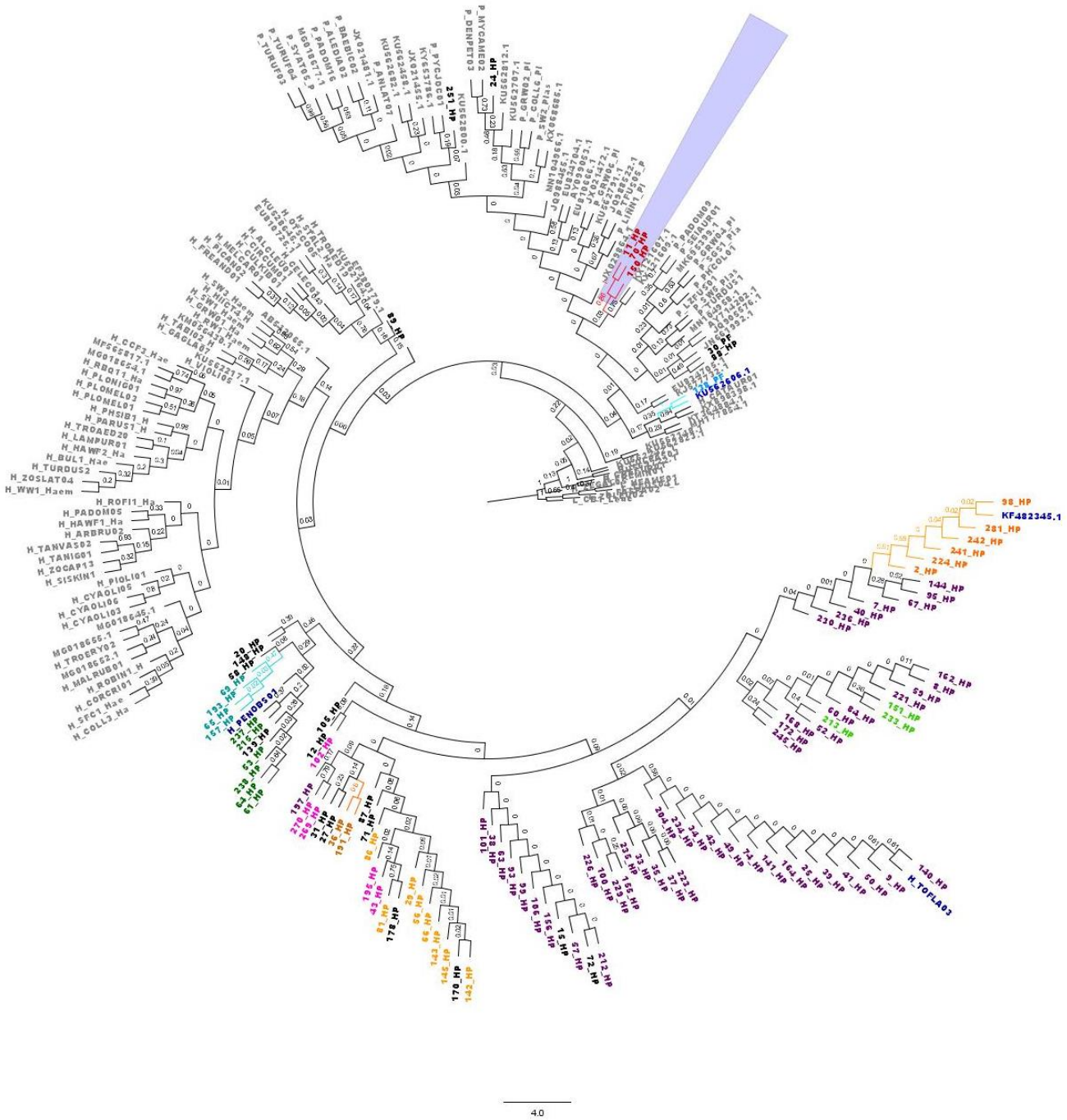

Figure 3.6. Maximum Parsimony phylogeny of *cytb* sequences representative of sequence clusters obtained with a 1% sequence divergence threshold. The tree was estimated with 100 bootstrap replicates and using a nucleotide substitution model. Reference sequences matching sequences obtained in this study are shaded in blue, the rest of them are shaded in grey. Sequences obtained in this study are shaded in different colours (red, blue purple, pink, yellow, orange, light green, dark green, brown, aquamarine); sequences grouped in the same cluster share the same colour. Sequences in black represent unique lineages (no other lineage was found in the same cluster). The highlighted cluster represents a possible new species of *Plasmodium*.

3.4 Discussion

This chapter has examined the prevalence and diversity of avian haemosporidian parasites in the Peruvian Amazonia based on samples provided by the subsistence hunting practices of the Yagua community. As a result, 106 parasite *cytb* sequences were obtained from 172 birds belonging to five different species collected in a nine-year period (2007-2015). The opportunistic nature of the sampling employed in this study resulted in the collection of only five bird species, which are the ones that the Yagua hunters consider to be the most nutritious. This way of sampling also impacted the number of samples collected by year, since the hunters catch birds only when bigger mammals prove harder to catch. This has limited the number of birds collected by year, by species and by a combination of both; however, it has provided a valuable insight into the species and lineages composition of haemosporidians in this remote and understudied area. New host-parasite associations were observed in this study in all the bird species collected; except for the Spix´s guan, for which Marzal (MalAvi, 2021) had already made a *Haemoproteus* spp. infection report. This is the first record for *Haemoproteus* spp. infection in the White-winged trumpeter and in the Razor-billed curassow. Additionally, this is the first report of haemosporidian parasites infecting the Great tinamou (*Plasmodium* spp.) and the Blue-throated piping-guan (*Haemoproteus* spp. and *Plasmodium* spp.).

Infections by *Haemoproteus* spp. and *Plasmodium* spp. were observed in 120 birds, which corresponds to a 69.8% prevalence. Both parasites have been recorded previously in the Amazonia, with an overall prevalence of 17.4% (Fecchio, 2018c) and 21.7% (Svensson-Coelho, 2013). The difference between prevalence observed previously and the high prevalence observed in this study could be attributed to 1) Sampling area, 2) Bird species composition and, 3) Host fitness. 1) Sampling area: previous studies were carried out in a much larger area than in this study. The Amazon region shares, on a big scale, numerous environmental traits; however, it is frequently divided by rivers, which creates different habitats and endemism areas (Fecchio, 2018c) that affect vector populations. 2) Bird species composition: this study

presented a limited number of bird species, whereas the other studies included a more diverse array of species. It is well-known that prevalence varies greatly among avian species because of differences in immune response (Calero-Riestra, 2016), physiology (Ortego, 2008), breeding season (Lachish, 2011) and nesting, foraging, and flocking behaviour (González, 2014). 3) Host fitness: previous studies were based on bird netting, whereas in this study, hunted birds were employed. It has been argued that nets catch mainly birds in good condition and that sick or weak birds are hardly ever trapped (Valkiūnas, 2005). Additionally, it has been observed that the locomotion activity of birds decreases when infected with haemosporidians, particularly during the peak of infection, making birds vulnerable to predation (Valkiūnas, 2005). In this study, it was not possible to test this hypothesis; however, future studies on avian blood parasites in the area should target the same bird species included here and compare haemosporidian prevalence in birds captured by other methods.

Prevalence between *Plasmodium* spp. and *Haemoproteus* spp. varied widely. The highest observed prevalence for *Haemoproteus* spp. was 64.5%, whereas for *Plasmodium* spp. was 5.3%. These results may reflect environmental conditions that favour the prevalence of one parasite species versus the other, as they are transmitted by different vectors. However, Svensson-Coelho (2013) and Fecchio (2018c) found a more similar prevalence between *Haemoproteus* spp. and *Plasmodium* spp. (6.4%, 9.6% and 2.4%, 15.3% respectively) in the Amazon region. Differences with the prevalence found in previous studies could be attributed to the sampling location. This study was done in a small region, whereas the other studies were done on multiple Amazonian areas, and host-parasite-vector interactions may differ due to environmental factors. In fact, Fecchio (2018c) found a significant difference in *Haemoproteus* spp. and *Plasmodium* spp. prevalence amongst the six areas of endemism they tested in the Amazonia.

Differences were also observed between prevalence by bird species, which has also been reported in birds in Western Amazonia (Svensson-Coelho, 2013). Interestingly, bird species with high *Haemoproteus* spp. prevalence had low *Plasmodium* spp. prevalence and vice versa. This pattern has already been described in Amazonian birds

(Svensson-Coelho, 2013), and it could be indicative of vector preferences over certain bird species, or different exposure rates of the hosts to vectors associated with bird behaviours (nesting type and height, social behaviour, foraging height, etc.) (Svensson-Coelho, 2013). The Spix´s guan represented the most captured bird in our sampling (n=70), as well as the one with the highest haemosporidian prevalence (86%). Despite this, no *Plasmodium* spp. infections were recorded in this bird species. Since the other bird species in the area were infected with *Plasmodium* parasites, the parasite must be established in the region. Hence, either this bird is refractory to *Plasmodium* spp. infection due to its biology or behaviour; or it is infected at such low prevalence that further sampling is required to uncover this association (Chagas, 2017). A similar array was observed between years, where the years with higher *Haemoproteus* spp. prevalence were also the years with the lowest *Plasmodium* spp. prevalence and the other way around. This pattern has not been observed in the area before and previous work assessing prevalence changes across years found no changes in *Haemoproteus* spp. prevalence with respect to their hosts (Svensson-Coelho, 2013). It has been suggested that haemosporidian prevalence might vary across years in tropical bird communities (Fecchio, 2017a) because of fluctuations in abundance of hosts, vectors, or both (Svensson-Coelho, 2013). However, in the present study, a low and disproportionate number of birds were caught yearly, which could have created a sampling bias.

Heamoproteus spp. presence was not influenced by collection year; but it was influenced by bird species. In this case, Blue-throated piping-guan and White-winged trumpeter were unlikely to be infected compared to the other species, and Spix´s guan could be more susceptible to the infection since the result was close to significance. For *Plasmodium* spp. infection, no significant influences were found, possibly due to the low number of birds infected with this parasite. For the same reason, the analysis of both parasites combined was very similar to the results of *Haemoproteus* alone. To better understand species susceptibility, complementary samplings including more birds species are needed, and pathological examinations could help to determine the pathogenicity of the parasite.

From the phylogenetic analysis, it was noticed that *Plasmodium* sequences were placed on well-supported branches in all the trees, whereas *Haemoproteus* sequences mostly arose from low-supported branches. Interestingly, matches to reference sequences from both, *Plasmodium* and *Haemoproteus*, were exclusively with sequences that had only been described in the Amazon region. This indicates that geographical barriers have so far impeded the exchange of parasites from different geographic areas (Chagas, 2017). *Haemoproteus* sequences obtained here clustered with sequences of the *Parahaemoproteus* subgenus, indicating that they belong to that subgenus. All the *Haemoproteus* spp. sequences obtained in this study originated from a main branch suggesting that they are highly related. The close grouping of *Haemoproteus* sequences might be related to their evolutionary speciation history, since these parasites showed low rates of speciation events (codivergence, duplication, and sorting along with high rates of host switching), and parasites from the *Parahaemoproteus* subgenus are known to have high host specificity (Fecchio, 2018a). With the available data from the trees, it was not possible to determine the number of *Haemoproteus* species represented by the sequences due to unresolved relationships among them. In addition, nodal support for most of the recorded *Haemoproteus* spp. lineages from this study was low. By contrast, nodes containing *Plasmodium* sequences had high nodal support; hence, it is likely that five different *Plasmodium* species were observed in this study. Of those, one species was previously recorded, and the rest were new. Noteworthy is the well-supported clade containing three *Plasmodium* sequences obtained from the same bird species (Great tinamou), which was distinct from any previously observed sequence strongly suggesting that this is a new species. Nevertheless, without blood smears to characterize the life stages of the parasites, new species cannot be properly established.

Some of the sequences obtained in this study clustered with reference sequences from Genbank and MalAvi (TOFLA03, PENOBS01, KF482345.1 and KU5626.1). The lineage TOFLA03 belonging to *H. paraortalidum* was the most frequently observed lineage in this study. This lineage was previously observed in Peru and Brazil on Galliformes and Passeriformes (MalAvi, 2021). The lineage PENOBS01 of the *H. ortalidum* species has been previously reported in Brazil in birds of the Galliformes order (Chagas, 2017). The

lineage KF482345.1 was reported in Peru (MalAvi, 2021), although the parasite species was not determined. The last matching lineage, KU5626.1, corresponds to a *Plasmodium* parasite (species not determined) (Fecchio, 2017b) recorded in the Brazilian Amazonia. Therefore, the areas and the birds on which the reference lineages were described are consistent with the findings from this study. Moreover, the reference lineages have only been reported in the mentioned bird orders and geographic locations. The rest of the sequences obtained here represented new lineages. A total of four new lineages were recorded for *Plasmodium* and 20 for *Haemoproteus*. Given the small sampling number of birds and bird species we had in this study, a high number of lineages were obtained, possibly reflecting the high diversity that is associated with the area. Neotropical regions, such as the Amazonia, are considered hotspots of avian diversity. The positive association between host and haemosporidian diversity indicates that the diversity of the latter could be great and that new species and lineages may be discovered as more studies are performed in the area (Chagas, 2017).

Most of the new lineages were found in a single bird species, suggesting that they are specialists. Similarly, Fecchio (2017b) found specialized haemosporidian lineages in Amazonian birds, and other studies in the region have only found *Haemoproteus* species that belong to the subgenus *Parahaemoproteus*, which has high host specificity (Fecchio, 2018a). Nevertheless, it should be considered that most of the lineages retrieved here had only one observation; so, these lineages might be found in more bird species over time (Chagas, 2017). On the other hand, it was found that the lineage HTOFLA03 is a generalist since it was found on four of the five bird species sampled in this study and throughout eight years. Other studies in this area have shown that many of the generalist parasites in this area have evolved through a host switching strategy (Fecchio, 2017b), and this could be reflected in the results observed here. Most of the new lineages were isolated from the Razor-billed curassow (Mih1 – Mih12), and it had significantly more lineages with regards to its numbers. This might suggest that this bird is exposed to a more diverse array of vectors due to its biological or behavioural characteristics. Previously, it has been pointed out that a bird range distribution is positively related to parasite diversity. However, this is not the case for the Razor-

billed curassow; in fact, this bird has the narrowest distribution range amongst the birds collected in this study, partially inhabiting three countries (Bolivia, Peru and Brazil) (IUCN, 2021); hence, other factors must be responsible for this pattern.

3.5 Conclusion

This study contributes to the knowledge of haemosporidian prevalence and distribution in the Peruvian Amazonia. Here, various new lineages and new host-parasite associations were recorded for the first time showing that there is a great diversity in Amazonian haemosporidian parasites and that more studies are required to fully describe it. The determination of distribution and diversity of avian blood parasites in the Peruvian Amazonia is an important first step to further differentiate evolutionary process that have shaped the host-parasite associations and their current assembly in contrast with other parts of the world. Therefore, understanding the natural parasite distribution and dispersal will provide useful information to predict and prevent infectious diseases. Results from this study were affected by sampling bias; therefore, future research in the area should target the same bird species with different sampling methods and a broader range of species to compare the patterns observed here. They should also benefit from studying vectors present in the area, their competence, their distribution, and their host preferences.

CHAPTER 4

A new non-invasive procedure to diagnose haemosporidiosis in birds: feathers

4.1. Introduction

Haemosporidian diagnosis is traditionally done from a blood sample; this implies capturing and handling birds. Animal restraining is known to induce stress, which could trigger underlying diseases on the birds. Hence, blood sampling is not done frequently to avoid stress induction on birds, and careful consideration is needed for diagnosis from weak or sick birds. Feathers might be an alternative to blood sampling since they are provided with a blood vessel and once they are molted some of that blood is preserved in the feathers´ shaft. As feathers are molted constantly, they can be picked up from a bird´s enclosure or nesting site without disturbing the birds.

Most common avian blood parasites (*Haemoproteus* spp., *Plasmodium* spp. and *Leucocytozoon* spp.) have not been reported to damage feathers, or to deposit in feather capillaries. However, a weak association between haemosporidians and feathers has been described; during an avian malaria outbreak of Mohuas (*Mohoua ochrocephala*), Alley (2008) observed ruffled feathers in one of five infected birds; similarly it has been observed that feather growth rate decreases in birds infected with blood parasites (Marzal, 2013).

Traditional methods to detect blood parasites (PCR, microscopy and ELISAs) require restraining and handling of birds in order to obtain a fresh blood sample (Motta, 2013), this stressful process could ultimately undermine the bird´s immune response. In that

sense, the collection of a blood sample requires careful thought, weighing the associated dangers to obtain it versus the value of the sample. Thus, it is often decided not to take the sample (Alley, 2008) and blood samples are obtained mainly opportunistically when birds are in hand for other veterinary requirements (Spottiswoode, 2020).

The limitations of blood sample collection interfere with prompt disease diagnosis and early treatments being applied as seen by Alley (2008). However, the use of non-invasive samples, such as feathers, that could potentially provide the same information as a blood sample, could solve this issue. Additionally, this may be the only way to obtain genetic material from endangered and/or elusive species in the wild (Segelbacher, 2002; Horváth, 2005) without disturbing or potentially harming them. Moreover, when compared to blood sampling, collection of feathers does not require training, imply handling birds (stress-free), or require the use of laboratory materials, and they are easier and cheaper to store (Harvey, 2006).

4.1.1 Feathers usage for disease diagnosis

Historically, feathers have been used for diagnostic purposes only for two diseases, the Psittacine beak and feather disease (PBFD), and Marek´s disease (MD), both of which are viral diseases that directly affect the feathers. The PBFD is caused by a circovirus, whose genome consists of a single-stranded circular DNA chain. The disease is characterized by symmetric feather dystrophy and loss, development of beak deformities, and eventually death. PBFD more commonly affects young birds during the first feather formation after neonatal down replacement (Ritchie, 1989). Feather and beak malformations present due to multifocal necrosis of epidermal cells, epidermal hyperplasia, and hyperkeratosis. The rachis, which is the main central feather shaft (Figure 4.1), presents multiple histological damage, such as inflammation and necrosis in the distal pulp cavities, the epidermal collar, and the epidermal basal and intermediate zones (Ritchie, 1989). To diagnose PBFD, Ritchie (1989) extracted DNA from feathers of infected and uninfected birds through a phenol/chloroform

extraction protocol, resulting in the virus being purified only from the extracts of infected individuals; therefore, successfully detecting the virus by molecular methods from the feathers.

Marek´s disease is characterized by the production of lymphomas in various organs. This is the only avian virus whose major route of excretion by infected bird is through the feather epithelium. Virus particles mature in the superficial layers of the feather follicle epithelium to later be released into the space between the follicular epithelium and the rachis. A considerable amount of cell-free viruses are released on active infections due to the keratinization process, which reduces lysosomal activity in the feather follicle epithelium cells. The virus is present along the feather rachis, but it tends to accumulate at the feather tip (2.5-3.0 cm from the point of insertion); hence, feather tips are used to diagnose and monitor the disease (Davidson, 1986). Different non-molecular based techniques were used to diagnose MD (i.e. ELISA and agar gel precipitation), and posteriorly, Davidson (1986) reliably detected the infection with a dot-blot DNA hybridization test, using extracted DNA from feather tips.

4.1.2 Feather´s molecular material source

Growing feathers contain soft tissue (Taberlet, 1991) or blood (Bello, 2001) from which DNA can be extracted. Growing and non-growing feathers contain pulp cells in the proximal section of the rachis, with the former containing a higher amount of pulp cells (Taberlet, 1991). Feathers receive nutrients from a single axial artery located in the mesenchymal pulp; during feather development, a narrow channel forms in the calamus wall through which the mesenchymal pulp crosses. Once the feather is developed; the mesenchymal pulp is reabsorbed from the calamus, leaving only keratinized pulp caps and a remnant of the axial artery, which is often visible as a blood clot on the superior umbilicus (Figure 4.1) (Horváth, 2005).

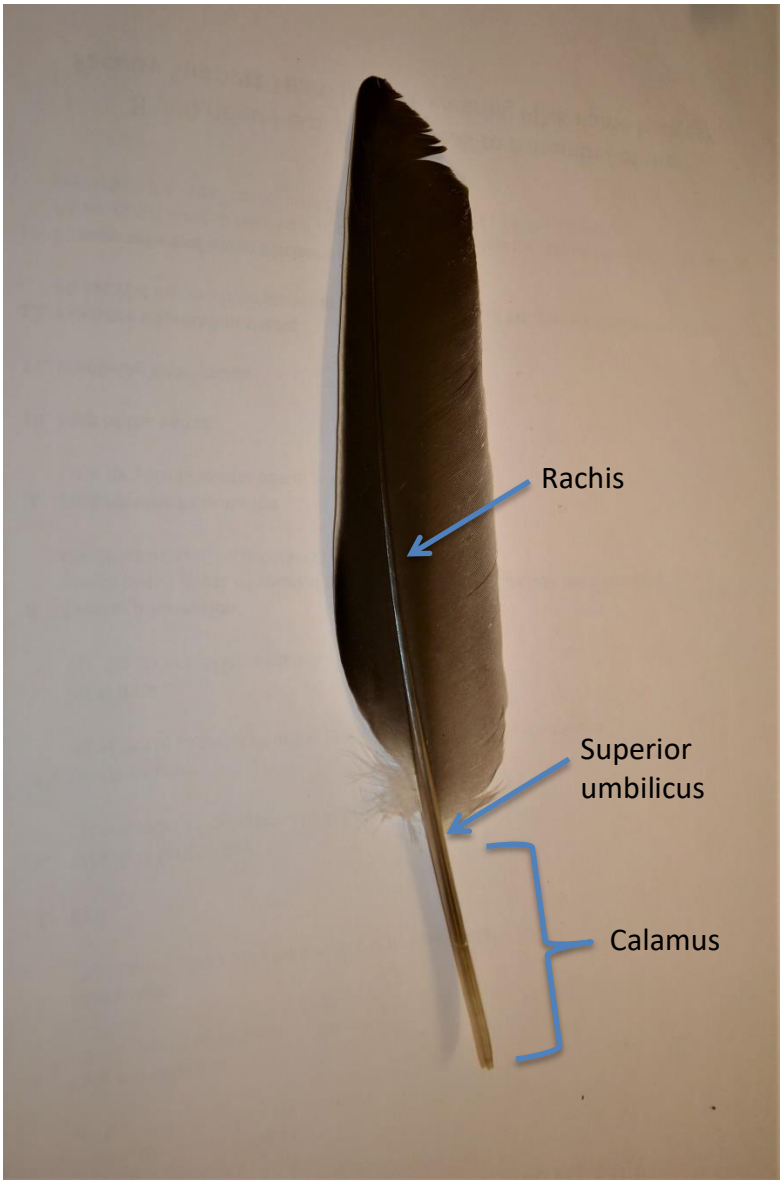

Figure 4.1. Representation of a flight feather. The parts of the feather are indicated.

As feathers stop growing the blood supply is suspended since the feather artery starts to be reabsorbed, and eventually the feather is moulted; however, residual blood cells remain inside the feather´s shaft. Molted feathers are exposed to degradation by microorganisms, hydrolysis, solar radiation and repeated freezing and thawing; however, the cells inside the shaft are partially protected from these factors (De volo, 2008). Hence, if sufficient blood remains in the feather shaft blood parasites could be detected from the feathers by molecular methods.

4.1.3 Feathers as a source of genetic material and its application in molecular analysis

Non-invasive sampling has been widely used for molecular genetic studies in wild animals to obtain information of population structure (Segelbacher, 2002), for species identification, and to perform intraspecific phylogeographic studies (Taberlet, 1999). Nevertheless, this DNA source has not been able yet to provide individual identification, relatedness estimates, pedigree reconstruction, estimates of census and effective population size, or the level of genetic polymorphism within or between populations (Taberlet, 1999).

For birds, non-invasive sampling, specifically of feathers, has been used for age determination, to analyze isotopes and trace elements (Harvey, 2006), to study genetic diversity (De volo, 2008) and population structure (Segelbacher, 2002), for sex determination of individuals (Harvey, 2006), for paternity testing (Bello, 2001), to identify parentage, to determine individual turnover at nests, and to estimate abundance in nonbreeding populations and interspecific phylogeny (De volo, 2008).

One disadvantage of using non-invasive samples for diagnosis is the low quality and quantity of the extracted DNA, (Segelbacher, 2002). For example, Segelbacher, (2002) often obtained lesser DNA amount from feather samples than from blood or tissue samples. Moreover, DNA from feathers can be degraded to some degree if it derives from cells that died when the feather stopped its development (Harvey, 2006). Nevertheless, more recent studies have achieved similar DNA yields to those obtained from blood and tissue (1 – 100 ng/μl) (Harvey, 2006; de Volo, 2008).

Previous studies have used different types of feathers for DNA extraction; large (rectrices or remiges; primaries, secondaries, or tertials) and small (coverts) (Segelbacher, 2002; Horváth, 2005; De volo, 2008) (Figure 4.2). Similarly, different parts of the feather have been used for the extraction; basal tip of the calamus (Taberlet, 1991; Segelbacher, 2002; Horváth, 2005; Bello, 2001), the superior umbilicus (Horváth, 2005), and full calamus (De volo, 2008) (Figure 4.1).

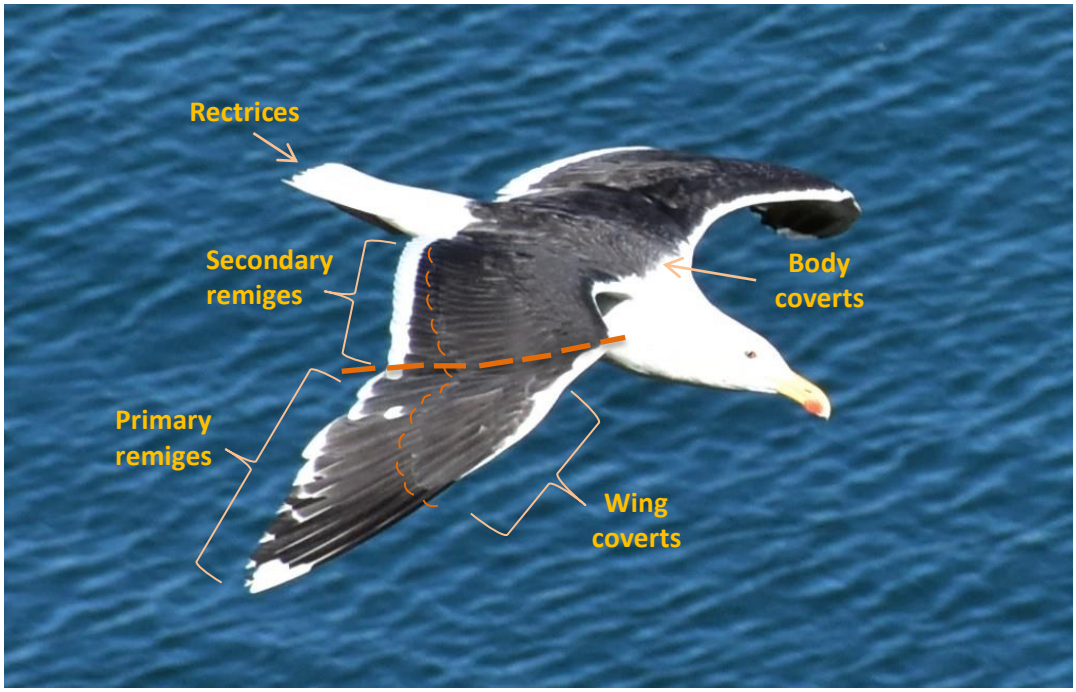

Figure 4.2. Image indicating the feather classification according to location.

Additionally, a variety of methods and modifications have been suggested for feather DNA extraction, including the use of commercial kits (Qiagen DNeasy tissue kit) (Horváth, 2005; Harvey, 2006; Segelbacher, 2002) and phenol:chloroform protocols (Bello, 2001; Taberlet, 1991). Another factor in the extraction methodology is the elution volume, which has been proposed to be 120 µl (Horváth, 2005), 60 – 100 µl (Segelbacher, 2002), and 50 – 500 µl depending on the feather size (Bello, 2001). Lastly, the incubation times also vary among studies; Bello (2001) proposed to do incubation overnight at 37°C to enhance the amount of DNA obtained, Segelbacher (2002) proposed to do incubation overnight at 55°C; while de Volo (2008) suggested incubating for 24 h or longer at 55°C.

Regardless of the variations in methodology, there is a general agreement that sufficient DNA for molecular analysis can be obtained from feathers (de Volo, 2008). However, the DNA quality obtained in different protocols varies and this is reflected in their results. In some cases limited nuclear DNA amplification has been observed (Horváth, 2005), but in some others, microsatellite amplification and Southern blotting testing have been successful (de Volo, 2008).

When genotyping from feathers, the efficacy of the assay is affected by the environmental exposure of the samples (Segelbacher, 2002). Feathers in good condition rendered high amplification success (De volo, 2008), while feathers that had been in the field for several months reached only 50% of success due to a high degree of degradation (Segelbacher, 2002). Likewise, Segelbacher (2002) recorded that larger feathers produced better genotyping results than small feathers; whereas de Volo (2005) observed no difference in microsatellite amplification by feather type.

Overall, an increase in the DNA yield has been observed in recent studies, possibly related to the use of large feathers since authors have reported that large feathers produce more DNA than small feathers (Bello, 2001; Horváth, 2005; de Volo, 2008). Alternatively, it might be attributed to the use of the superior umbilicus for the extraction, which produces higher DNA yields (Horváth, 2005; de Volo, 2008).

4.1.4 Objectives

This chapter explores the possibility of both, diagnosing haemosporidiosis in birds and detecting avian blood parasites from a non-traditional sample: feathers. Implementation of this diagnostic strategy would avoid handling of birds and therefore stress induction (Bello, 2001); in his way, frequent screening of blood parasites can be promoted. Feathers are shed commonly and can be picked up from zoo enclosures or nesting sites without disturbing the birds. Hence, this chapter aims to answer if haemosporidiosis can be diagnosed from feathers on any bird species and if so, how effectively. Having achieved that goal, it is also necessary to determine the DNA yield differences according to bird species and feather type. Likewise, it is important to establish optimal conditions for sample preservation and to determine the effects of environmental conditions on DNA retrieval. Lastly, it is intended to describe the optimised conditions for this new method to work.

4.1.5 Hypothesis

A variation in the DNA amount retrieved from feathers of different species influenced by their size is expected, given that each organism contains a certain quantity of blood in its body, which is relative to its body weight; therefore, it is predicted that the remnant blood in moulted feathers is proportional to the individual size. Hence, animals with larger body sizes are likely to produce bigger feathers that would provide more material for DNA extractions, and hence higher DNA outputs. The same logic is applied to the feather type, expecting the remiges and rectrices (referred to as flight feathers hereafter), which are generally larger, to produce higher DNA yields than the body coverts (referred to as body feathers hereafter).

Feather DNA is protected inside the shaft; however, progressive degradation when exposed to heat, erosion and moisture overtime is expected. The diagnosis of blood parasites from feathers is expected to be successful if the remaining blood in the feathers´ shaft produces the required DNA yield for PCR.

4.2. Materials and Methods

4.2.1 Intrinsic feather DNA variation

For a feather-based diagnostic method to be broadly applicable, feathers must routinely provide sufficient DNA after extraction; likewise, it is necessary to determine if all feathers are suitable for assay. Therefore, this section examined the ordinary variation in DNA quantities obtained among feathers. To avoid systematic effects of bird species and feather type, the same bird species was used and groups of the same type of feather were formed. From a group of crows that tested positive for

haemoparasites, five carcasses were selected and DNA was extracted from their feathers (details on the crows can be seen on section 4.6.2, and details on feather collection can be found on section 4.2.2). DNA was extracted from body and flight feathers establishing five different pooling amounts for the body feathers (1, 5, 10, 15 and 20) and three for the flight feathers (1, 2 and 3); performing five repeats per group of feathers. DNA was quantified using a Nanodrop 1000 spectrophotometer.

4.2.2 Diagnosis of haemoparasites from feathers of a single bird species

Feathers of birds proven to be infected with haemosporidians from blood or organ assays were examined to assess the plausibility of parasite diagnosis from feathers. Corvids were selected given that they are prone to haemosporidian infections (Schmid, 2017) and, given their predation of other, vulnerable birds, culling of corvids is permitted. Therefore, carcasses of corvids (Eurasian magpies (*Pica pica*), Carrion crow (*Corvus corone*) and Eurasian jackdaw (*Corvus monedula*)) were obtained. These were culled at the RSPB Burton Mere Wetlands from March to May of 2018 and 2019 as part of their regular population management. Body feathers from the breast and back were plucked from the birds, as well as flight feathers (remiges and rectrices), prior to post-mortem examination (PME). The feathers were placed in plastic bags and stored at -20°C until needed. At PME organ samples were taken, from which DNA was extracted and tested for haemosporidians by PCR. Methodology details of PMEs, organ sampling and DNA extraction can be found on section 2.2.6, and organ PCR details can be found in section 2.2.8. Feathers from birds that tested positive to haemosporidian parasites were subsequently tested by PCR, details on the procedure can be found on section 3.2.9.

4.2.3 Use of feathers to diagnose haemosporidian infection from multiple bird species

To explore how bird species may affect the success of haemoparasite detection, feathers of different dead bird species that tested positive for haemoparasites by organ assay were used. The same methodology presented above was applied for this analysis; however, the samples were obtained from a different source. Bird carcasses were collected by zoo staff at Flamingo Land (Annex 1) and at Chester Zoo during 2017 (Section 2.6.2) and 2018 (Section 2.6.4).

4.2.4 The effect of environmental factors on feather DNA yield and PCR results

Carcasses and other samples collected for this study spent considerable time exposed either to rain, heat or wind for variable amounts of time. This section examines how exposure to different environmental conditions affects the amount of DNA obtained. DNA was extracted from primary and secondary flight feathers (remiges) from a euthanized red breasted goose infected with *Plasmodium* spp. (information regarding the goose can be seen in section 2.3.6), after being exposed to three different environmental conditions. A total of 16 flight feathers were collected at the time of death and stored in a plastic bag at -20°C for two months. Passed this time, two feathers were taken for immediate DNA extraction and quantification to use as a control; and the rest of the feathers were separated into different groups and assigned to one of three locations: outdoors, room and freezer. Two feathers were placed in plastic bag with punctures in a garden (outdoors); six feathers were placed in a plastic bag with punctures and left in a room with an average temperature of 22°C (room); and the other six feathers remained in the plastic bag in the freezer at -20°C (freezer). After two months, two feathers from each location were taken for DNA extraction and measurement; passed four and six months, two feathers from room and freezer were taken for DNA extraction and measurement. Extraction was done individually for each feather using the content of the rachis (Figure 4.3). All extractions were tested for *Plasmodium* spp. according to Hellgreen´s (2004) nested PCR protocol.

To analyse the effect of environmental conditions on feather DNA yield, the amount of DNA obtained from feathers kept under different conditions was compared to the control, with a t-test for one sample, using average from the control as a theoretical value. A Mann-Whitney-U test was used to compare the amount of DNA obtained under two different conditions. For the feather location comparisons, 'feathers kept outside were excluded from statistical tests because too few observations were possible. Statistical analyses were done using R studio™ package lme4 (RStudio Team, 2020).

Figure 4.3. Rachis contents of a goose feather prepared for DNA extraction.

4.2.5 Feather DNA yield and PCR performance

To determine the number of feathers required to obtain sufficient DNA for the blood parasite PCR test, feathers in optimal preservation conditions were needed. The Gower bird Hospital in Wales was asked to provide carcasses of birds that died in their care or that, due to poor prognosis, were euthanized. Immediate freezing of carcasses after death was requested as well as a blood sample and three thin blood smears per

bird taken immediately after death. Bird carcasses and blood samples were sent to the lab and frozen at -20°C. Prior to post-mortem examination (PME); breast and back body feathers were plucked from the birds, as well as flight feathers (remiges and rectrices). The feathers were placed in plastic bags and stored at -20°C until needed. DNA was extracted from organs and blood samples and subsequently tested for haemosporidia by PCR (PME procedure, and organs DNA extraction can be found on section 2.2.6. Blood extraction method is described on section 2.2.5. Organ PCR procedure can be found on section 2.2.8). Blood smears were fixed, stained and screened for haemosporidia; when blood parasites were observed the intensity of infection was calculated (methodology for smears processing can be found on section 2.2.5). DNA was extracted from feathers pooling them as follows; body feathers: 1, 5, 10, 15 and 20; flight feathers: 1, 2 and 3; with five repeats per feather group. The extraction was done using an OMEGA DNA extraction kit and in the case of the flight feathers, the optional step of centrifuging and transferring the supernatant to a 1.5 ml eppendorf after the incubation, was done. All feather extractions were eluted in a 40 µl volume. Although fledging birds (Figure 4.4) were sent, these were not used; however, their blood and organs were tested for diagnostic purposes.

Figure 4.4. Carcass of a fledging bird. Arrows signaling growing feathers.

A binomial GLMM (Generalized linear mixed model) was done to establish an association between the PCR result and the DNA amount extracted. W6 was excluded from this test, as PCR was not done for this bird. A nested ANOVA was done looking for a relation between bird and DNA extracted amount; birds were considered as groups and feathers as subgroups; as the data did not show a normal distribution a Log10(n+1) transformation was used. To establish if the type of feather (body or flight) affected the DNA yield on the extraction, the mean extraction value by feather group was calculated, and posteriorly transformed to Log10(n+1) to create a linear model. Lastly, a linear model was used to determine if bird´s weight affected the DNA amount retrieved. The mean of the DNA concentrations per feather group was used and posteriorly transformed to Log10(n+1). Normal distribution after Log10(n+1) transformation was confirmed by a histogram and a plot of the model residuals. All statistical analyses were done using R studio™ package lme4 (RStudio Team, 2020).

4.2.6 Feather haemosporidian detection from a wild bird population

Once the detection of blood parasites from feathers was established, it was decided to test the efficacy of the method to detect infections in live birds under field conditions, without confirmation from blood samples. In Chapter Two, Eurasian blackbirds (*Turdus merula*) were found to have the highest haemosporidian prevalence amongst wild birds, and the literature reports them as commonly infected with these parasites (Dinhopl, 2015). Therefore, bird ringing-groups from Wales were contacted and asked to send by post any Eurasian blackbird feathers that came loose from birds while they were being handled. Varying amounts of feathers were received per bird inside paper envelopes; the samples were collected at different locations from February to June 2018; once collected they were kept at room temperature for varying amounts of time. The feathers received were mostly body feathers; but, for one bird, two flight feathers were received. Body feathers were extracted by pooling 1 to 23 calamus per bird (Figure 4.5) and the flight feathers´ calamus were cut into pieces and pooled with the calamus of the body feathers of the same bird.

Figure 4.5. Body feather tips pooled for DNA extraction.

DNA was extracted, quantified and tested by PCR to determine the presence of blood parasites. The Hellgreen (2004) PCR protocol was followed; but with optimization of the quantity of DNA template. Based on previous results, only samples above 4.5 ng/μl were tested. Testing of these samples was repeated on four occasions increasing the DNA amount used as template on each occasion and reducing the water volume, so that the final volume reached was 20 μl on the first part of the PCR. The first test used 1 μl of template and 6 μl of water; the second was 2 μl of template and 5 μl of water; the third was 3 μl of template and 4 μl of water; and the fourth was 4 μl of template and 3 μl of water (PCR details of the procedure can be found on section 4.3.9).

4.2.7 *Leucocytozoon* spp. detection from feathers of a captive bird population

A final test was done to measure the effectiveness of the method for diagnosing haemosporidia infections in live birds under a situation for which the method was intended. A Sumatran laughingthrushe (SL) (*Garrulax bicolor*) population from Chester Zoo was chosen for this test because, on previous screening, haemosporidan parasites were detected in their blood. Chester Zoo staff was asked to collect fresh moulted

feathers from the SLs enclosure and store them in plastic bags at -20°C. Complementary to this, they were asked to store at -20°C feathers that came loose from birds while they were handled. Identity of the bird was accurately registered when the feathers were obtained while the birds were in hand; but, when the feathers were collected from the enclosure the identity of the birds was estimated based on the proximity of the feathers to the nesting site. The feathers were sent to the lab and stored at -20°C, prior to DNA extraction and testing.

In preliminary tests, an average of 45 ng/μl DNA was retrieved from each flight feather of birds of similar body weight to the SLs; however, environmental exposure could have decreased that amount. Therefore, to ensure sufficient DNA for the PCR, two or three flight feathers (remiges or rectrices) were pooled for each extraction. For the body feathers the same reasoning was applied and 20 body feathers were pooled; although, if a smaller number of body feathers were sent, they were extracted nonetheless. For the flight feather DNA extraction, the basal rachis were cut into small pieces (Figure 4.6), and the optional step of centrifuging and transferring the supernatant to a 1.5 ml eppendorf after the incubation was carried out. All feather extractions were eluted in a 40 μl volume.

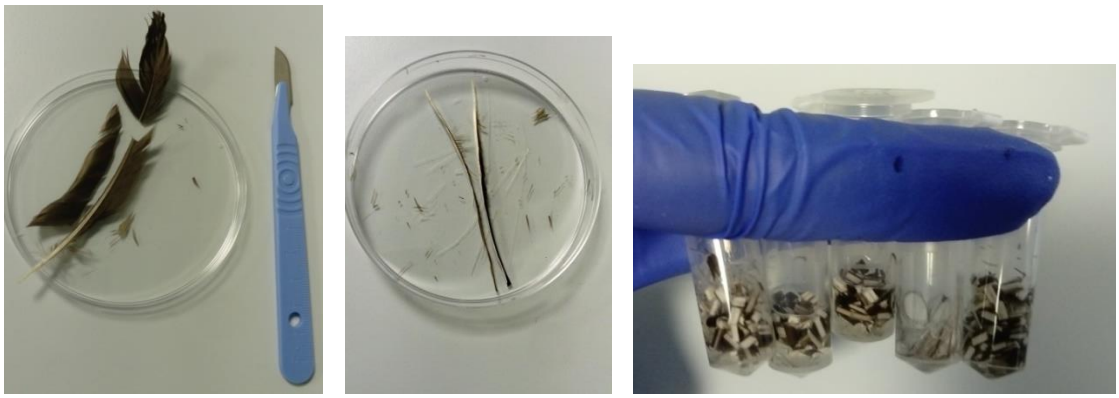

Figure 4.6. SLs flight feathers rachis cut for DNA extraction.

Finally, to guarantee that the PCR had sufficient material to run, the amount of template used was changed in the first part, from 1 μl to 5 μl; therefore, increasing the DNA concentration four times with respect to the original. The additional volume was subtracted from water volume, changing from 6 μl to 2 μl.

4.2.8 Feather DNA extraction

DNA from body feathers was extracted by pooling the calamus of up to 20 feathers from the same individual. In the case of flight feathers, the calamus of one feather was cut longitudinally and its content used. Each calamus was inspected so that if skin was still attached, it was removed using fine tweezers. Extraction was done with an OMEGA tissue and DNA extraction kit according to the manufacturer´s instructions with slight modifications. After adding the first lysis buffer and proteinase K, the sample was left incubating overnight at 55°C in a shaking incubator, and the samples were eluted with 100 μl of elution buffer. Finally DNA concentration of the extractions was measured using a Nanodrop 1000 spectrophotometer.

4.2.9 Polymerase chain reaction

DNA extracted from bird organ samples and feathers was tested by nested PCR using the method described by Hellgreen (2004). The original protocol requires a sample with a DNA concentration of 50 ng/μl, but most of the feather extractions here yielded lower concentrations. From the first experiments it was observed that the sample with the lowest DNA concentration that produced an amplicon had 4.5 ng/μl. Since then, this value was used as a threshold and any sample with a DNA concentration higher than 4.5 ng/μl was tested.

The PCR protocol is able to detect three different haemosporidian genera: *Haemoproteus* spp., *Plasmodium* spp. and *Leucocytozoon* spp. The nested PCR consist of two parts. The first part employs the primers HaemNF1 and HaemNR3. The second

part, occupies either HaemF and HaemR2 primers to detect *Plasmodium* spp. and *Haemoproteus* spp., or HaemFL and HaemR2L primers to detect *Leucocytozoon* spp. The amplicons were visualised on a 1.5% agarose gel. Molecular grade water was used as a negative control and genomic DNA from *Plasmodium bergei* ANKA or genomic DNA from *Leucocytozoon* spp. as a positive control. A positive and a negative control were used on every occasion. For the bird organs and feathers DNA, a T3 Thermocycler (Biometra®) was used, adding a negative and a positive control every 10 samples. For details of this procedure see section 2.2.8.

4.2.10 Gene sequencing

Positive PCR products from all birds´ organs and blood as well as positive feather PCR products from experiments of section 4.2.3, 4.2.4 and 4.2.7 were sent for sequencing in the forward direction with primer HaemF with the Sanger method. For the section 4.2.6, positive PCR products from two flight feathers and two body feathers per bird were randomly selected and sent for sequencing in both directions by the Sanger method with the primers HaemF and HaemR2. Sequence reads were compared to previously published avian haemosporidia in the GenBank nucleotide database (http://www.ncbi.nlm.nih.gov/genbank/) using BLASTn to identify the parasite genus.

Forward and reverse feather PCR products from birds in section 4.2.6 were merged and compared to the corresponding bird blood sequence. Samples form the bird W6 were not compared because its feathers were not tested by PCR. PCR feather products from the bird W1 were compared to its brain sequence, as the blood sample could not be extracted. For the birds W2 and W3, only forward feather sequences were used for comparison.

4.3 Results

4.3.1 Intrinsic feather DNA variation

In an effort to determine how DNA yield varied within individual feathers and within groups of feathers, DNA was extracted from feathers of the same bird species exposed to similar environmental conditions. No DNA could be extracted from any of the crows' body feathers (BF), neither from a single feather nor by pooling them into 5, 10, 15 or 20. Nevertheless, DNA was obtained from the flight feathers (FF); when one FF was used, DNA was recovered from three out of five crows with concentrations ranging from 0.4 to 7.3 ng/μl; when two FF where pooled DNA was obtained from the two tested crows yielding concentrations of 1.7 to 30.8 ng/μl; and when 3 FF were pooled DNA was retrieved from all but one crow with concentrations of 7.6 to 609.6 ng/μl. It should be mentioned that the latter came from an extraction where one of the feathers had visible blood (Table 4.1).

Table 4.1. DNA extraction from body and flight feathers of crows pooled into groups of increasing size.

ID	1 BF (ng/µl)	5 BF (ng/µl)	10 BF (ng/µl)	15 BF (ng/µl)	20 BF (ng/µl)	1 FF (ng/µl)	2 FF (ng/µl)	3 FF (ng/µl)
CR1	0	0	0	-	-	0	-	0
	0	0	0	-	-	0	-	0
	0	0	0	-	-	1.6	-	7.6
	0	0	0	-	-	7.3	-	20.2
	0	0	0	-	-	10	-	609.6
CR2	0	0	0	-	-	0	-	0
	0	0	0	-	-	0	-	0
	0	0	0	-	-	0	-	0
	0	0	0	-	-	0	-	0
	0	0	0	-	-	0	-	0
CR3	0	0	0	-	-	0	-	0
	0	0	0	-	-	0	-	5
	0	0	0	-	-	0	-	10.4
	0	0	0	-	-	0	-	16.9
	0	0	0	-	-	0	-	78.3
CR4	0	0	0	0	0	0	1.7	0
	0	0	0	0	0	0	11.5	0
	0	0	0	0	0	0	12.6	9
	0	0	0	0	0	0	16.1	23
	0	0	0	0	0	5.2	30.8	23.5
CR5	0	0	0	0	0	0	11.3	7
	0	0	0	0	0	0	20.4	14.5
	0	0	0	0	0	0.4	22.6	30.4
	0	0	0	0	0	5.4	42.2	35.3
	0	0	0	0	0	6.9	51.2	36.5

ID= Bird identification; BF= Body feather; FF= Flight feather; Numbers in red indicate samples with observable blood; - = Extraction was not carried out

In the cases where DNA was recovered after the extraction, considerable variation was observed. In most occasions the variation was higher than the mean value; the exceptions to this is observed on CR5 2FF and 3FF (Table 4.2). Post-mortem examinations and PCR results from the corvids received in 2019 can be seen in appendix 4.6.2.

Table 4.2. Mean and standard deviation of DNA extraction from body and flight feathers of crows pooled into groups of increasing size.

ID	1 BF (ng/µl)	5 BF (ng/µl)	10 BF (ng/µl)	15 BF (ng/µl)	20 BF (ng/µl)	1 FF (ng/µl)	2 FF (ng/µl)	3 FF (ng/µl)
CR1	0 ± 0	0 ± 0	0 ± 0	-	-	3.8 ± 4.6	-	127.5 ± 269.6
CR2	0 ± 0	0 ± 0	0 ± 0	-	-	0 ± 0	-	0 ± 0
CR3	0 ± 0	0 ± 0	0 ± 0	-	-	0 ± 0	-	22.1 ± 32
CR4	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1 ± 2.3	14.5 ± 10.5	11.1 ± 11.6
CR5	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	2.5 ± 3.3	29.5 ± 16.5	24.7 ± 13.2

ID= Bird identification; BF= Body feather; FF= Flight feather; - = Extraction was not carried out

4.3.2 Detection of haemosporidians from feathers of a single bird species

Body and flight feathers from multiple individuals of a single bird species, all of which had infections confirmed by PCR on organs, were tested to examine how reliably haemoparasites can be diagnosed from feathers. DNA from flight feathers was obtained from all corvids, while DNA from body feathers was recovered only from one corvid. Concentration of DNA from the flight feathers ranged from 6.4 ng/µl to 352 ng/µl. However, the higher value was obtained from a feather that contained dry blood in the calamus. The effectiveness to detect haemosporidian infection from feathers in these known-infected corvids was 73.3% (11/15). *Plasmodium* infection was detected from feathers in one corvid (100%); *Leucocytozoon* infections were corroborated in nine of 12 corvids (75%); and *Haemoproteus* infections were observed in two of four corvids (50%) (Table 4.3). Post-mortem and PCR data for all corvids can be found on appendix 4.6.1.

Table 4.3. Feather DNA extraction and PCR results of haemosporidia infected dead corvids from the RSPB Burton Mere Wetlands in 2018.

ID	Species	Weight (g)	Degradation	Parasite in organs	Body feather (ng/µl)	Flight feather (ng/µl)	Body feather PCR	Flight feather PCR
C10	Eurasian magpie	150	5	L	< 5*	14.4	-	n
C37	Eurasian magpie	151	5	L	< 5*	74.4	-	p
C13	Eurasian magpie	156	4	H	< 5*	24.9	-	p
C6	Eurasian magpie	164	3	L	< 5*	17.1	-	n
C12	Eurasian magpie	172	3	H, L	< 5*	32.9	-	p
C14	Eurasian magpie	176	4	H, L	< 5*	9.5	-	n
C35	Eurasian magpie	177	5	L	< 5*	12.1	-	p
C33	Eurasian magpie	180	5	L	< 5*	8.7	-	p
C31	Eurasian magpie	186	4	L	< 5*	41.5	-	p
C5	Eurasian magpie	187	4	L	< 5*	6.4	-	p
C20	Eurasian magpie	188	5	L	5.1	21.3	p	p
C11	Eurasian magpie	190	5	H	< 5*	52.8	-	n
C34	Eurasian magpie	195	5	L	< 5*	8.3	-	p
C42	Carrion crow	427	4	L	< 5*	15.6	-	p
C45	Carrion crow	462	5	P	< 5*	352ǂ	-	p

ID= Bird identification; PCR= Polymerase Chain Reaction; P= *Plasmodium* spp.; H= *Haemoproteus* spp.; L= *Leucocytozoon* spp.; n= Negative PCR result; p= Positive PCR result; *=DNA concentration was too low to do a PCR; ǂ = The feather calamus contained dried blood.

4.3.3 Haemosporidian detection from feathers of multiple bird species

To establish that diagnosis of haemosporidiosis from feathers is possible on any bird species, an attempt to corroborate positive infections from organs on free-living wild birds was made by testing body and flight feathers from the same individuals. It was not possible to retrieve DNA from all feathers, for some individuals DNA was obtained either from the body feathers or the flight feathers; but for two very light birds (Goldcrest (*Regulus regulus*) and Firecrest (*Regulus ignicapilla*), both under 6 g) DNA could not be obtained from any of the collected feathers. Overall, it was possible to corroborate infection in 78.6% (22/28) of the organ-infected birds from their feathers. Haemosporidian infection was detected from flight feathers in 67.9% (19/28) of the organ-infected birds; whereas for the body feathers it was 76.2% (16/21) of the cases. In terms of the parasite species; *Leucocytozoon* infections were detected in 85.7% (12/14) of the birds; *Plasmodium* infections were confirmed in 83.3% (5/6) of the birds; and *Haemoproteus* infections were seen in 70% (7/10) of the birds (Table 4.4).

4.3.4 The effect of environmental factors on feather DNA yield

The objective for this section was to analyse variations on feather DNA yield, after exposing feathers from the same individual to different environmental conditions. The feathers kept in the freezer and used as control yielded an average DNA concentration of 273 ng/μl; similarly, measurement of feathers kept under the same conditions for additional two, four and six months yielded an average DNA concentration of 242.5 ng/μl; these values do not vary significantly ($p=0.86$). The DNA quantity from the feathers kept in a room for two, four and six months averaged 47.6 ng/μl; which differs significantly from the control ($p<0.001$). The values of feathers kept in a room for two, four and six months vary slightly amongst each other. A comparison between feathers kept in the freezer and feathers in a room indicated a significant difference ($p=0.002$) (Figure 4.7). For the feathers kept outside, an average DNA concentration of 22.8 ng/μl was obtained, this differs from the observed in the other groups (freezer: 242.5 ng/μl; room: 47.6 ng/μl) and the control (273 ng/μl). It is worth mentioning that the feathers left outside were wet and their content appeared to have diminished in comparison with the other feathers. *Plasmodium* spp. was detected from the control feathers as well as those kept in the freezer and at room temperature (despite the significant decrease in DNA concentration in the latter case); however, *Plasmodium* spp. was not detected in feathers left outdoors (Table 4.5).

Table 4.4. Feather DNA extraction and haemosporidia PCR results from dead free-living wild birds found in Flamingo Land and Chester Zoo during 2017 and 2018.

ID	Species	Body weight (g)	Date	Parasite in organs	BF (ng/µl)	FF (ng/µl)	BF PCR	FF PCR
M37	Firecrest (*Regulus ignicapilla*)	3	2017	H	< 5*	< 5*	-	-
M35	Goldcrest (*Regulus regulus*)	5	2017	H	< 5*	< 5*	-	-
Me2	Northern wren (*Troglodytes troglodytes*)	6	2018	H	6.4	5.6	p	n
MF3	Common swift (*Apus apus*)	13	2017	H	< 5*	7.9	-	n
M103	Willow warbler (*Phylloscopus trochilus*)	15	2017	H	< 5*	12.4	-	p
MF5	Coal tit (*Periparus ater*)	16	2017	H	21.2	15.5	p	p
M25	Coal tit (*Periparus ater*)	16	2017	L	< 5*	61.9	-	p
M38	Europian greenfinch (Chloris chloris)	20	2017	L	< 5*	17.6	-	n
MF4	Song thrush (*Turdus philomelos*)	79	2017	H	< 5*	18.7	-	n
Me3	Eurasian blackbird (*Turdus merula*)	82	2018	P	7.6	12.6	n	n
M92	Eurasian blackbird (*Turdus merula*)	85	2017	H	< 5*	18.5	-	p
M89	Eurasian blackbird (*Turdus merula*)	88	2017	H, L	8.2	28.6	p	p
Me4	Eurasian blackbird (*Turdus merula*)	88	2018	P	5.1	12.9	p	p
M26	Eurasian blackbird (*Turdus merula*)	90	2017	P	29.3	19.7	p	p
M93	Eurasian blackbird (*Turdus merula*)	90	2017	H	33.9	37.9	p	p
M112	Eurasian blackbird (*Turdus merula*)	95	2017	P	24.2	13.2	p	p

Me5	Eurasian blackbird (*Turdus merula*)	95	2018	P	9.3	33.3	p	n
Me1	Eurasian blackbird (*Turdus merula*)	96	2018	P	< 5*	15.1	-	p
M90	Eurasian blackbird (*Turdus merula*)	100	2017	H L	16.3	10.6	p	p
M113	Eurasian blackbird (*Turdus merula*)	100	2017	L	63.9	27	p	p
M125	Eurasian magpie (*Pica pica*)	170	2017	L	8.7	22.7	n	p
Me7	Eurasian jackdaw (*Corvus monedula*)	170	2018	L	23.7	12.2	n	p
M84	Carrion crow (*Corvus corone*)	180	2017	L	5.7	7.1	p	p
M101	Common moorhen (*Gallinula chloropus*)	280	2017	L	11	9.3	p	n
MF9	Rock dove (*Columba livia*)	420	2017	L	59.5	29.2	p	p
M111	Rock dove (*Columba livia*)	450	2017	L	8.2	12	p	p
M127	Rock dove (*Columba livia*)	470	2017	L	50.4	14.4	p	p
MF10	Tawny owl (*Strix aluco*)	480	2017	L	6.9	25.6	p	p
M94	Eurasian buzzard (*Buteo buteo*)	790	2017	H	14.5	9.1	n	n
M7	Mallard (*Anas platyrhynchos*)	1000	2017	L	7.6	11.4	n	n

ID= Bird identification; BF= Body feather; FF= Flight feather; PCR= Polymerase Chain Reaction; P= *Plasmodium* spp.; H= *Haemoproteus* spp.; L= *Leucocytozoon* spp.; n= Negative PCR result; p= Positive PCR result; *=DNA concentration was too low to do a PCR

Table 4.5. Feather DNA extraction and PCR results from feathers kept at different temperature conditions for two, four and six months.

	26th September 2017		26th November 2017		26th January 2018		26th March 2018	
	DNA (ng/µl)	**PCR**	**DNA (ng/µl)**	**PCR**	**DNA (ng/µl)**	**PCR**	**DNA (ng/µl)**	**PCR**
Freezer	230 .2	(+)	324.6	(+)	336.4	(+)	147.2	(+)
	315.8	(+)	203.8	(+)	253.1	(+)	337.7	(+)
Room temperature			66.8	(+)	53.9	(+)	52.7	(+)
			28.4	(+)	64.9	(+)	39.0	(+)
Outdoors			16.4	(-)				
			29.1	(-)				

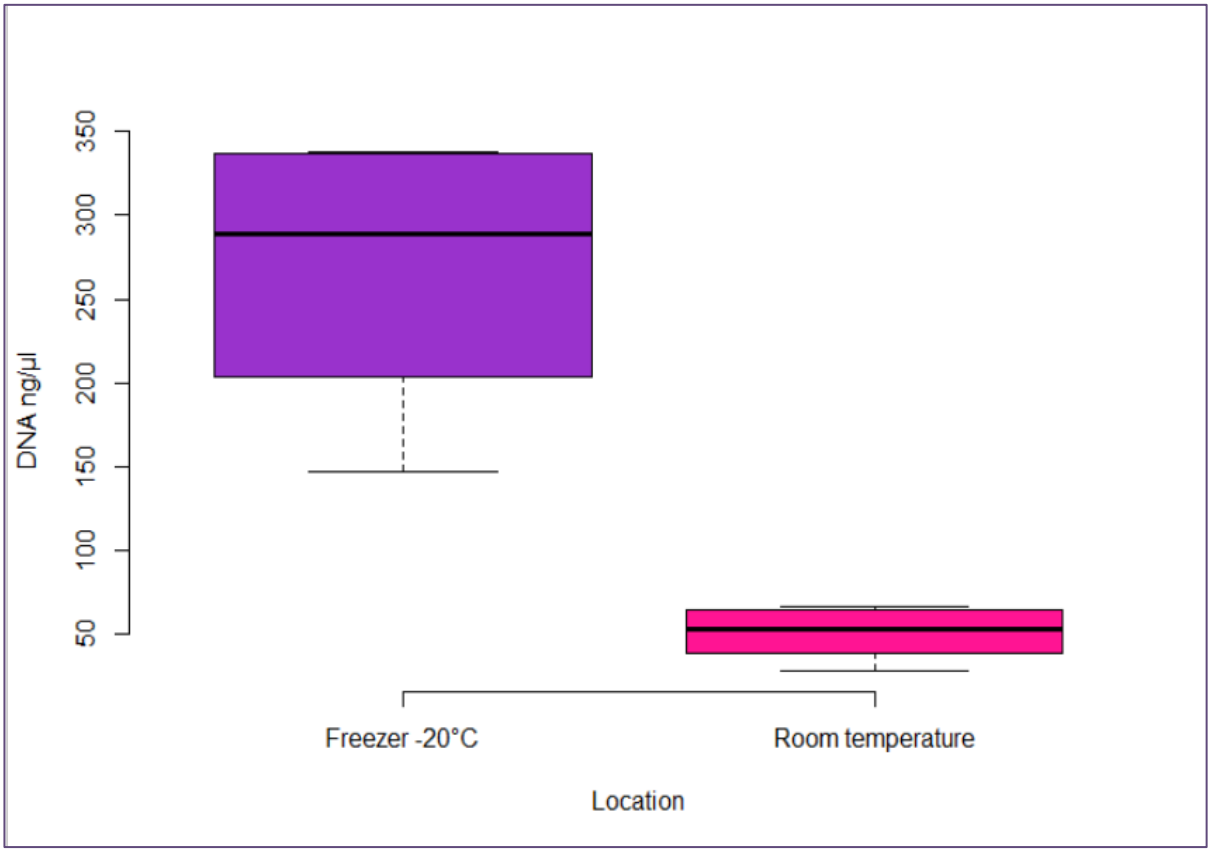

Figure 4.7. DNA concentration comparison from Red-breasted goose (*Branta ruficollis*) flight feathers kept at room temperature and at -20°C.

4.3.5 Feather DNA yield and PCR performance

This section aimed to accurately determine DNA yields for individual feathers and for groups of feathers of different bird species, and to establish how that DNA yield affected the PCR results. For this, a set of birds, eight non-fledging birds from seven different species preserved at optimal conditions, was acquired from the Gower bird Hospital. Haemosporidia infection was detected from the blood smears screening in a Eurasian blackbird, which presented a high intensity infection with *Haemoproteus* spp. (Figure 4.8a), and in a Carrion crow, which had a low intensity infection with *Leucocytozoon* spp. (Figure 4.8b). The blood PCR detected two birds infected with *Haemoproteus* spp. and five infected with *Leucocytozoon* spp. Lastly, the brain PCR uncovered four *Leucocytozoon* spp. infections, three mixed infections of *Haemoproteus* spp. and *Leucocytozoon* spp. and one mixed infection of *Plasmodium* spp. and *Leucocytozoon* spp. The *Haemoproteus* spp. infection detected from a blood smear was consistent with the blood PCR result. The results from the blood PCR were consistent with the PCR from the brain, except that the later detected four additional mixed infections (Table 4.6).

DNA was extracted from all body and flight feathers from the different birds; except for 1 BF of W2. DNA yield ranges for 1 BF, 5BF, 10BF, 15BF, 20BF, 1FF, 2FF and 3FF were 0.4 - 26.5 ng/μl, 2 - 52.5 ng/μl, 2.3 - 153.2 ng/μl, 2.8 - 199.2 ng/μl, 5.7 - 188.4 ng/μl, 10.3 - 2131.1 ng/μl, 11.9 to 584.5 ng/μl, and 23.5 - 623.9 ng/μl respectively.

On some occasions, dry blood was observed inside the feathers' shaft, distributed in different sections of the rachis (Figure 4.9). The extractions of these feathers generally produced higher DNA yields than the observed for the feathers within their group.

Table 4.6. Gower Bird Hospital birds´ data and haemosporidia tests results.

ID	Species	Capture site	Age	Body weight (g)	Admission date	Clinical history summary	Blood smear	Blood PCR	Brain PCR
W5	Song thrush (*Turdus philomelos*)	Parkmill	J	45	16/05/2019	Moribund, cold and emaciated	N	L	L
W6	Eurasian blackbird (*Turdus merula*)	Llanelli	A	59	03/06/2019	Emaciated Suspect cat attack	H HII	H	H / L
W4	Song thrush (*Turdus philomelos*)	Winchwen	J	69	24/05/2019	Spinal trauma Cat attack	N	H	H / L
W7	Mistle thrush (*Turdus viscivorous*)	Parkmill	A	106	25/04/2019	Moribund Missing rectrices and bald rump	N	L	L
W8	Eurasian magpie (*Pica pica*)	Newton	J	172	08/05/2019	Moribund Unable to stand	N	L	P / L
W2	Eurasian jackdaw (*Corvus monedula*)	Pnllergaer	A	224	14/04/2019	Puncture wound Left wing damaged	N	L	L
W1	Carrion crow (*Corvus corone*)	Kidwelly	A	425	07/04/2019	Right humerous broken	L LII	-	H / L
W3	Common raven (*Corvus corax*)	Cwmdu	J	953	05/06/2019	Traumatic injury in caudal spine	N	L	L

ID= Bird identification; A= Adult; J= Juvenile; N= Negative; P= *Plasmodium* spp.; H= *Haemoproteus* spp.; L= *Leucocytozoon* spp.; (n) = No parasites observed on the smear; HII= High intensity infection; LII= Low intensity infection; - = DNA extraction was not possible

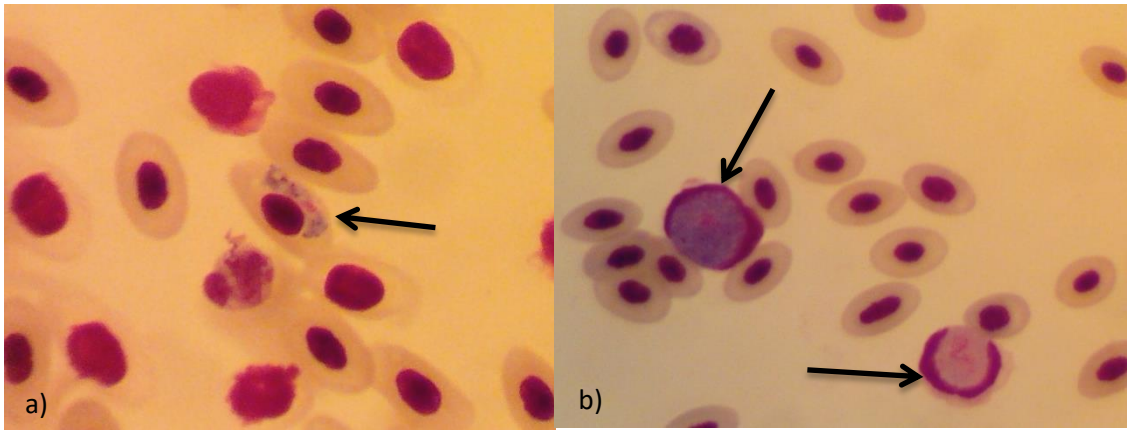

Figure 4.8. Haemosporidian infections of birds from the Gower Bird Hospital. a) *Haemoproteus* infection in a Eurasian blackbird, arrows point to a gametocyte b) *Leucocytozoon* infection in a Carrion crow, arrows point to gametocytes.

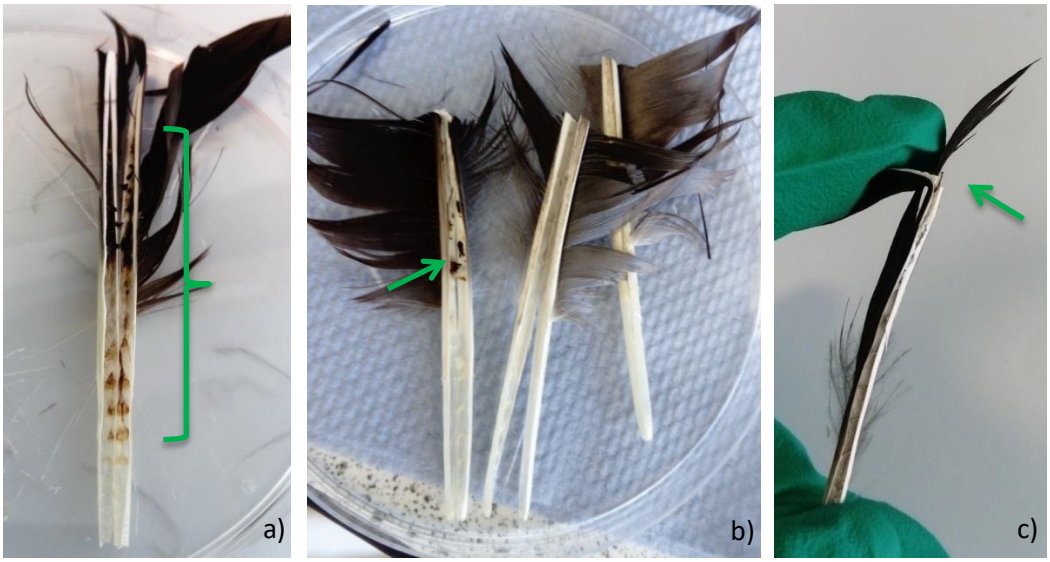

Figure 4.9. Feathers with dry blood inside the shaft. a) Blood is located from the superior umbilicus to the distal portion of the rachis, brackets indicate where blood is. b) Blood is located in a small section of the rachis from the superior umbilicus to the distal part of the rachis, arrow point the dry blood. c) Blood is only observable at the distal portion of the rachis, arrow point the dry blood.

PCR diagnosis from 1BF was possible in two of the seven infected birds; for 5BF a positive PCR result was obtained for six of seven birds; and for the following BF and FF groups the PCR diagnosis worked in all of the birds. Only in the Common raven (*Corvus corax*)(W3) was the PCR positive on every feather. The lowest PCR diagnosis success was seen in the Eurasian jackdaw (*C. monedula*) (W2) with only 10 positive feathers out of 40 (Table 4.7).

Table 4.7. DNA concentration after extraction of body and flight feathers pooled into different numbers with five replicates by feather group.

ID (g)	1 BF (ng/µl)	5 BF (ng/µl)	10 BF (ng/µl)	15 BF (ng/µl)	20 BF (ng/µl)	1 FF (ng/µl)	2 FF (ng/µl)	3 FF (ng/µl)
W2 Eurasian jackdaw (224)	0	2	2.3	2.8	3	17.8	33.3	66.1
	(-)	(-)	(-)	(+)	(-)	(-)	(+)	(+)
	0.3	2.1	2.5	3.6	5.6	21.1	35.2	68.60
	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(-)
	0.4	2.3	2.6	4.3	7.6	25.2	48.9	77.9
	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
	0.6	4.8	3.5	5	12.4	28.5	58.9	112.5
	(-)	(-)	(+)	(-)	(+)	(-)	(-)	(+)
	1.6	5.5	4.2	7.4	12.9	2131.1	62.3	155.1
	(-)	(-)	(-)	(+)	(-)	(+)	(-)	(+)
W4 Song thrush (69)	2.5	2.8	3.5	4.9	5.7	10.3	11.9	23.5
	(-)	(-)	(+)	(+)	(+)	(+)	(-)	(-)
	3.5	2.8	5	5.3	6.9	11	16.8	28.8
	(-)	(+)	(+)	(+)	(+)	(-)	(+)	(+)
	3.6	3	5.7	5.9	7.2	11.4	17.5	37
	(-)	(+)	(+)	(+)	(-)	(+)	(+)	(-)
	4.3	4.4	5.9	6	17	15.5	20	42.8
	(-)	(+)	(+)	(+)	(+)	(-)	(-)	(+)
	6.1	7	7	6.8	18.1	17.5	25.6	48.9
	(-)	(+)	(-)	(+)	(-)	(+)	(+)	(+)
W7 Mistle thrush (106)	4	5.2	5.1	9.3	14.2	34.3	39.8	106.4
	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(+)
	4.3	5.7	5.6	10.4	16.9	40.3	76.4	115.9
	(-)		(-)	(+)	(+)	(-)	(+)	(+)
	5.1	6.2	9.3	12.2	19	40.8	88.3	118.7
	(-)	(+)	(-)	(+)	(+)	(+)	(-)	(+)
	5.4	9.4	9.9	15.7	19	53.8	100.5	147.6
	(-)	(+)	(+)	(+)	(+)	(-)	(+)	(+)
	6.4	9.6	12.6	16.4	19.8	64.9	136	190.8
	(-)	(-)	(-)	(+)	(-)	(+)	(+)	(-)
W8 Eurasian magpie (172)	2.9	4.1	6.7	12.7	20.3	46.2	79.4	53.2
	(-)	(-)	(-)	(-)	(+)	(-)	(+)	(+)
	2.9	4.8	11.3	14.3	20.4	46.4	80.2	62.7
	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)
	3	5.2	12.1	19.4	21	70.3	99.2	79.5
	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(+)
	4.4	6	13	22.8	21.9	79.6	102.3	135.3
	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
	4.5	7.6	16.8	24.7	28.8	125.7	123.8	218.7

	(-)	(-)	(-)	(+)	(+)	(+)	(+)	(+)
W1 Carrion crow (425)	3.6	6.2	10.1	11.2	19.5	27.7	53	83.4
	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(+)
	3.8	8.3	12.3	12.6	20.2	33	66.5	102.6
	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)
	4.8	8.3	12.6	16.5	23.6	57.7	69.7	144.7
	(-)	(+)	(-)	(-)	(-)	(-)	(+)	(+)
	8.4	8.8	12.6	17.7	24.2	68.4	74.4	205.8
	(-)	(-)	(+)	(-)	(-)	(-)	(+)	(+)
	9	10.1	19.6	18.1	24.3	137.4	111.7	231.80
	(-)	(-)	(-)	(+)	(+)	(+)	(+)	(+)
W5 Song thrush (45)	1.6	4	7.1	7.9	11.6	87.4	127.3	158.5
	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
	1.7	5.6	8.4	8.2	11.9	89.6	158	221.5
	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(+)
	1.7	6.8	9.3	8.7	13.1	138.5	166.8	307.5
	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)
	2.9	7.6	10.1	10.6	43	148.4	308.4	623.9
	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(+)
	3.1	8.1	10.3	20	71.1	171.2	584.5	725.6
	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
W3 Common raven (953)	6.8	47.4	71.1	107.3	118.8	105.9	103.4	147
	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
	12	48.3	89.5	109.5	125	169.6	155.8	185.30
	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
	12.6	48.9	102.9	116.4	129.8	726.9	160.7	195.9
	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
	22.3	51	132.9	177.1	187.4	726.9	248	227.8
	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
	26.5	52.2	153.2	199.2	188.4	868.4	386	524.4
	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
W6* Eurasian blackbird (59)	2.5	4.5	6	8.2	9.8	12.1	29.6	40.8
	3.1	5.5	7.3	9.8	12.2	18.1	32.3	43.1
	3.7	6	8.1	10.6	15.8	23.4	37.5	55.3
	4	6.2	9	10.7	18.3	27.3	37.9	-
	5.5	7	10.4	18.1	25.2	29.4	41.5	-

ID= Bird identification; BF= Body feather; FF= Flight feather; *= This bird´s feathers were not tested by PCR; Numbers in red indicate samples with observable blood; - = the feathers were not extracted; (+)= Positive PCR result; (-)= Negative PCR result

The average DNA concentration obtained from the feathers of each bird was different; nevertheless, it can be observed that the DNA amount increases as the number of feathers used increases in all of the birds. Also, it seems that the FF produced a higher DNA concentration with respect to the BF. Interestingly, in both, the Common raven (W3) and the Eurasian jackdaw (W2), one FF yielded higher DNA concentration than two or three FF. (Figure 4.10)

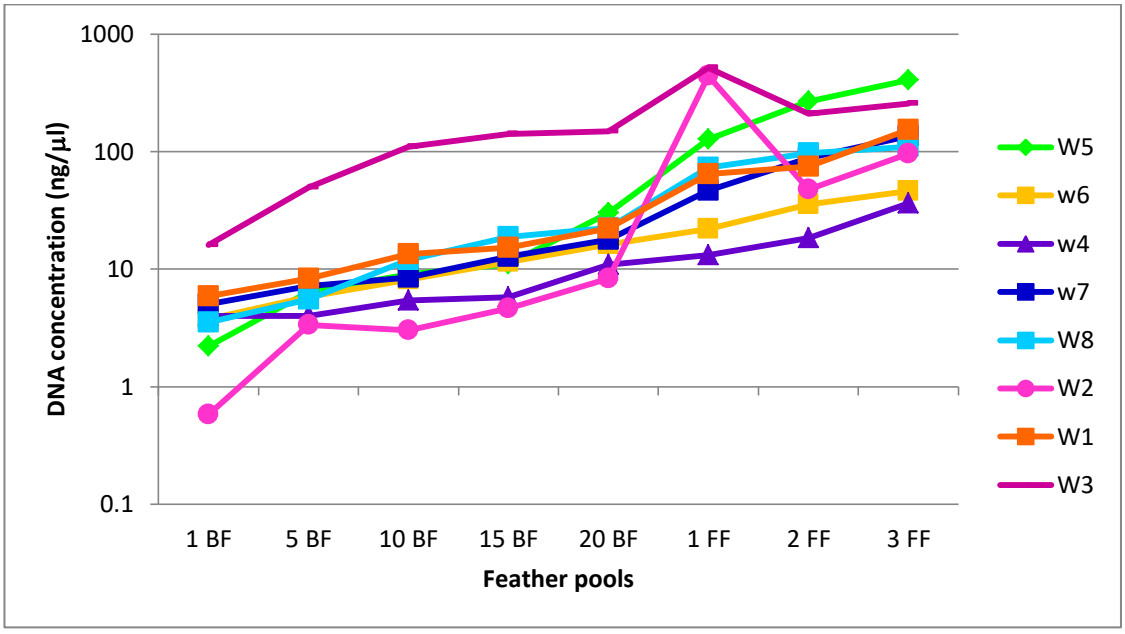

Figure 4.10. Mean DNA concentration extracted for each feather group in all birds coming from the Gower Bird Hospital. The plot is on base 10 logarithmic scale.

Variation in DNA concentration was observed on every group of pooled feathers. DNA concentration range and standard deviation range for each group were as follows: 1BF 0.6 – 16 ng/µl ± 0.6 - 8.1; for 5BF 3.3 – 49.6 ng/µl ± 0.9- 2.1; for 10BF 3 – 109.9 ng/µl ± 0.8 – 33.1; for 15BF 4.6 – 141.9 ng/µl ± 0.7 -43.1; for 20BF 8.3 – 149.9 ng/µl ± 2.3 -34.9; for 1FF 13.1 – 519.5 ng/µl ± 3.2 – 942.7; for 2FF 18.4 – 269 ± ng/µl 4.8 – 189.7; for 3FF 36.2 407.4 ng/µl ± 7.8 – 252.3 (Table 4.8). It is worth to mention that the high standard deviation value for 1FF originated from an unusually high DNA concentration from a feather with observable blood.

Table 4.8. Mean and standard deviation DNA concentration after extraction of body and flight feathers pooled into different numbers with five replicates by feather group.

ID	1 BF	5 BF	10 BF	15 BF	20 BF	1 FF	2 FF	3 FF
W2	0.6 ± 0.6	3.3 ± 1.7	3.0 ± 0.8	4.6 ± 1.8	8.3 ± 4.3	444.7 ± 942.7	47.7 ± 13.3	96.0 ± 37.9
W4	4.0 ± 1.3	4.0 ± 1.8	5.4 ± 1.3	5.8 ± 0.7	11.0 ± 6.0	13.1 ± 3.2	18.4 ± 5.0	36.2 ± 10.3
W7	5.0 ± 1.0	7.2 ± 2.1	8.5 ± 3.1	12.8 ± 3.2	17.8 ± 2.3	46.8 ± 12.4	88.2 ± 35.1	135.9 ± 34.3
W8	3.5 ± 0.8	5.5 ± 1.3	12.0 ± 3.6	18.8 ± 5.2	22.5 ± 3.6	73.6 ± 32.6	97.0 ± 18.3	109.9 ± 68.6
W1	5.9 ± 2.6	8.3 ± 1.4	13.4 ± 3.6	15.2 ± 3.1	22.4 ± 2.3	64.8 ± 43.9	75.1 ± 22.0	153.7 ± 64.1
W5	2.2 ± 0.7	6.4 ± 1.6	9.0 ± 1.3	11.1 ± 5.1	30.1 ± 26.5	127.0 ± 37.1	269.0 ± 189.7	407.4 ± 252.3
W3	16.0 ± 8.1	49.6 ± 2.0	109.9 ± 33.1	141.9 ± 43.1	149.9 ± 34.9	519.5 ± 354.0	210.8 ± 110.8	256.1 ± 152.7
W6	3.8 ± 1.1	5.8 ± 0.9	8.2 ± 1.7	11.5 ± 3.8	16.3 ± 6.0	22.1 ± 7.0	35.8 ± 4.8	46.4 ± 7.8

ID= Bird identification; BF= Body feather; FF= Flight feather

The DNA amount obtained from feathers was significantly different amongst birds ($p < 0.001$); with W3 being significantly different from the rest of the birds (Figure 4.11). DNA yield was significantly affected by feather type, being highest for FF than for BF. DNA retrieval was also affected by the amount of feathers, the more feathers used, the higher the DNA quantity ($p < 0.001$) (Figure 4.12).

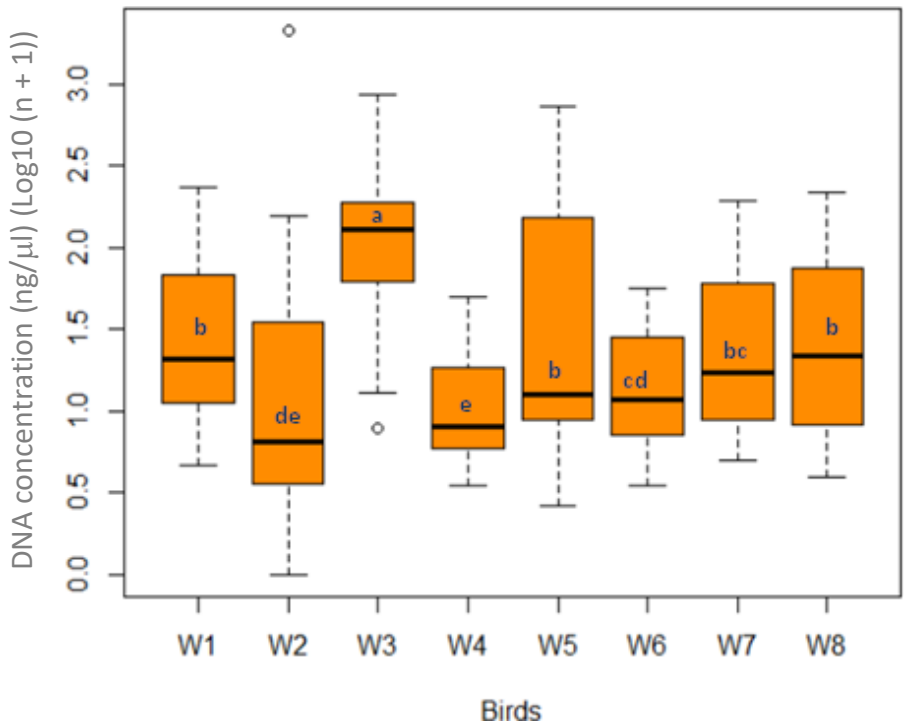

Figure 4.11. DNA concentration extracted for all feathers of each bird. Boxes with the same letters are not significantly different from each other. Data for this plot was transformed to Log10(n+1) prior to analysis.

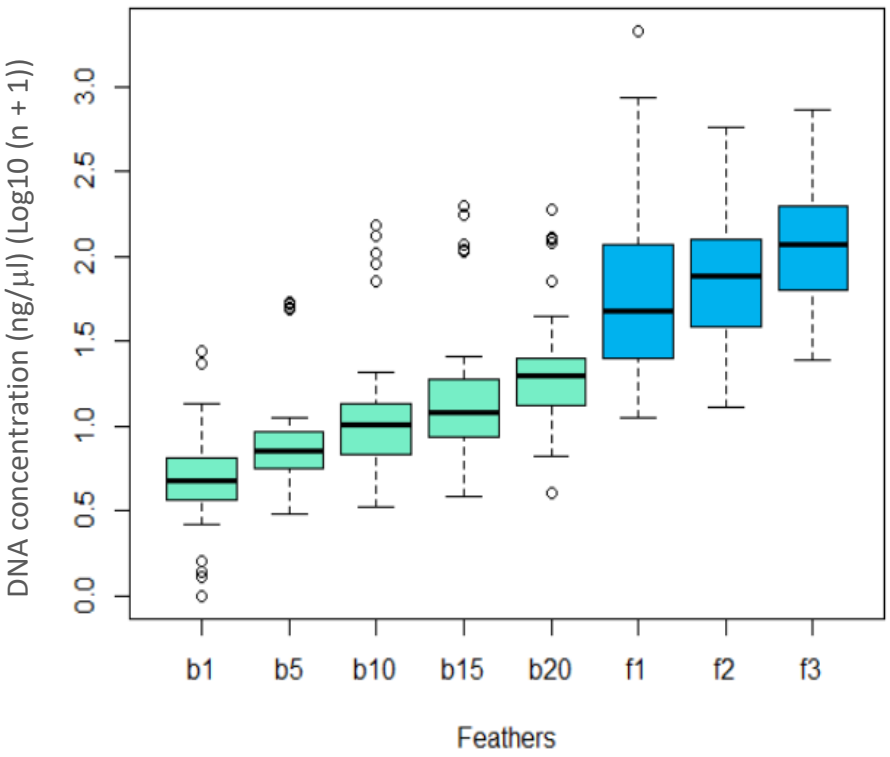

Figure 4.12. Box plots showing median DNA concentration extracted for each feather group of all birds. Data for this plot was transformed to Log10(n+1).

Birds´ body weight was found to influence the DNA amount that was obtained from the extraction, with the heaviest birds producing a higher DNA retrieval ($p < 0.001$) (Figure 4.13). Likewise, a significant difference in the PCR result depending on the DNA concentration was observed ($p < 0.001$), as the DNA increased, the PCR ability to detect haemosporidians increased.

The genetic analysis comparing Gower bird Hospital bird feathers sequences with their corresponding blood sequences revealed complete correspondence for some birds, and partial correspondence for others. For W8 and W4 blood reference sequences infected with *Leucocytozoon* spp. and *Haemoproteus* spp. respectively, there was 100% correspondence with the four feather sequences (two FF and two BF per bird) used for comparison. Similarly, W5 and W2 reference blood sequence were 99% and 99.3% identical to the four feather sequences selected respectively. However, for the rest of the birds only one of the four feathers had a high similarity score. W1 brain sequence was 100% identical to one feather sequence, 93.2% identical to another feather sequence and 92.2% identical to other two feather sequences. W3, blood sequence was 100% identical to one feather sequence, 97.1% identical to two feather sequences and 96.2% identical another feather sequence. W7 blood sequence was 98.6% identical to one feather sequence, and 92.1% identical to the other three feather sequences (Table 4.9).

4.3.6 Feather haemosporidian detection from a free-living wild bird population

The effectiveness of feathers to detect blood parasites infections was tested on feathers from live birds collected opportunistically. Feathers from 71 Eurasian blackbirds were received during 2018 from different locations in Wales, the number of feathers per individual ranged from 1 to 23. Only seven extractions reached over4.5 ng/μl, reaching DNA concentrations of 5.7 to 13.6 ng/μl, by employing 3 to 23 body feathers (Table 4.10).

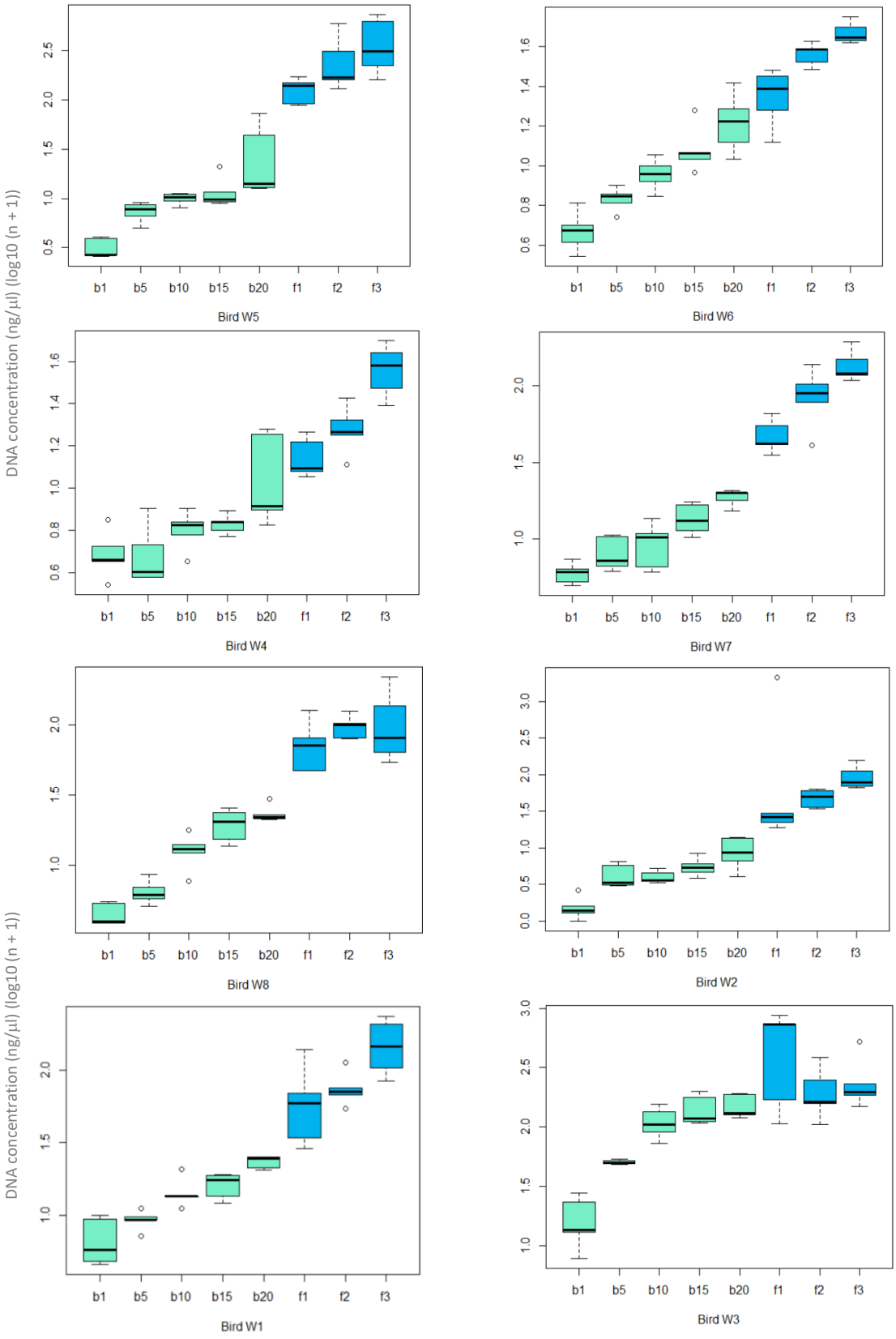

Figure 4.13. Box plots showing mean DNA concentration extracted for each feather group by bird. Data for this plot was transformed to Log(n+1). Bird´s weights are as follows from lightest to heaviest: W5 (45 g), W6 (59 g), W4 (69g), W7 (106 g), W8 (172 g), W2 (224 g), W1 (425 g), W3 (953 g).

Table 4.9. Comparison of organ or blood retrieved sequence against feather retrieved sequences.

ID	Species	Reference sequence origin	Detected blood parasite	Sequence length	Feathers sequence identity
W1	Carrion crow (*Corvus corone*)	Brain	*Leucocytozoon* spp	438 bp	20 BF4 (100%) 2FF1 (93.2%) 15BF3 (92.2%) 2FF3 (92.2%)
W2	Eurasian jackdaw (*Corvus monedula*)	Blood	*Leucocytozoon* spp	440 bp	15BF5 (99.3%) 20BF3 (99.3%) 1FF1 (99.3%) 2FF4 (99.3%)
W3	Common raven (*Corvux corax*)	Blood	*Leucocytozoon* spp	442 bp	1BF1 (100%) 1BF2 (97.1%) 1FF3 (97.1%) 1FF2 (96.2%)
W4	Song thrush (*Turdus philomelos*)	Blood	*Haemoproteus* spp	433 bp	20BF3 (100%) 20BF4 (100%) 3FF3 (100%) 3FF4 (100%)
W5	Song thrush (*Turdus philomelos*)	Blood	*Leucocytozoon* spp	423 bp	20BF1 (99%) 20BF5 (99%) 1FF1 (99%) 1FF2 (99%)
W7	Mistle thrush (*Turdus viscivorous*)	Blood	*Leucocytozoon* spp	418 bp	1FF1 (98.6%) 20BF2 (92.1%) 20BF4 (92.1%) 2FF5 (92.1%)
W8	Eurasian magpie (*Pica pica*)	Blood	*Leucocytozoon* spp	423 bp	15BF2 (100%) 15BF5 (100%) 1FF2 (100%) 1FF3 (100%)

ID= Bird identification; BF= Body feather; FF= Flight feather; bp = base pairs

From the 71 Eurasian blackbird feathers submitted only eight were tested, as they produced the minimum DNA amount required. When one, two and three microliters of template were used in the PCR reaction all samples tested negative; nevertheless, when four microliters of template were used one sample tested positive. Sequencing results from the positive sample identified a *Plasmodium* parasite (Table 4.11).

Table 4.10. Eurasian blackbird testing for haemoparasites

ID	Sampling date	Ringing site	Body feathers used (n)	DNA extraction (ng/μl)
BB1	22/02/2018	Close, Julian	14	< 5*
BB2	21/04/2018	Weaven valley	23	13.6
BB3	21/04/2018	Weaven valley	12	< 5*
BB4	21/04/2018	Weaven valley	23	< 5*
BB5	21/04/2018	Weaven valley	23	1.8
BB6	21/04/2018	Weaven valley	13	< 5*
BB7	08/04/2018	Woodford	5	< 5*
BB8	26/04/2018	Runcorn	23	< 5*
BB9	15/03/2018	Newmadls	6	< 5*
BB10	05/07/2017	Bromborough	23	11.4
BB11	13/03/2018	Bromborough	23	< 5*
BB12	11/03/2018	Woodford	3	< 5*
BB13	11/03/2018	Woodford	3	< 5*
BB14	11/03/2018	Woodford	8	8.1
BB15	11/03/2018	Woodford	3	< 5*
BB16	11/03/2018	Woodford	3	0.5
BB17	11/03/2018	Woodford	2	< 5*
BB18	11/03/2018	Woodford	3	< 5*
BB19	11/03/2018	Woodford	5	13.5
BB20	11/03/2018	Woodford	3	5.7
BB21	11/03/2018	Woodford	3	4.5
BB22	11/03/2018	Weaven valley	7	< 5*
BB23	11/03/2018	Burton	5	< 5*
BB24	10/03/2018	Burton	3	6.5
BB25	13/02/2018	Burton	8	< 5*
BB26	13/02/2018	Burton	16	< 5*
BB27	13/02/2018	Burton	6	< 5*
BB28	11/03/2018	Warrington	10	2.8
BB29	11/03/2018	Weaven valley	3	< 5*
BB30	06/03/2018	Houghton	16	< 5*
BB31	31/12/2017	Dobcross	5	< 5*
BB32	07/03/2018	Dobcross	6	< 5*
BB33	07/03/2018	Dobcross	7	< 5*
BB34	19/03/2018	Alderley Edge	4	< 5*
BB35	17/03/2018	Garden	17	< 5*
BB36	18/03/2018	Woodford	3	< 5*

BB37	18/03/2018	Woodford	5	< 5*
BB38	18/03/2018	Woodford	4	< 5*
BB39	18/03/2018	Woodford	3	< 5*
BB40	18/03/2018	Woodford	1	< 5*
BB41	18/03/2018	Woodford	4	< 5*
BB42	18/03/2018	Woodford	3	< 5*
BB43	18/03/2018	Woodford	4	< 5*
BB44	18/04/2018	Woodford	3	< 5*
BB45	18/03/2018	Woodford	4	< 5*
BB46	18/03/2018	Woodford	2	< 5*
BB47	18/03/2018	Woodford	2	< 5*
BB48	21/03/2018	Woodford	3	< 5*
BB49	26/05/2018	Higher Paynton	23	< 5*
BB50	26/05/2018	Higher Paynton	23	< 5*
BB51	26/05/2018	Higher Paynton	23	< 5*
BB52	01/04/2018	Woodford	2	< 5*
BB53	01/04/2018	Woodford	3	< 5*
BB54	01/04/2018	Woodford	4	< 5*
BB55	25/03/2018	Woodford	3	< 5*
BB56	25/03/2018	Woodford	3	< 5*
BB57	25/03/2018	Woodford	3	< 5*
BB58	25/03/2018	Woodford	2	< 5*
BB59	30/06/2018	Higher Paynton	13	< 5*
BB60	09/06/2018	Higher Paynton	23	< 5*
BB61	09/06/2018	Higher Paynton	23	< 5*
BB62	06/05/2018	Higher Paynton	2	< 5*
BB63	06/05/2018	Higher Paynton	4	< 5*
BB64	05/03/2018	Garden	5	< 5*
BB65	04/03/2018	Houghton	4[ж]	5.8
BB66	01/04/2018	Garden	18	< 5*
BB67	31/03/2018	Garden	9	< 5*
BB68	19/05/2018	Higher Paynton	5	< 5*
BB69	19/05/2018	Higher Paynton	4	< 5*
BB70	20/05/2018	Higher Paynton	3	< 5*
BB71	19/05/2018	Higher Paynton	5	< 5*

ID= Bird identification; [ж]= Two flight feathers were sent for this bird and their content was added to the extraction; * = Value was not recorded by Nanodrop.

Table 4.11. Eurasian blackbird feathers PCR testing for haemoparasites

ID	Sampling date	Ringing site	BF (n)	Extraction (ng/µl)	PCR template used			
					1 µl	2 µl	3 µl	4 µl
BB2	21/04/2018	Weaven valley	23	13.6	(-)	(-)	(-)	(-)
BB19	11/03/2018	Woodford	5	13.5	(-)	(-)	(-)	(-)
BB10	05/07/2017	Bromborough	23	11.4	(-)	(-)	(-)	**(+)**
BB14	11/03/2018	Woodford	8	8.1	(-)	(-)	(-)	(-)
BB24	10/03/2018	Burton	3	6.5	(-)	(-)	(-)	(-)
BB65	04/03/2018	Houghton	4*	5.8	(-)	(-)	(-)	(-)
BB20	11/03/2018	Woodford	3	5.7	(-)	(-)	(-)	(-)
BB21	11/03/2018	Woodford	3	4.5	(-)	(-)	(-)	(-)

ID= Bird identification; PCR= Polymerase Chain Reaction; (-)= Negative PCR result; (+)= Positive PCR result; * Two flight feathers were sent for this bird and their content was added to the extraction.

4.3.7 *Leucocytozoon* spp. detection from feathers of a captive bird population

The efficacy to detect haemosporidian infections using feathers was tested on feathers of live captive birds, collected opportunistically and stored under optimal conditions. BF samples from 11 different birds belonging to one bird species were employed. The number of BF sent for the Sumatran laughingthrushes (SL) were between 2 and 10 yielding DNA concentrations from 2.8 to 46 ng/µl. FF were sent for five different birds and five groups of birds. The FF extractions produced DNA concentrations of 4.8 to 337.9 ng/µl. PCR detected infections on four SLs (C17703, C14419, C18421 and C12640); and detected infections on three different groups on two occasions each (C15751/C15752/C181038, C14418/C16484 and C18663/C18857). All except one infection were detected from FF (Table 4.12).

Table 4.12. *Leucocytozoon* spp. detection from Sumatran laughingthrushes feathers

ID	Sampling date	BF used (n)	FF used (n)	DNA extraction (ng/µl)	PCR
C15751/ C15752/ C181038		9		2.8	
C18421		2		3	
C15752	02/11/18	10		3.6	
C17703	16/10/18	2		4.7	L
C14419/ C16484	01/01/19		2-3	4.8	
C18683			2-3	5.2	
C15751/ C15752/ C181038	01/01/19		2-3	6.9	L
C14419/ C16484	01/01/19		2-3	8.6	L
C15751	02/11/18		2-3	8.9	
C15751/ C15752/ C181038			2-3	9.6	
C15749/ C15750	16/10/18		2-3	9.8	
C15751/ C15752/ C181038	01/01/19		2-3	9.9	
C14419	20/11/18		2-3	10.7	L
C15749	17/10/18	10		10.7	
C181038	02/11/18	2		10.7	
C15751/ C15752/ C181038	01/01/19		2-3	11.2	
C15751/ C15752/ C181038	01/01/19		2-3	11.3	L
C18683		6		12.7	
C18421	21/11/18		2-3	13.1	L
C18663/ C18857	01/01/19		2-3	13.5	L
C15751/ C15752/ C181038	01/01/19		2-3	14	
C18538		2		15.3	
C15751/ C15752/ C181038	01/01/19		2-3	17	
C15751/ C15752/ C181038	01/01/19		2-3	17.2	
C15749/ C15750	16/10/18		2-3	17.3	
C15751/ C15752/ C181038	01/01/19		2-3	18.9	
C15751/ C15752/ C181038	01/01/19		2-3	19.3	
C18421	02/01/19		2-3	19.7	
C14419	20/11/18		2-3	20.2	
C14419/ C16484	01/01/19		2-3	22	L
C15751/ C15752/ C181038			2-3	22.1	
C18663/C18857	01/01/19		2-3	22.2	
C15751/ C15752/ C181038	01/01/19		2-3	22.4	
C18421	21/11/18		2-3	22.5	
C18663/ C18857	01/01/19		2-3	23.7	
C15751/ C15752/ C181038	01/01/19		2-3	23.9	
C18421	02/01/19		2-3	24.2	
C14419/ C16484	01/01/19		2-3	25.1	
C18421	21/11/18		2-3	26	

C18663/ C18857	01/01/19		2-3	27.6	
C18857		8		28.1	
C18663/ C18857	01/01/19		2-3	29.6	
C18857/C18683	21/11/18		2-3	32.5	
C18663/ C18857	01/01/19		2-3	33.3	
C18663/ C18857	01/01/19		2-3	38.6	L
C15751/ C15752/ C181038	01/01/19		2-3	40.1	
C15750	17/10/18	3		40.3	
C18857/ C18683	21/11/18		2-3	42.5	
C15751	02/11/18	8		46	
C18421	02/01/19		2-3	59.4	L
C16484	20/11/18	7		63.2	
C12640	06/11/18		2-3	337.9	L

ID= Bird identification; BF= Body feather; FF= Flight feather; L = *Leucocytozoon* spp.

4.4 DISCUSSION

This chapter aimed to show that feathers are a viable alternative to blood samples for diagnosis of blood parasites. Secondarily, it explored factors affecting the feather DNA yield, such as exposure to environmental conditions, preservation conditions, bird´s body weight, feather type and feather intrinsic variation.

Overall, DNA extraction was possible for most of the feathers sampled. A few exceptions where it did not work were possibly related to the state of preservation of the sample, especially in cases where the samples were plucked from dead birds that remained on site for an unknown period of time, and for feathers stored at room temperature for prolonged periods.

The majority of the feather extractions yielded an adequate DNA concentration to be used for PCR. Blood parasite detection was possible in most cases; however, it was seen that PCR performance depended on DNA concentration, which was subject to the bird´s weight, type of feather and sample preservation.

This chapter shows that the DNA amount extracted from feathers decreases by about 80% if they are stored at room temperature (22°C) from two months onwards. It was also shown that feathers that remained outdoors for more than two months, have a similar decrease in DNA yield, and will give poor amplification results. This is supported by Taberlet (1999), who states that samples should be collected soon as possible, for if they stay long in the field DNA will degrade. Nevertheless, it should be considered that in the present study only two replicates per treatment were used; hence, further testing with bigger sample sizes is required.

After storing feathers at room temperature, the first DNA extraction and measurement were done after two months; at that point, it was noticed that DNA yield was lower compared to control feathers. Degradation of feathers stored at room temperature might have started before two months; but, due to the small number of feathers received it was not possible to test this. After storing the feathers at room temperature for two months, a further decrease in the DNA concentration was not observed by extending the storage period at room temperature for an additional two and four months. Keeping feathers for longer than that at room temperature; could lead to a slow DNA decrease, or based on Horváth (2005), who extracted DNA from feathers of museum specimens up to 115 years old, DNA might not decrease further to a certain point. Nevertheless, without data characterizing DNA recovery at longer exposure periods of time, it cannot be determined.

This chapter also shows that feathers are best keep at -20°C for up to six months at least, since their DNA concentration does not significantly differ after storage for two, four and six months at freezing temperatures. Improved feather sample preservation by freezing has also been suggested by Harvey (2006). It is possible that feather DNA concentrations remain unaltered for longer by keeping them at -20°C.

Another methodological aspect directly linked to DNA retrieval is the means of feather collection. Feathers can be obtained by plucking them directly from live birds (Segelbacher, 2002; Harvey, 2006) or carcasses (Segelbacher, 2002; Horváth, 2005); alternatively, moulted feathers can be picked up from nesting sites (Bello, 2001; Horváth, 2005; de Volo, 2008) or floor enclosures. This study employed plucked feathers from dead birds and moulted feathers from live birds. DNA concentrations from feathers of dead birds varied; for instance, for the crows little to no DNA was recovered, whereas for the birds coming from the Gower bird Hospital DNA rates were considerably higher. This result could be due to the storage of the samples; the bird hospital samples were immediately frozen, whereas the crows remained exposed to the environment for some days. Regarding the moulted feathers, they came from two sources, Eurasian blackbirds (EBs) being ringed and Sumatran laughingthrushes (SLs) whose feathers were collected from the enclosure floor or while birds were in hand. In the first case, sufficient DNA concentrations (for the established threshold: 4.5 ng/μl) for the PCR were obtained from 11.6% of the birds, whereas for the SLTs 90.4% of extractions produced sufficient DNA. However, for the EBs mostly body feathers were employed, whereas for the SLs body feathers represented only 23.1% of the samples. Considering just body feathers for the SLs, sufficient DNA for PCR was achieved in 66.7% of the extractions. The difference in these two groups could be the result of sample storage; SLs feathers were immediately kept at -20°C after collection, while EBs feathers were kept at room temperature for up to seven months.

Concerning how to obtain feathers from live birds less invasive approaches are advised. Therefore, collection of moulted feathers is recommended first, followed by gathering of fallen feathers of captured birds while on hand, and lastly plucking body feathers from captured birds. Likewise, it is urged not to pluck flight feathers (remiges or rectrices) from live birds. As mentioned by Harvey (2006) no studies of feather sampling fitness consequences are available; but, as the flight feathers tips´ are considerably wide and as they are provided with a nerve, plucking these feathers may be quite painful for the birds and it may produce a more profuse bleeding.

Some studies have found that the amount of DNA extracted from feathers is lower than that obtained from blood or tissue samples (Segelbacher, 2002). Other studies have found the contrary to be true; but, only if feather samples were under optimal preservation conditions (Horváth, 2005). In this study, it was observed that the DNA yield from some feather extractions was the same or higher than what was observed in the past for blood samples and organs. Superior DNA yields from feathers over other DNA sources could be related to the way in which the samples were collected and stored. Segelbacher (2002) and Horváth (2005) suggest that longer permanence of feather samples in the field could result in high DNA degradation; similarly, Taberlet (1999) reports that DNA will be degraded if the method of preservation is not appropriate. Some of the feathers used in this study remained no longer than four days outdoors, and they were frozen at -20°C immediately after collection; such conditions may be considered optimal. Whereas, in the study that obtained lower DNA concentrations from feathers than from other tissues (Segelbacher 2002), the samples remained in the field for a long time and were stored for 1-20 months at room temperature.

A direct comparison of feathers against blood samples extraction (Harvey, 2006) showed that extracts of blood samples contained significantly more DNA (30.9 +/- 18.8 ng/μl) than the feathers (1.2 +/- 0.7 ng/μl). Here, those two DNA sources were not compared; nevertheless, the DNA concentrations obtained from a single flight feather from different species (10.3 - 2,131.1 ng/μl), in some occasions were higher than what has been previously seen for blood samples (30 - 40 ng/μl). Mainly, this difference could be attributed to the storage conditions and to the species body weight from which samples were obtained. Feathers obtained for this study were immediately stored at -20°C; whereas feathers collected by Harvey (2006) were stored at room temperature. Harvey (2006) sampled an 11 g bird species, while we sampled species between 45 and 953 g. A significant difference in DNA concentration by bird species was found, which probably derived from the bird‘s body weight. A significant difference on DNA quantity was also observed by body weight of the birds, with the heaviest birds producing higher DNA amounts; therefore, the lower amount of DNA on Harvey´s samples (2006) compared to the

DNA amounts obtained in this study could be also attributed to the lower weight of the bird species he sampled.

In agreement with Horváth (2005) and de Volo (2008), it was observed in this study that flight feathers yielded higher DNA concentrations than body feathers. This observation could be explained by the higher amount of tissue contain in flight, relative to body feathers (Bello, 2001). Furthermore, de Volo (2008), recorded an average low DNA concentration for body feathers (<10 ng/μl), similar to the results observed in this study for a single body feather of a bird with a similar weight (W3: 16 ng/μl) to the species sampled. In general, for this study, the body feather DNA yield was low, particularly for light birds; therefore, to overcome this problem, body feathers were pooled for extraction, which increased the DNA yield. Implementation of this approach would depend on the objective of the study; for disease diagnosis, this seems suitable, but for genotyping it may not.

For body feathers all research agrees on using the tip; however, debate exists on what part of flight feathers should be used. Some authors have suggested the use of the feather´s tip, as its pulp and follicle cells represent a good DNA source (Harvey, 2006), particularly on growing feathers (Taberlet, 1991). Others suggest using the feather calamus, as long as it includes the superior umbilicus, for it contains a small blood clot which provides DNA even in body feathers (Horváth, 2005). However, the superior umbilicus is not the only part where blood can be found in feathers; de Volo (2008) observed dried blood inside of the feather calamus and reported high DNA yields (1,500 ng/μl) after the extraction. Likewise, in this study high DNA concentrations from feathers with observable blood (up to 2,131 ng/μl) were recorded. Although, it should be mentioned that blood was observed not only in the calamus, but in some occasions, all along the rachis and sometimes only at the superior rachis; therefore, and given that the objective of this study was to replace blood samples with feathers, it is recommend to employ the superior umbilicus. Moreover, it is highly suggested to longitudinally cut the rachis along its full length and use its contents; although, if the feather is too small for that, then the recommendation is to cut all of the rachis into small pieces.

If a low DNA yield is expected, approaches such as pooling feathers (Taberlet, 1999) and decreasing the final DNA elution volume (de Volo, 2008) can be used to increase the DNA amount and concentration. Furthermore, if DNA yield after the extraction is considered to be low for the required test, a number of strategies can be used to overcome this issue. DNA can be concentrated in the sample by an ethanol precipitation protocol; a vacuum centrifuge can be used to reduce liquid volume from the sample, or as done in this study, volumes of water and template can be exchanged in the PCR mix. Additionally, PCR cycles can be increased to compensate for the low DNA yield (Harvey, 2006).

Performance of genetic analysis with feather derived DNA is variable across studies. Preceding studies reported a 1.1% error on the PCR results (Segelbacher, 2002). However, more recent works, showed complete consistency for sex determination between blood and feather extractions (Harvey, 2006), and de Volo (2008) successfully used feather DNA for microsatellite amplification. In the present study, detection of the most common avian haemosporidia (*Plasmodium*, *Leucocytozoon* and *Haemoproteus*) from feathers has been successful. Noteworthy, is that previous protocols amplifying DNA extracted from feathers (Segelbacher, 2002; Harvey, 2006; de Volo, 2008) were aiming to amplify the bird DNA itself whilst this study aimed to amplify haemoparasite DNA which represents a low proportion of the sample. For instance, a high intensity infection by these parasites represents typically 0.5% of infected erythrocytes or more (Benedikt 2006).

Detection of blood parasites, even in severely affected birds with high intensity infections, is difficult (Fix, 1988). One contributing factor to this situation is possibly the short period on which the parasites can be seen in circulating blood (2-4 weeks) (Valkiūnas, 2005). Difficulty of diagnosis added to the associated risks with capturing and handling weaken birds, may lead to the decision of not taking blood samples (Alley, 2008). Under this scenario, shed feathers could be an invaluable source of genetic material, as they could provide the same information of a blood sample without posing any danger for the birds; therefore, allowing a fast and reliable diagnosis, which could translate into an early treatment.

Haemosporidian parasites are usually diagnosed from blood, yet some studies have diagnosed them from organs of dead birds. By employing only feather samples from birds that tested positive to blood parasites in organs, this chapter has tested whether detection from feather DNA has a similar success rate to that from organs. Detection of haemoparasites from carcasses of zoo birds and crows from feathers coincided with organs on average 75% of the time; however, blood parasites in organs indicate mainly chronic infections. In acute infections the parasites appear in circulating blood for a short period of time, subsequently moving to the organs producing considerable damage; if the bird survives this stage, then the parasites remain in the organs for long time, even for life (Valkiūnas, 2005). Chronic infections may not be detected from the remnant blood inside feathers that was used in this study, a more suitable comparison would be between a blood sample and a feather´s blood remnant. For that reason, a subset of samples indicative of acute infections was obtained from the Gower bird hospital; this is because all of the birds used tested positive by blood. Additionally these samples were optimally preserved; they represented different bird species and different amounts of feathers. Complete consistency in haemosporidia diagnosis between blood and feathers was observed in this group of birds; however, detection was not successful on each testing category, possibly due to the DNA concentrations. From this experiment it can be concluded that all acute haemosporidia infections will be detected from feathers, as long as the extraction provide sufficient DNA yield for the test.

The Gower bird hospital feather´s experiment showed that detection of blood parasites was enhanced by higher DNA yields, which was increased when heavier birds, flight feathers and more feathers were used. Data from this experiment can be used as a guide for planning the type and amount of feathers to use, based on the bird´s weight to be studied, to obtain the desired DNA yield for a chosen molecular analysis. It should be noted that the feathers used here were kept at optimal conditions and eluted in a final volume of 45 µl. Based on the obtained data, to obtain sufficient DNA for haemosporidia diagnosis it is recommended to use: one flight feather or 1-5 body feathers for birds above 1 kg; 1 -2 flight feathers

or 10 – 20 body feathers for birds between 1 kg and 100 g; and 2 – 3 flight feathers or 20 body feathers for birds under 100 g. As flight feathers produce higher DNA yields, it is recommended to use them whenever possible; however, if only few body feathers are available, they still should be used given that multiple strategies can be used to compensate for their low DNA yields.

Lastly, comparison of body and flight feather isolated genotypes versus the corresponding organ or blood genotypes showed two different scenarios. On the first one, a 98.2 – 100% match was observed in four out of seven birds. On the second, one body or flight feather matched the correspondent blood or organ genotype in 98.6 – 100%; while a lower match, 92.8 – 97.1%, was observed for the other three feathers. A small difference between genotypes could be due to sequencing errors, which can be interpreted as having found the same genotype. However, larger differences could be attributed to low quality of the sample, or they could represent different genotypes. The feathers used for this comparison were kept under optimal conditions; therefore, it is assumed that the extractions produced were of good quality. Hence, it is concluded that different feathers from the same bird may possess different genotypes, possibly indicating that feathers can identify not only current haemosporidia infections (i.e. infections observed in blood); but also, previous and latent infections (i.e. infections observed in organs).

4.5 CONCLUSION

This chapter demonstrates that feathers obtained in a non-invasive way provide good quality and sufficient DNA for infection detection and diagnosis, and possibly for other molecular analysis. DNA is protected inside the feathers´ shaft; however, the protection is not unlimited and is subjected to a series of conditions. Therefore, optimal collection conditions and preservation methods should be used for the technique to work reliably.

The information obtained in this chapter is a first approach to predict how much DNA can be extracted from feathers based on the type of feather and bird´s body weight. However, further research including more bird species with a wider range of weights is needed for a complete standardization of the method.

One of the most critical aspects for the use of feathers as a reliable DNA source is the preservation of the sample; once the feathers are collected they should be stored at -20°C or lower. If the feathers are collected in the field, an effort to collect fresher samples is recommended, since some degree of degradation will occur quickly from exposure to the environment. Special care should be taken with feathers collected from the field that were exposed to water; as DNA might be still extracted from them, but it might not have the adequate quality for molecular tests.

Conservation programs for endangered species and studies of elusive bird species may benefit from this technique. Regular screening diagnostic tests in captive birds and prompt disease diagnosis in fit and weakened birds could be facilitated; therefore, improving disease control and management of captive populations without increasing the stress of handling the individuals for conventional samplings. For wild populations, this technique can be used in a wide range of molecular analysis to gain information about individuals, species and populations and diseases, without causing major disturbances.

4.6 Appendixes

4.6.1. Post-mortem examinations on dead corvids from RSPB Burton Mere Wetlands from 2018 and PCR results for haemosporidian in organs.

ID	Species	Site	PME Date	Weight (g)	Degradation	Haemosporidia
C1	Carrion crow	Rhug Estate Corwen	24/03/18	435	2	n
C2	Carrion crow	Rhug Estate Corwen	25/03/18	500	5	n
C3	Carrion crow	Rhug Estate Corwen	24/03/18	452	4	n
C4	Eurasian magpie	Rhug Estate Corwen	24/03/18	174	3	L
C5	Eurasian magpie	Rhug Estate Corwen	25/03/18	184	4	L
C6	Eurasian magpie	Rhug Estate Corwen	24/03/18	166	3	L
C7	Eurasian magpie	Shotton Steal Works	04/04/18	194	2	L
C8	Eurasian magpie	Shotton Steal Works	04/04/18	200	3	H
C9	Eurasian magpie	Shotton Steal Works	04/04/18	186	5	H
C10	Eurasian magpie	Shotton Steal Works	04/04/18	204	3	L
C11	Eurasian magpie	Shotton Steal Works	06/04/18	221	4	H
C12	Eurasian magpie	Shotton Steal Works	06/04/18	176	4	H, L
C13	Eurasian magpie	Shotton Steal Works	06/04/18	170	3	H
C14	Eurasian magpie	Shotton Steal Works	06/04/18	188	4	H
C15	Eurasian magpie	Corwen	08/04/18	201	5	H, L
C16	Eurasian magpie	Rhug Estate Corwen	08/04/18	199	3	H
C17	Eurasian magpie	Rhug Estate Corwen	08/04/18	169	4	L

C18	Eurasian magpie	Rhug Estate Corwen	08/04/18	206	3	L
C19	Carrion crow	Rhug Estate Corwen	08/04/18	416	5	n
C20	Eurasian magpie	Rhug Estate Corwen	10/04/18	174	3	L
C31	Eurasian magpie	Rugh Estate Corwen	25/04/18	186	4	L
C32	Eurasian magpie	Rugh Estate Corwen	25/04/18	160	4	n
C33	Eurasian magpie	Rugh Estate Corwen	25/04/18	180	5	L
C34	Eurasian magpie	Rugh Estate Corwen	25/04/18	195	5	H
C35	Eurasian magpie	Rugh Estate Corwen	25/04/18	177	5	L
C36	Eurasian magpie	Rugh Estate Corwen	25/04/18	173	5	n
C37	Eurasian magpie	Rugh Estate Corwen	25/04/18	151	5	L
C38	Eurasian magpie	Rugh Estate Corwen	25/04/18	155	5	n
C39	Carrion crow	Rugh Estate Corwen	25/04/18	520	3	L
C40	Carrion crow	Rugh Estate Corwen	25/04/18	584	4	n
C41	Carrion crow	Rugh Estate Corwen	25/04/18	545	4	n
C42	Carrion crow	Rugh Estate Corwen	25/04/18	420	4	L
C43	Eurasian jackdaw	Eastham	25/04/18	388	3	n
C44	Carrion crow	Eastham	25/04/18	456	3	n
C45	Carrion crow	Eastham	25/04/18	466	3	P
C46	Eurasian magpie	Eastham	10/05/18	186	2	n
C47	Carrion crow	Llanrwst	10/05/18	437	3	n
C48	Carrion crow	Llanrwst	10/05/18	532	3	n
C50	Carrion crow	Rhug Estate Corwen	10/05/18	425	3	n
C51	Carrion crow	Rhug Estate Corwen	10/05/18	502	4	n

C52	Eurasian magpie	Eastham	10/05/18	215	5	n
C53	Eurasian magpie	Eastham	10/05/18	180	4	n
C54	Eurasian magpie	Eastham	10/05/18	199	5	P

ID= Bird identification; PME= Post-mortem examination; P= *Plasmodium* spp.; H= *Haemoproteus* spp.; L= *Leucocytozoon* spp.; n= Negative. Degradation was scored from 1 to 5, with one being fresh and 5 highly autolysed.

4.6.2. Post-mortem examinations on dead corvids from RSPB Burton Mere Wetlands from 2019 and PCR results for haemosporidian in organs.

ID	Species	PME Date	Weight (g)	Degradation	Haemosporidia
CR1	Carrion crow	23/05/19	435	2	L
CR2	Carrion crow	23/05/19	500	2	L
CR3	Carrion crow	23/05/19	452	2	L
CR4	Carrion crow	23/05/19	452	3	L
CR5	Carrion crow	23/05/19	416	2	L
CR6	Carrion crow	23/05/19	406	3	L
CR7	Carrion crow	23/05/19	503	2	L
CR8	Carrion crow	23/05/19	536	3	L
CR9	Carrion crow	23/05/19	498	2	L
CR10	Eurasian magpie	23/05/19	204	3	L, H
CR11	Eurasian magpie	23/05/19	221	4	L, H
CR12	Eurasian magpie	23/05/19	176	3	L, P
CR13	Eurasian magpie	24/05/19	178	3	L
CR14	Carrion crow	24/05/19	532	3	L
CR15	Carrion crow	24/05/19	549	3	L
CR16	Carrion crow	24/05/19	559	3	L
CR17	Carrion crow	24/05/19	556	3	L
CR18	Carrion crow	24/05/19	478	3	P
CR19	Carrion crow	24/05/19	416	3	n
CR20	Carrion crow	24/05/19	542	3	P
CR21	Carrion crow	17/06/19	420	1	-
CR22	Carrion crow	17/06/19	369	3	-
CR23	Carrion crow	17/06/19	427	2	-
CR24	Carrion crow	17/06/19	516	2	-
CR25	Carrion crow	17/06/19	499	3	-
CR26	Carrion crow	17/06/19	572	2	-
CR27	Carrion crow	17/06/19	423	3	-
CR28	Carrion crow	17/06/19	480	2	-
CR29	Carrion crow	18/07/19	520	3	-
CR30	Carrion crow	18/07/19	584	3	-
CR31	Carrion crow	18/07/19	545	4	-
CR32	Carrion crow	18/07/19	420	4	-
CR33	Carrion crow	18/07/19	520	3	-
CR34	Carrion crow	18/07/19	456	3	-
CR35	Carrion crow	18/07/19	466	3	-
CR36	Carrion crow	18/07/19	570	2	-
CR37	Carrion crow	26/07/19	437	3	-
CR38	Carrion crow	26/07/19	532	3	-
CR39	Carrion crow	26/07/19	425	3	-

CR40	Carrion crow	26/07/19	502	2	-

ID= Bird identification; PME= Post-mortem examination; P= *Plasmodium* spp.; H= *Haemoproteus* spp.; L= *Leucocytozoon* spp.; - = Untested samples; Degradation was scored from 1 to 5, with one being fresh and 5 highly autolysed.

Chapter 5

General Discusion

5.1 Challenges in the haemosporidian parasites field

The aim for this thesis was to shed light on the diversity, host-parasite associations and surveillance of haemosporidian parasites in captive and free wild birds. The study of such parasites in wild birds is challenging for many reasons, one being that following the course of disease in wild birds requires the continuous monitoring of health status, from the moment the animal is infected with the parasite, until it resolves (Chagas, 2017). There are only a few documented cases describing the pathology of haemosporidian species on certain hosts in that way (Garvin, 2003; Palinauskas, 2013; Ilgunas, 2016; Chagas, 2017). Hence, the real impact of haemosporidians on wild bird populations is yet unknown (Palinauskas, 2013). Likewise, the true diversity of avian haemosporidians has not been described, since it requires the collection and screening of samples from hundreds to thousands of individuals (Bell, 2015) from all bird species at their natural distribution sites. To this day, there are still numerous bird species and regions that have not been examined for haemosporidian parasites, mostly due to their isolation (Chagas, 2017). Another challenge seen in this field of research has arisen from the recent application of molecular diagnostic techniques (i.e. PCR) which has partially replaced microscopy. This has allowed for the establishment of new lineages that have no correspondence to a morphospecies, and therefore, lack all morphospecies-related information, such as life cycle, pathology, pathogenicity, and host species; knowledge that has been accumulating since the end of the 19th century and that is required to adequately understand these pathogens.

5.1.1 Challenges of haemosporidians study in this thesis

All of those aspects were observed in this thesis; the pathogenicity derived from an unseen host-parasite interaction (Humboldt penguins and *P. matutinum*), as well as the prevalence and diversity of haemosporidian parasites in a UK zoo were described. This work also studied the prevalence and diversity of haemosporidians from the Peruvian Amazonia, an area that had been little studied; additionally, an alternative method for diagnosing blood parasites in birds was explored. The prevalence and diversity of avian blood parasites on multiple geographic regions have been understudied, in consequence multiple bird species have not yet been tested (Yabsley, 2018), particularly endangered species (Carmona-Isunza, 2020). The detection of new host-parasite associations as well as new parasite species and lineages after screening a relatively small number of samples from the Peruvian Amazonia reinforces the view of this region as relatively unexplored. By contrast, all the haemosporidian species observed in Chester Zoo, had already been described, as Europe is a fairly well-studied region. In both regions studied in this thesis, bird sampling presented different complications. In Chester Zoo, a home office licence and specific training lasting over a year was required to sample live birds. In the Amazonia, access to the site and facilities to preserve samples were limited. For those reasons, sampling was opportunistic in both situations by necessity. This type of sampling created similar biases, in both cases low numbers of birds were collected and the abundance and diversity of the birds in the area was not reflected in the sample. Nevertheless, a baseline description of haemosporidian parasites circulating in the area was obtained from both sites.

5.1.2 Lack of standards for haemosporidian classification

Perhaps the main challenge in this thesis concerned the criteria to define haemosporidian lineages and species. The taxonomy of haemosporidian parasites presents multiple difficulties that need to be resolved urgently. For instance, at the moment, there are proposals to elevate the *Haemoproteus* and *Parahaemoproteus*

subgenera of *Haemoproteus*, as well as the *Akiba* and *Leucocytozoon* subgenera of *Leucocytozoon*, to genus level, since some phylogenies place them on separate branches, possibly reflecting the fact that these subgenera are transmitted by different vectors (Yabsley, 2018). Similarly, based on their position within the *cyt b* phylogeny, the recently described *H. antigonis* and *H. cathartic*, might be classified within a novel genus (Yabsley, 2018). Furthermore, the validity of morphological characters to describe species has been challenged, with some authors stressing the lack of observable distinguishable characteristics between haemosporidian species. Walther (2016) argued that *L. californicus* gametocytes are identical to those of other leucocytozoid parasites, highlighting the importance of molecular information for the identification of this species. However, gametocytes present only a proportion of the 133 morphological characters displayed in blood stages to differentiate species, and morphological descriptions of new species do not consistently provide information on the same set of characters (Hernández-Lara, 2018). Hence, the morphological description of *L. californicus* is incomplete and it may be possible to find distinctive characters in other life stages. Likewise, the recent molecular study of *L. toddi* showed a great genetic divergence in the *cytb* sequences, suggesting that it is a complex of cryptic species; however, a revision to the 1970s description and a molecular analysis of certain sequences by Valkiūnas (2010) showed that they are not cryptic species, as there were readily distinguishable morphological characters among some of the sequences placed in the "complex" which are now to be named as *L. mathisi* and *L. buteonis* (Walther, 2016). Hence, morphology has been proven to contribute relevant information for the identification of haemosporidians when applied correctly. Therefore, its use is still suggested in combination with molecular methods, and efforts to link the molecular data with morphospecies information are continuing (Argilla, 2013; Palinauskas, 2013; Palinauskas, 2016; Fecchio, 2018b; Lotta, 2019). Nevertheless, most recent research on haemosporidian diversity has been relied solely on molecular methods, excluding microscopy, and this has raised the problem of the definition of purely genetic lineages. The number of genetic lineages, for which we only know the *cytb* genetic sequence, is considerably higher than the number of

reported morphospecies, and correspondence between the two is found only in a few cases (Fecchio, 2018b).

The *cytb* gene has been used as a barcode to identify haemosporidian sequences, and a specialized database (MalAvi) concentrates all avian haemosporidian molecular data (Carmona-Isunza, 2020) with additional information on geographic location, host species and associated morphospecies. However, there is a lack of consensus on the naming of new or unique lineages based on *cytb* sequences; different authors have used different criteria in naming these lineages (Chaisi, 2019). For instance Chaisi (2019) used single nucleotide differences to define new lineages; whereas Levin (2013) used a sequence divergence of more than two base pairs and Hellgren (2004) described new lineages as those differing by one or more bases. However, some authors mantain that the difference in a few base pairs is probably indicative of variations on a single lineage (Ellis, 2019). The *cytb* gene fragment that is typically used for haemosporidian barcoding is 479 bp long (Ciloglu, 2019); hence a one, two and three base-pair difference corresponds to a 0.2%, 0.4% and 0.6% sequence divergence respectively (Galen, 2018b). Based on the data obtained here, the usage of different divergence thresholds leads to a different interpretation of the results. What would be differentiated as a new lineage based on a 0.2% divergence from already reported lineages, would not be by a 0.6% divergence, and the greater the threshold chosen, the more dissimilar are the results obtained.

Choosing a divergence threshold for defining genetic uniqueness does not only affect the establishment of lineages; ultimately, our understanding of species richness and diversity results would be affected, as well as geographic ranges, host utilization and host-parasite associations. The clustering analysis in Chapter 2 indicates that, if the divergence threshold used to differentiate lineages is too high, it would affect the assignment of well-established morphospecies, causing them to be considered synonymous. For instance, the use of any divergence threshold above 1.5% places *P. matutinum* and *P. lutzi* on the same cluster, indicating they are the same species; which would imply a case of geographic expansion given that *P.*

lutzi has only been described in America whilst *P. matutinum* is a well-known European species (Valkiūnas, 2017). In addition, the recorded diversity of haemosporidian parasites has been shown to vary greatly. While some morphospecies can be distinguished by a *cytb* cutoff of 0.5–0.6%, species like *P. relictum* and *P. elongatum* exhibit much greater *cytb* divergence (Svensson-Coelho, 2013; Palinauskas, 2013). Moreover, marked morphological differences on parasites with negligible *cytb* sequence differences have been observed (Palinauskas, 2013); by contrast, parasites with no apparent morphological differences have presented considerable genetic variation (cryptic speciation) (Galen, 2018b); hence, the establishment of a universal divergence threshold might not be possible.

Alternatively, the genetic divergence in haemosporidian lineages has been evaluated from other genes. Hellgreen (2014) used the MSP1 (Merozoite surface protein), a non-conserved gene to evaluate the genetic variance within *P. relictum* lineages. Each *P. relictum* lineage defined by *cytb* divergence (SGS1, GRW11, GRW4), presents a characteristic geographic distribution. However, when the MSP1 gene was used, the geographic pattern changed and considerable genetic variation across lineages was observed. These results suggest that *P*. *relictum* lineages actually represent different populations. For example, SGS1 is transmitted in Europe and Africa; nevertheless, different MSP1 alleles were found in Europe and Africa for this lineage. Different taxonomic classifications to establish haemosporidian lineages have also been used; Svensson-Coelho (2013) applied a 1% divergence cut-off in combination with host species affiliation, Galen (2018a) used a multigene approach using 21 different nuclear genes, and Hernández-Lara (2018) incorporated 133 morphological characters with molecular characters of the *cytb*, cox3 and coxI genes. Inconsistencies are present across all phylogenies (Martinsen, 2008) as they are dependent on taxon sampling, molecular characters included and analytical approach used (Galen, 2018a). Nevertheless, it has been proposed that the best way to estimate haemosporidian phylogenies is by incorporating data from broad taxon, multiple genes (Galen, 2018a) and morphological characters (Hernández-Lara, 2018).

The objective of this thesis was not to define criteria to determine haemosporidian lineages or species, but to identify the parasites. However, due to all the taxonomy issues mentioned previously, a more advantageous methodology than the others and at the same time was easy to apply to the dataset was not found; hence, like other researchers addressing the topic, a unique approach was developed to delimit the observed diversity. This procedure aimed to respect the morphospecies description as well as to preserve the previous lineages established by using a rather conservative divergence threshold. It is the case, therefore, that the results presented here should be taken with caution, since it should be considered that they could change under a different criterion.

5.1.3 Limited knowlende of specific haemosporidian life cycles

Another critical issue in the interpretations made here was the lack of information regarding the life cycles of different haemosporidian species and the pathogenicity produced on specific host-parasite associations. Currently, detailed descriptions of life cycles are only available for a limited number of haemosporidian species, and most of the assumptions that are made on pathogenicity of haemosporidians are based on the life cycles of the most representative species on each genus (Chagas, 2017). In the absence of detailed biological knowledge of parasite lineages, we assume that the pathogenicity caused by one parasite species is similar on all its hosts, all lineages of the same species produce comparable damage, and parasitaemia patterns are alike for all parasites within the same genus. This derives in practical implications for diagnosis, such as the timing for sample collection or the choosing for the organ to sample. Yet, there are good grounds for accepting that none of these assumptions are valid.

On live birds, diagnosis is made by screening blood samples; although, as it is often assumed that haemosporidian infection appear in blood only for a short period, sometimes clinicians are dissuaded to sample in this way for these parasites (Alley, 2008). However, from recent experimental infections, it is known that parasitaemia

patterns differ greatly between species, for instance, *H. danilewskyi* was visible in blood after 14 days of infection and its intensity declined drastically after eight weeks (Garvin, 2003). By contrast, parasitaemia by *P. homocircumflexum* was present through the entire course of the disease; and its intensity declined after three weeks. In addition, it has been observed that infection intensities vary significantly amongst different bird species infected with the same parasite (Ilgunas, 2016). In this thesis, it was not possible to determine strategic dates to sample the Humboldt penguins during the avian malaria outbreak that occurred at Chester Zoo, because the length of the prepatent period of infection by *P. matutinum* and the period on which it can be detected in blood has not been reported. In a similar way, selection of specific organs to sample from was not viable, since infections of Humboldt penguins with *P. matutinum* have not been described and thus; a full screening of all typically infected organs by *Plasmodium* spp. had to be done. Haemosporidian diagnosis in dead birds implies the screening of organs affected by the parasites; however, organs affected depend not only of the parasite species involved, but also on the host-parasite association. If the organ selected is not the most representative of the disease, the infection could be missed and the cause of death could be misdiagnosed. For example, infections with *H. danilewskyi*, produces schizonts exclusively in the lungs (Gavin, 2003), *L. caulleryi*, proliferates mostly in ovaries and oviducts (Lee, 2016), *P. elongatum* reproduction occurs mainly in bone marrow (Palinauskas, 2016), *P. homocircumflexum*, multiplication takes place predominantly in the brain capillaries (Ilgunas, 2016), and in the lineage LINN1 of *P. matutinum* the reproduction occurs more often in heart, lung, and brain (Himmel, 2020).

Similarly, the pathogenicity of haemosporidian species and lineages has been reported to change in different hosts. For example, *P. elongatum* phanerozoites typically develop in cells of the haematopoietic system; but on unusual hosts, like penguins, phanerozoites have been observed in the heart, lungs, brain, kidney and muscles (Palinauskas, 2016). Likewise *P. matutinum* LINN1, regularly produces high parasite burdens in Eurasian blackbirds, but in song thrushes it develops low burdens, illustrating that the same parasite lineage can show different levels of

virulence in related bird species (Himmel, 2020). Moreover, in an experimental infection with *P. elongatum* on different bird species, a morphological character presented only in one host, suggesting it is a host-dependent character (Palinauskas, 2016). Most of the information about haemosporidian pathogenicity and life cycles has been obtained from infections in captive birds (Palinauskas, 2013; Chagas, 2017) or from experimental studies (Garvin, 2003; Ilgunas, 2016; Himmel, 2020). Experimental infections might be the best way to characterize haemosporidian parasite life cycles because all development stages are present and they can be evaluated morphologically and molecularly. In addition, this method is useful to gain information on new species and to complement data on previously described species (Palinauskas, 2013; Chagas, 2017). Nevertheless, this method has ethical constraints; hence, it is essential to record in detail the pathogenicity observed in diverse host-parasite associations that occur naturally.

5.2 Conclusion

This thesis has contributed to the knowledge of the pathogenicity observed in host-parasite associations, describing the *P. matutinum*-Humboldt penguin relationship for the first time. This was a partial description and some of the details on this association remain unknown; most importantly, it is not clear if Humboldt penguins are competent host for *P. matutinum* as gametocytes were not observed in blood. Different regions of the world have been understudied for haemosporidians; hence, it is expected that new parasite species and lineages will continue to emerge. Their impact on wild bird populations is unknown, and so we must continue to study their effects on host fitness and survival, particularly on endangered species. This work contributes as a first approach to describe haemosporidians diversity in a poorly studied area of the Peruvian Amazonia. The discovery of novel lineages corroborates the high diversity of this group of parasites, some of which potentially belong to multiple new species. Additionally, in this work an alternative diagnosis method to the stress-inducing and invasive blood collection technique was explored, proving that the diagnosis of haemosporidians can also be achieved using

feathers. This is a novel approach that has not been tried for bird pathogens others than viral diseases that directly damage the feather. Therefore, this technique has the potential to be applied for the diagnosis of other bird pathogens and it could become a reliable option with the advantage of causing less stress and not requiring special equipment or training. Haemosporidians are a multifaceted group of parasites which are highly diverse and have intricate relationships with their vectors and hosts, which is reflected in their genetic composition. Disentangling the haemosporidian diversity requires novel approaches, extensive surveillance and standardization of methods. Yet, a better understanding of parasite diversity will lead to improved epidemiological and ecological management of disease and the conservation of susceptible species in captive or free life populations. This thesis contributed to this goal in diverse bird groups and proposed a novel diagnosis technique; however, further efforts are still required.

GLOSSARY

Disease: any harmful deviation from the normal structural or functional state of an organism generally associated with certain signs and symptoms.

Diagnosis: investigation or analysis of the cause or nature of a condition.

Haematozoa: subclass of blood parasites of the Apicomplexa clade, in this case is used to refer specifically to three genera *Haemoproteus*, *Plasmodium* and *Leucocytozoon*.

Haemosporidia: group of intraerythrocytic parasites belonging to the Apicomplexa clade, in this case is used to refer to three genera in particular *Haemoproteus*, *Plasmodium* and *Leucocytozoon*.

Host competence: Tha ability of a host to develop all the necessary life stages of a parasite required to be transmitted to the next susceptible host.

Infection: Entry and development or multiplication of a pathogenic agent in the body of animals, in this case birds.

Lineage: A unique genetic sequence used to define units of relevant taxonomic value

Morphospecies: A parasite species that has been defined based solely on its morphological and life cycle characteristics.

ANNEXES

Annex 1

Avian malaria screening in Flamingo Land

INTRODUCTION

Transmission is a key epidemiological process for understanding host–pathogen dynamics and predicting the effects of disease on populations. Under natural conditions, transmission is influenced by biotic factors like; host age, sex or abundance, and by abiotic factors, such as microclimate and landscape. Vector transmitted pathogens are affected by environmental traits, which determine vector abundance and their temporal and spatial distribution (Lachish, 2011). Annual climatic variation, responsible for alterations on the vector´s microhabitat, shapes fluctuations of vectors population abundance, which ultimately define the force of infection exerted on the hosts. When annual variation is favourable for vector´s breeding, the rate of infection may increase producing an observed raise in new infections and little recoveries; contrary, if the conditions on a year are unfavourable for the vectors, fewer new infections and more recoveries will be observed (Lachish, 2011). Thus, outlining infection prevalence yearly variation.

Avian malaria is a vector-borne disease of birds produced by protozoans of the genus *Plasmodium* spp. with a worldwide distribution. Mosquitoes of the Culicidae family are recognized as the vectors of the disease. These parasites have been shown to cause severe damage in domestic, wild, and captive birds sometimes leading to fatalities (Cocumelli, 2021). A wide number of birds have been affected by the disease, particularly exotic captive birds exposed to local *Plasmodium* strains. The observed susceptibility might be due to the lack of selective pressure for hosts to evolve appropriate immune responses to these parasites, or it may originate

from the stress associated to captivity, which limits the immune response of the birds (Cocumelli, 2021). Within the birds in captivity, one group stands out for their susceptibility to avian malaria, the penguins (Sphenicidae) (Grilo, 2016).

During 2016, an avian malaria outbreak was recorded at Flamingo Land, reporting 30 fatalities of Humboldt penguins (The mirror, 2016). Thus, in order to determine the source of infection, the presence of *Plasmodium* parasites in the mosquito vector population, free-living wild birds, and birds from Flamingo Land collection was investigated during 2017.

MATERIALS AND METHODS

Sampling location

From April to December 2017, we collected mosquitoes, dead free-living wild birds, and dead zoo birds in Flamingo Land resort, which is located in Kirby Misperton, North Yorkshire, UK. Flamingo Land has been operating since 1959 and it comprises an area of 152 ha housing 140 different species and over 1000 animals, including 17 bird species. The zoo is permanently open, but the attractions park is only accessible from April to December (Flamingo Land Ltd., 2020).

Mosquito screening

Mosquitoes were collected at Flamingo Land from May to November 2017 employing two types of traps; BG-Mosquitaire traps and CDC Gravid traps (for details on the traps see section 2.2.2), because of their efficacy at catching *Culex* spp. mosquitoes (Hernández-Colina, 2019). BG-Mosquitaire traps were operating continuously all weekdays and two sets of samples were collected. The first one gathered the mosquitoes captured during a six days and the second one represented the mosquitoes captured during a 24 hours period. The CDC Gravid traps operated with an oviposition medium and they were turned on once a week for 24 hours, after which mosquitoes were collected.

Mosquito sampling sites

Four sampling areas were established in or near the bird enclosures at the zoo to trap adult mosquitoes; these areas were protected from direct sunlight, artificial lighting, wind, and rain, but near vegetation and, whenever possible, near to water bodies. Sampling areas were 30 m in diameter and contained one trap of each kind, separated by at least 10 m to avoid interference between traps and pseudo-replicates. For trapping set up, Flamingo Land staff were consulted to confirm that each site complied with the following requirements: access to electricity (for the BG-mosquitaires), ease of access, avoiding sites requiring access grant; safety for animals, ensuring that traps and all its elements would not be dangerous or stressful for animals; and safety for visitors, making sure that the traps were not in plain sight of visitors. Sites that complied the mentioned requirements were A1 Penguins, A2 Capybaras, A3 Lemurs and A4 Camels (Figure 1).

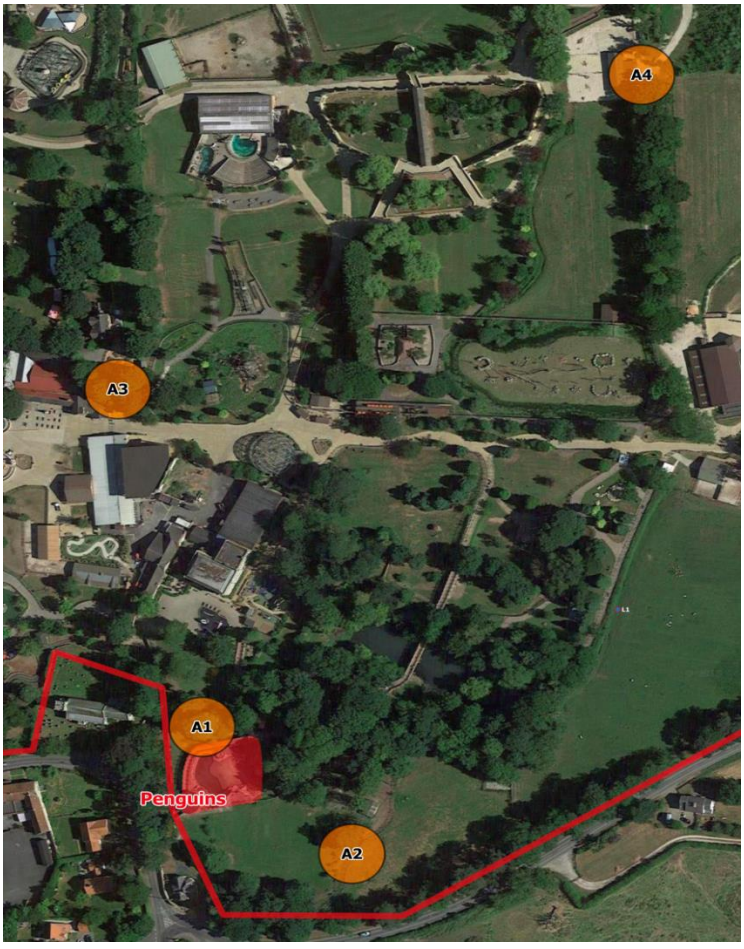

Figure 1. Flamingo Land satellite map indicating the location of the sampling areas. Red lines represent the limits of the zoo.

Mosquito screening

Mosquitoes from both traps were killed and preserved at -20 °C to posteriorly be counted and identified morphologically using the key from Cranston (1987). Mosquitoes from the *Culex* complex were identified to the species level by extracting the DNA from a mosquito leg with OMEGA kits and performing a PCR following Hesson (2015) protocol. DNA extractions for avian malaria testing were performed only on female mosquitoes and carried out in 96 deep well plates (1.5ml), placing mosquitoes individually in each well. The Livak extraction method (Livak, 1984) was used with the following modifications, half of the lysis buffer volume was added to macerate the mosquitoes with autoclavable pistils inserted in a homogenizer, then the remainder of the lysis buffer was added, the plate was sealed with a mat and incubated as required. The rest of the extraction was done using 96 deep well plates and the centrifugation steps were carried on in an Eppendorf 5810R plate centrifuge. For the elution step, 200 μl of nuclease free water was added to each well and the elute was deposited individually in 1.5 ml tubes.

Bird screening

Plasmodium spp. prevalence was evaluated in organ samples from dead free-living wild birds found in the zoo grounds. Carcasses found on the zoo grounds were picked up and stored at -20°C degrees until they could be transported to the laboratory to perform post-mortem examinations (PME), which were carried out assessing body condition, conservation state, weight, sex and cause of death. Brain and liver samples, consisting of a small piece (1 cm^3), were taken from each bird at PME and stored in 1.5 ml microcentrifuge tubes at -20°C. Organs were additionally tested for *Haemoproteus* spp. and *Leucocytozoon* spp. For the DNA extraction, a 0.5 cm^3 piece of each organ was taken and dissected into smaller pieces with disposable scalpels in a petri dish. For each organ a different scalpel and clean sections on the petri dish were used to avoid contamination. The extractions were done with a QIAGEN DNeasy kit following the manufacturer´s instructions.

PCR Protocol

DNA extracted from female mosquitoes and bird samples was tested by nested PCR using the protocol described by Hellgreen (2004). Used primers amplify a conserved 479-bp fragment of the cytochrome b (*cytb*) gene located in the mitochondrial genome. The reaction can detect *Plasmodium*, *Leucocytozoon* and *Haemoproteus* parasites. The nested PCR consisted of two parts. For the first part, each reaction included 1 μl of DNA template, 1 μl of forward primer HaemNF1, 1 μl of reverse primer HaemNR3, 10 μl of Bioline mix, 1 μl of BSA (Bovine Serum Albumin) and 6 μl of nuclease free water, to reach a final volume of 20 μl. The PCR profile was 22 cycles at 94°C for 3 min, 94°C for 30 s, 50°C for 30 s, 72°C for 45 s, followed by an extension at 72°C for 10 min. The second part, intended to detect *Plasmodium* spp. and *Haemoproteus* spp., was performed using 2 μl of PCR product from the previous reaction, 1 μl of forward primer HaemF, 1 μl of reverse primer HaemR2, 10 μl of Bioline mix, 1 μl of BSA and 5 μl of nuclease free water, to reach a final volume of 20 μl. The profile for the second part was 36 cycles at 94°C for 3 min, 94°C for 30 s, 50°C for 30 s, 72°C for 45 s, followed by an extension at 72°C for 10 min. To detect *Leucocytozoon* spp. the exact same procedure was followed; but, using the primers HaemFL and HaemR2L instead. The number of cycles in the first reaction was increased from 20 to 22 relative to the original protocol. Molecular grade water was used as a negative control; for *Plasmodium* spp. and *Leucocytozoon* spp., genomic DNA from *Plasmodium bergei* ANKA and *Leucocytozoon* spp. isolated from a Mallard (*Anas platyrhynchos*) were used as positive controls respectively. A positive and a negative control were used on every occasion. Amplicons were visualised on a 1.5% agarose gel stained using the SYBR Safe DNA gel satin (Thermo Fisher Scientific).

Amplification of *cytb* from mosquito DNA was done using the same protocol but in 96-well plates in a Techne TC-412 Thermal Cycler. Mosquito DNA samples (n = 2-4) were pooled per well, plus eight negative controls and four positive controls per plate. In the event of a PCR-positive well, all individual samples from the well were tested individually. For the bird samples, a T3 Thermocycler (Biometra®) was used, adding a negative and a positive control every 10 samples.

Gene sequencing

Positive PCR products were sent for sequencing in the forward direction (primer HAEMF) with the Sanger method. Sequence reads were compared to previously published avian haemosporidia in the GenBank nucleotide database using BLASTn to identify the genus of the parasite.

RESULTS & DISCUSION

Flamingo Land mosquito screening

In total 1,588 mosquitoes were captured, these were identified as *Anopheles maculipennis*, *Culex pipiens*, *Cx*. *torrentium* and *Culiseta annulata*; being *Cx. pipiens* the most abundant (70.78%) (Hernández-Colina, 2019). From the total of captured mosquitoes, 1,270 were females (80%). An additional 138 and 22 overwintering female mosquitoes (Figure 2) were caught in November and December respectively; most of these were identified as *Cx. pipiens*/torrentium (n=152), but *A*. *maculipennis* (n=4) and *C*. *annulata* (n=1) were observed too (Hernández-Colina, 2019).

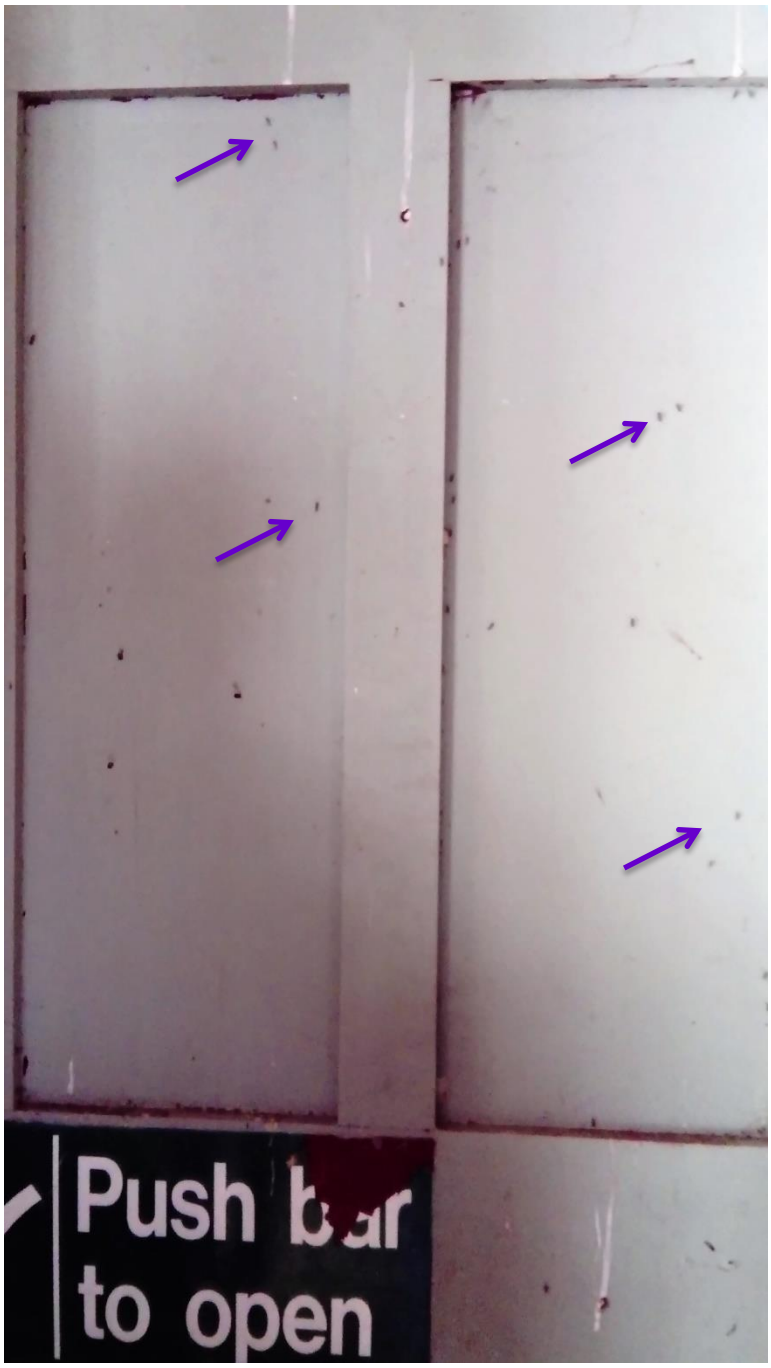

Figure 2. Door from the tapir´s night house in Flamingo Land with a considerable number of overwintering mosquitoes. Arrows point some of the overwintering mosquitoes

After excluding the overwintering mosquitoes, 1109 females were tested for *Plasmodium* spp., presenting a 0.45% prevalence (n=5) from mid-June to late November (Table 1). This result is contrasting with the 10% *Plasmodium* spp. prevalence recorded at Chester Zoo in that same year. Although the same site

selection criteria, type of traps and molecular testing were employed; the number of collected mosquitoes in Chester Zoo (n=7,888) and Flamingo Land (n=1,588) were contrasting as well, possibly due to two factors. First, the number of established sites and in consequence the number of traps used in Chester Zoo (10 and 20 respectively) and Flamingo Land (4 and 8 respectively) were not the same. Second, the sites themselves, even when both were selected under the same criteria, Chester Zoo sites fitted the characteristics better than the Flamingo Land sites, and Chester Zoo traps were placed on more ideal spots (i.e., closer to water bodies and dense vegetation protecting the traps from wind, light and rain) than in Flamingo Land due to accessibility (Figures 3, 4). Microclimate has been found to influence the transmission rate of *Plasmodium* spp., particularly the proximity to water bodies, with *Plasmodium*-transmitting vectors exhibiting a higher prevalence to bread alongside large water bodies (Lachish, 2011). Another factor to consider is the location of both zoos, since Flamingo Land is situated further north than Chester Zoo, which makes climatic conditions different between zoos. It is known that temperature and humidity impact on vector-borne diseases prevalence, as they modulate the vector´s development (Valkiūnas, 2005). Since Chester Zoo presented a more favourable climate conditions for the vectors, a higher amount of mosquitoes and *Plasmodium* spp. prevalence is expected there.

Table 1. Date and area where *Plasmodium* spp. infected mosquitoes were observed in Flamingo Land during 2017.

***Plasmodium* spp. (n)**	**Date**	**Area**
1	24/07/2027	A2 Capybaras
1	22/08/2017	A2 Capybaras
1	29/08/2017	A3 Lemur
1	29/08/2017	A1 Penguin
1	04/09/2017	A3 Lemur

Figure 3. Mosquito trapping in Flamingo Land. A) Sampling area A4 Camels, showing a small non-permanent water body surrounded by little vegetation. B) Site where traps for area A4 were placed, trap is not protected from sunlight, wind or rain. Arrow signals the BG-mosquitaire trap.

Figure 3. Mosquito trapping in Chester Zoo. A) Sampling area A1 Flamingo´s pond, showing a large permanent water body surrounded by abundant vegetation. B) Site where traps for area A1 were placed, trap is protected from sunlight, wind and rain. Arrow signals the BG-mosquitaire trap.

Flamingo Land bird screening

In 2017, ten free-living wild birds from the Flamingo Land belonging to five different orders, eight families and nine species were received; none of the birds were infected with *Plasmodium* spp.; however, infections with *Leucocytozoon* spp. and *Haemoproteus* spp. were detected with a prevalence of 20% and 40% respectively. Additionally, one bird (*Phoenicopterus chilensis*) from the zoo collection was sampled and it tested positive for *Leucocytozoon* spp. (Table 2). Registered *Haemoproteus* spp. infections in Flamingo Land birds were mainly observed on migratory birds, whereas *Leucocytozoon* spp. infections were seen only in resident birds. This pattern is consistent with the temperature preferences for these parasites, *Leucocytozoon* spp. is associated to cold climates (Valkiūnas, 2005), like the present in Flamingo Land, and *Haemoproteus* spp. is more commonly observed in temperate to warm climates, like those where migratory birds travel to. The role of migratory birds as disease bearers and drivers has been described previously (Chaisi, 2019); however, it should be noted that the sample size in this case was very low; hence, to confirm this pattern it is necessary to study the bird´s prevalence in the area with a larger sampling size. Lastly, absence of *Plasmodium* spp. in Flamingo Land birds does not indicate the lack of active transmission in the area; this situation is probably also resulting from the limited number of individuals that were tested.

Table 2. Blood parasites prevalence in dead captive and free-living wild birds from Flamingo Land during 2017

Order	Family	Species	H	P	L	n	%
Anserifirme	***Anatidae***	***Branta canadensis*** $^{W, M}$				**1**	**0**
Caprimulgiformes	***Apodidae***	***Apus apus*** $^{W, M}$	**1 (100)**			**1**	**100**
Columbiformes	***Columbidae***	***Columba livia*** ^{W}				**2**	**0**
		Columba palumbus ^{W}			**1 (100)**	**1**	**100**
Passeriformes	***Muscicapidae***	***Erithacus rubecula*** ^{W}				**1**	**0**
	Sylvidae	***Sylvia curruca*** $^{W, M}$	**1 (100)**			**1**	**100**
	Phylloscopidae	***Phylloscopus trochilus*** $^{W, M}$	**1 (100)**			**1**	**100**
Phoenicopteriformes	***Phoenicopteridae***	***Phoenicopterus chilensis*** ^{Z}			**1 (100)**	**1**	**100**
	Turdidae	***Turdus philomelos*** ^{W}	**1 (100)**			**1**	**100**
Strigiformes	***Strigidae***	***Strix aluco*** ^{W}			**1 (100)**	**1**	**100**
		Total	**4 (40)**		**2 (20)**	**10**	**60**

H = *Haemoproteus* spp.; L = *Leucocytozoon* spp.; P = *Plasmodium* spp.; ^{Z} = Captive wild birds; ^{w} = Free-living wild birds; ^{M} = Migratory

Conclusion

Both, the low *Plasmodium* spp. prevalence observed in the mosquitoes, and the absence of *Plasmodium* spp. in Flamingo Land birds, most likely reflect methodological issues; therefore, to uncover the real situation of avian malaria within the zoo it is necessary to increase the bird sampling size, and to place mosquito traps on more suitable sites to establish where mosquitoes are breeding, the mosquito species involved in *Plasmodium* spp. transmission, and then to control them by modifying their preferred areas.

It has been observed that application of preventive measures have resulted in the decline of new infections in captive birds (Beier, 1980; Chaisi, 2019). One way to prevent the disease is achieved by reducing or eliminating the vector´s population (Grilo, 2016), this can be done by modifying or if possible by eliminating the mosquitoes favorite sites making them less appealing as it was done in Chester Zoo after the 2017 avian malaria outbreak. Likewise, limiting the stressful events for birds can also help to prevent the onset of the disease; hence a combination of both is recommended at Flamingo Land.

Annex 2

Wolbachia associations with avian *Plasmodium* species

INTRODUCTION

Wolbachia is a bacterial endosymbiont of arthropods transmitted maternally; this organism is known to be either beneficial or detrimental to their hosts by modulating the development of secondary organisms that infect the host at the same time (Zélé, 2014a). In nature, disease transmitting vectors get infected by multiple microorganisms at once. Such co-infections have the ability to shape diseases ecology, as it has been recorded in natural infections with *Wolbachia*, in which, the latter posed either no effect on pathogen development, or it facilitated or blocked pathogen replication (Zélé, 2014a).

In *Wolbachia* co-infections, parasites may interact in different ways. In some occasions they may suppress each other because they are in competition for a resource in limited supply; whereas, at other times they may cooperate to facilitate their mutual development. The latter strategy is most evident when one of the parasites immunosuppresses the host (Zélé, 2014a).

Studies conducted on *Plasmodium* suggest that the outcome of the co-infection depend mostly on the particular *Wolbachia-Plasmodium* combination, some combinations seem to have an inhibiting effect while others facilitate the parasite's development (Zéle, 2014b). Artificial combinations of *Wolbachia* with *Plasmodium* spp. infected mosquitoes have shown different results; for instance, in *An. gambiae* mosquitoes transfected with the wAlbB strain of *Wolbachia* and infected with *P. falciparum* (human malarial parasite), the *Wolbachia* strain decreases *Plasmodium* parasitaemia. By contrast, when mosquitoes are infected with a rodent malaria *Plasmodium* (*P. berghei*) prevalence increases (Zélé, 2014a).

In the case of avian malaria, it has been shown that in *Cx. pipiens* carrying the native wPip *Wolbachia* strain and infected with *P. relictum*; *Wolbachia* either had no effect, or significantly increased *Plasmodium* prevalence in the mosquito by increasing the number of oocysts present in the mosquito (Zélé, 2014a). Likewise, wPip-III *Wolbachia* strain has been shown to facilitate infection of the *P. relictum* SGS1 lineage in *Cx. pipiens* (Zélé, 2014b).

Within *Cx. pipiens*, one of the main vectors for avian malaria worldwide, *Wolbachia* infections are fixed in wild populations, these infections are polymorphic; thus individual mosquitoes are infected by one of the five recognized Wolbachia wPip strains (Zélé, 2014b). Given this situation and the varied reactions on *Plasmodium* spp. prevalence associated to the particular combination of *Plasmodium* and *Wolbachia*, it is necessary to record *Plasmodium* and *Wolbachia* strains occurring in a natural system to determine the effects of their interaction. Therefore, this work aims to find an association between avian *Plasmodium* parasites occurring naturally at Chester Zoo and *Wolbachia*, the diversity of *Wolbachia* strains circulating in the area, and the probability of mosquitoes being infected by *Plasmodium* depending on the *Wolbachia* strain.

MATERIALS AND METHODS

Mosquito collection

During 2017 mosquitoes were collected at Chester Zoo, UK, employing two types of traps, BG-Mosquitaire traps and CDC Gravid traps. Nets from both traps were collected every week and nets from the BG-Mosquitaire traps were also collected one day after the weekly collection, hence obtaining mosquitoes on a weekly and on a daily basis. Mosquitoes were caught from ten sampling areas located inside or near bird enclosures´ at the zoo (For details on the traps and sampling areas see sections 2.2.2 and 2.2.3).

Mosquito screening

Mosquito species were determined using the Cranston (1987) morphological key; posteriorly DNA was extracted individually from females. Extractions were performed employing OMEGA Bio-Tek E.Z.N.A® Tissue DNA kits (n=2072) and the Livak method (Livak, 1984) (n=5816). For details of the extraction process consult section 2.2.4.

***Plasmodium* spp. PCR Protocol**

DNA extracted from female mosquitoes was tested for Haemosporidians with the nested PCR protocol described by Hellgreen (2004). The first part of the reaction included 1 μl of DNA template, 1 μl of forward primer HaemNF1, 1 μl of reverse primer HaemNR3, 10 μl of Bioline mix, 1 μl of BSA (Bovine Serum Albumin) and 6 μl of nuclease free water, to reach a final volume of 20 μl. The PCR profile was 22 cycles at 94°C for 3 min, 94°C for 30 s, 50°C for 30 s, 72°C for 45 s, followed by an extension at 72°C for 10 min. The second part was performed using 2 μl of PCR product from the previous reaction, 1 μl of forward primer HaemF, 1 μl of reverse primer HaemR2, 10 μl of Bioline mix, 1 μl of BSA and 5 μl of nuclease free water, to reach a final volume of 20 μl. The profile for the second part was 36 cycles at 94°C for 3 min, 94°C for 30 s, 50°C for 30 s, 72°C for 45 s, followed by an extension at 72°C for 10 min. The number of cycles in the first reaction was increased from 20 to 22 relative to the original protocol. Molecular grade water was used as a negative control and genomic DNA from *Plasmodium bergei* ANKA was used as a positive control. Amplicons were visualised on a 1.5% agarose gel stained using the SYBR Safe DNA gel satin (Thermo Fisher Scientific). Positive PCR products were sent for sequencing in the forward direction (primer HAEMF) with the Sanger method. Sequence reads were compared to previously published avian haemosporidia sequences in the GenBank nucleotide database using BLASTn to identify the genus of the parasite.

Ace-2 gene PCR protocol

A qPCR targeting a 208 bp conserved region of the mosquitoes´ acetylcholinesterase ace-2 gene was done using a small set of ten *C. pipiens* mosquitoes infected with *Plasmodium* spp. to analyse the quality of the sample. Mosquito samples were diluted 1/10 and 1/100 with nuclease free water. The qPCR reaction mix consisted of 10 μl of SYBR green dye, 1 μl of forward (59GCAGCACCAGTCCAAGG39) and reverse (59CTTCACGGCCGTTCAAGTAG39) primers, 1 μl of sample DNA and 7 μl molecular grade water to a total volume of 20 μl. Two negative controls were included in every run as well as eight subsequent dilutions in duplicate of standardized controls to calibrate the instrument. DNA quantity was estimated after each PCR cycle by measuring the fluorescent dye incorporation in the PCR product. The thermocycling protocol consisted in an activation step at 95°C for 10 minutes followed by 35 amplification cycles at 95°C for 15 seconds, 55°C for 30 seconds and 72°C for 15 seconds. Standard curves were performed for each light cycler run, such that the cycle number to which the log profile of a given sample intercept the noise band used to establish these standard curves can be directly converted to a concentration (Weill, 2000).

***Wolbachia* PCR protocol**

A 438 bp conserved fragment of the *Wolbachia* 16S rRNA gene was targeted. Numerous PCR procedures and primers exist for the amplification of *Wolbachia*; however, the primers W-Specf/W-Specr (5'-CAT ACC TAT TCG AAG GGA TAG-3' and 5'- AGC TTC GAG TGA AAC CAA TTC3') were chosen for their ability to detect most *Wolbachia* strains infecting insects. The qPCR reaction mix consisted of 10 μl of SYBR green dye, 1 μl of forward and reverse primers, 1 μl of sample DNA and 7 μl molecular grade water to a total volume of 20 μl. Two negative controls were included in every run as well as eight subsequent dilutions in duplicate of standardized controls to calibrate the instrument. The thermocycling protocol consisted in an activation step at 95°C for 15 minutes followed by 45 amplification cycles at 95°C for 15 seconds, 58°C for 15 seconds and 72°C for 20 seconds. PCR products were characterized by analyzing the amplicon melt curve (Sawasdichai, 2019). DNA quantities in each sample were automatically calculated according to

the standard curves and amplification efficiencies generated from the C_t values and quantities of standard samples. At the end of the runs the amplification curves were checked to obtain threshold cycles (C_t) of each sample with default settings and results were only accepted if the controls did not exhibit fluorescence curves that crossed the threshold line. Melting curves were also inspected to determine false positives.

Primers design

To establish a *Wolbachia-Plasmodium* ratio it was required to perform a quantitative PCR for *Plasmodium* parasites based on nuclear genes. PCR has been used on multiple occasions for the detection of *Plasmodium* parasites; however, a literature review revealed no universal primers of nuclear origin, nor genus-specific primers for the species we identified in Chester Zoo (Table 1); hence, they had to be designed. A gene search on PlasmoDB looking for highly conserved, single copy, nuclear genes from the parasites genome was done. From the genes that fulfilled these characteristics, eight different genes for which available sequences of at least *P. relictum* and *P. gallinacem*, and with a length above 200 bp were available were selected. All of the available sequences for each selected gene were aligned with Bioedit, from the alignment eight different priming sites (four forward and four reverse) were selected for each gene and posteriorly formed into the best matching pair according with their melting temperature, degree of degeneration, GC content, product size, and hybridization energies of homo and heterodimers. The selected primer pairs were tested with a range of annealing temperatures to ensure optimal assay conditions for selective amplification of the genes (Biedrzycka, 2015). A first temperature gradient (44°C, 44.3°C, 45°C, 46.1°C, 47.4°C, 49.0°C, 50.6°C, 52.1°C, 53.5°C, 54.7°C, 55.6°C, 56°C) and a second temperature gradient (45°C, 45.3°C, 46°C, 47.1°C, 48.4°C, 50°C, 51.6°C, 53.1°C, 54.5°C, 55.7°C, 56.6°C, 57°C) were used. Primers were tested using DNA samples extracted from penguin´s blood know to be infected with *P. relictum* and *P. matutinum*. When multiple bands were observed on the gel, the Thermofisher gel extraction kit was employed to purify the right size band and the purified product was sent for sequencing.

Table 1. Primers used for *Plasmodium* spp. detection on different PCR protocols.

Reference	Target gene	Primers	Notes
Fallon (2003) Ishtiaq (2017) Huang (2018)	SSU, LSU rRNA	343F / 496R	Lineage specific RTqPCR
Huang (2018)	Phosphoenolpyruvate Circumsporozoite	017-Fb/017Rb, 017-Fc/ 017Rc CTRAP-R2 / CTRAP-W2	RTqPCR
Schoener (2017)	*Cytb*	Plas56F/PlasrevRT	HRM qPCR
Bell (2015) Chaisi (2019)	rRNA	R330F/R480RL	RTqPCR
Feldman (1995)	18s rRNA	90/89	Simple PCR
Friedl (2012) Sijbranda (2017)	LSU-rRNA	Plasmo474For/Plasmo558Rev	RTqPCR
Zehtindjiev (2008)	*Cytb*	GRW2-8F / GRW2-9R GRW4 / GRW4-11R	Lineage specific
Knowles (2011)	*Cytb*	L9 / NewR	
Njabo (2011)	*Cytb*	PlasHRM.F / PlasHRM1.2R	HRM PCR

RESULTS AND DISCUSSION

Mosquito collection and *Plasmodium* screening

A total of 7,888 mosquitoes were collected at Chester Zoo during 2017; from these, 6,814 (87.1%) individuals were females, which were used for avian malaria testing since only they feed on blood and are therefore vectors. Further details on mosquito collection and *Plasmodium* testing can be found in section 2.3.1 and 2.3.3.

Ace-2 gene PCR

Given that the Ace-2 gene is constitutive for mosquitoes, amplification of it in all tested samples was expected and indeed, report of the qPCR showed positive amplification in all cases, with an average gene copy number ranging from 270 to 48,800. Copy number estimation for the mosquitoe´s Ace-2 gene was accurate, since there were no unspecific products detected by melting curve analysis, the log of fluorescence increased linearly along PCR cycles for the dilution range, and all the linear parts of the log curves were parallel (Figure 1).

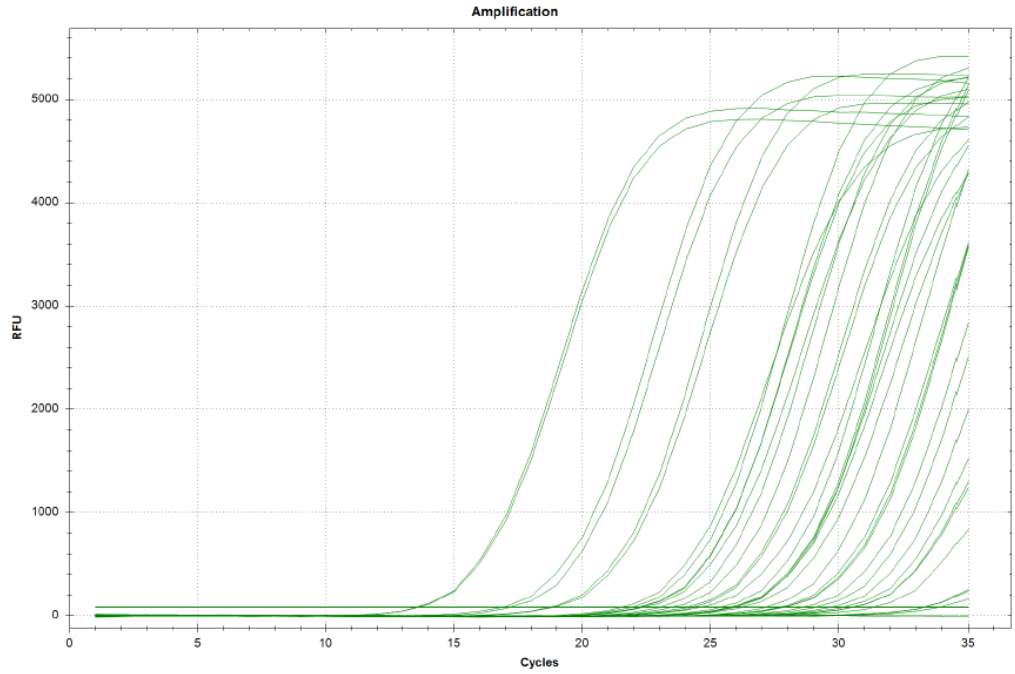

Figure 1. Mosquito actin melting curves for each tested sample and controls by PCR cycle.

Likewise, the reaction efficiencies were between 85 and 100% and the coefficient of determination was 0.995 for this test. A linear correlation was found between the measured concentration and the input concentration of the controls at each dilution (Figure 2); therefore, all parameters of the qPCR fall within generally accepted ranges indicating an efficient and reliable assay.

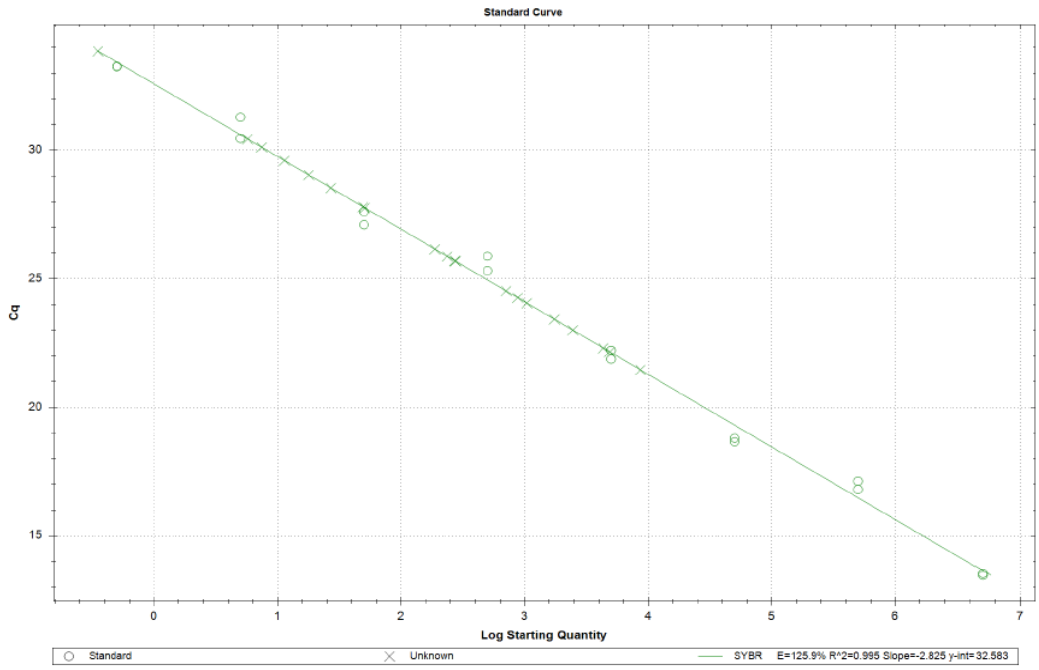

Figure 2. Standard curve regression for the Ace-2 gene showing the expected and actual copy numbers obtained.

Wolbachia PCR

Positive amplification of *Wolbachia* gene 16S rRNA was observed in all tested samples (n=10), which were infected with *Plasmodium*. This result is consistent with previous findings that suggest that *Wolbachia* is either present or absent in a given population of insects; however, the small sample size used for this testing might be misleading and testing of a larger number of individuals is required for a definitive result. Copy numbers of the 16S rRNA gene ranged from 10350 to 621122, and similarly to the previous assay, accuracy was confirmed by the lack of unspecific products in the melting curve analysis, the linear increase in log fluorescence along PCR cycles for the dilutions employed, and the parallelism of all linear parts of the log curves (Figure 3).

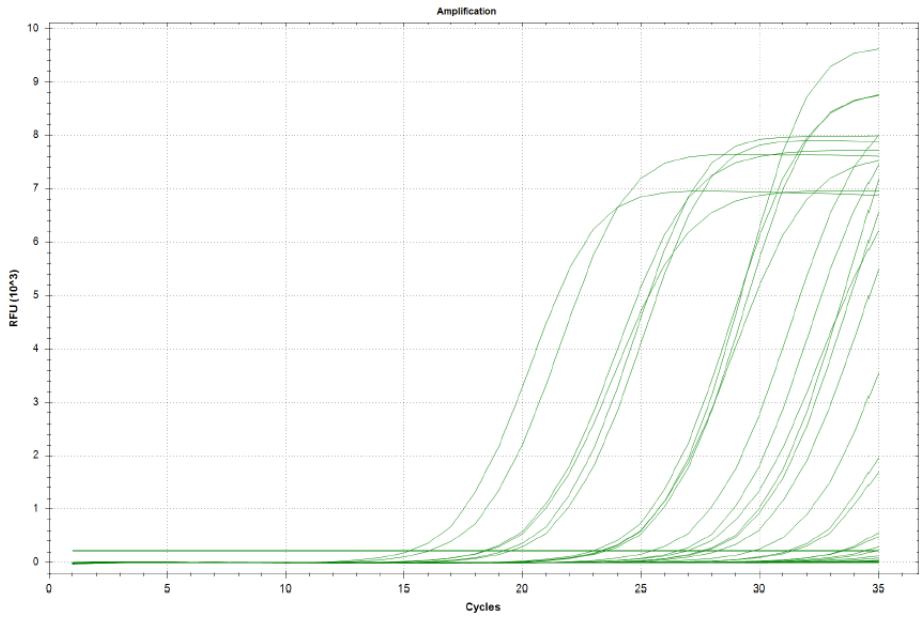

Figure 3. *Wolbachia* melting curves for each tested sample and controls by PCR cycle.

Similarly, the coefficient of determination for this assay was 0.994 and the reaction efficiencies were between 83 and 100%. The measured copy concentration and the input concentration of the controls showed a linear correlation at each dilution (Figure 4); hence, the obtained results are reliable. Further determination of the wPip strain and screening of all infected and not infected mosquito samples for the presence and intensity of infection with *Wolbachia* did not continue because off the lack of a suitable qPCR protocol to quantify *Plasmodium* infection intensity.

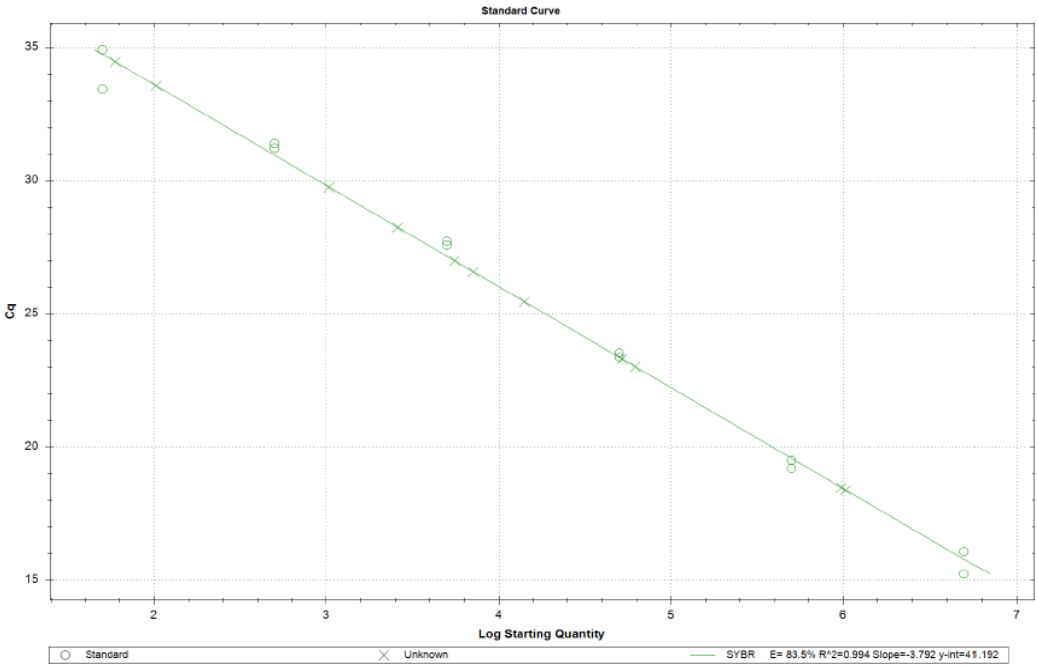

Figure 4. Standard curves regression for the *Wolbachia* gene showing the expected and actual copy numbers obtained

Primers design

Matching primer pairs were produced for all of the selected genes, with the exception of S18 and S27. The obtained primer pairs were tried out in a PCR assay where an amplification product by the Cloroquine, GPI antigen, MSA 180 and MSP 7 primers was observed (Figures 5, 6 and 7). None of the amplified products match the expected fragment size; nevertheless, they were sent for sequencing and the NCBI blast did not identify the gene they were designed for (Table 2). Therefore, the observed bands produced in the PCR must have been due to unspecific interaction between the components.

Table 2. Selected primers pairs of different genes tested with *P. relictum* and *P. matutinum* infected samples with a temperature gradient

Gene	Tested primers	PCR Result	Expected product size	Sequencing result
Cloroquine	F (GCTTTACAATTRAGCATACCAAT) R (GTCATGYTTGAGAAACATAATGG)	700 bp band on *P. matutinum*. From 44°C to 46.1°C	470 bp	No matches found
GPI antigen	F2 (TGGAAGCARTACAAAGTAGA) R4 (TCTACATCTCCAGACAAR)	500bp band on both species From 44°C to 49°C	2000 bp	Golden eagle DNA
MSA 180	F1 (CTCYCGATGATTTAAAGA) R2 (GCCCATGCAAYCATTATTCTGAC)	1000bp band on both species From 44°C to 47.4°C	1700 bp	Golden eagle DNA
S18	No suitable primer pair found			
S27	No suitable primer pair found			
ROP 148	F2 (ACTCCCTTATCTRATTTATTTAG) R2 (TGATGCYAAATGAAGATG)	No bands produced		
MSP 7	F (AAGAGCAAGAATTACTTAACG) R (TTTGAAGAACTACAGAATATTCCT)	500bp band on both species From 45°C to 50°C	600 bp	No matches found
MSP 10	F (GTYTATATAAGCTCGAACAA) R (ATCCATTATTAACAGAACAAR)	No bands produced		

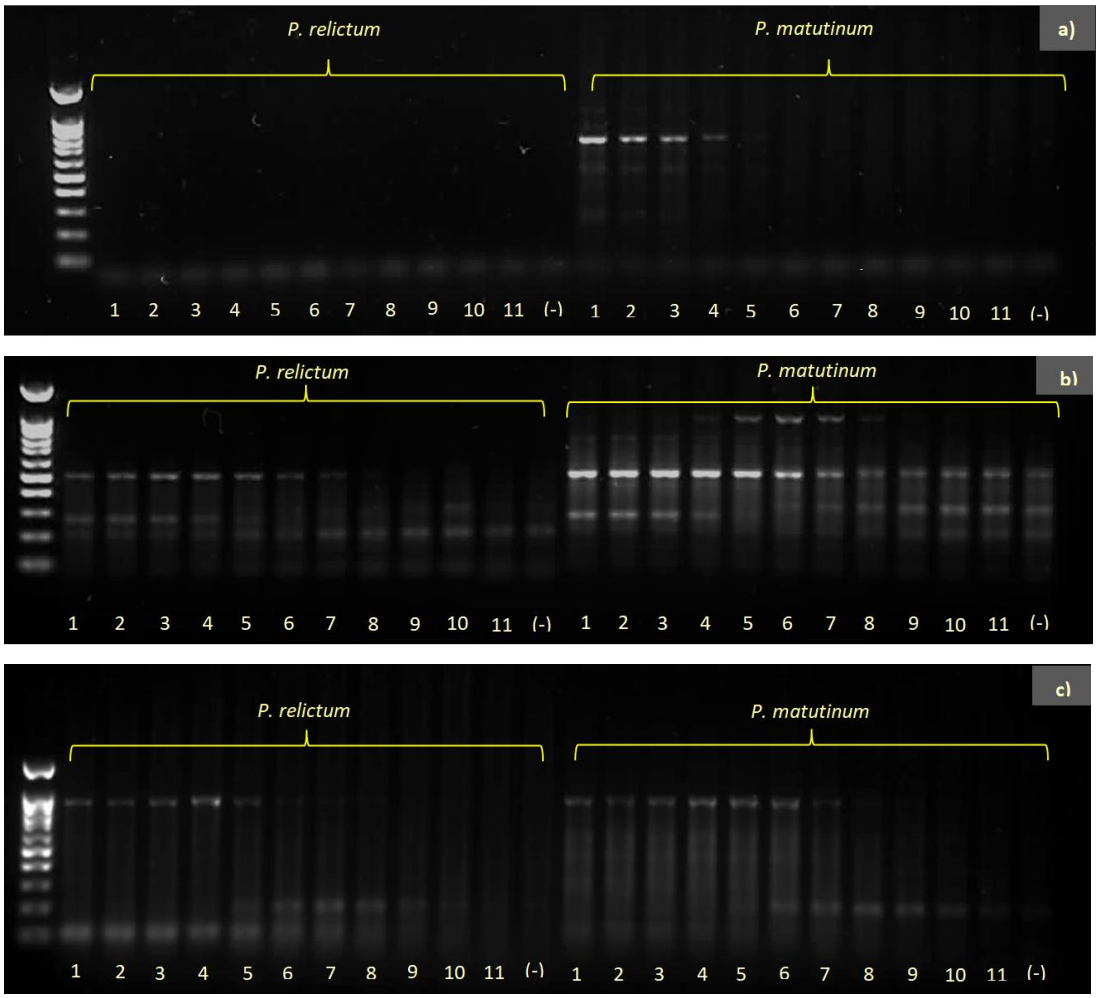

Figure 5. *P. relictum* and *P. matutinum* gradient PCR. 1= 44°C; 2= 44.3°C; 3= 45°C; 4= 46.1°C; 5= 47.4°C; 6= 49°C; 7= 50.6°C; 8= 52.1°C; 9= 53.5°C; 10= 54.7°C; 11= 55.6°C; (-)= Negative control. a) Cloroquine. b) GPI Antigen. c) MSA 180.

CONCLUSION

Genetic material of malarial parasites have a high AT content making it difficult to design effective primers; additionally, designing universal primers complicates further by sequence variation in different *Plasmodium* species and the lack of full genomes for the majority of known avian malaria species. It is likely that the Chester Zoo mosquito

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