UNIVERSITY OF GHANA
COLLEGE OF BASIC AND APPLIED SCIENCES
SCHOOL OF BIOLOGICAL SCIENCES

IDENTIFICATION OF AVIAN MALARIA PARASITES CAUSING DISEASE IN GHANAIAN BIRDS

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THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF DOCTOR OF PHILOSOPHY DEGREE IN MOLECULAR CELL BIOLOGY OF INFECTIOUS DISEASES

WEST AFRICAN CENTRE FOR CELL BIOLOGY OF INFECTIOUS PATHOGENS, DEPARTMENT OF BIOCHEMISTRY, CELL AND MOLECULAR BIOLOGY

JANUARY 2023

DECLARATION

I, Constance Agbemelo-Tsomafo, do declare that except for the references to other people's work, which I have duly acknowledged. This thesis is the product of my research work carried out under the supervision of Dr. Yaw Aniweh, Prof. Kirk W. Deitsch, and Prof. Asamoah Kusi. I do further declare that no part of this thesis has been previously submitted for a degree or any other qualification.

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ABSTRACT

Avian malaria is a blood parasite disease that occurs in various species of birds worldwide except in Antarctica. The avian malaria parasites of the genus *Plasmodium* belong to a group of apicomplexan protozoan parasites of the order *Haemosporida* and are closely related genetically to two other protozoan parasites of the genera *Haemoproteus,* and *Leucocytozoon*. Bloodsucking dipteran insects of the families *Culicidae*, *Hippoboscidae*, *Simuliidae*, and *Ceratopogonidae* transmit the three haemosporidian parasites. These parasites cause malaria, haemosporidiosis, and leucocytozoonosis diseases in wild and domestic birds, and cause asymptomatic to fatal cases in birds. The haemosporidian parasites have been extensively studied in many parts of the world, especially in temperate regions. However, little is known about these parasites in Ghana. This study sought to identify the species of avian malaria and related haemosporidians that prevail in Ghanaian birds and establish their genetic diversity.

The study was conducted in Sunyani and Aburi in the Brong Ahafo and Eastern regions of Ghana. Birds were sampled from poultry farms and wild habitats around the poultry farms. One thousand one hundred and ten birds were collected, comprising free-range, confined/caged, and wild birds. Blood samples were collected from the brachial veins of birds by needle puncture. Thin blood smears were prepared and stained with Giemsa solution for microscopy. DNA was extracted from whole blood and molecular screening of haemosporidian parasites was done by qPCR and nested PCR protocols. Sanger sequencing was used to identify haemosporidian parasite genera. Microbial DNA enrichment and whole genome amplification were performed on DNA samples to enrich AT biased genome. Phylogenetic analysis was performed to establish the genetic diversity of the parasites.

The results reported an overall haemosporidian prevalence of 21.5% for parasites belonging to all the three genera. At the level of individual genus prevalence, *Plasmodium* had a higher prevalence (18.9%) than *Leucocytozoon* (10.4%) and *Haemoproteus* (2.1%). Genus co-infections were also observed in most categories of birds studied. The study results found a 9.8% *Plasmodium* and *Leucocytozoon* co-infection mostly observed among domestic birds.

In this study, we observed that most of the samples that tested positive were detected using molecular methods. The morphological identification of the parasites was almost not possible in most of the blood smears examined. Extensive screening of blood smears obtained from known positive samples did not reveal parasite morphology, and this provides evidence suggesting that parasitaemia was extremely low and/or the infections were mostly abortive.

Histological preparations obtained from an infected turkey did not reveal parasite tropism in any of the organs studied. This could be a confirmation of abortive infections, or parasite development could be found in organs other than those examined.

Our study compared the prevalence of haemosporidian parasites belonging to each of the three genera in three categories of birds: confined, free-range, and wild birds. The prevalence of *Plasmodium* and *Leucocytozoon* was higher in free-range and wild birds than confined birds.

The results from the targeted Sanger sequencing revealed 24 new mitochondrial cytochrome b lineages of the haemosporidian parasites and we deposited them in the NCBI GenBank. The new lineages are composed of 6 *Leucocytozoon*, 13 *Plasmodium*, and 5 *Haemoproteus* and *Parahaemoproteus* lineages. Some of the parasite lineages found within all three genera of the parasites were host and habitat generalist or specialist, and lineage spillover was observed among the different bird categories.

Phylogenetic analyses revealed close genetic relatedness between our parasite lineages and others from the GenBank. A few of our new lineages were closely related to *Plasmodium globularis, Plasmodium juxtanucleare*, *Plasmodium bennettinia,* and *Leucocytozoon schoutedeni*. *Haemoproteus* parasites morphologically identified in wild passerine birds of the family *Ploceidae* were similar to the *Haemoproteus nucleofascialis.* The most prevailing haemosporidian lineages found in our study sites were the *Plasmodium* lineage PGHA708, *Leucocytozoon* lineage LGHA146, and *Haemoproteus* lineage PHGHA989.

For future comparisons of new parasite genomes to the few existing avian malaria genomes, we successfully enriched haemosporidian parasite DNA from naturally infected birds, and amplified the AT-biased genome for further genomic analysis.

This study provided evidence that avian malaria and related haemosporidian exist in the Ghanaian poultry industry, and there is a community sharing of the parasites among wild birds, free-range birds, and confined birds. This is the first study reporting *Leucocytozoon* in domestic birds in Ghana and the first describing haemosporidian parasite interactions among wild birds, free range, and confined birds in Ghana.

DEDICATION

To God be the Glory.

To my loving husband and children.

To all who played diverse roles in this study.

ACKNOWLEDGEMENT

I thank the Almighty God for grace and sustenance throughout this Ph.D. work. My sincere appreciation goes to my supervisors; Dr. Yaw Aniweh (principal supervisor) of the West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), Department of Biochemistry, Cell and Molecular Biology (BCMB). I cannot forget how he went with me to the field the first time and all his inputs in this Ph.D. research. I appreciate Prof. Asamoah Kusi (co-supervisor) of the Noguchi Memorial Institute for Medical Research, who makes time amidst his busy schedule to put me back on track; and Prof. Kirk Deitsch (co-supervisor) of the University of Cornell, who has always been ready to listen and encourage me. I would like to show extreme gratitude to Prof. Ellin Videvall, Prof. Phyllis Addo, Dr. Patrick Arthur, Prof. Lydia Mosi, and Prof. Osbourne Quaye for their help at the beginning of my study. I am also grateful to all members of the department of Animal Experimentation, Noguchi Memorial Institute for Medical Research (Daniel Amoah, Richard Obeng-Kyeremeh, Shirley Adu-Poku, David Barnes, Emmanuel Kodua, Priscilla Frimpong, Paul Padi, Dr. Samuel Adjei, Mr. Maxwell Quartey) for their immense support in every stage of my study. I appreciate the staff of NAMRU, Noguchi Memorial Institute for Medical Research (Selasie, Mawuli, and Clara). I am also extremely grateful to Mr. Anaba, Asare, and all the poultry farmers who helped tremendously in obtaining samples for this study. I also want to thank Emefa, Marian, and Anthony from the WACCBIP account and Sara from WACCBIP procurement, who have been very helpful in my Ph.D. journey. I am grateful to all staff and colleagues of WACCBIP and BCMB. I am also grateful to Prof. Gordon Awandare and WACCBIP for the fellowship, which was the major funding for this study.

I am most grateful to my loving husband Jonathan Forson and my children Kayla Forson and Kekeli Forson for their immense sacrifice, understanding, and support throughout my study period.

I appreciate my sisters (Daniela, Irene, and Larissa), and especially my parents, Dr. Proper Mensah Kounouho Agbemelo-Tsomafo and Mme Jeanne Adjanyo who have invested a lot of resources into my education.

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LIST OF ABBREVIATIONS

FAO	Food and Agriculture Organization
B.C.	Before Christ
P	*Plasmodium*
H	*Haemoproteus*
C	*Culex*
Spp	species
PCR	Polymerase chain reaction
L	*Leucocytozoon*
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
Cyt	cytochrome
U.S.	United States
SM	Sunyani municipality
NMIMR	Noguchi Memorial Institute for Medical Research
IACUC	Institutional Animal Care and Use Committee
EDTA	Ethylenediaminetetraacetic acid
bp	base pair
rDNA	ribosomal Deoxyriboncleic acid

qPCR	quantitative polymerase chain reaction
min	minute
UV	Ultraviolet light
rpm	revolution per minute
MIT cyt b	mitochondrial cytochrome b
df	degree of freedom
Plas	*Plasmodium*
Haemo	*Haemoproteus*
Leuco	*Leucocytozoon*
NCBI	National Centre for Biotechnology information
WGA	Whole genome amplification

CHAPTER ONE

INTRODUCTION

1.1 Background

Pathogens of zoonotic importance have become an issue of global concern. Considering global health studies, it has become important to link the health of different animal species. Factors such as urbanisation, agricultural activities, road construction, and human population expansions leading to encroachment of the natural habitat of animals have contributed to increased risk of emerging and re-emerging zoonotic pathogens in our world (White & Razgour, 2020). One of the major concerns of global health now is the possibility of disease spill overs in humans and animal species. Many human bacterial and viral pathogens have been transmitted to domestic and wild animals, resulting in various degrees of outbreaks (Epstein & Price, 2009). Some of these pathogens which infect multiple hosts have been implicated in wildlife populations' decline and extinction (Van Riper III & Van Riper, 1986). New host shifting in these multi-host pathogens can have negative implications on wild and domestic animals (Ricklefs & Fallon, 2002). This research will focus on studying avian malaria and other closely related haemosporidians in domestic and wild bird populations within poultry farming communities.

Haemosporidians are groups of protists found infecting animals and humans (Valkĭunas, 2005). These organisms are transmitted through the bites of dipteran insects (Valkĭunas, 2005). They are very well-studied groups because they include the malaria parasites which threaten humans (Valkĭunas, 2005) especially in the tropics.

Malaria is a global threat to public health around the globe. The disease is vector-mediated and affects mammals and other vertebrates (Valkĭunas, 2005). In 1884, Danilewsky published an article 'About Blood Parasites (*Haematozoa*)' which was very useful in studying haemosporidians (Valkĭunas, 2005). When Danilewsky was working on reptiles, amphibians, and birds, he discovered the morphology of the developmental stages of the blood parasites, which he did not differentiate taxonomically (Valkĭunas, 2005). He made suggestions that favoured joining the discovered parasites to the group *Haemocytozoa*, which includes the human malaria parasites (Valkĭunas, 2005). Subsequently, other scholars studied these haemosporidian parasites and assigned them to different groups. The first haemosporidian genus, *Plasmodium*, was established by Marchiafava and Celli (1885), and he attributed human malaria parasites to it (Valkĭunas, 2005). The genus *Haemoproteus* was established by Kruse based on the crescent-shaped description of the parasites (Valkĭunas, 2005). *Leucocytozoon smithi*, was described by Laveran as the first haemosporidian parasite causing severe disease in domestic birds (Valkĭunas, 2005).

The work by these scholars revealed three haemosporidian genera: *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* (Garnham, 1966). The haemosporidian groups infect mammals, amphibians, reptiles, and birds. However, the most diverse group within these parasites recovered is the bird haemosporidian (Bensch *et al*., 2007, 2009). The importance of bird haemosporidians has been extremely underestimated.

Bird malaria is the earliest experimental model used to investigate *Plasmodium* parasites' biology and transmission in all vertebrates (Rivero & Gandon, 2018). Since avian malaria was discovered in 1885, it has been used to study the malaria parasites in humans s(Hewit, 1940). It was also useful in testing and developing antimalarial drugs and the very first vaccines (Rivero & Gandon, 2018).

When the initial protocols for the detection and characterization of the lineages of bird malaria parasites using molecular techniques were designed (Bensch *et al*., 2000), the new era of avian malaria research, provided a lot of information about the distribution, host range, diversity, and prevalence of bird malaria all over the world " (MalAvi database http://mbio-serv2.mbioekol.lu.se/Malavi/)" (Hellgren *et al*., 2009). These molecular studies have identified a significant diversity within the lineages of the avian malaria parasites, that matches a rich phenotypic diversity (Rivero & Gandon, 2018). Avian malaria re-emerged as a model to study the ecology and evolution of malaria parasites (Rivero & Gandon, 2018). This was possible because the parasites are phenotypically diverse, with a high prevalence, and are easy to obtain (Rivero & Gandon, 2018).

There are approximately 220 morphological species and 2,876 avian haemosporidian lineages described, and their diversity may be greater or equal to the diversity of their hosts (Ricklefs & Fallon, 2002). Indeed, there are new avian haemosporidian species not yet found. Avian haemosporidian parasites have certain basic morphologies and life cycles in common. However, they have different insect and vertebrate host associations (Atkinson & Van Riper III, 1991; Garnham, 1966; Valkĭunas, 2005).

Dipteran insect vectors transmit the parasites (Valkĭunas, 2005). Avian haemosporidian infections can affect the fitness of the host, ranging from their physical condition and success in reproduction to lowered rates of survival, and a source of an emerging disease in non-endemic areas (Coon, Brown & Strand, 2016). The disease has been extensively studied in the temperate regions with deficits in the Afrotropics (Clark *et al*., 2014) even though tropical regions have the highest avian species richness. There is, therefore, the need to recover more haemosporidian parasites in the tropical regions.

1.2 Rationale

1.2.1 Problem and Justification

Avian malaria and related haemosporidians have been studied extensively in many parts of the world, majorly in wild birds. Wild birds are involved in pathogen transmission to humans and other animals, including domestic birds. Wildlife is increasingly getting closer to humans and domestic animals because of increasing populations resulting in land use changes (Devictor *et al*., 2008). These land use changes alter not only biodiversity but also ecological and evolutionary processes (Ryall & Fahrig, 2006), including hosts and parasite interactions. This implies that parasites of wild birds could pose great danger to domestic birds almost sharing the same ecological habitats (Chasar *et al*., 2009), and this becomes a significant concern for the poultry industry. Poultry is very important in playing a critical role in providing animal protein to man and generating revenue (Nnadi & George, 2010). However, it has been reported that productivity could decrease and mortality rate will rather increase especially in young birds, e.g. 57% mortality in Mali (Kuit, Traore, & Wilson, 1986). Various factors involved include mismanagement, malnutrition, diseases, and predation (Minga *et al*., 1989; Negesse, 1993).

One of the greatest factors known to threaten poultry production is parasitism (Adene & Dipeolu, 1977). It has been reported that some haemosporidian parasites causing avian malaria worldwide induce mortality of up to 90% in domestic chickens (Sprinter, 1991). *Plasmodium gallinaceum* also reportedly caused over 80% mortality in poultry. The disease leads to economic and agricultural loss, such as poor quality and quantity of eggs and meat in the poultry industry (Permin *et al*., 2002). What is the situation in Ghana? Which species predominantly infect our birds? Information regarding the epidemiology of these important parasites in Ghana is limited (Poulsen

et al., 2000; Wink & Bennett, 1976). Therefore, more information on the prevalence of these parasites in birds will help to understand the epidemiology of the diseases.

1.3 Hypothesis

Avian malaria and related haemosporidians that infect wild birds are the same species found in domestic birds in Ghana.

1.3.1 Research questions

The hypothesis will be tested by answering the following questions:

1. What species of avian malaria and other haemosporidian parasites are found in domestic birds in Ghana?
2. Are the same haemosporidian parasites present in wild birds, confined and free-range birds?

1.4 Aims

The aims of this study are:

1. To identify avian malaria and related haemosporidian parasites predominantly infecting birds in Ghana.
2. To evaluate the tissue tropism of the haemosporidian parasites in domestic birds.
3. To identify the specific parasite species of avian malaria and related haemosporidian parasites infecting birds in Ghana.

1.5 Specific objectives

1. To determine the prevalence of malaria and related haemosporidian parasites among both domestic and wild birds.

2. To assess malaria and related haemosporidian parasites interaction at the host tissue level.

3. To assess the correlation between blood parameters and tissue-stage haemosporidian infections.

4. To determine the genetic diversity of malaria and related haemosporidian parasites among both domestic and wild birds.

CHAPTER TWO

3.1 Literature review

With over 249 million cases (WHO, 2023), malaria is one of the deadliest diseases in the world (Lutz *et al*., 2015). The illness is probably older than humans, having been first identified in people in China about 2700 before Christ (B.C). The transmission of modern malaria by mosquitoes about 20 million years ago has been suggested by fossil data (Poinar, 2016). According to a recent investigation of the disease's prehistoric origins, the sickness may have existed in earlier times carried by biting midges and dating back at least 100 million years (Poinar, 2016). Therefore, malaria was not only infecting humans, because biting midges were not transmitting malaria to humans. Over 500 known parasite species from 15 genera in the order Haemosporida (phylum Apicomplexa) have been discovered by systematic parasitologists; these species infect mammals, reptiles, and birds and transmit their infections through dipteran vectors from at least seven families (Martinsen *et al*., 2007). These parasites are found in all the warm continents (Lutz *et al*., 2015).

The parasites that cause avian malaria are numerous, diversified, and easy to sample without disrupting the host populations (Lutz *et al*., 2015). There is debate among parasitologists, ecologists, and evolutionary biologists on the phrase "malaria parasites" (Pérez-Tris & Bensch, 2005). However, the terms "malaria parasites" are typically used to refer to the genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* (Pérez-Tris & Bensch, 2005). The first reports of avian malaria appeared in the late 19th century, shortly after human malaria (Pigeault *et al*., 2015). Molecular studies of the diseases have shown an unexpected level of diversity among the haemosporidian parasites (Beadell *et al*., 2006; Bensch *et al*., 2000; Ricklefs & Fallon, 2002; Ricklefs *et al*., 2004). Danilewsky was the first to look into the pathology of avian malaria, and

his findings included severe anaemia, enlarged liver and spleen, an accumulation of pigment and parasites, and infected erythrocytes in the phagocytes of these organs (Valkĭunas, 2005). He connected ecological observations to the seasonal dynamics of bird infections and concluded that prevalence increased during the warm seasons, parasitaemia corresponded with environmental temperature, and vectors contributed to the spread of the parasites (Valkĭunas, 2005).

After M. Laird established the International Reference Center for Avian Malaria Parasites in 1968, with the official backing of the World Health Organization (WHO), avian malaria research became increasingly active (Valkĭunas, 2005). Studies on avian malaria have largely been focused on naturally infected bird populations since the turn of the 20th century, and significant data have been gathered in the fields of ecology, molecular biology, distribution, prevalence, diversity, and phylogeny (Beadell *et al*., 2006; Bensch *et al*., 2000; Hellgren *et al*., 2007).

2.2 Malaria and related haemosporidians

The study discusses the three genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* of avian haemosporidian parasites. The specific genus of each of these parasites is described in depth in this section.

2.2.1 *Plasmodium*

Plasmodium parasites cause avian malaria (Atkinson *et al*., 2009; Valkiunas *et al*., 2016). There are at least 55 species of avian *Plasmodium* described (Valkiunas *et al*., 2004), of which some are generalist parasites that can parasitize a vast range of bird species, while others are host-specific (Dimitrov *et al*., 2010). According to the description by Atkinson and colleagues, *Plasmodium* parasites differ in pathogenicity, host range, geographic distribution, and vectors (Atkinson *et al*., 2009). They share *Plasmodium* share similar traits with the avian *Leucocytozoon* and

Haemoproteus parasites, except that they exhibit asexual reproduction (merogony) in circulating erythrocytes. Several reports have documented acute and pathogenic *Plasmodium* infections in individual birds (Atkinson *et al*., 2009). Reports from Hawaii suggested that introducing one of the most prevalent species of bird malaria, *Plasmodium relictum*, into the bird population served as a factor that limits the distribution and abundance of the native forest birds in Hawaii (Beadell *et al*., 2006; Woodworth *et al*., 2005)(Woodworth et al., 2005).

2.2.2 Synonyms of *Plasmodium* parasites

Plasmodium and *Haemoproteus* genera have been referred to as avian malaria parasites in several studies due to their close relatedness, and this makes it difficult to identify the genus being discussed (Atkinson *et al*., 2009). However, the distinct life cycle traits of these genera justify their division (Valkĭunas, 2005).

2.2.3 History of avian malaria

From the years 1890s to the 1940s, avian malaria was used to study the physiology and chemotherapy of human malaria (Slater, 2005). A lot of breakthroughs were achieved in the study of human *Plasmodium* species using bird parasites (Atkinson *et al*., 2009). The description of the abnormal pathologies of bird malaria in 1889, the discovery of mosquitoes as vectors for *P. relictum* in 1898, and the discovery of the exoerythrocytic merogony *Plasmodium elongatum* in reticuloendothelial cells in bone marrow and other organs in 1934 are part of the early descriptions of *Plasmodium* abnormalities in birds (Atkinson *et al*., 2009).

Avian malaria incidence reports have been useful in studying the ecology and evolution of sexual selection and the consequences of parasitic infections in wild birds (Gilman, Blumstein & Foufopoulos, 2007; Kilpatrick *et al*., 2006). In some of these studies, mitochondrial lineages of the parasites have been traced to determine their geographic distribution and host specificity using

genetic approaches (Ricklefs *et al*., 2005). After the Second World War, rodent malaria was used to replace avian malaria as the most productive animal model (Aviado & Cambar, 1969) for studying human malaria.

2.2.4 Distribution of avian *Plasmodium*

All zoogeographic zones of the world, apart from Antarctica, where the dipteran insects necessary for *Plasmodium* transmission are absent, harbour many species of avian *Plasmodium*. The parasite has been reported in Australia, however, these reports are notably fewer than others (Bennett *et al*., 1993; Valkĭunas, 2005). It is unknown whether this is because the area was not well sampled or whether a true distributional abnormality exists (Bennett, Bishop & Michael, 1993). The parasites were morphologically detected in over 250 species of birds (Rivero and Gandon, 2018). The many morphospecies revealed a significant phenotypic variety in the life history features of the parasites, which helped researchers understand the parasite's biology, life cycle, and mode of transmission (Rivero & Gandon, 2018).

2.2.5 *Plasmodium* host range

Plasmodium has been found in all bird orders, except Trogoniformes, Coliiformes, and Struthioniformes (Atkinson *et al*., 2009). The most diverse collection of bird *Plasmodia* was found in the orders Passeriformes, Columbiformes, and Galliformes (Valkĭunas, 2005). Avian *Plasmodium* was found in more than 800 species of birds belonging to 90 bird families, and 25 bird orders (Rivero & Gandon, 2018). *P. relictum*, which naturally occurs in 70 distinct bird families, has a wider host range than the other bird *Plasmodia* (Atkinson *et al*., 2009). Although certain species of avian *Plasmodium* seem to have restricted host ranges in wild birds, most species have a wide host range (Rivero & Gandon, 2018). For example, *P. kempi* and *P. hermani* are

morphologically different species, but were found infecting wild and domestic turkeys (*Meleagris gallopavo*) in North America (Atkinson *et al*., 2009). In the laboratory, *P. kempi* can infect members of the orders Galliformes and Anseriformes, but its sole known natural host is the wild turkey (Christensen *et al*., 1983).

2.2.6 Diversity and distribution of avian malaria

Many *Plasmodium* genetic lineages have been discovered following the application of molecular techniques in the study of avian malaria (Bensch *et al*., 2004). Since the introduction of the molecular methods of avian malaria over two decades ago, there has been increase in publications that describe the genetic diversity of the mitochondrial cyt-b gene lineages (Rivero & Gandon, 2018). These publications provide information on the distribution, host range, diversity, and prevalence of avian malaria (Clark *et al*., 2014). There is more genetic diversity in avian malaria parasites than in any other vertebrate host (Rivero & Gandon, 2018). Many different cyt-b lineages of avian *Plasmodium* are reported in the "MalAvi Database" (Bensch *et al*., 2009), and new lineages are continually being added (Rivero & Gandon, 2018). Variations in the prevalence of the disease across host species, lineages, and geographical areas exist (Knowles *et al*., 2011; Loiseau *et al*., 2013). Sometimes avian malaria prevalence could go as high as 80% in a host species (Loiseau *et al*., 2013). Most of *Plasmodium* lineages were discovered in passerine birds, probably because they represent over half of bird species described globally, and are simple to catch (Rivero & Gandon, 2018). It could also be because of the disproportionate efforts in sampling passerine birds (Rivero & Gandon, 2018). Some of the bird *Plasmodium* lineages were only isolated from a few host species in a specific geographic location, while others, like the *Plasmodium* lineage pSGS1, can prey on more than a hundred bird species worldwide (Rivero & Gandon, 2018). The major research gap now is the lack of information on the variety, distribution, and host range of

the avian malaria vectors (Rivero & Gandon, 2018). Very few avian *Plasmodium* lineages have been matched to a potential vector, and only a few mosquito species have been recognized as spreading avian malaria (Zélé *et al*., 2014).

2.2.7 Etiology of avian *Plasmodium* parasites

About 55 known species of avian *Plasmodium* have been reported. However, this number varies because of the synonymization of existing species, increased understanding of their biological traits, and the description of new species (Atkinson *et al*., 2009). There are mitochondrial gene sequences available for many lineages (Bensch *et al*., 2004), but, in most of these lineages, the morphology of the erythrocytes, the insect vectors, or other life-history traits are unknown (Valkiūnas & Zehtindjiev, 2007). Atkinson *et al*., reported that there are proposed initiatives to match the life history data of the five *Plasmodium* subgenera (*Huffia, Haemamaoba, Novyella, Bennettinia, and Giovannolaia*) with molecular data. They also found that the exceedingly unusual characteristics of the subgenera *Haemamoeba*, *Huffia*, and *Bennettinia* are all consistent with their monophyletic origins. These characteristics include large, round gametocytes and substantial host nucleus displacement (Atkinson *et al*., 2009). In contrast, *Giovannolaia* and *Novyella* form a clade that includes individuals from both subgenera, demonstrating that these parasites' less distinguishing morphology may have changed over evolution (Martinsen *et al*., 2007).

2.2.8 Epizootiology of avian *Plasmodium*

Clay Huff and colleagues used *P. gallinaceum* as the foundation to show the intricate life cycles of avian *Plasmodium* species. They gave mosquito bites to chickens and other birds, which were then periodically observed to see where and how the parasites looked. These investigations explain the development of the parasites (Atkinson *et al*., 2009).

The life cycle of *P. gallinaceum* was described in the review by Atkinson and colleagues (Atkinson *et al*., 2009). During a blood meal, a vector injects infectious sporozoites into a susceptible host, and this begins the life cycle (Figure 1). The sporozoites invade macrophages and fibroblasts close to the bite site and undergo the first stage of asexual development (merogony) to become cryptozoites. The second generation of merogony begins, and the capillary endothelial cells of the primary organs, and circulating erythrocytes can both be invaded by merozoites, which are released as metacryptozoites. The first two merogony generations are known as the pre-erythrocytic stages of infection. Phanerozoites are merogony-producing merozoites that persist in stationary host tissues through a third-generation. They are known as exoerythrocytic meronts after they penetrate capillary endothelial cells and multiply by asexual merogony. When liberated from exoerythrocytic meronts, merogony can persist in stationary tissues by reinvading endothelial cells or invading circulating erythrocytes. Capillary endothelial cells have round, elongated, or branched exoerythrocytic meronts that resemble the thin-walled meronts of *Haemoproteus*. When merozoites infiltrate circulating erythrocytes, they undergo merogony and, within 24 to 48 hours, either mature meronts with 8 to 32 ovoid merozoites or mosquito vector-infecting gametocytes emerge. Meronts produce a huge number of merozoites, which may only be found in a specific type of *Plasmodium* and can have a sphere or an elongated shape. In contrast to gametocytes, which are spherical or elongated in shape and have a single nucleus, merozoites usually feed on their host erythrocytes when they are released (Atkinson *et al*., 2009).

Giemsa stain typically leaves a pink stain on male gametocytes (microgametocytes) and a pale blue stain on female gametocytes (macrogametocytes) (Atkinson *et al*., 2009). The parasites breakdown hemoglobin as they ingest host erythrocyte cytoplasm while developing inside the erythrocyte. Malarial pigment, also known as haematozoin, is created as a by-product of the

breakdown of haemoglobin which can form granules that are black or golden brown in colour, and are found in the cytoplasm of the parasite (Atkinson *et al*., 2009). The circulating erythrocytes may continue to undergo merogony indefinitely, and some erythrocytic merozoites can re-invade tissues and continue to develop into phanerozoites (Garnham, 1966).

Parasites in the subgenus *Huffia* including *P. elongatum* undergo exoerythrocytic merogony in the host's haematopoietic tissues (Garnham, 1966). Atkinson *et al*., also reported that all avian *Plasmodium* species gametocytes remain in the bloodstream and do not continue to develop until an arthropod vector ingests them (Atkinson *et al*., 2009). They depart from their host cells once within the midgut of an appropriate mosquito vector, where they go through gametogenesis to create gametes. Male gametocytes undergo exflagellation, and produce microgametes. When one microgamete fertilizes a macrogamete, a motile zygote develops into an oocyst and emerges within 24 hours. The asexual process of sporogony is used by oocysts to produce thousands of sporozoites. Sporozoites find their way into the salivary glands and enter a new avian host with the saliva of a mosquito that has just consumed blood, where they begin a fresh infection. Only a few naturally occurring mosquito vectors are known, although over 60 different mosquito species can sustain the experimental development of a range of *Plasmodium* species from avian hosts (Atkinson *et al*., 2009), and only three *Culex* species (*C. tarsalis*, *C. stigmatasoma* and *C. quinquefasciatus*) are natural carriers of *Plasmodium relictum* (Lapointe *et al*., 2005).

The severity of *Plasmodium* infection is affected by the availability of food (Appleby *et al*., 1999), concurrent parasitic infections, and vulnerability to predators (Navarro *et al*., 2004; Wright *et al*., 2005), and immune system modifications caused by stress and reproductive effort (Atkinson *et al*., 2009).

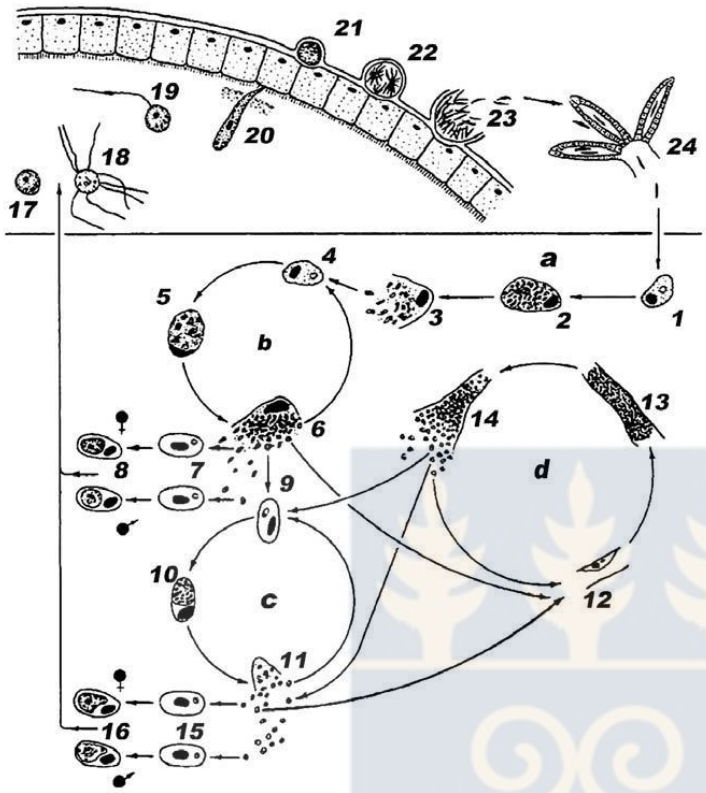

Figure 1: Diagram showing the life cycle of avian *Plasmodium* parasite

(Valkiunas, 2005). "Legend: Upper part (18-24), Upper part (18-24), in vector; lower part, in bird: a, b - primary exo-erythrocytic merogony; c -erythrocytic merogony; d - secondary exoerythrocytic merogony; 1 - sporozoite in reticuloendothelial cell; 2, 3 - cryptozoites; 4 - merozoite in macrophage; 5, 6 - metacryptozoites; 7 - merozoites in erythrocytes; 8 - gametocytes; 9 - merozoite in erythrocyte; 10, 11 - erythrocytic meronts; 12 - merozoite in endothelial cell of capillaries; 13, 14 -phanerozoites; 15 - merozoites in erythrocytes; 16 - gametocytes; 17 - macrogamete; 18 - exflagellation of microgametes; 19 - fertilization of macrogamete; 20 - ookinete penetrating the peritrophic membrane; 21 - young oocyst; 22, 23 - sporogony; 24 - sporozoites in the salivary glands of vector " (Valkiunas, 2005).

2.2.9 Difference between avian and mammalian *Plasmodium* life cycle

The haemosporidian parasite life cycle involves both blood cells and tissues (erythrocytic and exo-erythrocytic cycles). Before the blood cells are infected, and gametocytes are produced, the parasites go through exo-erythrocytic merogony in tissue cells (Valkiunas & Iezhova, 2017).

The avian *Plasmodium* life cycle is complex. The parasite alternates between sexual and asexual reproduction in a vertebrate host and a mosquito (Machado *et al*., 2021). The life cycles of *Plasmodium* species found in mammals, birds, and humans all have certain fundamental similarities (Krizanauskiene *et al*., 2006). The nutritional properties of non-human *Plasmodium spp*. are identical to those of the species that infect humans (Schuster, 2002). When a mosquito transmits *Plasmodium* into the bloodstream of a vertebrate host, the sporozoites move into the exoerythrocytic phase of development (Schuster, 2002). Unlike in birds, where the exoerythrocytic stage happens in the reticuloendothelial cells, the exoerythrocytic stage in mammals, occurs in the hepatic cells (Trager, 1986). The merozoite, the invasive stage for erythrocytes in mammals, develops in a series of stages that start with the ring stage and result in a multinucleate schizont (Schuster, 2002). The schizont develops and produces many merozoites, which infect additional erythrocytes (Schuster, 2002). However, in avian malaria, the merozoites can either re-invade the reticuloendothelial cells or penetrate the erythrocytes after leaving the cells of the reticuloendothelial system (Schuster, 2002). Parasites can be retrieved from the peripheral bloodstream of the vertebrate host, where they undergo a cycle of repeated erythrocyte invasion with distinctive synchronization to raise the amount of parasitaemia in the host bloodstream (Schuster, 2002).

2.2.10 Sexual stages of mosquito transmission of the avian *Plasmodium* parasites

Lots of efforts were made to describe the various avian malaria parasites' blood stages (Hewit, 1940). MacCallum discovered the sexual stages of the parasite (MacCallum, 1897). He collected blood from an infected bird and left it in the open at room temperature for many minutes, and observed that the flagellated forms of the parasite behaved similarly to the spermatozoids of higher animals (Rivero & Gandon, 2018). These findings had a significant impact on the field of parasitology.

Ronald Ross was the first to show that avian malaria parasites were transmitted, by mosquitoes (Ross, 1902). He was a military doctor who studied mosquitoes that preyed on malaria patients. After having difficulties in finding enough willing human subjects for his trial, He focused on bird malaria. He demonstrated the complete malaria cycle in laboratory-bred *Culex* mosquitoes, that were fed on either malaria-infected or uninfected birds. He discovered uninfected birds contracted the parasite when bitten by mosquitoes carrying thread-like versions of the parasites in their salivary glands (Ross, 1902). This meant that for a mosquito to transmit malaria to a new host, it would need to feed again on blood from an uninfected individual (Rivero & Gandon, 2018). This finding altered people's perceptions of how vector-borne diseases are transmitted and refuted the theory that humans contract parasites from contaminated water after drinking mosquito carcasses (Ross, 1902).

2.2.11 Clinical signs of avian malaria

Extremely pathogenic infections have been noted during acute episodes with *P. relictum*, *P. gallinaceum*, and *P. juxtanucleare* in domestic hens, *P. juxtanucleare* in penguins, *P. elongatum* in penguins, and *P. durae* in domestic turkeys (Williams, 2005). The sick birds typically exhibit

anorexia, anaemia, and ruffled feathers (Atkinson *et al*., 2009). Hematocrits may decrease by over 50% (Atkinson *et al*., 2009). Domestic chickens with *P. juxtanucleare* and *P. gallinaceum* infections were reported as sedentary, with their combs being pale, passing green stool, diarrhea, and complete or incomplete paralysis (Garnham, 1966). Young turkeys infected with *P. durae* display relatively little clinical symptoms and later experience severe convulsions soon before passing away (Garnham, 1966). Because of hypoxic pulmonary arterial hypertension, infected adult birds exhibit anorexia, and inactive behaviour (Huchzermeyer *et al*., 1988). Adult birds can also have gangrenous wattles and edematous legs (Atkinson *et al*., 2009).

2.2.12 Pathology and pathogenesis of *Plasmodium* infection in birds

Chickens infected with *P. gallinaceum* exhibit the initial clinical symptoms between days 5 and 7 after the inoculation in experimental trials (Williams, 2005). This lowers hematocrit and raises peripheral parasitaemia (Atkinson *et al*., 2009). When infected and uninfected red blood cells hemolyze, the breakdown of haemoglobin causes excess biliverdin to be formed and is eliminated through faeces (Williams, 2005). According to Atkinson *et al*., infected birds pass green faeces, four days after infection. In the phase I, the faeces appear normal because of the green pigment In phase II, thin, mucoid, vivid green diarrhea appears on day 5 and lasts two days in birds that recover from the infection (Atkinson *et al*., 2009). Phase III marks the beginning of the birds' recovery from the infection, and the green colouring of the faeces shifts to a level of intensity halfway between phases I and II. The green colour of the faeces disappears when parasitaemia is no longer evident, (Williams, 2005). The acute stage of *P. gallinaceum* infection in chickens was associated with elevated cloacal temperatures (Williams, 2005). Atkinson *et al*., also explained that avian malaria acute infections cause anomalies such as thin blood, liver, and spleen enlargement, as well as liver and spleen coloration. The organs, especially the spleen, can develop

thrombi or emboli in severe lethal infections. Secondary shocks could be observed when many infected and uninfected erythrocytes are destroyed in the final stages of several acute illnesses (Atkinson *et al*., 2009).

They described an experiment where canaries were infected with *P. cathemerium* (Atkinson *et al*., 2009). The birds developed inflammatory myopathy in their skeletal muscles, capillary, and muscle fiber degeneration, and mononuclear cell infiltrates. Though *Plasmodium*'s pre-erythrocytic meronts cause little to no host reaction, species, such as *P. gallinaceum* and *P. durae*, have exoerythrocytic meronts that can partially or totally block capillaries, causing leaking of plasma proteins, edema, and bleeding. These lesions can affect the brain, kidney glomeruli, heart, and lungs. Neurologic symptoms may develop, leading to death when they happen in the brain (Atkinson *et al*., 2009).

2.2.13 Diagnosis of avian malaria

Avian malaria is diagnosed using Giemsa-stained thin blood smears, as the gold standard for diagnosing the infection (Atkinson *et al*., 2009). Individual species are defined based on the morphology of gametocytes and meronts, the number of merozoites produced, changes in the morphology of avian erythrocytes, and other biological traits such as host range, susceptibility to mosquito vectors, morphology, and location of exoerythrocytic meronts (Valkiunas, 2005). *Plasmodium* infections are typically persistent, with very low intensities in some hosts, making parasite identification below the subgenus level almost impossible (Atkinson *et al*., 2009).

Other methods developed to diagnose low parasitaemia infections include molecular diagnostics using polymerase chain reaction (PCR) with primers to amplify ribosomal and mitochondrial genes as this may provide a solution to low parasitaemia detections (Atkinson *et al*., 2009). Despite their exceptionally high sensitivity, PCR methods can still miss infections with extremely low

parasitaemias (Jarvi *et al*., 2002), therefore real-time methods have recently been developed to address these issues (Boonma *et al*., 2007).

2.2.14 Immunity for *Plasmodium* infection in birds

Birds infected with *Plasmodium* produce strong antibody and cell-mediated immune responses against the erythrocytic parasites, but these immune responses do not destroy the parasites entirely (Van Riper III *et al*., 1994). Investigations of infection with *P. hermani* in domestic turkeys and canaries, and *P. relictum* in Hawaii Amakihi showed that infected birds may carry chronic parasite infections forever, thereby strengthening their immune system against reinfection with different strains of the same parasite species (Jarvi *et al*., 2002). Atkinson *et al*., described this phenomenon as premunition. If the host immune system is weakened by stress or infection with other pathogens, subclinical infections may persist, making birds susceptible to the resurgence of the parasites. It is also an indirect indicator of how expensive it is to develop an immune response (Atkinson *et al*., 2009). For instance, the prevalence of malarial illness was higher in male Great Tits that put in more effort to raise larger broods (Richner *et al*., 1995).

2.2.15 *Plasmodium* infections in domesticated birds

Domestic poultry can exhibit multiple species of avian *Plasmodium*. However, they have the most severe consequences when infection comes from wild reservoir hosts (Atkinson *et al*., 2009). Domestic chickens, especially European breeds introduced into avian malaria endemic regions are very susceptible to *P. gallinaceum* (Atkinson *et al*., 2009). Malaria mortality rate from *P. durae* can reach 90% in young poults, making it a serious threat to domestic turkeys (Atkinson *et al*., 2009).

2.2.16 *Plasmodium* infections in wild bird populations

Severe death in natural populations of wild birds may occur. However, there is little proof that such epizootic die-offs are caused by avian *Plasmodium* species (Atkinson *et al*., 2009). Over 5,000 studies on avian blood parasites were reviewed, and only about 4% showed bird mortality or pathogenicity in the wild, with the majority affecting domestic birds or birds in zoological collections (Atkinson *et al*., 2009; Bennett *et al*., 1993).

The reviews revealed evidence of both direct and indirect impacts of acute and chronic infections on the lifelong reproductive performance of the birds (Atkinson *et al*., 2009). Behavioral research showed that *Plasmodium* and *Leucocytozoon* infections affected the consistency of the song of White-crowned Sparrows (*Zonotrichia leucophrys oriantha*) (Atkinson *et al*., 2009). Judging from the playback of recorded songs, *Plasmodium*-infected birds sang less frequently than uninfected birds (Atkinson *et al*., 2009). This may influence the infected individuals' choice of mate and ability to reproduce (Atkinson *et al*., 2009). Acute infections can also increase the risk of predators attacking the infected birds (Atkinson *et al*., 2009).

2.2.17 Treatment and control of avian malaria

Medications that proved effective against avian malaria include primaquine phosphate, chloroquine phosphate, mefloquine, and a combination of pyrimethamine-sulfadoxine. They have shown effectiveness against avian malaria in canaries, penguins, and raptors (Atkinson *et al*., 2009). Anticoccidial medications such as sulfamonomethoxine, sulfachloropyrazine, and halofuginone are also successful in treating *P. durae* infections in domestic turkeys and may also be beneficial in treating *P. gallinaceum* (Atkinson *et al*., 2009). After the emergence of circulating parasites, sulfamonomethoxine has been shown to lower parasitaemia but does not fully protect

against mortality (Atkinson *et al*., 2009). Sulfachloropyrazine decreases mortality but does not affect parasitaemia, showing some effectiveness against exoerythrocytic schizonts (Atkinson *et al*., 2009). Halofuginone only slightly inhibits parasitaemia (Atkinson *et al*., 2009).

Birds were first experimented on to develop vaccines against *Plasmodium* parasites, but there are no methods have been discovered to immunize wild birds yet, and this is the biggest barrier to reducing avian malaria infection using vaccines (Atkinson *et al*., 2009). However, there have been numerous experimental vaccinations tested (Atkinson *et al*., 2009). These include synthetic vaccines based on parasite surface chemicals and sporozoites, merozoites, and gametes that have been irradiated, formalin-inactivated, and UV light-inactivated (Atkinson *et al*., 2009).

Using Jackass Penguins and canaries exposed to the natural transmission of *P. relictum* at a zoological park (Grim *et al*., 2004; McCutchan *et al*., 2004), two DNA vaccines based on the circumsporozoite protein of *P. gallinaceum* and *P. relictum* have been tested. Both offered protection against naturally occurring *P. relictum* exposure. However canaries' immunity was transient, and after a year of exposure to mosquito vectors, the birds were no longer protected and were just as susceptible as the unvaccinated controls (Atkinson *et al*., 2009).

It has been shown in human malaria research that decreasing mosquito populations can limit *Plasmodium* transmission, but this strategy has not been widely applied to manage avian malaria infections (Atkinson *et al*., 2009). Reduced larval habitat for the invasive mosquito *Culex quinquefasciatus* has been the primary goal of efforts to prevent avian malaria in Hawaiian forest birds (Reiter & Lapointe, 2007). Housing cage birds in screened, mosquito-proof structures or placing them in locations away from wild reservoir hosts are the most affordable control strategies for domestic or captive birds (Atkinson *et al*., 2009).

2.2.18 Avian malaria management implications

The risk of exposure to avian malaria should be considered when threatened or endangered species are transported outside of their natural habitats and kept in establishments where they can come in contact with new vectors and/or locally transmitted *Plasmodium* strains (Atkinson *et al*., 2009). Although penguins were at risk, species of birds from remote areas that might not have been exposed to these parasites should also be monitored (Atkinson *et al*., 2009). To prevent the emergence of novel host-parasite relationships that may be highly dangerous, it is also important to prevent the unintended transfer of parasites and mosquito vectors to new ecosystems (Lapointe *et al*., 2009).

2.2.19 Importance of avian malaria in the studies of human malaria parasite biology

Avian haemosporidian parasites studies have provided many fundamental discoveries about human malaria (Atkinson *et al*., 2009). *P. relictum* sporozoites were observed within the oocysts and salivary glands of *C. fatigans* mosquitoes (Ross, 1898). This led to the first report, which proved that *Plasmodium* was transmitted by mosquitoes (Ross, 1898). Subsequently, Étiene Sergent also conducted an experimental immunization research using avian malaria where he showed canaries were resistant to mosquito-transmitted malaria after vaccination with attenuated sporozoites of *P. relictum* (Sergent, 1910). Before analogous research with human *Plasmodia* were carried out, the extra-erythrocytic stages of malaria parasites were found using an avian *Plasmodium*, and in vitro growth of the blood stages of malaria parasites was established with avian *Plasmodia* (Atkinson *et al*., 2009). *P. gallinaceum* was used to cultivate the mosquito stage of malaria parasite in vitro (Warburg & Miller, 1992).

Humans could not contract avian malaria, but infected avian *Plasmodium* vectors can transfer sporozoites to people during their subsequent blood meal (Atkinson *et al*., 2009). The sporozoites

then remain in the blood circulation and, infect more vectors (Santiago-Alarcon *et al*., 2012). These vectors serve as a bridge between two hosts, birds serving as reservoirs and humans serving as dead-end hosts for avian *Plasmodium* (Nourani *et al*., 2020).

2.2.20 Synchronicity and periodicity of malaria

The biology behind symptoms of human malaria patients was resolved by avian malaria research (Richard & Kamini, 2002); an example is the periodic chills and fevers, recurring at regular 48 hours or 72 hour intervals (Rivero & Gandon, 2018). Through avian malaria research, it was also shown that the rhythmic appearance of symptoms results from periodic phenomena in the cycle of *Plasmodium* parasites within the blood of the host (Riveros & Gandon, 2018).

Periodicity and synchronicity were two distinct occurrences discovered in the schizogony of asexual parasites in the blood (Wolfson, 1936). These two characteristics were displayed differentially in several species of avian malaria (Rivero & Gandon, 2018). While all stages of the avian malaria parasite were present in the blood for some of the species that displayed asexual parasitaemia peaks around the same time each day, other species showed the same developmental stage of the parasite (Hewit, 1940). Early studies claimed that most species of avian malaria followed 24-hour rhythms, or multiple (Hewit, 1940). However parasitaemia peaks could happen at different times of the day (Rivero & Gandon, 2018). Malaria periodicity is a parasite genetic disorder (Taliaferro, 1928), which can be experimentally changed by changing the physiology of the host (Boyd, 1929).

2.2.21 Exoerythrocytic stages and relapses

When it was revealed that the asexual cycle of the avian malaria parasites may occur in blood cells other than erythrocytes, avian malaria caused a paradigm change in malariology (Huff, 1947).

Prior research on *P. gallinaceum* malaria showed that the reticuloendothelial system had an exoerythrocytic phase before the parasites become visible in the blood (Cox, 2010). Soon after this finding, a second exoerythrocytic form that was exclusive to avian *Plasmodium*, and was formed from the blood stages was discovered (Huff & Coulston, 1946). A connection between long latencies and malaria relapses during dormant exoerythrocytic malaria stages was established (Raffaele & Marchiafava, 1944). Relapses can happen with *P. vivax*, a human malaria parasite, and in bird malaria parasites (Rivero & Gandon, 2018). Malaria parasite relapse studies in birds, showed that after the blood is cleared of the parasites, the bone marrow and spleen continue to harbor the disease and, relapses could be triggered by administering either ultraviolet light or adrenaline injections (Ben-harel, 1923).

2.2.22 The role of avian malaria in human malaria immunity

Prior research on avian malaria was very interested in the great defense that birds mount against the malaria parasites (Rivero & Gandon, 2018). Birds that had recovered from a *Plasmodium* infection might get re-infected, and birds that had chronic infections could no longer be successfully immunized with the same parasite species (Moldovan, 1912). This experiment was possible, with the observation that avian malaria parasites lingered in bird blood for years after the first infection (Rivero & Gandon, 2018). Other experiments were performed where, birds with a chronic parasite infection were vaccinated with the same parasite strain, a new strain of the same parasite species, or a different parasite species (Hewit, 1940). These investigations led to the first immunization trials using serum or attenuated parasites (Rivero & Gandon, 2018). Some of these studies showed that canaries could be protected against future infections, by being injected with a significant volume of *P. circumflexum*-infected bird serum (Manwell & Goldstein, 1938). However, the level of protection was highest when the serum came from birds afflicted with the

same parasite strain (Rivero & Gandon, 2018). Although the concentrations may be quite low, these studies showed humoral antibodies in the blood serum of birds which had avian malaria (Hewit, 1940).

The feasibility of using inactivated sporozoites of malaria parasites for immunization was first established in avian malaria (Sergent, 1910). Birds that were inoculated with *P. gallinaceum* sporozoites that had undergone radiation treatment, exhibited some resistance to a future infection (Russell & Mohan, 1942). These studies showed the efficacy of sporozoite vaccines, which inspired research on rodent malaria (Nussenzweig *et al*., 1967) and malaria in humans (Clyde, 1973). These studies also aided in the development of the first sporozoite-based malaria vaccines against *P. falciparum*, which have undergone field trials in several African countries (Richie *et al*., 2015).

2.2.23 Drug therapy and drug resistance studies using avian malaria

To investigate the effectiveness of chemical compounds other than quinine, in treating malaria in humans, avian malaria parasites were used as the experimental host (Hewit, 1940). When the Indonesian quinine supply during World War I was cut off, it became urgent to produce synthetic antimalarials (Rivero & Gandon, 2018). Avian malaria-infected canaries were used for the in vivo efficacy testing of the first synthetic antimalarials, plasmoquin and atabrin (Rivero & Gandon, 2018). *P. gallinaceum*, was used to develop a program for screening and clinical testing of antimalarials in the USA (Coatney *et al*., 1953). Avian malaria was also used to illustrate how *Plasmodium* parasites might quickly develop drug resistance (Rivero & Gandon, 2018). Researchers successfully created *P. relictum* strains that were resistant to quinine, by giving infected canaries increasing dosages of the medication over many successive passes (Bishop & Birkett, 1947). Studies also showed an experimental evolution of drug resistance in *P. gallinaceum*

to the human malaria medicines proguanil and pyrimethamine (Bishop & Birkett, 1947; Greenberg & Bond, 1954).

2.2.24 The molecular era and empirical field studies

The morphology of the blood stages has played a significant role in the classification of avian malaria parasites (Rivero & Gandon, 2018). There are many morphospecies of avian malaria parasites, which has made it difficult for researchers studying the disease to distinguish between different parasite species solely based on their morphology (Rivero & Gandon, 2018). Only a few highly skilled laboratories have been able to identify the parasites that cause avian malaria (Rivero & Gandon, 2018). The field of avian malaria attracted ornithologists from around the world when the first molecular protocols were developed for the characterization of avian malaria lineages based on the amplification of about a 500 bp partial region of the mitochondrial cytochrome-b gene, marking the beginning of a new era in avian malaria research (Bensch *et al.*, 2000).

2.2.25 Origin and maintenance of malaria diversity

The diversity of parasites that cause avian malaria requires evolutionary justification (Rivero & Gandon, 2018). What ecological factors influence the evolution and conservation of genetic diversity and the coexistence of malaria lineages with narrow (specialist) or broad (generalist) host ranges (Rivero & Gandon, 2018)? A thorough phylogenetic reconstruction of the diversity of parasite species and their bird hosts in the New World was conducted in order to identify reasons for the speciation processes in avian malaria parasites (Ricklefs *et al*., 2014; Ricklefs & Fallon, 2002; Ricklefs, Fallon, & Bermingham, 2004). Since there is little evidence of cospeciation between avian malaria parasites and their hosts, it is possible that the parasite lineages diverged from those that gave rise to most bird taxa (Ricklefs *et al*., 2014; Ricklefs & Fallon, 2002). Some

claim that frequent bird host change is the primary factor controlling how avian malaria diversifies (Ricklefs & Fallon, 2002). This is because closely related parasite lineages are typically linked to hosts that are far apart phylogenetically (Ricklefs & Fallon, 2002).

However, it is still unclear how diversification works (Rivero & Gandon, 2018). The work by Javier Pérez-Tris *et al*. in 2007 raised the potential that parasite divergence could take place in sympatry (Javier Pérez-Tris *et al*., 2007). Ricklefs *et al*. in 2014 claimed that as most known avian malaria vectors are extremely generalists, this will break local reproductive isolation and favor gene transfer across parasites in sympatric hosts (Ricklefs *et al*., 2014). Therefore, speciation will entail local host-pathogen coevolution and the adaptation of parasites to new hosts in allopatry, which happens as bird hosts spread to new geographic areas (Rivero & Gandon, 2018). Allopatric speciation followed by secondary sympatry would cause the development of closely related parasite lineages (Pérez-Tris *et al*., 2007; Ricklefs *et al*., 2014). Exploring the evolutionary processes that resulted in the diversification of parasites in nature is made possible by the unprecedented diversity of the avian malaria pathogen and hosts (Rivero & Gandon, 2018). Variations in host resistance to avian malaria parasites may produce a selective pressure that keeps the genetic diversity of malaria populations at high levels (Rivero & Gandon, 2018). This was shown by studies on the population genetics of Hawaiian amakihi, which revealed signs of genetic structuring among bird populations in response to various pressures from avian malaria (Foster *et al*., 2007). Some of the most exciting instances of how allelic variability in the major histocompatibility complex (MHC) genes is created and maintained in infected hosts come from research on avian malaria (Bonneaud *et al*., 2006; Rivero & Gandon, 2018). A correlation between the prevalence of *Plasmodium* and the quantity of MHC class I alleles has been shown in avian malaria studies (Westerdahl, 2005). Similar observations were made in wild sparrows as well. It

was shown that two different bird populations were protected from the same *Plasmodium* lineage by two different MHC alleles, which supported the existence of local adaptation (Bonneaud *et al*., 2006). The coexistence of specialized and generalist parasite lineages has also been a contentious issue and a focus of ongoing research in avian malaria (Drovetski *et al*., 2014; Hellgren, Pérez-Tris, & Bensch, 2009; Medeiros, Ellis, & Ricklefs, 2014; Svensson-coelho *et al*., 2016).

The trade-off hypothesis states that host specialization gives parasites an advantage because specialist parasite lineages grow more adept at exploiting their hosts for a narrower host range (Rivero & Gandon, 2018). The niche breadth hypothesis suggests that "the ability to exploit various hosts permits parasites to gain larger local abundances and greater global distributions," which highlights the advantage of generalist parasites (Rivero & Gandon, 2018). Both hypotheses have been addressed in many studies (Rivero & Gandon, 2018). According to the trade-off hypothesis, specialist lineages are more common than generalist parasites in shared host species, and this was demonstrated using an ensemble of avian malaria parasites from North America (Medeiros *et al*., 2014). However, Hellgren *et al*. demonstrated that European avian malaria specialists are less common than generalist parasites in their hosts (Hellgren *et al*., 2009). Being a generalist, according to researchers, may be helpful in the fight against avian malaria since it will enhance the variety of hosts that the parasites would inhabit when they are spread by generalist vectors (Rivero & Gandon, 2018). A generalist has more opportunities for transmission than a specialist, which leads to a higher parasite incidence in the host group (Rivero & Gandon, 2018). Experimental research is required to determine the advantages and disadvantages of being an specialist or generalist in avian malaria (Rivero & Gandon, 2018).

2.2.26 Avian malaria as an experimental model for the ecology and evolution of *Plasmodium*

After *Plasmodium* parasites were found in Central African rats in 1948, avian malaria studies decreased (Rivero & Gandon, 2018). Several, if not all, experimental studies on malaria transitioned from using birds to rodents (Rivero & Gandon, 2018). However, avian malaria has recently come back into the spotlight as a novel and highly practical experimental system used to study the evolutionary ecology of malaria in both the lab and the field (Rivero & Gandon, 2018). The unprecedented phenotypic and genetic diversity of avian malaria that has been documented to date provides many opportunities for studying the selective pressures under which hosts and parasites develop (Rivero & Gandon, 2018). Because of a variety of other advantages associated with avian malaria, birds make up a unique animal model for the study of malaria ecology and evolution (Rivero & Gandon, 2018). The high prevalence of avian malaria has enabled field ornithologists a relatively easy access to infected birds, the ability to mark and recapture birds, and the ability to measure and alter the immunity, physiology, and behavior of birds infected with various parasite lineages (Rivero & Gandon, 2018). Avian malaria has been reintroduced as a potential experimental malaria model, which provides a more accurate representation of the disease as it manifests itself in the field, and laboratory studies (Rivero & Gandon, 2018). Both the *Plasmodium* parasites and its primary natural vector, the *Culex pipiens* mosquito, are widespread and simple to keep alive in laboratory settings (Rivero & Gandon, 2018). Recent investigations have used avian malaria experiments in the field or in the lab to answer several evolutionary problems (Rivero & Gandon, 2018).

2.2.27 Parasite virulence

Avian malaria parasites have the potential to place a significant selective pressure on their bird hosts because they are frequently pathogenic (McKnight *et al*., 2017). *Plasmodium* infections in

birds present side effects such as reduced red blood cell counts, enlarged spleen and liver, occasionally necrosed spleen and liver, brain hemorrhages, and edemas (Hewit, 1940). Avian malaria can have severe effects on hosts, especially when new parasite lineages are unintentionally introduced into a population of uninfected hosts (Rivero & Gandon, 2018). In contrast, endemic locations typically experience sublethal effects of avian malaria on host fitness (Rivero & Gandon, 2018).

Wild bird population studies also showed significant correlations between malaria infection and bird survival, behavior, mate choice, and reproductive success (Lachish *et al*., 2011; Lapointe *et al*., 2012). However, because there was no information on the direction of the effect, it was difficult to use the available data to draw conclusions about fitness effects (Knowles *et al*., 2010). Two alternative approaches were used in field trials to examine the impact of avian malaria on bird reproductive success (Rivero & Gandon, 2018). Using antimalarial medications, the effects of persistent malaria infections on fitness in a population of breeding blue tits was assessed (Knowles *et al*., 2010). The findings showed that the offspring of treated females significantly outperformed those of their untreated counterparts for hatching and fledging success (Knowles *et al*., 2010). Other studies, which involved changing the size of the great and blue tits' respective broods, demonstrated a strong negative association between parental investment and malaria infection (Knowles *et al*., 2010; Richner *et al*., 1995). The findings showed that birds with greater reproductive pressure had higher malaria prevalence, showing a trade-off between parental and immunological investment (Knowles *et al*., 2010; Richner *et al*., 1995).

Studies showed that birds with malaria have significantly shorter lifespans than birds without the disease, in long-term research using great reed warblers from Sweden (Asghar *et al*., 2015). They also proved that a higher rate of telomere shortening in the blood and internal tissue cells of

Plasmodium-infected individuals is the underlying reason for the shorter lifetime reported (Asghar *et al*., 2015; Asghar *et al*., 2016). Later, this telomere research from birds applied to humans with malaria, and it was discovered that *P. falciparum* infections are linked to faster cellular aging (Asghar *et al*., 2018). The virulence of malaria parasites to their mosquito vectors has been a topic of contention, although the effects of malaria transmission and epidemiology are quite clear (Ferguson & Read, 2002).

The potential adaptive nature of *Plasmodium* pathogenicity to mosquitoes in avian malaria has also been studied (Rivero & Gandon, 2018). In mosquitoes infected with *P. relictum*, there is a trade-off between longevity and fecundity (Vézilier *et al*., 2012). Infected mosquitoes with *Plasmodium* have much lower fecundity and significantly longer lifespans (Rivero & Gandon, 2018). The parasite used the decreased fecundity as an adaptive strategy to improve mosquito survival and the continuity of the parasite's life cycle (Rivero & Gandon, 2018).

2.2.28 Parasite plasticity

As an adaptation to environmental variability, many organisms develop plasticity (Cornet *et al*., 2014). Avian malaria served as the first illustration of how *Plasmodium* parasites alter their transmission tactics in response to the host environment (Rivero & Gandon, 2018). To adapt to unfavorable environmental conditions, parasites are predicted to plastically change their life cycle features (Rivero & Gandon, 2018). This was shown in a study on *P. gallinaceum*, where it was demonstrated that a considerable rise in the proportion of gametocytes was observed in hens treated with an antimalarial (Bishop 1954). This finding has been classified as a plastic technique used by the parasite to avoid the negative effects of the medicine and was validated in rodent and human malaria parasites (Buckling *et al*., 1999). *Plasmodium* parasites could alter the male to female ratio of gametocytes in response to a negative host environment (Paul, 2000). According

to the local mate competition theory, *P. gallinaceum* gametocytes have a male to female sex ratio at the start of an infection, but as the infection advances and the host condition deteriorates, the male to female sex ratio increases (Rivero & Gandon, 2018). The deterioration of the environment causes a change to a more male-biased sex ratio (Paul, 2000). The shift in the sex ratio can be used as an adaptive method to ensure that all female gametocytes are fertilized (West *et al*., 2002).

It was also shown through research on avian malaria that *Plasmodium* relapses result from a plastic reaction to insects (Rivero & Gandon, 2018). *Plasmodium* parasites increased their within-host replication as a plastic response to the biting of uninfected vectors, which led to higher rates of transmission to the mosquito vector (Cornet *et al*., 2014; Pigeault *et al*., 2018).

The first comprehensive transcriptome of an avian malaria species also offered new information regarding the adaptability of the parasite (Rivero & Gandon, 2018). Using a clonal strain of *P. ashfordi* (pGRW2), four wild-caught siskins (Carduelis spinus) were infected (Videvall *et al*., 2017). At two distinct stages of the infection, the parasite's gene expression was measured (Videvall *et al*., 2017). The findings showed significant changes in the parasite transcriptomes between the four hosts (Videvall *et al*., 2017). The most significant genes were found to be those involved in cytochrome oxidase c, heat shock proteins, and aminopeptidases, which are involved in stress response, haemoglobin digestion, and parasite energy metabolism (Videvall *et al*., 2017). These results could be the consequence of the parasite being modulated differently by each host or, the parasites changing their gene expression levels to adapt to the environment of the hosts (Rivero & Gandon, 2018).

2.2.29 Parasite manipulation

Many pathogens that are spread through vectors can change how their vectors behave to increase their own transmission (Rivero & Gandon, 2018). An uninfected vector must bite an infected host before an infected vector may then bite an uninfected host for malaria transmission to occur successfully (Anderson *et al*., 1999; Lacroix *et al*., 2005). Nevertheless, there is evidence to support the theory that *Plasmodium* may control its vectors during both the infected and uninfected phases of its life cycle (Anderson *et al*., 1999; Lacroix *et al*., 2005). As part of their research on the avian malaria system, Cornet *et al*. examined whether *Plasmodium* parasites could make birds more attractive to mosquitoes (Cornet *et al*., 2012). In order to accomplish this, scientists gave mosquitoes the option to choose between a bird that was *Plasmodium*-infected or not (Rivero & Gandon, 2018). The findings showed that, although infected blood meals are expensive for the vector (Vézilier *et al*., 2012), both infected and uninfected mosquitoes were found to feed more frequently on infected birds than uninfected birds (Cornet *et al*., 2012). In 2018, Gandon examined how mosquitoes' host-choice preferences have evolved and suggested that the behavior shown in avian *Plasmodium* may have changed because of the pathogen's capacity to alter the odor of infected birds, making them more noticeable to the vectors (Gandon, 2018).

In order to better understand how malaria parasites have evolved to manipulate mosquitoes, avian malaria was used as an excellent experimental model for investigating plausible reasons for these parasites' behavioral tendencies (Rivero & Gandon, 2018). Many parasites now exist that can influence their host's immune system (Frank & Schmid-Hempel, 2008). According to some researchers, *Plasmodium* parasites can also influence the immune system of their invertebrate host (Mahanta *et al*., 2018). After ingesting a blood meal containing *P. gallinaceum* parasites, it was demonstrated that mosquito immune responses decreased (Boëte *et al*., 2002).

2.3 Why study avian malaria?

Instead of using models like *P. berghei* in mice or *P. falciparum* in Aotus monkeys, which are investigated in artificial hosts, researchers can analyze the interaction between malarial parasites and their natural host, which has naturally evolved immunity. Other benefits of studying malaria in birds include the lower cost of chickens compared to mice and the capacity to get a larger amount of chicken blood for investigation and organism culture (Nagao *et al*., 2008). Compared to the malaria parasites of rodents, phylogenetic analyses showed a closer link between the avian malaria parasite *P. gallinaceum* and *P. falciparum* (Nagao *et al*., 2008), which will be very useful in understanding the biology of the human *P. falciparum* parasite (Rivero & Gandon, 2018). *P. gallinaceum* is significantly simpler to maintain than rodent parasites, which has led to its continued usage in fundamental malaria research (Nagao *et al*., 2008). *P. gallinaceum* may also infect a variety of avian hosts and typically exhibits significant parasitaemia, which are crucial aspects in its application to investigations in cell biology and biochemistry (Nagao *et al*., 2008). Health researchers and veterinarians are particularly interested in avian malaria because it can affect non-adapted birds (Rivero & Gandon, 2018). Its vectors share a phylogenetic relationship with human malaria species, and it has the potential to spread other vector-borne zoonotic diseases to humans (Adouchief *et al*., 2016; Aguilar *et al*., 2011; Brugman *et al*., 2018; Campbell *et al*., 2011; Delfraro *et al*., 2011; Endy & Nisalak 2002; Hassing *et al*., 2010; Iwamoto *et al*., 2003; Molaei *et al*., 2013; Neumayr *et al*., 2012; Pecorari *et al*., 2009; Rocco *et al*., 2005; Yang *et al*., 2004; Yu *et al*., 2016). Aside from malaria, mosquitoes can spread other vector-borne illnesses such as chikungunya, dengue, Japanese encephalitis, lymphatic filariasis, Usutu virus, Rift Valley fever, and west nile virus (WNV) (Takken & Verhulst, 2013). Because the mosquito vectors also

act as bridge carriers of bacteria and viruses, the disease is a very significant aspect in research of human zoonotic diseases (Armstrong & Andreadis, 2010; Hamer *et al*., 2008).

2.4 Avian *Haemoproteus*

Approximately 200 species of the most prevalent haemosporidian parasites, *Haemoproteus*, have been identified in 1700 bird species (Nardoni *et al*., 2020). The real malarial parasites that infect vertebrates are closely linked to the avian *Haemoproteus* species (Atkinson *et al*., 2009). Atkinson *et al*., also clarified that contrary to *Plasmodium* parasites, *Haemoproteus* parasites do not reproduce asexually in tissues but in the circulating erythrocytes. These parasites are among the most prevalent blood parasites in wild birds. However it is uncertain how important they are to wild bird populations. The parasites are used by avian ecologists to investigate how disease affects host fitness and sexual selection, but their effectiveness is significantly hampered by a lack of understanding of their life cycles, vectors, and etiology (Atkinson *et al*., 2009). According to Atkinson *et al*. (2009), several highly pathogenic *Haemoproteus* species can make avian hosts suffer from painful, exhausting, and weak muscles because of severe myositis. However, there are just a few documented occurrences of this illness (Cardona *et al*., 2002). *Haemoproteus* parasite infections are frequently referred to as hemosporidiosis (Atkinson *et al*., 2009). Although there are significant life history traits that set them apart from *Plasmodium* species, avian haemosporidiosis is more commonly known as avian malaria (Valkĭunas, 2005).

2.4.1 History of *Haemoproteus* parasites

Haemoproteus parasite was first observed by the Russian zoologist Danilewsky, in unstained blood smears from infected birds (Atkinson *et al*., 2009). He described the parasites as having clear, colorless, transparent vacuoles with various shapes and sizes and contained several refractile glossy black granules (Atkinson *et al*., 2009).

The diversity and wide host range of these parasites were seen by avian malaria researchers after the discovery of Giemsa staining to visualize parasites (Garnham, 1966), but information about their life cycles, host specificity, and transmission vectors was still lacking (Atkinson *et al*., 2009). According to reports by Atkinson *et al*., there was a lot of misunderstanding over what made up the *Haemoproteus* species, and the taxonomy of the genus has been undergoing a steady change for more than a century. The study of the asexual phases of the parasite inside the invertebrate vector and the pre-erythrocytic stage within the vertebrate host was prompted by the discovery of hippoboscid and ceratopogonid flies as insect vectors of *Haemoproteus*. Over the past 50 years, most investigations on bird *Haemoproteus* species have been surveys and taxonomic descriptions. Because these parasites make excellent models for testing evolutionary ideas, avian ecologists and evolutionary biologists were interested in them during the first two decades of the twentieth century. There is currently some accumulating data regarding how these parasites affect host survival and reproduction (Atkinson *et al*., 2009).

2.4.2 Distribution of avian *Haemoproteus*

The temperate and tropical regions of the world are home to a variety of avian *Haemoproteus* species (Atkinson *et al*., 2009). The varied environments of the ceratopogonid and hippoboscid flies are most likely related to the distribution of the parasites (Greiner *et al*., 1975). The parasites have been discovered in most regions of the world where the vectors exist, including remote islands in the Central Pacific (Padilla *et al*., 2004). The Holarctic, Ethiopian, and Oriental zoogeographic regions had the highest *Haemoproteus* species diversity and abundance, whereas the Neotropical and Australian regions had the lowest number of species recorded (Mendes *et al*., 2005). Due to a lack of adequate vectors, haemoproteids are absent from the high arctic tundra in North and South America (Greiner *et al*., 1975).

2.4.3 Etiology of *Haemoproteus* parasites

The parasites of the genus *Haemoproteus* are members of the phylum Apicomplexa, order Haemospororida, and family *Plasmodiidae* (Valkiūnas & Iezhova, 2022). These parasites show intraerythrocytic development, black pigment granules produced by the breakdown of the host haemoglobin, and a lack of asexual reproduction in circulating erythrocytes (Atkinson *et al*., 2009). The morphology of circulating gametocytes, the host specificity, and the distinctive alterations in host erythrocyte morphology are used to identify all species of *Haemoproteus* (Atkinson *et al*., 2009). Five morphologically distinct *Haemoproteus* gametocytes can be identified by their sizes, shapes, and distance from the erythrocyte nucleus (Atkinson *et al*., 2009). Based on phylogenetic analysis using mitochondrial gene sequences, *Haemoproteus* was classified as a polyphyletic group and assigned to the same clade as *Plasmodium* (Perkins & Schall, 2002). A second analysis using four genes divided avian haemoproteids into two clades: a clade of columbiform parasites that use hippoboscid flies as vectors and a clade of ceratopogonid fly-borne parasites (Martinsen *et al*., 2007). Following these analyses, the parasites were subsequently separated into two genera: *Haemoproteus*, which hippoboscid flies transmit, and *Parahaemoproteus*, transmitted by ceratopogonid flies (Martinsen *et al*., 2007).

2.4.4 Epizootiology of avian *Haemoproteus* parasites

Haemoproteus reproduces through both sporogony (in its vector) and merogony (in its bird host) (Figure 2). From the description of the *Haemoproteus* life cycle reported by Atkinson *et al*., when a blood meal from an infected host is taken, it contains female macrogametocytes and male microgametocytes, which are the mature sexual stages of the parasite. The sexual stages produce an ookinete, a motile zygote, by gametogenesis and fertilization in the vector's midgut. During the asexual sporogonic cycle, ookinetes penetrate the midgut wall and develop as spherical oocysts

beneath the midgut basal lamina. The parasite develops similarly in both vectors, but the size of the oocysts, the quantity of sporozoites generated, and the length of the sporogony are different. Oocysts then burst, releasing sporozoites into the insect's haemocoel. During the subsequent blood meal, they enter the salivary glands and exit through the salivary ducts. It is unclear what influences the growth of a specific *Haemoproteus* species in a specific vector species. There is no proof that ceratopogonid flies may spread the same *Haemoproteus* species as hippoboscid flies (Atkinson *et al*., 2009).

Hippoboscid fly populations are more stable in tropical and subtropical locations, due to high *Haemoproteus* prevalences and transmission rates (Sol *et al*., 2000). *Haemoproteus* transmission cycles are influenced by the migration behavior of the long-distance migrants that spread parasites both inside and between continents (Hasselquist & Bensch, 2007). Some avian *Haemoproteus* species are transmitted on breeding grounds, whereas others may be transmitted on both breeding grounds and wintering areas in the tropics and subtropics (Atkinson *et al*., 2009). This implies that geographic locations or connections between vectors and parasites may be implicated in transmission (Garvin *et al*., 2004; Garvin *et al*., 2003; Hasselquist & Bensch, 2007; Hellgren *et al*., 2007).

The intensity of *Haemoproteus* infection is influenced by levels of host immunity, seasonal variations in photoperiod, and reproductive hormone fluctuations (Atkinson *et al*., 2009). Infection intensity can also be influenced by immune system alterations caused by stress related to reproductive efforts (Siikamäki, 1998), food availability (Appleby, Anwar & Petty, 1999) concurrent parasitic infections (Cox, 1987), and sometimes exposure of the birds to predators.

Vector abundance and distribution may also be affected by extrinsic factors, including habitat, geographic location, and season (Mendes *et al*., 2005; Sol *et al*., 2000). That different *Haemoproteus* species can be found in both vast and small landscapes (Sol *et al*., 2000; Wood *et al*., 2007) suggesting that vector distribution and abundance may be the key factor influencing prevalence (Atkinson *et al*., 2009). Other variables that may induce variations in parasite prevalence include winter mortality in affected birds, new infections connected to insect vectors, and transmission to young birds which were not infected (Atkinson *et al*., 2009).

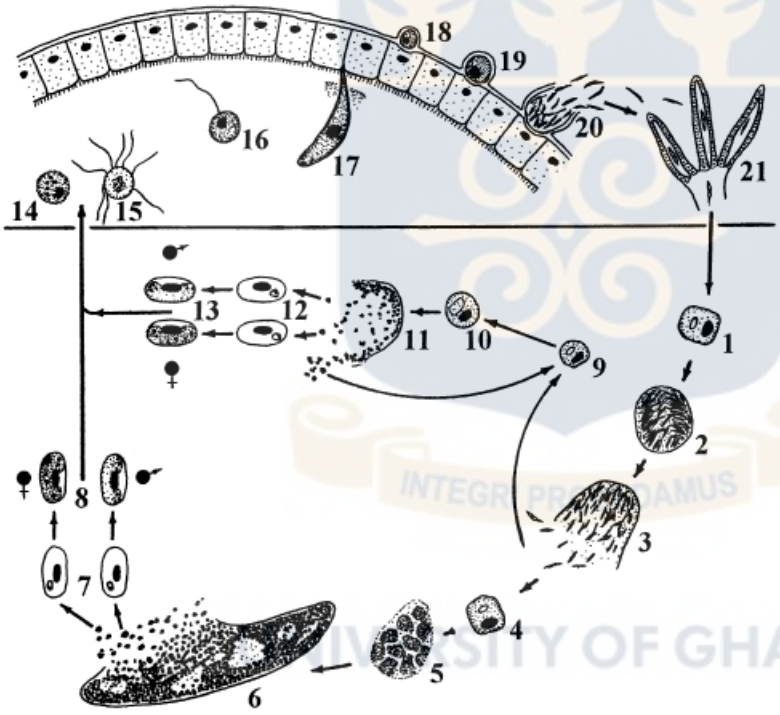

Figure 2: Diagrammatic representation of the life cycle of bird haemoproteids (*Haemoproteus mansoni*)

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"Upper part, in vector; lower part, in bird: *1*–sporozoite in endothelial cell; *2*, *3*–exoerythrocytic meronts of the first generation with elongated merozoites; *4*–merozoite in endothelial cell; *5*, *6*–growing and mature megalomeronts in skeletal muscles, respectively; *7*–merozoites in erythrocytes; *8*–mature gametocytes; *9*–merozoite in reticuloendothelial cell in spleen; *10*, *11*–growing and mature meronts in spleen, respectively; *12*–merozoites in erythrocytes; *13*–mature gametocytes; *14*–macrogamete; *15*–exflagellation of microgametes; *16*–fertilization of macrogamete; *17*–ookinete penetrating the peritrophic membrane; *18*–young oocysts; *19*, *20*–sporogony; *21*–sporozoites in the salivary glands of vector "(Valkĭunas, 2005).

2.4.5 Clinical signs of avian *Haemoproteus* infections

Clinical symptoms of *Haemoproteus* infections in birds appear only when the illness is in its acute phase, and the number of tissue meronts and erythrocytic parasitaemia increase (Atkinson *et al*., 2009). This was shown in Northern Bobwhites with naturally occurring *H. lophortyx* infection which showed apprehension to move, ruffled feathers, a downcast expression, loss of balance, and trouble walking (Cardona *et al*., 2002). It was also seen in infected Rock Pigeons that showed starvation, anaemia, and weakness (Coatney, 1933). Both naturally and experimentally infected birds had an increase in the number of blood cells as lymphocytes, heterophils, basophils, eosinophils, and monocytes; this could result from a cell-mediated response to both erythrocytic and pre-erythrocytic phases of the parasite (Garvin *et al*., 2003). The development of anaemia in host species of birds with *Haemoproteus* infection has been documented, but the mechanisms causing this are unknown (Atkinson *et al*., 2009). Anaemia can happen when an infected host lacks the physiological capacity to replace contaminated blood cells because of reproductive stress or a lack of sufficient nutritional sources (Atkinson *et al*., 2009).

2.4.6 Pathogenesis and pathology of avian *Haemoproteus* infections

Haemoproteus infections' pathogenesis is unknown (Atkinson *et al*., 2009). This is accounted for by a lack of knowledge regarding their development in both natural and experimental hosts (Atkinson *et al*., 2009). Certain host reactions were linked to thin-walled branching meronts, which were common in lung tissue (Garnham, 1966). In experiments conducted on Blue Jays infected with *H. danilewskii*, there no correlation found between host responses and pre-erythrocytic meronts on day 31 post infection (Atkinson *et al*., 2009). Juvenile jays displayed lesions in the liver, spleen, and lung tissue on day 57 following infection (Atkinson *et al*., 2009). The abnormalities comprised lymphocytic infiltrates and epithelial hyperplasia around the tertiary bronchi in the lung tissue, as well as cell necrosis in the liver (Atkinson *et al*., 2009). Hyperplasia, lymphocyte necrosis at random sites, and an increase in the quantity of macrophages, plasma cells, and Mott cells were among the histological abnormalities in the splenic tissue (Garvin *et al*., 2003). In several avian species, severe myositis has been linked to thick-walled megalomeronts (Atkinson *et al*., 2009).

2.4.7 Diagnosis of *Haemoproteus* infections

Atkinson *et al*., reported that the gold standard for diagnosing *Haemoproteus* is traditional microscopy. This is performed on a Giemsa-stained thin blood smear, which can show the absence of *Plasmodium spp*., specific erythrocytic meronts, and erythrocytic gametocytes with conspicuous golden-brown or black pigment granules. Individual species can be identified by their host specificity and the morphology of the gametocyte. Certain parasite lineages are identified using molecular techniques. Although these are genus-specific, the high sensitivity of molecular approaches helps detect very low-intensity infections (Atkinson *et al*., 2009). Species-specific molecular identification may become possible (Hellgren *et al*., 2007; Valkiūnas & Zehtindjiev,

2007). Atkinson *et al*., also reported that, *Plasmodium* and *Haemoproteus* are difficult to identify from one another, particularly in chronic infections with low gametocyte counts. It is also challenging to determine whether *Plasmodium*'s intracellular meronts are present or absent. It is challenging to diagnose *Haemoproteus* infections just based on the appearance of tissue stages. The tissue stages of *Leucocytozoon* and *Plasmodium* resemble the thin-walled oval or branched meronts that are typical of some species of columbiform haemoproteids. *Haemoproteus* megalomeronts are difficult to identify from *Leucocytozoon* megalomeronts (Atkinson *et al*., 2009). There have been many reports of aberrant *Leucocytozoon* infections caused by *Haemoproteus* megalomeronts (Borst & Zwart, 1972).

Many primers have been developed for the amplification of specific regions of the mitochondrial genome all *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* parasites (Hellgren *et al*., 2004), or distinguishing all three genera from each other using restriction digests (Beadell & Fleischer, 2005). The identification of specific parasite lineages and the determination of phylogenetic relationships depend on the sequencing of the PCR results (Atkinson *et al*., 2009).

2.4.8 Immunity for *Haemoproteus* infections

It is unknown how the immune system responds to *Haemoproteus* infections (Atkinson *et al*., 2009). The infected birds typically experience prolonged infection, develop spontaneous relapses that may become less frequent, and eventually recover (Coatney, 1933). Experimental data from several research suggests that birds with persistent infections have an immune response that boosts resistance to reinfection (Coatney, 1933). The persistent tissue stages of the parasites may cause the relapses linked to chronic infections (Atkinson *et al*., 2009).

2.4.9 *Haemoproteus* infections in domesticated birds

Reviews from Atkinson *et al*., shows that in domestic birds, *H. meleagridis*, a parasite of wild turkey, has been a potential threat to turkey production. There has not been evidence of this species infecting domestic turkeys because most commercial poultry facilities are far from habitats where wild turkeys range. There have also been outbreaks of *H. lophortyx* which naturally infects California quail reported in northern bobwhite quails raised in California. All these cases occurred because of the introduction of captive birds into areas outside of their natural ranges (Atkinson *et al*., 2009). *Haemoproteus* outbreaks were mostly seen in warm weather because of the increase in ceratopogonid flies populations (Cardona *et al*., 2002).

2.4.10 *Haemoproteus* infection in wild bird populations

In wild birds, it is difficult to assess the effects of individual *Haemoproteus* infections (Atkinson *et al*., 2009). There are a lot of co-infections observed in wild birds, where most *Haemoproteus*-infected avian hosts also carry *Leucocytozoon*, *Plasmodium*, and *Trypanosoma* (Atkinson *et al*., 2009). Wild birds that have haemosporidiosis, are difficult to locate for sampling or recover for necropsy, suggesting that *Haemoproteus* infections death may occur considerably more frequently than recorded (Atkinson *et al*., 2009; Bennett *et al*., 1993). It becomes highly challenging or impossible to record epizootics in locations where dead infected passerine birds are scavenged (Bennett *et al*., 1993). Birds infected with *Haemoproteus* species have been shown to have decreased survival, adverse effects on immunity, health, and reproductive success (Dawson & Bortolotti, 2000; Hõrak *et al*., 2001; Sol *et al*., 2003). Infections with *Haemoproteus* might have an indirect effect on host reproduction (Atkinson *et al*., 2009). There is evidence of a trade-off between illness resistance and reproductive effort, since there are only so many resources that can be allocated to each of these things (Atkinson *et al*., 2009). While many studies have been able to

show the effects of *Haemoproteus* infections on avian hosts, other studies also have demonstrated the association between infection with *Haemoproteus* and survivorship, mating success, reproductive success, host condition, or clinical chemistry (Dawson & Bortolotti, 2000).

2.4.11 Treatment and control of avian *Haemoproteus*

Some antimalarial drugs have been effective in treating *Haemoproteus* infections (Atkinson *et al*., 2009). In both domestic and wild birds, some antimalarial medications have shown effectiveness in reducing the severity of parasitaemia (Atkinson *et al*., 2009). These include atovaquone and proguanil hydrochloride combination, atebrine, plasmochin, chloroquine, primaquine, and mefloquine (Chand *et al*., 2018; Lee *et al*., 2018; Remple, 2004). Buparvaquone, an antitheilerial medication, was also efficient (Joshi *et al*., 2017). Housing captive birds in screened, Culicoides-proof buildings and dusting birds to prevent hippoboscid flies from feeding on them can help control *Haemoproteus* infections (Atkinson *et al*., 2009).

2.4.12 Management implications of avian *Haemoproteus*

Atkinson *et al*., reported that there are no current methods for preventing or controlling *Haemoproteus* infections in wild birds. According to them, reducing the number of vectors can reduce the spread of some *Haemoproteus* species. However, this method is not practical, because of the large number of ceratopogonid fly species that have made homes for their larvae in moist soil and tree cavities and are likely to evade any management measures. There are also hippoboscid ectoparasitic flies present on the wild birds, and cannot be managed (Atkinson *et al*., 2009).

2.5 Avian *Leucocytozoon*

Leucocytozoon species, the least researched haemosporidian (Nardoni *et al*., 2020) is usually reported in the Northern temperate areas, where both the parasite and its vectors are common

(Nardoni *et al*., 2020). *Leucocytozoon* is a protozoan parasite that causes vector-borne leucocytozoonosis disease in birds (Atkinson *et al*., 2009). Several species of *Leucocytozoon* have been documented, but only few are pathogenic (Atkinson *et al*., 2009). Waterfowl, pigeons, galliforms, raptors, and ostriches are among the bird species that are susceptible to leucocytozoonosis (Valkĭunas, 2005). A number of *Leucocytozoon* species are to blame for domesticated duck and poultry deaths (Atkinson *et al*., 2009). At the level of bird orders, all *Leucocytozoon* species are host-specific; some are at the family level (*L. simondi*), while others are at the species level (*L. caulleryi* and *L. smithi*) (Atkinson *et al*., 2009). Their life cycles are comparable to those of the genera, *Plasmodium* and *Haemoproteus* (Atkinson *et al*., 2009). Except for *L. caulleryi*, which is spread by biting midges of the family *Ceratopogonidae*, they are all transmitted by distinct black flies of the *Simuliidae* family (Valkĭunas, 2005).

2.5.1 Synonyms of leucocytozoonosis

Other names for leucocytozoonosis include Bangkok hemorrhagic illness (which specifically refers to *L. caulleryi* infection in domestic chickens in South and Southeast Asia), haemosporidian disease, *Leucocytozoon* disease, and haematozoan disease (Atkinson *et al*., 2009).

2.5.2 History of avian *Leucocytozoon*

In a morphological analysis of leucocytozoids of crows, magpies, and rooks in the Russian state of Georgia in 1893, Sakharoff made the first official description of *Leucocytozoon* (Atkinson *et al*., 2009). Besides being the first to stain blood films and describing the species *as L. danilewskyi*, he also displayed a color image of the *Leucocytozoon* gametocytes (Atkinson *et al*., 2009). In 1908, Sambon first defined the genus *Leucocytozoon*, and Berestneff first used the name *Leucocytozoon* in 1904 to identify several species of owls, rooks, and crows (Atkinson *et al*., 2009). *Leucocytozoidae* was later established by Fallis and Bennett (Fallis & Bennett, 1961). The genus

Akiba was established in 1965 to describe a leucocytozoid species (*Akiba caulleryi*) that was spread by biting midges rather than black flies (Bennett *et al*., 1965). Currently, the genus *Akiba* is thought to be a subgenus of the genus *Leucocytozoon* (Valkĭunas, 2005). *Leucocytozoon* was originally chosen as the name for these parasites, since the parasites only lived in leukocytes. It was later discovered that the gametocytes of this parasite were also infecting erythrocytes (Atkinson *et al*., 2009).

2.5.3 Distribution of avian *Leucocytozoon*

Except for Antarctica, *Leucocytozoon* species are found all around the world (Valkiunas, 2005). The presence or absence of running streams and rivers, where the vectors develop, affects the spread of *Leucocytozoon* parasites within various zoogeographic zones (Valkiunas, 2005). The Holarctic, Ethiopian, and Oriental zoogeographic regions include all known species of *Leucocytozoon*, with a few species also present in the Neotropical and Australian regions (Atkinson *et al*., 2009). The Holarctic has the biggest diversity of species, the highest prevalence of leucocytozoids, and the greatest number of species that are unique to a given zoogeographic region (Atkinson *et al*., 2009).

2.5.4 Host range of avian *Leucocytozoon*

In 2000, Clements found species of *Leucocytozoon* in 113 of the 204 avian families and 22 of the 28 orders of birds studies (Atkinson *et al*., 2009). The highest numbers of *Leucocytozoon* species were documented in Passeriformes (8 species), Galliformes (7), and Coraciiformes (4). *Leucocytozoon* parasites were discovered in about 30% of the bird species studied for blood parasites, which represents about 45% of all bird species worldwide (Valkĭunas, 2005). *Leucocytozoon* records in certain bird orders may be unintentional, as reported by Atkinson *et al*., about the Common Loon (*Gavia immer*) in the Gaviiformes order. According to them, the loon

was in captivity in an outdoor pen for some time, exposed to arthropod vectors, and was suffering from aspergillosis, which impaired its immune system. This immunocompromised condition is likely to make the bird susceptible to infection by a *Leucocytozoon* parasite from other birds within and around the facility (Atkinson *et al*., 2009). They reported a similar case where infections with *L. struthionis* were found only in chicks of ostriches (*Struthio camelus*) up to 7 weeks old, but never in adult birds. Leucocytozoonosis occurs mainly in birds of the families *Anatidae* (ducks, geese, and swans) and *Columbidae* (pigeons and doves), and less often in birds of the families *Accipitridae* (hawks, eagles, and kites) and *Falconidae* (falcons and caracaras) (Atkinson *et al*., 2009).

2.5.5 Etiology of avian *Leucocytozoon*

Protozoan parasites known as *Leucocytozoon* species are categorized as belonging to the phylum Apicomplexa, class Coccidea, subclass Coccidia, order Haemosporida, and family *Leucocytozoidae* (Atkinson *et al*., 2009). A huge number of *Leucocytozoon* species were described by early researchers using the theory that "a new host equals a new species", many of which had minimal or no morphometric differences (Atkinson *et al*., 2009). Two morphological forms of gametocytes described were round or elongated, occurring in the blood of the avian host (Atkinson *et al*., 2009).

2.5.6 Epizootiology of avian *Leucocytozoon*

According to Atkinson *et al*., leucocytozoids use biting dipteran flies as vectors throughout their life cycle (Atkinson *et al*., 2009). Except for *L. caulleryi*, which employs biting midges of the genus *Culicoides*, all are *Simuliidae* (black fly) species. The life cycle of *L. simondi* has received the most attention (Figure 3). When flies bite birds during a blood meal, sporozoites are introduced into the bloodstream of the avian hosts. These sporozoites enter the hepatic cells, where they

transform into meronts of the first generation. These meronts develop into larger cells during 4 or 5 days, go through several nuclear divisions, and split into many distinct units known as cytomeres. These subsequently develop into multinucleated syncytia and uninucleated merozoites. Some of these syncytia and merozoites get into the bloodstream. After entering erythrocytes, these merozoites transform into gametocytes (round forms). Blood carries syncytia to a variety of organs, including the spleen, lymph nodes, liver, brain, and others, where macrophages engulf them and form megalomeronts. These megalomeronts contain many merozoites, which break off and enter leukocytes and lymphocytes before developing into gametocytes (fusiform or elongated forms). The dipteran vector is infected by the round or fusiform gametocytes (male gametocytes or microgametocytes and female gametocytes or macrogametocytes). The gametocytes undertake sexual reproduction and develop into a zygote, which then develops into an ookinete, after being consumed by a blood-feeding vector. The ookinete enters the vector's midgut, proceeds through sporogony, and creates sporozoites. When the insect vector ingests blood, the sporozoites move into the salivary glands, where they might be injected into the next bird (Atkinson *et al*., 2009). All leucocytozoids have first-generation meronts in the parenchymal cells of the liver, except for *L. caulleryi*, which develops in the endothelial cells of the capillaries of many organs (Valkiunas, 2005).

Atkinson and colleagues also reported that vectors are known for some leucocytozoid species. Some black fly species can transmit more than one species of *Leucocytozoon*, while others transmit more than one species of *Leucocytozoon*. The global distribution of the parasite is constrained by the range of the susceptible vectors, besides other ecological and behavioral considerations. It was discovered that the absence of flowing, oxygenated freshwater necessary for the flies to breed made the Curonian Spit free of *Simuliidae* vectors. Abiotic factors, such as favorable climatic

conditions, especially temperature, rainfall, humidity, and the presence or absence of flowing water, are also important in transmitting the leucocytozoids. Black flies need running water to reproduce, and this necessity affects leucocytozoids' ability to spread (Atkinson *et al*., 2009).

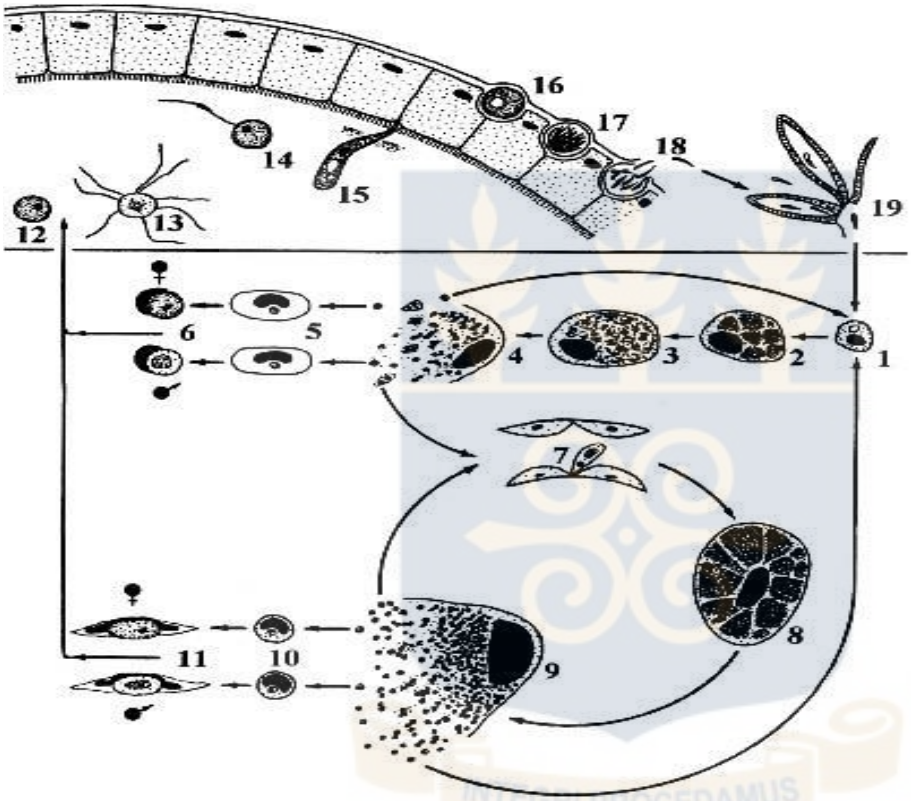

Figure 3: Diagrammatic illustration of the life cycle of *L. simondi*

"Upper section of the illustration represents events that occur in the vector and lower section represents events that occur in the bird. 1, sporozoite or merozoite in hepatocyte; 2–4, hepatic meronts; 5. merozoites in erythrocytes; 6, gametocytes in round host cells; 7, syncytia of merozoites in reticuloendothelial cells; 8 and 9, megalomeronts; 10, merozoites in mononuclear leukocytes; 11, gametocytes in fusiform host cells; 12, macrogamete; 13, microgamete that is exflagellating; 14, fertilization of macrogamete; 15, ookinete penetrating the peritrophic

membrane of the vector's gut wall; 16, young oocyst; 17 and 18, sprogony; 19, sporozoites in the salivary glands of the vector." (Atkinson *et al*., 2009).

2.5.7 Clinical signs of leucocytozoonosis

Atkinson *et al*., described leucocytozoonosis as typically not having a clear-cut clinical symptoms (Atkinson *et al*., 2009). Young ducks and geese are the most susceptible hosts to leucocytozoonosis, and they may pass away quickly after infection, but the sickness rarely claims older birds. Anaemia is the most significant clinical symptom. Some birds display anxious behaviours, including intense excitation and convulsions (Atkinson *et al*., 2009). Anorexia, lethargy, difficulty breathing, and diarrhea are further symptoms (Wobeser, 1997).

2.5.8 Pathogenesis and pathology of *Leucocytozoon* infection in birds

According to reports by Atkinson *et al*., the *Leucocytozoon* species *L. simondi*, *L. marchouxi*, and *L. toddi* are all harmful to wild birds. They also suggested that there is evidence that *L. danilewskyi* may cause harm to owls, although there is a lack of conclusive information. According to literature, *L. danilewskyi* infection may have decreased egg production, but necropsies, clinical, and histopathologic tests were not performed to confirm this (Atkinson *et al*., 2009). Waterfowl with fatal leucocytozoonosis have gross abnormalities, such as an enlarged spleen and liver, pale tissues, and thin blood (Wobeser, 1997).

2.5.9 Diagnosis of leucocytozoonosis

It is important to distinguish between the diagnosis of *Leucocytozoon* infections and the diagnosis of the disease brought on by *Leucocytozoon* species (leucocytozoonosis) (Atkinson *et al*., 2009). By looking at stained thin blood films generated from peripheral blood and identifying the distinctive gametocytes, *Leucocytozoon* spp. infections can be quickly and easily identified

(Atkinson *et al*., 2009). Leucocytozoonosis should be diagnosed by looking for the right clinical symptoms, including anaemia, as well as the characteristic gross and histologic lesions, and finding gametocytes of *Leucocytozoon* species in the blood (Wobeser, 1997). However, it must be kept in mind that young birds might die even in the absence of parasitaemia and that birds can be parasitized without having leucocytozoonosis (Wobeser, 1997). Other diagnostic methods include enzyme-linked immunosorbent assays (ELISA), immunoblot analysis, and latex agglutination tests employing recombinant R7 antigen, as well as serological tests such as agar gel precipitation, counter-immunoelectrophoresis, and immunofluorescence (Ito & Gotanda, 2004). To detect blood parasites in birds, additional molecular genetic assays, mostly PCR tests that focus on mitochondrial cytochrome b genes or small unit (18S) ribosomal RNA fragments have been described (Bensch *et al*., 2000; Fallon *et al*., 2003; Jarvi *et al*., 2002). However, none of these tests can distinguish *Leucocytozoon* beyond the genus level (Atkinson *et al*., 2009).

2.5.10 Natural resistance and immunity to *Leucocytozoon* infection

There is little information on naturally occurring leucocytozoid resistance, although there is some information on experimental *L. simondi* infections of domestic ducks, American black ducks, and mallards (Atkinson *et al*., 2009). Very little is known regarding acquired immunity to leucocytozoids, except for *L. caulleryi* infections in chickens and *L. simondi* infections in waterfowl (Atkinson *et al*., 2009).

2.5.11 Leucocytozoonosis in domestic birds

Significant disease in domestic birds is caused by six different *Leucocytozoon* species (Atkinson *et al*., 2009). These include *L. simondi*, which affects domesticated waterfowl in the United States, Canada, and Europe; *L. smithi*, which affects domesticated turkeys in the United States and Canada; *L. macleani*, which affects chickens in Southeast Asia; *L. struthionis*, which affects

captive ostriches in South Africa; *L. schoutedeni*, which affects chickens in sub-Saharan Africa and Southeast and Southern Asia; and *L. caulleryi* in chickens of many Southeast and Southern Asian countries (Valkĭunas, 2005). *L. smithi* and *L. caulleryi* cause significant economic losses in poultry in certain areas of the U.S. and Asia (Bennett *et al*., 1993). *L. smithi* causes mortality of poults and adult turkeys, but also causes a decrease in production and hatchability of eggs in those that survive (Jones *et al*., 1971).

2.5.12 Impacts of leucocytozoonosis in wild bird populations

According to Atkinson *et al*., it is unclear how leucocytozoonosis affects wild bird populations (Atkinson *et al*., 2009). Three species are of concern (*L. simondi* in ducks, *L. marchouxi* in doves and pigeons, and *L. toddi* in raptors), and there may be additional pathogenic species that are unknown. Avian populations may suffer negative sublethal effects from chronic infections. A wide range of additional infectious and noninfectious illness agents are frequently concurrently infected in wild birds. *Leucocytozoon* infections may have cumulative effects or interact with these disease agents synergistically, impairing behavior or health. Leucocytozoid infections may not directly cause death, but they might make hosts more vulnerable to predators or other disease agents, or they can make hosts less suitable for migration or reproduction. More research is required to understand the physiological and ecological implications of leucocytozoid infections (Atkinson *et al*., 2009).

2.5.13 Prevention, treatment, and control of leucocytozoonosis

Many methods have been used to prevent and treat clinical disease in domestic fowl caused by *L. caulleryi* and *L. smithi* (Atkinson *et al*., 2009). To protect hens from leucocytozoonosis, two vaccines have been created against the megalomeronts of *L. caulleryi* (Atkinson *et al*., 2009). One is a second-generation megalomeront-containing vaccine that has been formalin-killed, while the

other is a recombinant vaccine built around a second-generation megalomeront protein (Ito & Gotanda, 2004). Leucocytozoonosis can be treated with pyrimethamine or sufamonomethoxine combined with pyrimethamine (Atkinson *et al*., 2009). Clopidol is efficient at lowering the blood levels of *L. smithi* gametocytes in domestic turkeys, but it does not completely eradicate infections (Siccardi & Rutherford, 1974). Atkinson *et al*., reported that the application of *Bacillus thuringiensis israelensis* to running streams, the source of black fly vectors, has been used to investigate vector control in regions where domestic turkey populations are at risk of infection with *L. smithi*. However, it is challenging to prevent, treat, and control Leucocytozoonosis in free-living populations of wild birds (Atkinson *et al*., 2009).

2.5.14 Management implications of leucocytozoonosis

Leucocytozoon infections are presumably of little consequence for most free-ranging bird populations (Atkinson *et al*., 2009). Atkinson *et al*., also reported some avian populations that may be at risk because of the pathogenic strains of *L. simondi*, *L. marchouxi*, *L. toddi*, and other species. For the majority of wild populations, there is not much that can be done to lessen the effects of leucocytozoonosis. It may be wise to treat infections in situations involving small populations, fragments of endangered species, or rehabilitation facilities. When a large number of individuals can be easily collected, treating or immunizing a free-ranging population may be necessary. Small populations of birds that are endangered, or threatened might benefit from this strategy. It may also be helpful to treat streams with chemicals or biological control agents, to eradicate or minimize the vectors of black flies. To stop the transmission of a harmful strain of *Leucocytozoon* to other populations, they proposed that it could be wise to limit or eradicate any subpopulations of birds that carry it (Atkinson *et al*., 2009).

2.6 Haemosporidian research in Africa

The African continent harbours some of the highest bird diversity in the world. The avian malaria parasites found in these birds are as diverse as their hosts (Clark *et al*., 2014). This was confirmed by several studies conducted within the African subregions (Lauron *et al*., 2014; Loiseau *et al*., 2012; Lutz *et al*., 2015; Okanga & Cumming, 2013). Morphological examination of approximately 11 500 blood smears of birds from Africa found 70 *Haemoproteus* (subgenera *Haemoproteus* and *Parahaemoproteus*) and 13 *Plasmodium* species (Valkĭunas, 2005) with only 15 *Parahaemoproteus* and *Haemoproteus*, and 2 *Plasmodium* morpho-species endemic to Africa (Outlaw *et al*., 2017). From the year 1970 through the early 1990s, haemosporidian research was conducted on a vast species of avian hosts in Sub-saharan Africa using microscopy (Outlaw *et al*., 2017). Haemosporidian prevalence in Africa ranged from 11·5% in Senegal, 13% in Cameroon and 19·1% across sub-Saharan Africa, to 37% in East African savannah regions (Bennett *et al*., 1992). Two studies, one focusing on birds in the western African rainforests (Sehgal *et al*., 2004) and the other on birds in Uganda (Valkiūnas *et al*., 2005) highlighted the diversity of haemosporidian parasites in Africa using microscopy techniques. These two studies together screened 1276 individual birds and identified haemosporidian parasites in 27 bird families (Outlaw *et al*., 2017). Haemosporidian parasite prevalence in these two studies ranged from 28·6% to 61·9%, with Uganda recording the highest (Outlaw *et al*., 2017). Phylogenetic surveys on haemosporidians showed a higher parasite diversity and prevalence compared to microscopy (Outlaw *et al*., 2017). Haemosporidian parasite molecular surveys reported 45% *Plasmodium* infections in Western Congolian rainforests (Møller *et al*., 2011).

Most of the molecular parasite diversity in birds has focused on a few bird families, namely the sunbirds (*Nectariinidae*) and bulbuls (*Pycnonotidae*) from the Western African subregion

(Bonneaud *et al*., 2009; Chasar *et al*., 2009; Hellgren *et al*., 2007; Iezhova *et al*., 2011; Loiseau *et al*., 2010). The assessment of haemosporidian diversity in the Saharan region was seen in only one study in northeastern Nigeria for a few avian species (n = 9) (Waldenström *et al*., 2002). Other studies on the global parasite diversity listed sub-Saharan Africa as a biodiversity hotspot for haemosporidian parasites (Clark *et al*., 2014). Other studies showed a great diversity of *Plasmodium* parasite lineages from African sunbirds (Lauron *et al*., 2014).

A lot of variations observed in haemosporidian diversity across Africa could be attributed to research biases such as inconsistency in the collection and laboratory protocols (Outlaw *et al*., 2017). Many of the studies in Africa focused on the genera *Haemoproteus* and *Plasmodium* (Beadell *et al*., 2009; Bonneaud *et al*., 2009; Durrant *et al*., 2007; Karamba *et al*., 2012; Pérez-Tris *et al*., 2007; Ricklefs & Fallon, 2002; Waldenström *et al*., 2002), and others also focused only on *Plasmodium* (Beadell *et al*., 2006; Loiseau *et al*., 2012; Valkiūnas *et al*., 2009). Few studies were looking out for the parasites of the genus *Leucocytozoon*. This was partly because most existing primers targeted primarily *Plasmodium* and *Haemoproteus* and were not well adapted for *Leucocytozoon* (Outlaw *et al*., 2017).

CHAPTER THREE

3. MATERIALS AND METHODS

3.1 Study Area

The study was conducted in poultry farms in the Sunyani municipality, Berekum in the Brong Ahafo region, and Aburi in the Eastern region of Ghana. These sites were selected because they are a hub for commercial poultry activities. Secondly, most poultry farms have a lot of remnant pristine vegetation surrounding them.

3.1.1 Sunyani

There are 123 administrative districts in Ghana, and one of them is the Sunyani Municipality (SM) (Opare *et al*., 2018). Its total land area is 829.3 square kilometers, and it is between Latitudes 70 20'N and 70 05'N and Longitudes 20 30'W and 20 10'W (Mensah *et al*., 2016). In Ghana's rainy Semi-Equatorial Climatic Zone, sometimes referred to as the Transition Zone, which has historically mean annual rainfall averages of around 88.98 cm and mean annual humidity ranges between 70% and 80%, Sunyani Municipality has relatively high temperatures and high humidity (Mensah *et al*., 2016). The chosen areas are home to several poultry farms of varied sizes that raise a variety of bird species for their meat and eggs.

3.1.2 Berekum

Berekum, which has a total area of 1635 m^2, is bordered to the northeast by Tain District, to the northwest by Jaman South, to the southwest by Asunafo North District, and the southwest by Sunyani-west. It is between latitudes 7'15°S and 8'00°N and longitude 2'25^o and 2'50^o West. The city of Berekum is in the semi-arid climate zone. A lot of sunshine and rain results in a warm,

muggy climate. In the northern regions of the municipality, there are some patches of roofed savannah.

3.1.3 Aburi

The area is located in the eastern region of Ghana. It is situated along the former Accra-Koforidua road around 38 kilometers northeast of Accra, 22 kilometers from Nsawam, and 48 kilometers from Koforidua. Rising to a height of 370 to 460 meters above sea level, it provides a view of the coastal plain (Ofori & Njomaba, 2021). The region is situated in between the South and North Districts of Akwapim. These districts have a bimodal rainfall pattern and are located in the west semi-equatorial climate zone. Because of its location in the woodland zone, the area is said to have a tropical climate (Ofori & Njomaba, 2021). On average, the temperature is 23.7°C. 1278 mm of rain falls on average, every year throughout the two wet seasons. The first rainy season, which produces 67% of the yearly precipitation, starts in March and finishes in mid-July; the second rainy season starts in mid-August and lasts until October. With only 25 mm of rain, January is the driest month. With an average of 220 mm, June receives the majority of the rainfall. August has the lowest annual average temperature (21.7°C). The warmest month is March, with a temperature of roughly 25.1°C (Ofori & Njomaba, 2021).

3.2 Study design

This study was designed as both field and laboratory-based research. All the laboratory investigations to detect malaria and related haemosporidian parasites from avian blood samples were conducted in the department of Animal Experimentation, Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana.

The research team obtained approval from the regional office of Agriculture in Sunyani to contact poultry farmers. All farmers who agreed to participate in the study met the research team to discuss the details of the project. Thirteen farms located within poultry farm communities in Sunyani, Berekum, and Aburi were enrolled in the study. The study began with an initial meeting with all stakeholders, ensuring that the aim was well communicated to and understood prior to starting the sample collection. The process of bird restraining, and blood collection was also explained, and assurance was given to the farmers about the health and safety of their birds.

3.3 Ethics and consent

Ethical approval for this study was sought from the Institutional Animal Care and Use Committee (IACUC) of the University of Ghana. The study was explained to the farmers and onsite veterinarians for their approval before sampling the birds.

3.4 Sample collection

The sample collection occurred between November and December 2019, and September to November 2020. These sampling periods were chosen, because haemosporidian infections are not seasonal, and the study did not focus on seasonal patterns of infections.

3.4.1 Sample collection from wild birds

Birds were caught using mist nets (Figure 4). Two mist nets of 18m lengths were set in, and around poultry farms. The mist nets were opened between the hours of 05:30 – 10 am, and 3:00 - 05:00 pm. Nets were checked frequently for catches. The trapped birds were carefully removed from the nets and kept in holding bags before processing. Processed birds were marked with a stain before

release so that the sample was not taken from any recaptured bird. The birds were identified using the "Birds of Ghana" field guide (Borrow & Demey, 2010).

Each bird was removed from the holding bag and the area around the brachial vein was sterilized by swabbing with 70% alcohol, moistening the surrounding feathers of the brachial vein, making it more accessible before puncturing. Using a sterile 26-gauge needle, the area was pricked and squeezed gently to obtain a large drop of blood. The blood was collected using a 200 µL pipette tip fixed to a micropipette aid. Approximately 100 µL of whole blood was drawn from each bird, 2-3 µL was used to prepare a thin smear on each of 3 clean grease-free microscope slides for traditional microscopy, and the rest was kept in EDTA tubes for further processing.

Figure 4: Mist netting. Wild bird trapping and blood collection.

3.4.2 Sample collection from domestic birds

Free-range chickens were caught and kept in confinement overnight and sample collection was performed early the next day before the birds were released. Birds kept in the poultry sheds were caught by farm attendants and manually restrained on a table by technical assistants before blood collection (Figure 5). Sampled birds were marked to prevent resampling of the same bird.

About 1 -2 mL of whole blood was drawn from the brachial vein of the birds using 2 mL syringes with needle gauges between 21G and 23G depending on the size of the bird. The area around the brachial vein was sterilized by swabbing with 70% alcohol, moistening the surrounding feathers of the brachial vein, making it more accessible prior to puncturing. Three thin blood films were prepared using 2-3 µL of blood on each grease free microscope slide for traditional microscopy, about 200 µl kept on Whatman paper for long-term storage, 40 µL was kept in Trizol reagent in a ratio of 1:3 for RNA extraction and the rest was kept in EDTA tubes for further processing. Blood collected on Whatman paper was air-dried and kept in ziplock bags, and all other samples were kept on ice on the field and transferred later to -20 degrees in the laboratory.

Figure 5: Domestic bird sampling. Blood collection from the brachial vein of domestic birds

3.5 Detection of avian malaria parasites

Frosted end clean grease-free microscope slides were labeled using a grease pencil before preparation of blood smears. For each bird, 2 -3 µL of blood was dropped on each of three ready-to-use microscope slides. The blood was spread into a thin film using a smooth edge slide spreader. The smears were air-dried within 5-10 sec after their preparation, and subsequently fixed in absolute methanol for 1 min. The fixed slides were air dried, and packed into slide boxes, so that they did not touch each other. All smears packed into slide boxes were sent to the laboratory for further processing. In the laboratory, fixed slides were stained with 10% Giemsa solution for 20 min and examined using a light microscope equipped with a camera. At least 200 microscopic fields were examined at 1000 magnification using oil-immersion. Parasite species identification followed the "Avian Malaria parasites and other haemosporidia" identification guide (Valkĭunas, 2005).

3.6 Molecular detection of avian malaria parasites

3.6.1 DNA Extraction

Deoxyribonucleic acid (DNA) was extracted from whole blood following a Quick-DNA Miniprep Plus kit protocol (Zymo Research) according to manufacturer's protocol.

Ten (10) µL of nucleated blood was added to a 1.5 mL microcentrifuge tube. 200 µL BioFluid & Cell Buffer, 20 µL Proteinase K**,** and 200 µL DNA Elution Buffer were added to the blood. The sample was mixed thoroughly by pipetting up and down. The tubes were incubated at 55^{o}C for 20 min. One volume of Genomic Binding Buffer was added to each tube and mixed thoroughly by pipetting up and down or vortexing. Once the samples were homogenous, the mixtures were transferred to Zymo-Spin™ IIC-XLR Column in collection Tubes. The tubes were centrifuged at ≥ 12,000 x *g* for 1 minute. The collection tubes with the flow through were discarded. 400 µL DNA Pre-Wash was added to each spin column in new collection tubes. The tubes were centrifuged at ≥ 12,000 x g for 1 minute. The collection tubes were emptied and 700 µL g-DNA wash buffer was added to each spin column, and the tubes were centrifuged at ≥12,000 x *g* for 1 minute. The collection tubes were emptied again and 200 µL g-DNA wash buffer was added to each spin column directly on the matrix. The columns were centrifuged at ≥ 12,000 x *g* for 1 minute. The collection tubes with the flow through were discarded. The spin columns were transferred to clean microcentrifuge tubes and 100 µL DNA Elution Buffer was added directly to the matrix of each column. The samples were incubated for 5 min at room temperature, then centrifuged at top speed for 1 minute to elute the DNA. The eluted DNA was stored at -30^{o}C for future use. To confirm DNA, 2 µL of the extract was run on 1.5% agarose gel electrophoresis, post-stained with Diamond Nucleic Acid dye (Promega Ribose Nucleic Acid corporation), and visualized under ultraviolet light (UV).

3.6.2 Real-Time PCR analysis.

This method was used to screen for haemosporidian parasites of the genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*. This method amplified 182 bp of ribosomal DNA (rDNA) in all three haemosporidian genera under study using the primers R330F and R480RL previously described (Bell *et al*., 2015). All reactions were carried out using 2x Luna Universal qPCR master mix in a real-time thermocycler (ABI 7300). The total volume of the reaction was 10 µL, with 5 µL of 2x Luna qPCR master mix, 0.4 µL of each primer (10 µM concentration), 2.2 µL of molecular grade water, and 2 µL of DNA template. The following cycling conditions were used to run the reaction: 95 °C for 3 min, followed by 40 cycles of 95 °C for 15s and 53 °C for 35s (with a plate read) followed by a dissociation analysis using instrument default settings. Positive and negative controls were included in all runs. The positive controls were samples that tested positive from previous studies and sterile nuclease-free water was used as a negative control. The positive control was expected to produce a melt curve peak at 78.5 °C, like *P. relictum* (Bell *et al*., 2015). For verification of procedure, real-time PCR products were run on 1.5% agarose gels, post-stained with Diamond Nucleic Acid dye (Promega corporation), and visualized under ultraviolet light (UV) (Bell *et al*., 2015). The band sizes of 182 bp (Bell *et al*., 2015) confirmed positive samples.

3.6.3 Gel extraction

Gel extraction was performed using a Qiagen gel extraction Kit. The agarose gel was placed in a UV transilluminator and DNA fragments at the expected band size were excised from the gel with a clean scalpel. The gel slice was weighed in a colorless tube. Three volumes of buffer QG were added to 1 volume of gel. The mixture was incubated at 50°C for 10 min (or until the gel slice was completely dissolved), and vortexed every 2-3 min until the gel was completely dissolved. One gel volume (100 µL) of isopropanol was added to the sample mix. The mixture was transferred to

a spin column and centrifuged at 13000 rpm for 1 minute. The flow-through was discarded. 500 µL buffer QG was added to the spin column and centrifuged at 13000 rpm for 1 minute and the flow-through was discarded. In addition, 750 µL of buffer PE was added to the column and allowed to stand for 5 min to wash. The column was centrifuged at 13000rpm for 1 minute to remove the residual wash. The column was transferred to a 1.5 mL microcentrifuge tube. To elute DNA, 30 µL buffer EB was added to the center of the membrane, allowed to stand for 1 minute, and centrifuged at 13000 rpm for 1 minute. The DNA extracted was kept at -20 degrees for further processing.

3.6.4 Conventional PCR for amplification of a conserved region of the ribosomal DNA (rDNA)

This method was used to confirm that the gel DNA extract was the targeted rDNA gene of the haemosporidian parasites. A conventional PCR method that amplifies a conserved region (182 bp) of the ribosomal DNA was used. The primers R330F and R480RL used in the real-time PCR protocol proposed by Bell *et al*. were used. All reactions were carried out using One Taq quick load 2x master mix on a thermocycler (ABI 2720). The total volume of the reaction was 10 µL, with 5 µL of the master mix, 0.6 µL of each primer (10 µM concentration), 0.8 µL of molecular grade water, and 3 µL of DNA template. The following cycling conditions were used to run the reaction: 95 °C for 3 min, followed by 40 cycles of 95 °C for 30sec, 53 °C for 30sec, 68°C for 1 min, and a final elongation at 68°C for 5 min, and holding at 4°C. There were no positive controls included since all the samples excised from agarose gel were positive and sterile nuclease-free water was used as a negative control (Valkiūnas *et al*., 2009). The primer sequences used in this experiment are provided in Table 1. The PCR amplicons were run on 2% agarose gels, post-stained with Diamond Nucleic Acid dye (Promega corporation), and visualized under ultraviolet light (UV) (Bell *et al*., 2015). The band sizes of 182 bp (Bell *et al*., 2015) confirmed positive samples.

Table 1: Primers used for PCR and Sequencing

Real Time PCR on *Haemoproteus*, *Plasmodium* and *Leucocytozoon*	Primer
R330F (Bell *et al*., 2015)	5'- CGTTCTTAACCCAGCTCACG - 3'
R480RL (Bell *et al*., 2015)	5'- GCCTGGAGGTWAYGTCC - 3'
Sequencing *Haemoproteus* and *Plasmodium*	
FIFI (Videvall *et al*., 2017)	5' – GGGTCAAATGAGTTTCTGG – 3'
R2 (Videvall *et al*., 2017)	5' – GCTGTATCATACCCTAAAGG – 3'
Sequencing – *Leucocytozoon*	
L45F (Lutz et al., 2015)	5' – ACAAATGAGTTTCTGGGGA - 3'
L825R (Lutz et al., 2015)	5' – GCAATTCCAAATAAACTTTGAA – 3'

3.6.5 Nested PCR for amplification of a partial region of the mitochondrial cytochrome b gene

For the detection of *Plasmodium* and *Haemoproteus* spp., a nested PCR method that amplifies a partial region (479 bp) of the mitochondrial cytochrome b gene was used. The primers HaemNFI and HaemNR3 were used as outer primers to amplify all three genera of haemosporidians in the first round of nested PCR. All reactions were carried out using One Taq quick load 2x master mix on a thermocycler (ABI 2720). The total volume of the reactions was 10 µL, with 5 µL of the master mix, 0.6 µl of each primer (10 µM concentration), 1.8 µL of molecular grade water, and 2 µL of DNA template. The following cycling conditions were used to run the reaction: 95 °C for 3 min, followed by 20 cycles of 95 °C for 30sec, 50 °C for 30 sec, 68°C for 1 min, and a final elongation at 68°C for 5 min, and holding at 4°C. The positive controls included were taken from

positive samples from previous studies, and sterile nuclease-free water was used as a negative control in place of the DNA template (Valkiunas *et al*., 2009). The negative controls were used to control for false amplification because of the high sensitivity of the nested PCR. The primers HaemF (5'ATGGTGCTTTCGATATATGCATG3') and HaemR2 (5'GCATTATCTGGATGTGATAATGGT3'), designed by Bensch *et al*. (2000), were used as inner primers to amplify either the *Plasmodium* or *Haemoproteus* genus. The primers HaemFL and HaemR2L were used as inner primers to amplify the *Leucocytozoon* genus. The following conditions were used for the second round of the nested PCR amplification: initial denaturation at 95 °C for 3 min, followed by 20 cycles of 95 °C for 30sec, 50 °C for 30sec (for *Leucocytozoon*), and 55 °C for 30 sec (for *Plasmodium* and *Haemoproteus*), 68°C for 1 min and final elongation at 68°C for 5 min and holding at 4°C. The reaction mixture was run in a total volume of 10 µL, with 5 µL of the One Taq quick load 2x master mix, 0.6 µL of each primer (10 µM concentration), 1.8 µL of molecular grade water, and 2 µL of the amplicon obtained from the first round of PCR. The amplified DNA (5 µL) was then submitted to electrophoresis in 2% agarose gel and detected by post-staining with diamond nucleic acid dye (Promega corporation) and visualized by UV trans-illumination. The expected target size of 479 bp for *Plasmodium* or *Haemoproteus* and 480 bp for *Leucocytozoon* was measured using a 50 bp DNA ladder (Biolabs). The primer sequences used in this experiment are provided (Table 1).

3.6.6 Modified nested PCR for amplification of a more conserved region of the mitochondrial cytochrome b gene.

This protocol was performed because the standard nested PCR method described above did not successfully amplify the haemosporidian cyt b gene in known positive samples.

For the detection of *Plasmodium* and *Haemoproteus* genera, a nested polymerase chain reaction (PCR) method that amplifies a more conserved region of the mitochondrial cytochrome b gene was used. These primers were used by Bell, 2015 for sequencing a region within the amplified 479 bp region of the mitochondrial cyt b gene. The primers FIFI and R2 (Videvall *et al*., 2017) were used to target the genera *Haemoproteus* and *Plasmodium* while the primers L45F and L825R (Lutz *et al*., 2015) targeted the genus *Leucocytozoon*. The primer sequences used in this experiment are provided (Table 1).

The primers HaemNFI and HaemNR3 were used as outer primers to amplify all three genera of haemosporidians in the first round of nested PCR. All reactions were carried out using One Taq quick load 2x master mix on a thermocycler (ABI 2720). The total volume of the reaction mixture was 10 µL, with 5 µL of the master mix, 0.6 µL of each primer (10 µM concentration), 1.8 µL of molecular grade water, and 2 µL of DNA template. The following cycling conditions were used to run the reaction: 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 sec, 50 °C for 30 sec, 68°C for 1 min, and a final elongation at 68°C for 5 min and holding at 4°C. The positive controls included were taken from positive samples from previous studies, and sterile nuclease-free water was used as a negative control in place of the DNA template (Valkiunas *et al*., 2009). The negative controls were used to control for false amplification because of the high sensitivity of the nested PCR. The primers FIFI and R2, were used as inner primers to amplify either *Plasmodium* or

Haemoproteus genus. The primers L545F and L825R were used as inner primers to amplify *Leucocytozoon* genus. In the second round of nested PCR amplification, the following conditions were used: initial denaturation at 95 °C for 3 min, followed by 20 cycles of 95 °C for 30 sec, 50 °C for 30 sec, 68°C for 1 min, and a final elongation at 68°C for 5 min and holding at 4°C. The reaction mixture was run in a total volume of 10 µL, with 5 µL of the One Taq quick load 2x master mix, 0.6 µL of each primer (10 µM concentration), 1.8 µL of molecular grade water, and 2 µL of the amplicon from the first round of PCR. The amplified DNA (5 µL) was then submitted to electrophoresis in 2% agarose gel and detected by post-staining with diamond nucleic acid dye (Promega corporation), and visualized under UV trans-illumination. The expected band sizes of 186 bp for *Plasmodium* and *Haemoproteus*, and 280 bp for *Leucocytozoon* were measured using a 50 bp DNA ladder (Biolabs). The second round of nested PCR was repeated only on positive samples in a 20 µL reaction mixture, to get enough amplicons for subsequent analyses. The positive amplicons were shipped to Macrogen (Macrogen Europe) for purification and sequencing.

3.6.7 DNA sequencing

Amplicons from positive samples detected from modified nested PCR were dispensed into 96 well PCR plates and covered with cap strips. The plates were well packaged with the order details with primers enclosed and shipped at room temperature to the Macrogen company (Macrogen Europe) for sequencing. A bidirectional Sanger sequencing was performed on all the amplicons using the forward and reverse primers FIFI and R2, respectively for the *Plasmodium* and *Haemoproteus* genera and, L545F and L825R for the *Leucocytozoon* genus. The raw sequence data was obtained for further analysis.

3.6.8 Whole genome amplification of parasite DNA

This protocol was followed to concentrate parasite DNA for downstream whole genome sequencing analysis of the parasite genome. This was necessary because the haemosporidian parasites live in nucleated cells. This results in more host DNA (>99%) than parasite DNA. There is therefore the need for host DNA depletion before further omics analysis. This protocol employs enrichment and selective whole genome amplification (WGA) procedures, respectively to concentrate the parasite DNA. The enrichment procedure uses a methylation technique to deplete host DNA while the selective WGA procedure further selects for and amplifies AT bias genome.

3.6.9 DNA enrichment protocol

This procedure was performed using the NEBNext Microbiome DNA Enrichment Kit (NEB #E2612S/L). DNA extracts of the dominant parasites of the genera *Plasmodium, leucocytozoon* and *Haemoproteus* were sorted. The samples were quantified using nanodrop and the volume that will make 1 µG was calculated for each sample. The sample volumes were normalized to contain 1µg DNA in a starting volume of 50 µL of solution. A total of 20 mL Nuclease-free water was dispensed into each of two 50 mL falcon tubes, then 5 mL bind/wash buffer was added to each tube, and 16 µL MBD2 protein FC and 160 µL of protein magnetic beads were added to each tube. The mixture was pipetted up and down 10-15 times. The protein bead mix was mixed by putting it on a mixer for 10 min at room temperature. The tubes were briefly spined and placed on the magnetic rack until the beads have collected to the wall of the tube and the solution was clear. The supernatant was carefully removed with a pipette without disturbing the beads. One (1) ml of 1X Bind/wash Buffer (kept on ice) was added to the tube to wash the beads, and the mixture was pipetted up and down until the beads were completely homogeneous. The beads were mixed on a rotating mixer for 3 min at room temperature. The tube was briefly spined and placed on the

magnetic rack until the beads collected to the wall of the tube and the solution was clear. The supernatant was carefully removed with a pipette without disturbing the beads. The wash steps were repeated. The tubes were removed from the rack and 160 µL of 1X Bind/wash Buffer (kept on ice) was added to resuspend the beads and the mixture was mixed by pipetting up and down a few times. One (1) µG input DNA was added to the tube containing the 160 µL of MBD2-Fc-bound magnetic beads. Undiluted Bind/wash Buffer (5X) was added to make a final concentration of 1X and mixed to obtain a homogenous solution. The tube was agitated on a rotating mixer for 15 min at room temperature.

The tubes were briefly spined and placed on the magnetic rack for 5 min, until the beads have collected to the wall of the tubes, and the solution was clear. The supernatant was carefully removed with a pipette without disturbing the beads and transferred to clean microcentrifuge tubes. The supernatant containing the target microbial DNA was stored at -20°C till the next day. The samples were purified by AMPure XP bead cleanup.

AMPure XP/SPRIselect beads were vortexed to resuspend. A total of 1.8X volume of resuspended AMPure XP beads was added to the sample. The sample was mixed well by pipetting up and down at least 10 times. All the liquid was carefully expelled out of the tip during the last mix. The samples were incubated for at least 5 min at room temperature. The tubes were placed on the magnetic stand to separate the beads from the supernatant. After 5 min, the supernatant was carefully removed and discarded without disturbing the beads that contained DNA targets, and 400 µL of freshly prepared 80% ethanol was added to the tube/plate while in the magnetic stand. The sample was incubated at room temperature for 30 sec, and the supernatant was carefully removed, and discarded without disturbing the beads that contain DNA targets. The ethanol step was repeated once in two washes. All visible liquid was removed after the second wash. The beads

were air-dried for 5 min while the tubes were on the magnetic stand with the lid open. The tubes were removed from the magnetic stand. The DNA target was eluted from the beads by adding 50 µL of 1X TE. The sample was mixed well by pipetting up and down 10 times and incubated for at least 2 min at room temperature. The tubes were placed on the magnetic stand. After 5 min the eluate was transferred to new microcentrifuge tubes. The sample was kept at -30°C for whole genome amplification.

3.7 Whole genome amplification protocol

WGA was performed using REPLI-g Mini kit (Qiagen) using the manufacturer's instructions (Oyola *et al*., 2014). The following modifications were performed in developing optimized conditions for the REPLI-g Mini kit: nuclease-free water and all tubes were UV-treated before use (Oyola *et al*., 2014). WGA reactions were performed in 0.2 mL PCR tubes. Buffer D1 stock solution (Qiagen) was reconstituted by adding 500 µL of nuclease-free water, and a working solution was prepared by mixing the stock solution and nuclease-free water in the ratio of 1: 3.5, respectively (Oyola *et al*., 2014). Unmodified Buffer N1 was reconstituted by mixing Stop solution (Qiagen) and nuclease-free water in the ratio of 1: 5.7 (Oyola *et al*., 2014). Modified buffer N1 was prepared by including tetramethylammonium chloride (TMAC) at a concentration of 60 mM (Oyola *et al*., 2014). To denature DNA templates, 5 µL of the DNA solution was mixed with 5 µL of buffer D1 (working solution prepared as described above) (Oyola *et al*., 2014). The mixture was vortexed and centrifuged briefly before incubating at room temperature for 3 min (Oyola *et al*., 2014). Denatured DNA was neutralized by adding 10 µL of unmodified or modified buffer N1 (Oyola *et al*., 2014). Neutralized DNA was mixed by vortexing and centrifuged briefly (Oyola *et al*., 2014). To amplify the DNA template, denatured and neutralized sample was mixed with 29 µL of REPLI-g Mini Reaction Buffer and 1 mL of REPLI-g Mini DNA polymerase to obtain a

final reaction volume of 50 µL. The reaction mixture was incubated in a heat block at 30°C for 16 h. Amplified DNA was cleaned using Agencourt Ampure XP beads (Beckman Coulter) using a sample-to-bead ratio of 1:1 and eluted with 20 µL of EB (Qiagen) (Oyola *et al*., 2014).

3.8.1 Statistical analyses

The results were analyzed using KRUSKAL-WALLIS test and Fisher's exact test in Graph Pad PRISM statistical tool. KRUSKAL-WALLIS test was performed to evaluate the haemosporidian prevalence among bird species. Dunn's comparison test was used for pairwise comparisons of parasite prevalence between bird species. Fisher's exact test was used to compare prevalence of the three haemosporidian parasites, and prevalence among the bird categories. The results obtained were presented as graphs and tables.

Sequence identities were verified using a local BLAST against the MalAvi and National Centre for Biotechnology Information (NCBI) databases (Bensch, Hellgren, & Pérez-Tris, 2009) and parasite genus was assigned based on close homology.

3.8.2 Sequence analysis

Raw sequences obtained were cleaned using Chromas software (Version 2.6.6), edited, and assembled in Bioedit software to produce a consensus sequence from the forward and reverse sequence pairs. The consensus sequences were then subjected to the NCBI Basic Local Alignment Tool and MalAvi database to search for homologous sequences. Related sequences were downloaded from NCBI and aligned with new sequences obtained from this study using Bioedit. The aligned sequences were converted to the Molecular Evolutionary Genetics Analysis (MEGA) format and used in constructing a phylogenetic tree.

3.9 Assessing tissue tropism

Blood samples were collected from turkeys in farms in Aburi, the Eastern region of Ghana. About 2 mL of blood was collected from each bird and kept in EDTA tubes. The birds were given numbers on tags attached to their feet. Three thin blood smears were prepared for individual birds. Microscopy was performed in the laboratory to screen for positive birds. DNA was extracted, and qPCR and modified nested PCR were performed on all samples. The positive hosts were identified and purchased for further analysis. The purchased turkey was sacrificed (Figure 6). Major organs such as the brain, liver, lungs, spleen, and heart were harvested. The organs were fixed immediately in 10% formalin and sent to a histology laboratory for processing. The slides were embedded, sectioned, and mounted onto glass slides according to the procedures of the histology laboratory and returned for examination. The slides were examined using the x20 objective lens of an Olympus light microscope.

Figure 6: organ harvesting. Naturally infected turkey sacrificed, and organs harvested for histology

CHAPTER FOUR

4.0 RESULTS

4.1 Avian malaria and related haemosporidian parasites among domestic birds

We sampled 1110 birds comprising 1082 domestic and 28 wild birds from commercial poultry farms, backyard farms, and wild habitats surrounding the sampling areas. The birds belonged to 17 species comprising 7 species of domestic birds and 10 species of wild birds. The bird species belonged to 14 families, and 4 orders (Table 2). Birds of the order Galliformes and the family *Phasianidae* were most represented compared to birds of the other two orders (Table 2). The most sampled bird species was *Gallus gallus* (domestic chicken) (Table 2). We sampled these species both among confined/caged birds and free-range birds (Table 2).

Table 2: Number of individuals sampled indicating bird host species, families, and orders

Order/Common name	Family	Scientific name	Number of sampled	Proportion of birds sampled (%)
Galliformes				
Confined chicken	*Phasianidae*	*Gallus gallus*	588	53
Free range Chicken	*Phasianidae*	*Gallus gallus*	30	2.7
Quail	*Phasianidae*	*Coturnix coturnix*	90	8.1
Turkey	*Phasianidae*	*Meleagris gallopavo domestica*	90	8.1
Guinea fowl	*Numididae*	*Numida meleagris*	87	7.8
Anseriformes				
Duck	*Anatidae*	*Anas platyrhynchos domesticus*	178	16
Goose	*Anatidae*	*Anser anser domesticus*	9	0.8
Struthioniformes				
Ostrich	*Struthionidae*	*Struthio camelus*	10	0.9
Passeriformes				
Vieillot's black weaver	*Ploceidae*	*Ploceus nigerrimus*	18	1.6
Heuglin's-masked weaver	*Ploceidae*	*Ploceus heuglini*	1	0.09
Olive sunbird	*Nectariniidae*	*Cyanomitra olivacea*	2	0.18
Copper sunbird	*Nectariniidae*	*Cinnyris cupreus*	1	0.09

Table 2: Number of individuals sampled indicating bird host species, families, and orders (continued)

Little greenbul	*Pycnonotidae*	*Eurillas virens*	1	0.09
Common bulbul	*Pycnonotidae*	*Pycnonotus barbatus*	1	0.09
Village indigobird	*Viduidae*	*Vidua chalybeata*	1	0.09
Bearded barbet	*Lybiidae*	*Lybius dubius*	1	0.09
Black crowned Tchagra	*Malaconotidae*	*Tchagra senegala*	1	0.09
Coraciiformes				
Sao-Tome kingfisher	*Alcedinidae*	*Corythornis thesis*	1	0.09
Total			**1110**	

4.1.1 Morphological identification of parasites

We observed *Haemoproteus spp*. in thin blood smears of Vieillot's black weavers (Figure 7). We did not observe the morphological stages of *Plasmodium* and *Leucocytozoon* species in the birds sampled. The stages seen by microscopy were not enough for the morphological identification of the parasites at the species level. The picture below (Figure 7) shows circumnuclear forms of microgametocytes of *Haemoproteus spp*. (A, B, C, D, F) (Valkiūnas & Iezhova, 2022), similar to *Haemoproteus nucleofascialis*.

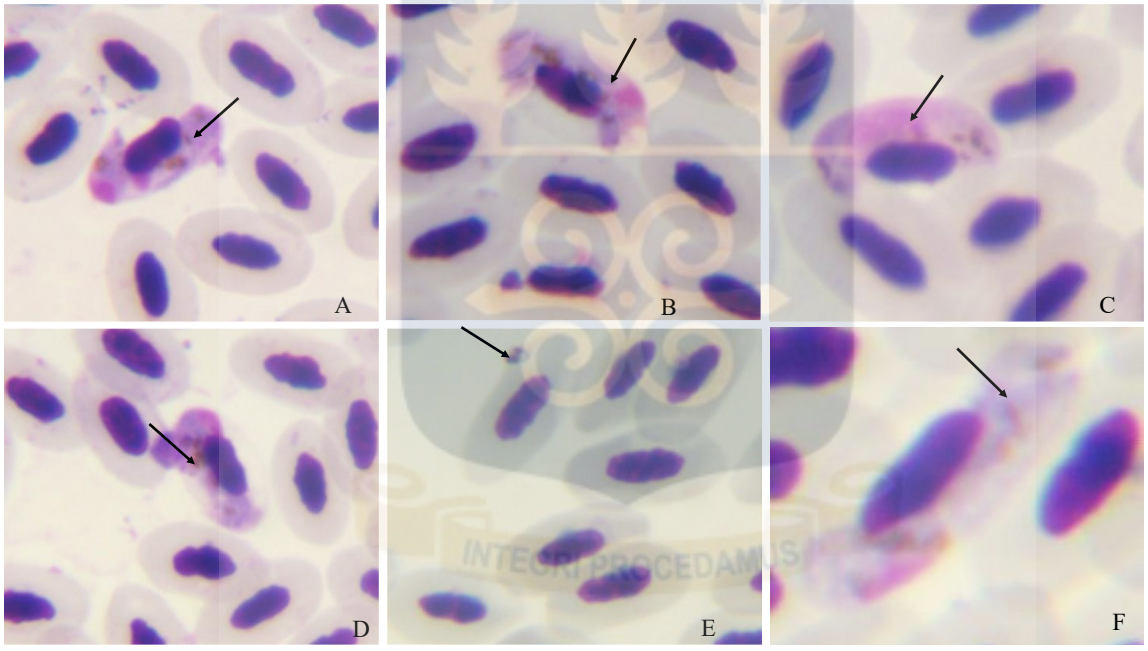

Figure 7: Microscopy results. A picture of fully grown microgametocytes of *Haemoproteus* sp. (A, B, C, D, and F) in the blood of vieillot's black weaver, with features similar to *H. nucleofascialis*. "*H. nucleofascialis* microgametocytes show main haemosporidian sexually dimorphic characters. Parasite nuclei diffuse in growing gametocytes. In fully grown gametocytes, nuclei are markedly compressed, and usually assume band-like shape, closely associated with parasite pellicle, located close to the erythrocyte envelope; these 2 attributes of gametocyte nuclei are distinctive morphological characters of this species." (Described in (Iezhova *et al*., 2011).

4.1.2 Haemosporidian parasites screening using the qPCR method

We used the real-time PCR protocol as a selective screening tool for any of the three haemosporidian genera. The purpose was to screen many samples in a short time for further processing to be done. We performed the protocol on all avian DNA samples to amplify a182 bp region of the ribosomal DNA (rDNA) gene, which is conserved in all three avian haemosporidian genera. There were multiple peaks observed on the dissociation curve results (Figure 8A) suggesting the amplification of mixed species, off-targets, or contaminants. Because of the multiple peaks, the samples were run on 1.5% agarose gel and visualized under a UV transilluminator to confirm the desired products. Agarose gel electrophoresis confirmed the product of interest and other products (Figure 8B).

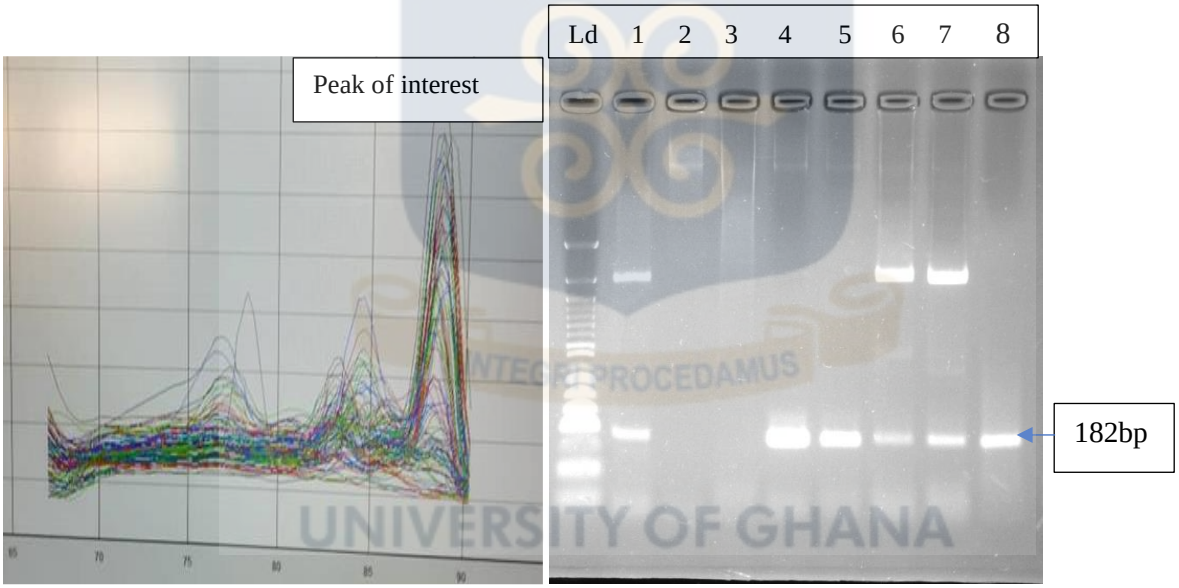

Figure 8: qPCR results showing amplification of multiple products. qPCR plot showing multiple peaks (Figure 8A). Agarose gel electrophoresis showing amplified qPCR amplicons confirming the presence of the target DNA (Figure 8B)

4.1.3 Gel DNA extraction

We excised amplified products of interest that were present on the qPCR gels shown in Figure 8B. The DNA extracted from the excised bands was used in a conventional PCR assay, using the qPCR primers. Agarose gel electrophoresis confirmed the successful amplification of the targeted 182 bp fragment of the ribosomal DNA (rDNA) gene (Figure 9).

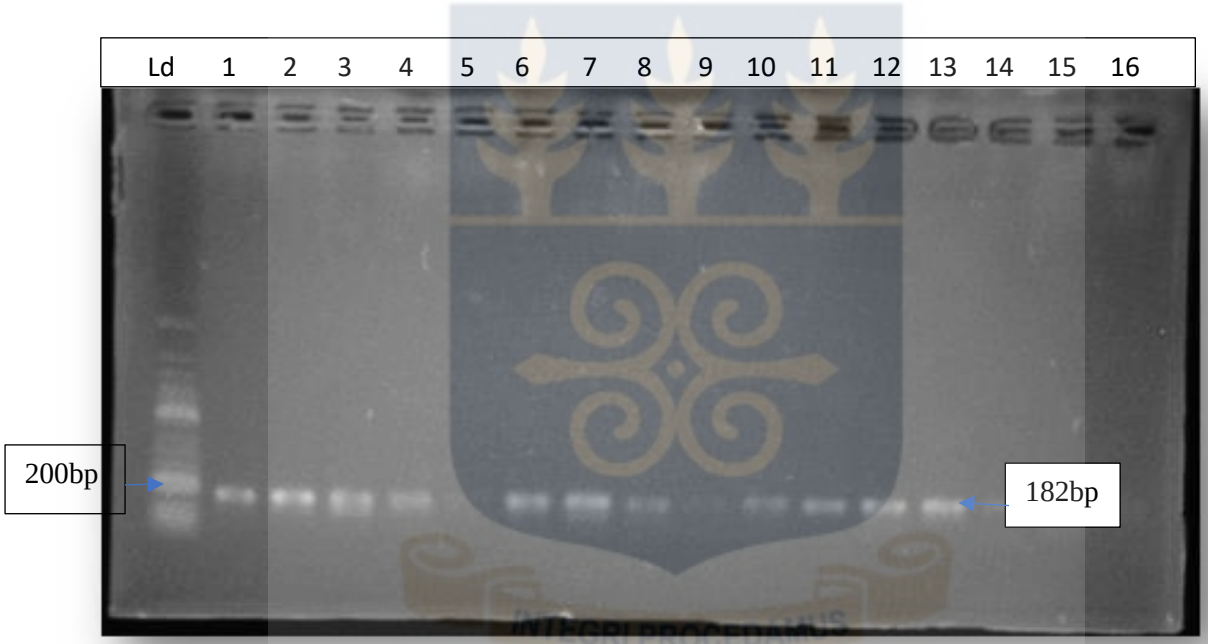

Figure 9: Agarose gel electrophoresis. Gel picture showing positive bands at 182 bp for conventional PCR amplification of gel extracts from qPCR excised gel

4.1.4 Haemosporidian screening using Nested and modified nested PCR protocols.

We performed a nested PCR that used the outer primers HaemNFI and HaemNR3 (Hellgren, *et al*., 2004) and inner primers HaemF and HaemR2; HaemFL and HaemR2L (Bensch *et al*., 2009) to amplify a partial region of the Cyt b gene on all DNA samples. Only 10 (1.75%) samples out of 571 tested positive with this method. However, when we employed a nested PCR protocol with a modification introduced by this study using the outer primers HaemNFI and HaemNR3 (Hellgren *et al*., 2004) and inner primers FIFI and R2; and L545F and L825R (Bell *et al*., 2015), we amplified a shorter region of the MIT Cyt b gene in many more samples compared to the existing nested protocol. This method recorded 66 (11.5%) positives out of the total samples screened. We then employed this method for the complete study since the focus was to amplify the MIT Cyt b gene for comparison, not the rDNA gene. We performed the modified nested PCR designed by this study on 16 positive samples and showed amplification of all the samples (Figure 10A). However, the same samples processed using the existing nested PCR showed the amplification of only 1 sa mple (Figure 10B).

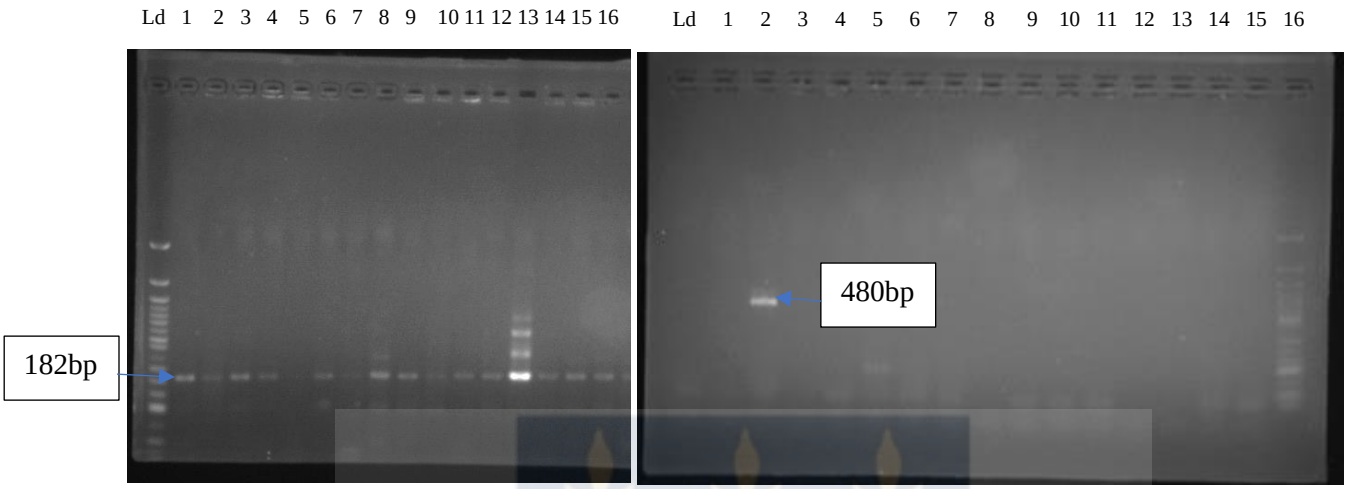

Figure 10: Comparative analysis of two nested PCR protocols. A modified nested PCR shows more amplification of *Leucocytozoon* positive samples (A) compared to the standard nested PCR protocol (B). The same samples were amplified in the gel A and B.

4.1.5 Comparison of the three molecular methods optimized for the detection of avian haemosporidian in poultry.

There are existing molecular primers to identify avian haemosporidian in birds. The most widely used primers were used to optimize the study protocol. The overall prevalence of haemosporidian parasites was determined to be 36.4%, 1.75%, and 11.5% by qPCR, nested PCR, and modified nested PCR. The overall detection rate of haemosporidian parasites using the qPCR and modified nested protocols was significantly higher than that of the standard nested PCR ($P<0.001$). qPCR parasite detection was also significantly higher than modified nested PCR ($P<0.001$). qPCR and modified nested PCR detected parasites in all bird species. The existing nested PCR detected parasites in only three bird species (Figure 11). For the amplification of a partial region of the MIT Cyt b gene of the haemosporidian parasites, the study preferred the modified nested PCR.

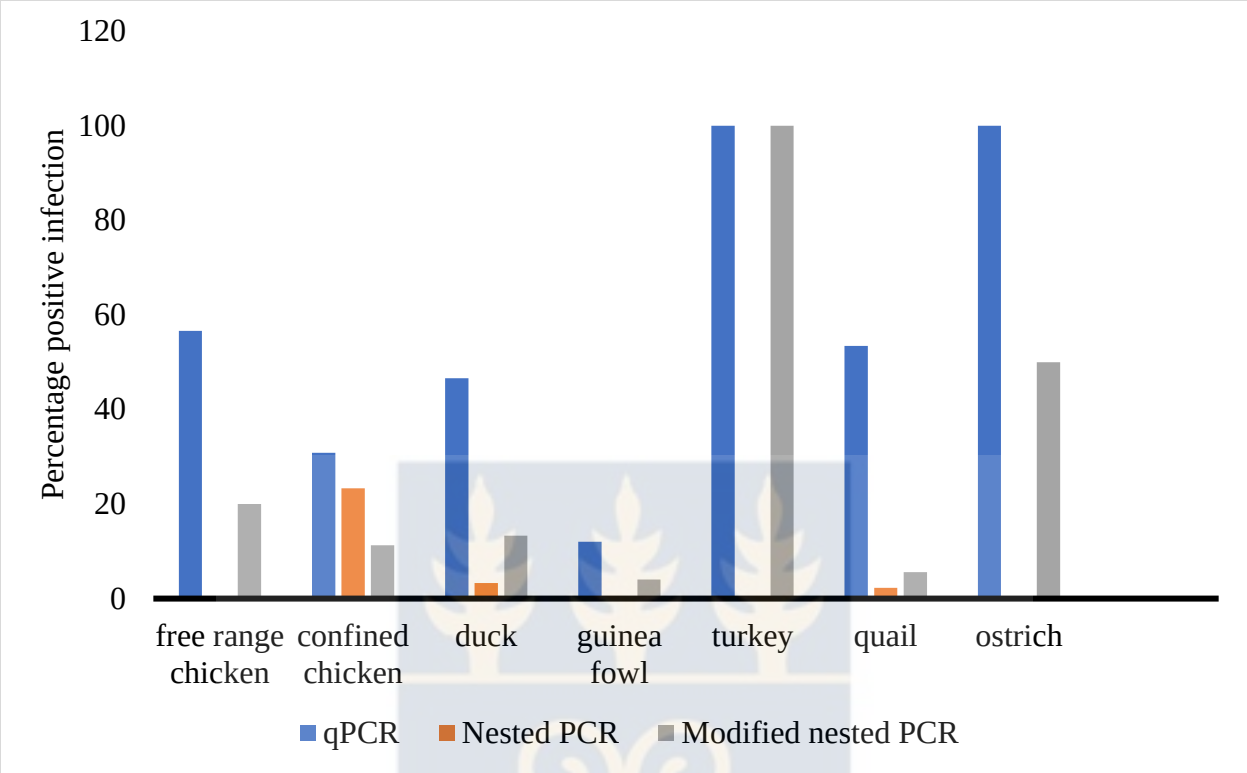

Figure 11: optimization of molecular methods. A higher parasite detection rate was observed in qPCR and modified nested PCR protocols compared to nested PCR in all bird categories except for confined chicken.

4.2 General prevalence of avian malaria and related haemosporidian parasites

This study found 233 out of 1082 domestic birds (21.5%) infected with three genera of avian haemosporidian parasites using PCR (Table 3). Haemosporidian prevalence for single parasite genus infection was 8.9%, 2.1%, and 0.5% for *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*, respectively. A prevalence of 9.9% for *Plasmodium* and *Leucocytozoon*, species co-infections was found. We assessed haemosporidian infections with each of the three parasite genera in individual domestic bird species, families, and orders to evaluate the host specificity of the parasites found in this study. Different bird species had a differing prevalence of haemosporidian parasites. We observed higher prevalences among the individual parasite genera when counting every coinfection as one infection. Significant differences were observed in *Plasmodium* (18.9%), *Leucocytozoon* (10.4%), and *Haemoproteus* (2.1%) prevalences ($P<0.001$).

Table 3: Prevalence of three genera of avian haemosporidian in domestic and wild birds using PCR

Order/Common name	Scientific name/Family	No. sampled	Infected (%)	Haemo (%)	Plas (%)	Leuco (%)	Plas / Leuco (%)
Galliformes	***Phasianidae***						
Free range chicken	*Gallus gallus*	30	5 (16.7)	0 (0)	1 (20)	0 (0)	4 (80)
Confined chicken	*Gallus gallus*	588	102 (17.3)	15 (14.7)	21 (20.6)	4 (4)	62 (60.8)
Quail	*Coturnix coturnix*	90	6 (6.7)	0 (0)	3 (3.3)	0 (0)	3 (3.3)
	Numididae						
Guinea fowl	*Numida meleagris*	87	6 (6.9)	2 (33.3)	2 (33.3)	2 (33.3)	0 (0)
	Meleagrididae						
Turkey	*Meleagris gallopavo domestica*	90	53 (58.9)	1 (1.9)	33 (62.3)	0 (0)	19 (35.8)
Anseriformes	***Anatidae***						
Duck	*Anas platyrhynchos domesticus*	178	53 (29.8)	5 (9.4)	29 (54.7)	0 (0)	19 (35.8)
Geese	*Anser anser domesticus*	9	3 (33.3)	0	3 (33.3)	0 (0)	0 (0)
Struthioniformes	***Struthionidae***						
Ostrich	*Struthio camelus*	10	5 (50)	0	5 (100)	0	0
Total (domestic birds)		**1082**	**233 (21.5)**	**23 (2.1)**	**97 (8.9)**	**6 (0.5)**	**107 (9.8)**
Passeriformes	***Ploceidae***						
Vieillot's black weaver	*Ploceus nigerrimus*	18	9 (50)	0	4 (44.4)	2 (22.2)	2 (22.2)
Heuglin's masked weaver	*Ploceus heuglini*	1	1 (100)	1 (100)	0	0	0
	Nectariniidae						
Olive sunbird	*Cyanomitra olivacea*	2	1 (50)	0	0	1	0
Copper sunbird	*Cinnyris cupreus*	1	0 (0)	0	0	0	0
	Pycnonotidae						
Little greenbul	*Eurillas virens*	1	0 (0)	0	0	0	0
Common bulbul	*Pycnonotus barbatus*	1	0 (0)	0	0	0	0
	Viduidae						

Table 3: Prevalence of three genera of avian haemosporidian in domestic and wild birds using PCR (continued)

Village indigobird	*Vidua chalybeata*	1	0 (0)	0	0	0	0
	Lybiidae						
Bearded barbet	*Lybius dubius*	1	0 (0)	0	0	0	0
	Malaconotidae						
Black-crowned Tchagra	*Tchagra senegala*	1	0 (0)	0	0	0	0
Coraciiformes	***Alcedinidae***						
Sao Tome kingfisher	*Corythornis cristatus thomensis*	1	0 (0)	0	0	0	0
Total birds		**1110**	**244 (22)**	**24**	**101**	**9**	**109**

4.2.1 Genus-specific prevalence of avian malaria and related haemosporidian parasite

This study recorded *Leucocytozoon* infection in 113 out of 1082 (10.4%) domestic birds of five species, guinea fowls (*Numida meleagris*), quails (*Coturnix coturnix*), ducks (*Anas platyrhynchos*), confined chickens (*Gallus gallus*), free-range chickens (Gallus gallus), and turkeys (*Meleagris gallopavo*), with the prevalence of 2.3%, 3.3%, 10.7%, 11.2%, 13.3%, and 21.1% (Figure 12). Dunn's post hoc test showed that *Leucocytozoon* prevalence was higher in turkeys (*M. gallopavo*) than quail (*C. coturnix*) and guinea fowls (*N. meleagris*) ($P < 0.001$) (Figure 12). The *Leucocytozoon* prevalence differences observed among the other pairs of bird species were not significant.

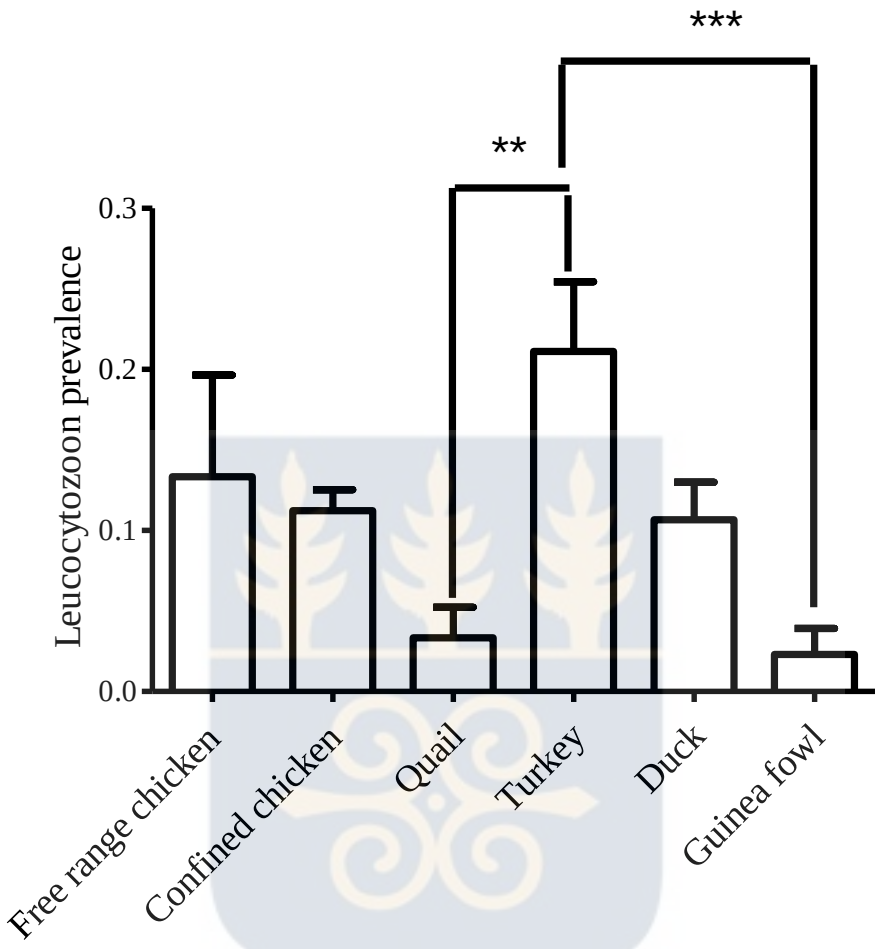

Figure 12: *Leucocytozoon* prevalence in domestic birds

We observed *Plasmodium* infections with an overall prevalence of 18.9% in all species of domestic birds sampled (Figure 13). Dunn's post hoc test showed higher *Plasmodium* prevalence in turkeys compared to free-range chickens ($P < 0.0001$). Prevalence in turkeys was significantly higher than in quail, guinea fowl, and duck. We also observed a significantly higher prevalence in duck compared to quail and guinea fowl ($P < 0.0001$).

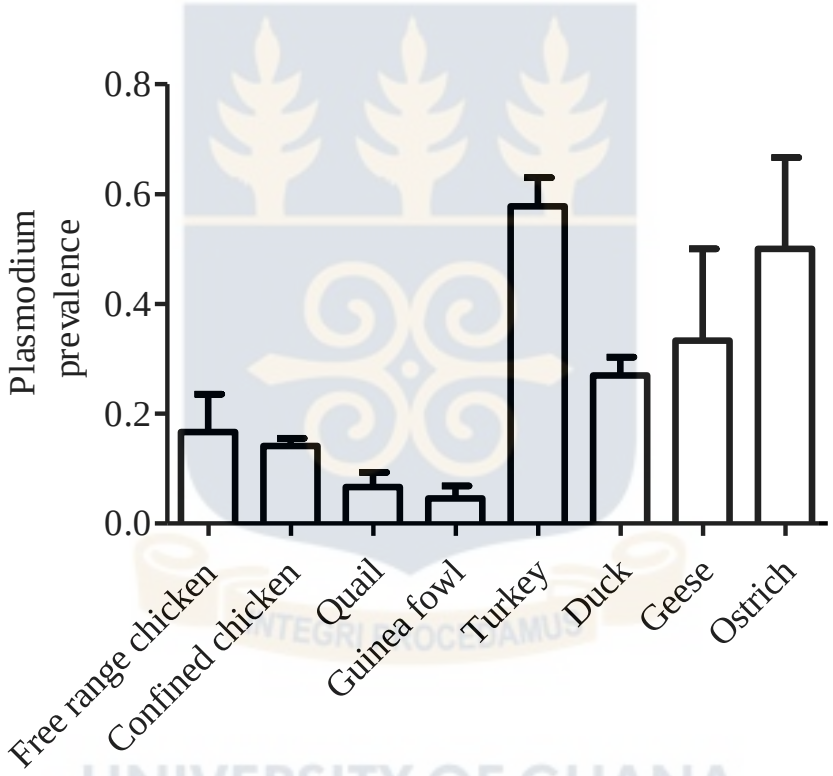

Figure 13: *Plasmodium* prevalence in domestic birds

Haemoproteus infections occurred in four species of birds (Figure 14). The study reported an overall prevalence of 2.1% which was much lower than 10.4% and 18.9% for *Leucocytozoon* and *Plasmodium*, respectively. We observed no significant difference in prevalence in all four infected species (Figure 14).

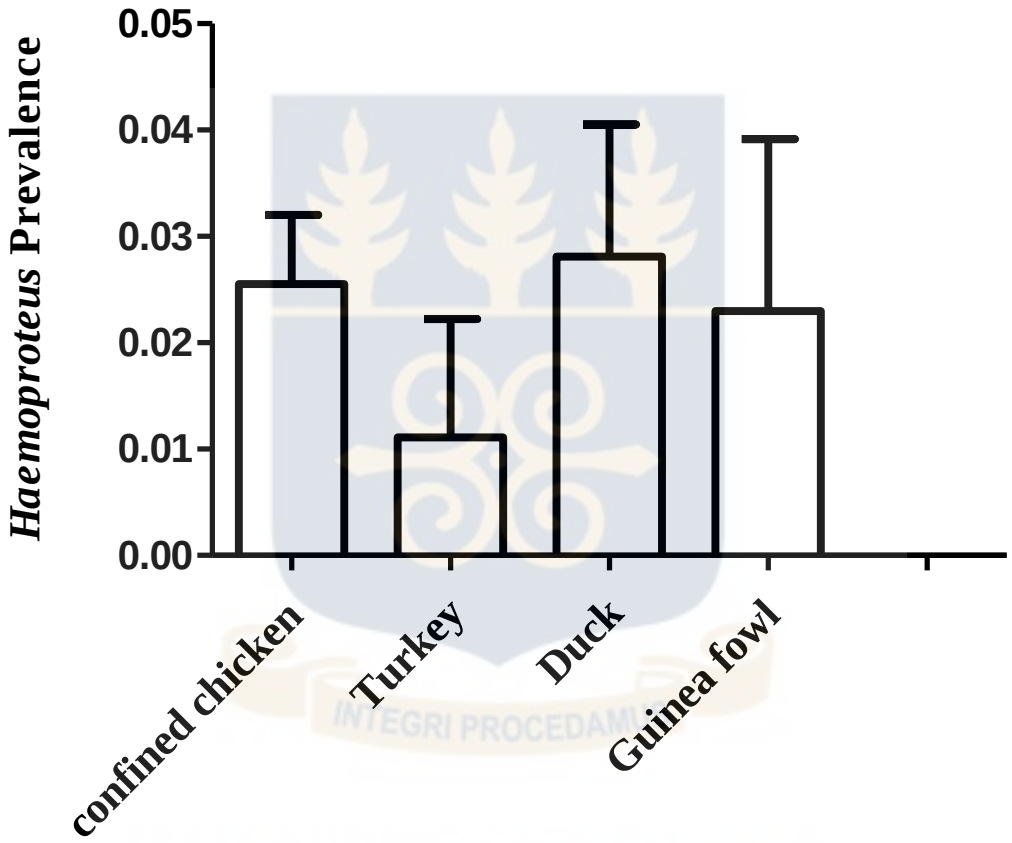

Figure 14: *Haemoproteus* prevalence in domestic birds

4.2.2 Coinfection of *Plasmodium* and *Leucocytozoon* across bird species

This study reported *Plasmodium* and *Leucocytozoon* coinfection in four species of domestic birds (Figure 15). Coinfection prevalence was 21.1% in turkeys, 13.3% in free-range chicken, 10.6% in ducks, 10.5% in confined chicken, and 3.3% in quails (Figure 15). Coinfection prevalence was significantly higher in turkeys than in quails and confined chickens (P = 0.0044) (Figure 15).

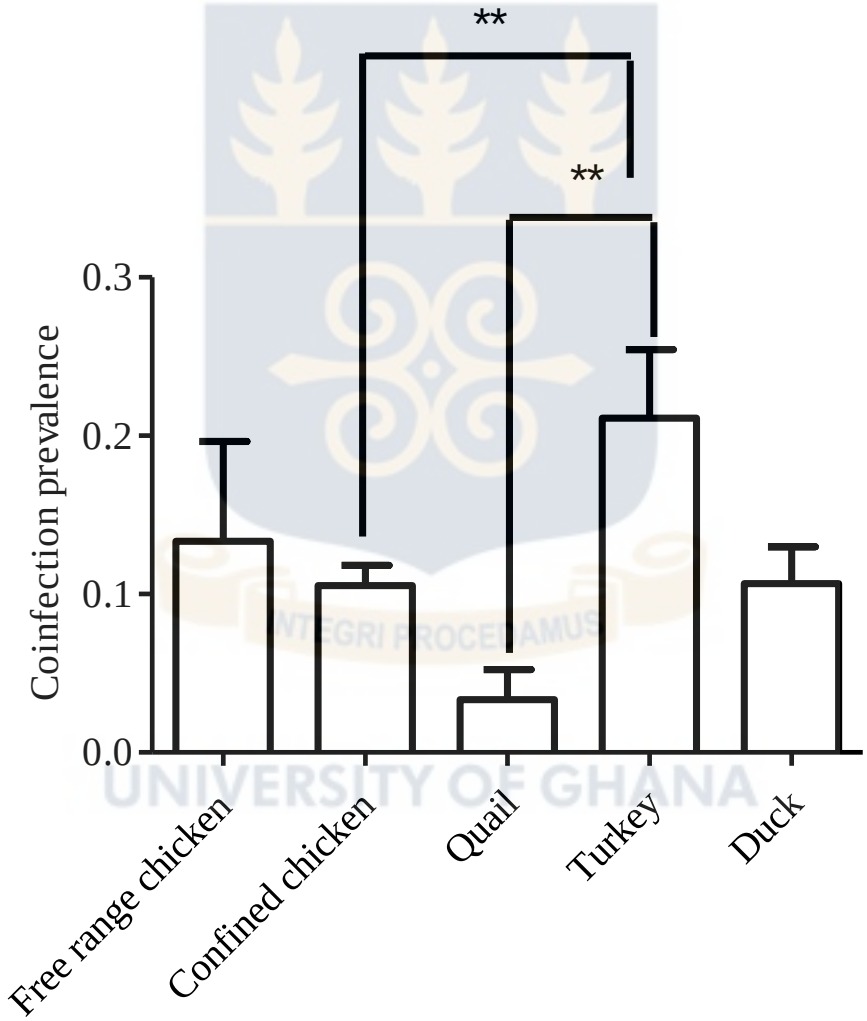

Figure 15: Coinfection of *Plasmodium* and *Leucocytozoon* in four species of domestic birds

4.2.3 Haemosporidian prevalence in host families

There was a significant difference in *Plasmodium* infections among the bird families with the families *Struthionidae* (eg. ostrich), *Meleagridae* (eg. turkey), and *Ploceidae* (eg. vieillot's black weaver) showing higher prevalences ($P < 0.001$) (Figure 16). *Plasmodium* and *Leucocytozoon* coinfection were significantly different among the four host families observed (Figure 16). The families *Meleagridae* and *Ploceidae* had a higher coinfection prevalence than the others ($P = 0.013$).

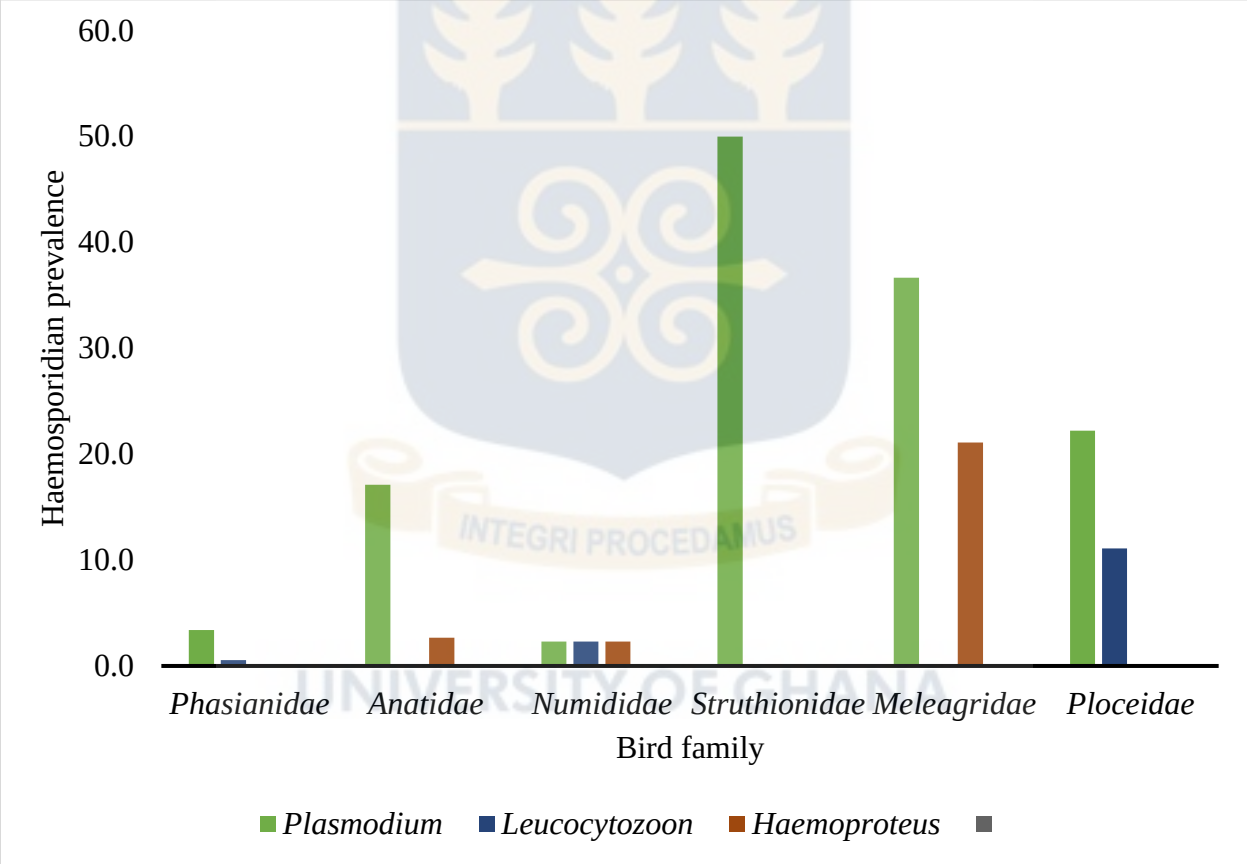

Figure 16: Haemosporidian prevalence in bird families. *Plas/Leuco* = *Plasmodium* and *Leucocytozoon* co-infections.

4.2.4 Haemosporidian prevalence in confined, wild, and free-range birds

Overall haemosporidian prevalence was significantly higher in free-range birds and wild birds than confined birds ($P < 0.001$). There was a significant difference in *Plasmodium* prevalence observed in free-range, and wild birds ($P < 0.001$). The differences observed in *Leucocytozoon* infections among the three categories of birds were also significant ($P = 0.045$). We observed no significant difference among the bird groups in terms of *Haemoproteus* infections (Figure 17).

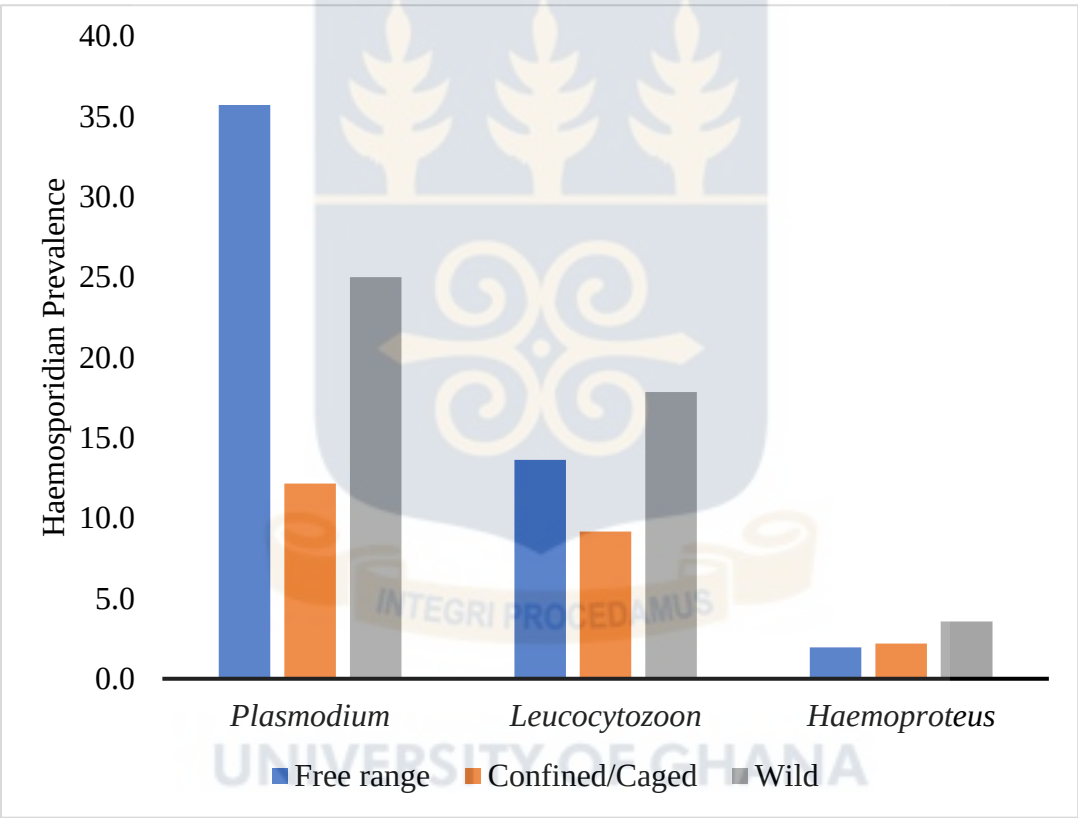

Figure 17: Haemosporidian prevalence in three bird categories

4.3 Tissue level host-parasite interaction

4.3.1 Evaluation of tissue tropism of haemosporidian parasites in turkeys

This study has confirmed avian haemosporidian infections in exotic poultry and backyard poultry farms in the Ghanaian poultry industry. There is little knowledge about the interaction of these parasites with their hosts, and therefore, the study further sought to assess parasite interaction at the host tissue level. The histology results shown below (Figure 18), which were obtained from a turkey that was naturally infected with *Plasmodium* parasites, revealed no haemosporidian parasite in the processed tissues of the bird.

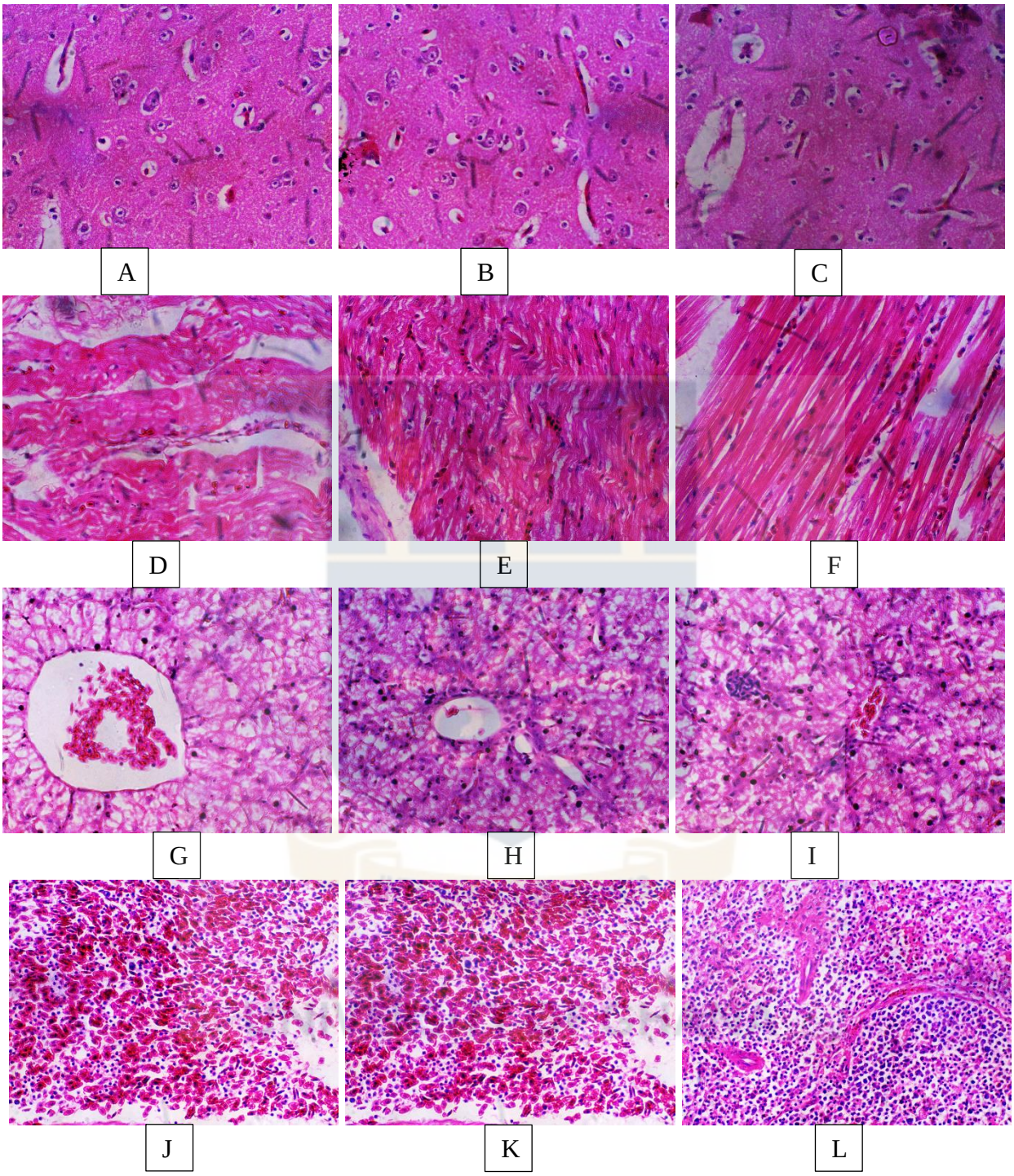

Figure 18: Pictures of histological sections from an infected turkey. There were no tissue-stage haemosporidian parasites seen. A–C (brain tissue), D–F (heart tissue), G–I (liver tissue), J–L (spleen tissue)

4.3.2 Hematology

We assessed hematological parameters for the blood sample obtained from the naturally infected turkey to assess the correlation between blood parameters and tissue-stage haemosporidian infections. The hematology test values obtained were compared with a turkey reference range. The hematology results showed very high leucocyte and lymphocyte counts (Table 4). Haemoglobin concentration was slightly lower than the reference range for turkey, and the total red blood cell (RBC) count was also low (Table 4). The total leucocyte count recorded was very high compared to the reference range (Table 4).

Table 4: Hematological parameters

Parameters	Reference Range	Test value	Remarks
Haemoglobin concentration (g/dl)	10.42 -15.2	9.6	Haemosporidian may have been feeding on the host haemoglobin (Valkiunas, 2005)
Total RBC(x10^6/µl)	2.95 - 5.35	2.14	Haemosporidians damaged RBCs during infection
Mean cell volume, MCV (fl)	90 - 140	151.4	A lot of immature RBCs present
Total leucocyte count (x10^3 /µl)	2.4 - 4.35	218.2	Stress syndrome, inflammatory processes (Krams *et al*., 2013)
Mean cell haemoglobin, MCH (pg)	33 - 47	44.9	Normal
MCHC (g/dl)	26 - 35	29.6	Normal
Lymphocyte (%)	45 - 70	93.4	Chronic inflammatory condition

4.4 Lineage diversity and phylogenetic analysis

This study confirmed that Avian malaria and related haemosporidian parasites belonging to the genera *Plasmodium*, *Leucocytozoon*, and *Haemoproteus* were present in the study area. *Plasmodium* and *Leucocytozoon* parasites occurred more than *Haemoproteus* parasites. The molecular methods used could only identify the parasites at the genus level. We employed sequencing for further classification, possibly at the species level. The results found haemosporidian cytochrome b gene sequences that had close matches to parasite lineages in the GenBank but did not share 100% homology with them. The study identified them as new lineages and we deposited them in the NCBI Genbank. These lineages did not match any already described species and therefore are suspected to be new species.

We identified 24 new mitochondrial cytochrome b gene lineages of the three haemosporidian genera in this study. There were six different *Leucocytozoon* lineages found infecting the birds sampled (Table 5). We found four *Leucocytozoon* lineages, LGHA73 (OM643343), LGHA83 (OM643344), LGHA146 (OM643346), and LGHAAS2 (OM643347) (Table 5) in domestic chicken (*G. gallus*), two lineages LGHA111 (OM43345) and LGHA146 (OM643346) recorded in *A. platyrhynchos* and LGHA29Q (OM643342) recorded only in *C. Coturnix* (Table 5). The most prevalent lineage LGHA146 (OM643346) was found in three bird species *G. gallus*, *A. platyrhynchos,* and *N. meleagris*.

Table 5: Lineage diversity of haemosporidian parasites

Lineages	Parasite species	Host species	Accession number
PGHA708	*Plasmodium sp.*	*Gallus gallus* (free range)	OM643371
		Gallus gallus (caged)	
		Numida meleagris	
		Meleagris gallopavo	
		Anser branta	
		Ploceus niggerrimus	
PGHA954	*Plasmodium sp.*	*Gallus gallus* (free range)	OM643374
PGHATDF24	*Plasmodium sp.*	*Gallus gallus* (free range)	OM643382
		Gallus gallus (confined)	
PGHA40Q	*Plasmodium sp.*	*Coturnix coturnix*	OM643361
PGHA58Q	*Plasmodium sp.*	*Coturnix coturnix*	OM643362
PGHAOS7	*Plasmodium sp.*	*Numida meleagris*	OM643380
		Struthio camelus	
PGHAOS2	*Plasmodium sp.*	*Struthio camelus*	OM643378
PGHATK2	*Plasmodium sp.*	*Meleagris gallopavo*	OM643383
PGHAAS37	*Plasmodium sp.*	*Gallus gallus* (confined)	OM643360
PGHA205	*Plasmodium sp.*	*Gallus gallus* (confined)	OM643367
PGHAAS10	*Plasmodium sp.*	*Gallus gallus* (confined)	OM643375
PGHA149	*Plasmodium sp.*	*Gallus gallus* (confined)	OM643365
PGHA70	*Plasmodium sp.*	*Gallus gallus* (confined)	OM643363
PGHA843	*Plasmodium sp.*	*Gallus gallus* (confined)	OM643372

Table 5: Lineage diversity of haemosporidian parasites (continued)

HGHA982	*Haemoproteus sp.*	*Meleagris gallopavo*	OM643356
		Gallus gallus (confined)	
HGHA3W	*Haemoproteus sp.*	*Cyanomitra olivacea*	OM643350
HGHA1W	*Haemoproteus sp.*	*Ploceus niggerrimus*	OM643348
PHGHA989	*Parahaemoproteus sp.*	*Gallus gallus* (confined)	OM643357
		Meleagris gallopavo	
		Anas platyrynchos	
LGHA29Q	*Leucocytozoon sp.*	*Coturnix coturnix*	OM643342
LGHA73	*Leucocytozoon sp.*	*Gallus gallus* (confined)	OM643343
LGHA83	*Leucocytozoon sp.*	*Gallus gallus* (confined)	OM643344
LGHA111	*Leucocytozoon sp.*	*Anas platyrynchos*	OM643345
LGHA146	*Leucocytozoon sp.*	*Gallus gallus* (confined)	OM643346
		Anas platyrynchos	
		Numida meleagris	
LGHAAS2	*Leucocytozoon sp.*	*Gallus gallus* (confined)	OM643347

The *Leucocytozoon* lineage LGHA146 (OM643346), the most prevalent (93.8%) among the six lineages reported recorded a prevalence of 59.6%, 20.17%, and 14.03% prevalence in *G. gallus, A. platyrhynchos* and *M. gallopavo* respectively (Figure 19). The lineage LGHA29Q (OM643342) which was found only in *C. coturnix* recorded a 2.6% prevalence (Figure 19).

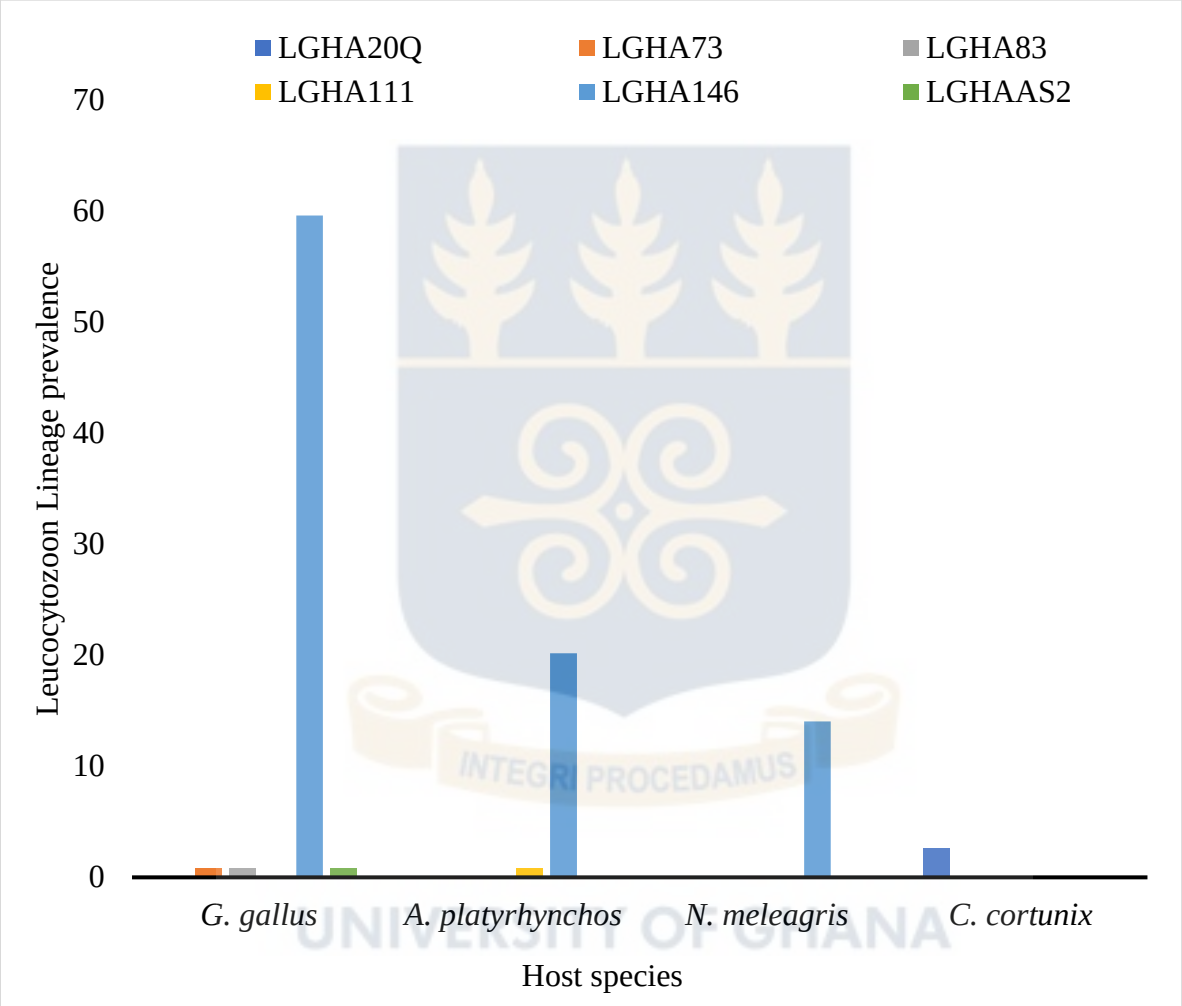

Figure 19: *Leucocytozoon* lineage diversity. This graph shows the prevalence of each Leucocytozoon sp. lineage within different bird species.

Plasmodium PGHA708 and *Parahaemoproteus* PHGHA989 were the most abundant parasite lineages found in infected birds (Figure 20). We observed the *Plasmodium* lineage PGHA708 in six bird species except for quail and ostrich (Figure 20). This study recorded thirteen new *Plasmodium* lineages in 213 birds, with lineages PGHA708 and PGHAAS37 being the most abundant. We found three new *Haemoproteus sp*. lineages and two *Parahaemoproteus sp*. lineages in 23 birds with lineages HGHA982 and PHGHA989 being the most abundant *Haemoproteus* and *Parahaemoproteus* lineages.

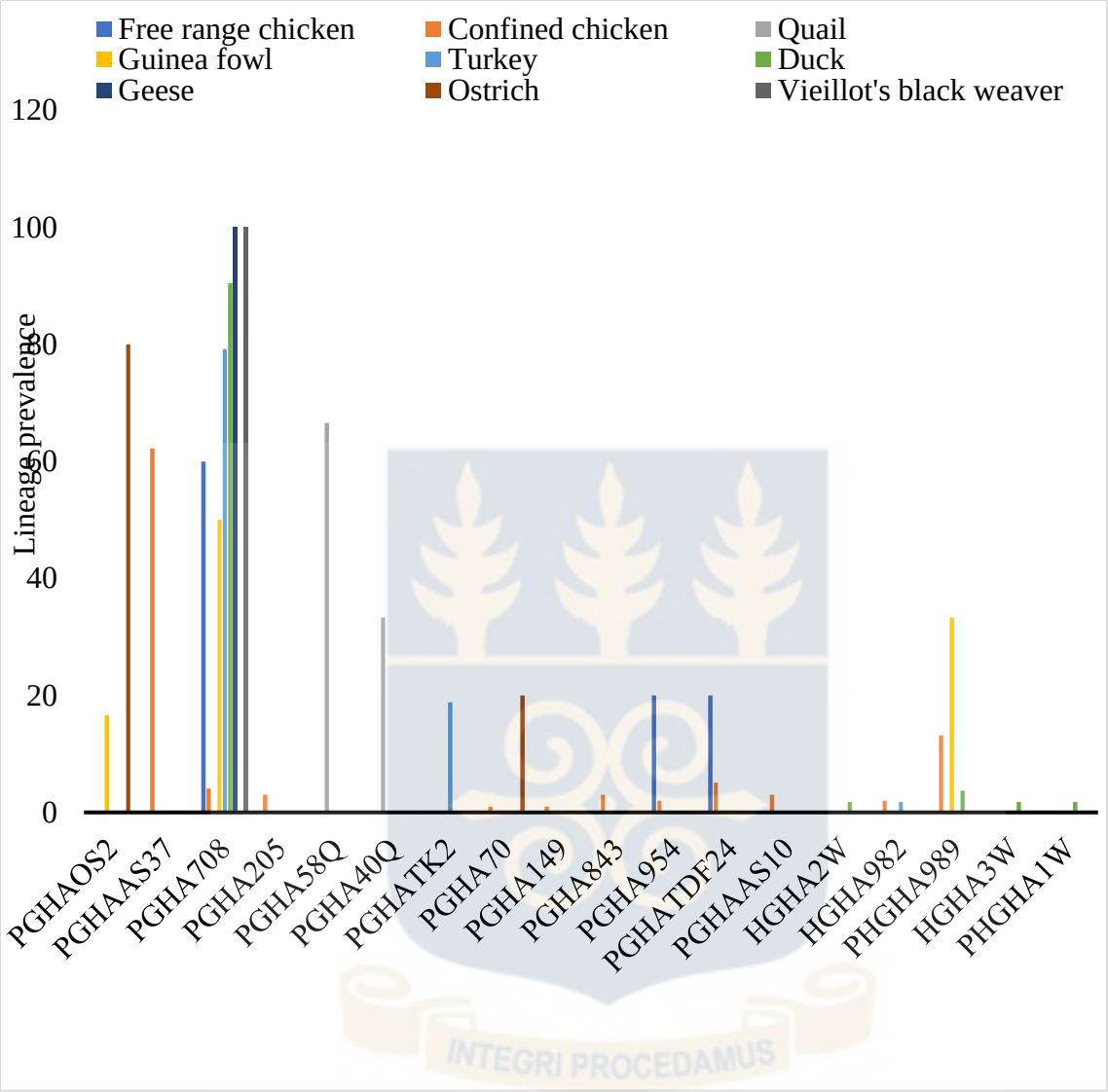

Figure 20: Lineage prevalence. Prevalence and diversity of *Plasmodium spp*. and *Haemoproteus spp*. lineages within infected bird categories.

Five *Leucocytozoon* lineages from this study (Figure 21) cluster together forming orthologs and are closely related to the clade of *L. schoutedeni* isolate 23-379, *L. GALLUS06,* and *Leucocytozoon* isolates AS4613 (Figure 21). The lineages in this study share the same ancestor with the *L. schoutedeni* clade. The lineage LGHA83 is distantly related to the other lineages in this study.

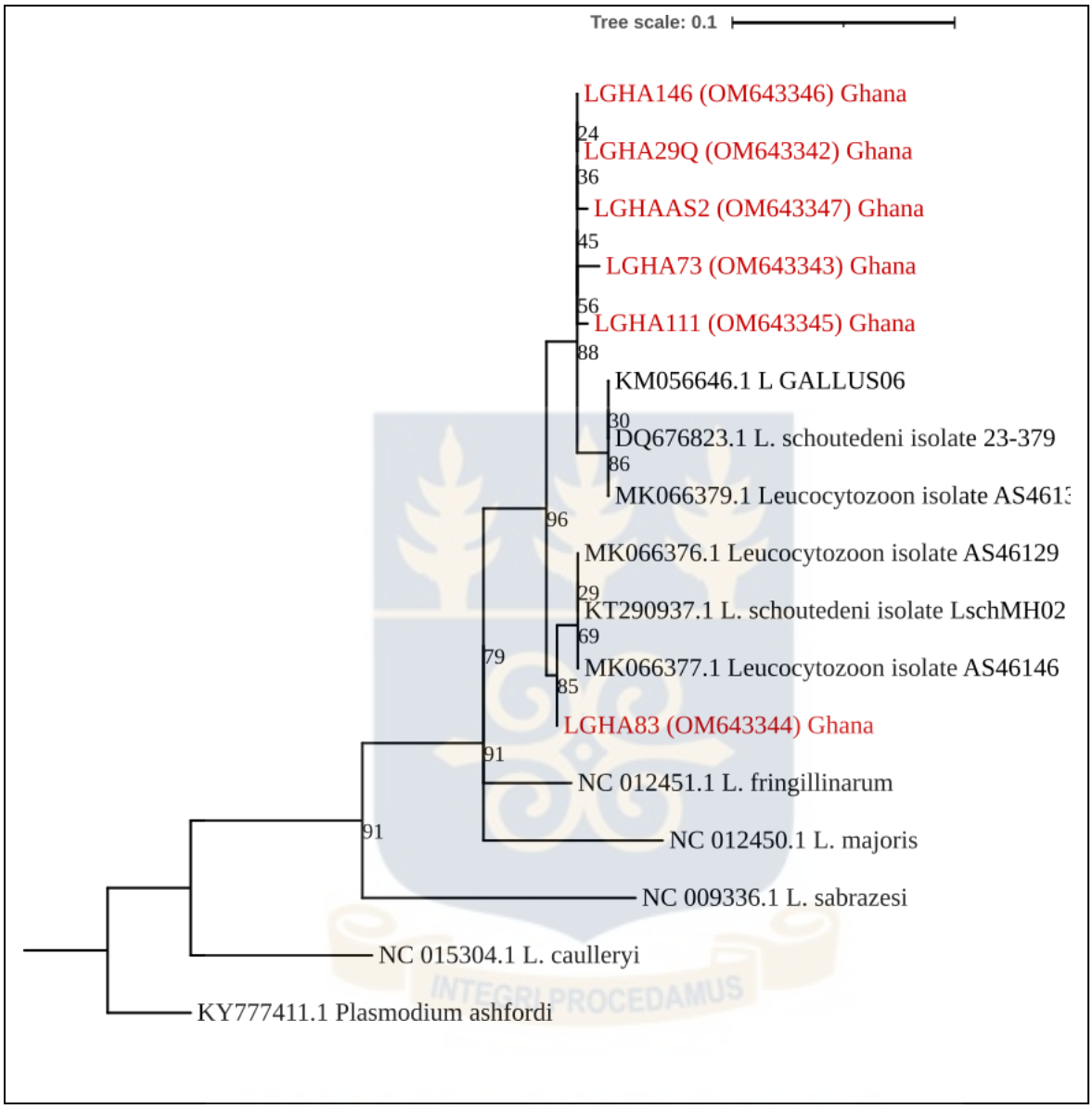

Figure 21: A maximum-likelihood tree on *Leucocytozoon*. This phylogenetic tree was constructed with partial Cytochrome b gene of six *Leucocytozoon* lineages in this study (shown in red) and others obtained from NCBI GenBank. Values on nodes denote bootstrap analysis based on 1000 replicates.

We found 18 new *Plasmodium*, *Haemoproteus* and *Parahaemoproteus* lineages in all birds sampled. The lineages comprised 13 *Plasmodium spp*. lineages (PGHA708, PGHA954, PGHATDF24, PGHA40Q, PGHAOS2, PGHA205, PGHA843, PGHAAS37, PGHA58Q, PGHA70, PGHA149, PGHATK2, PGHAAS10) and 5 *Haemoproteus* and *Parahaemoproteus* lineages (PHGHA989, HGHA982, PHGHA1W, HGHA2W, HGHA3W) (Table 5). We found *Plasmodium sp*. lineages were closely related and shared a common ancestor with previously described lineages (Figure 22).

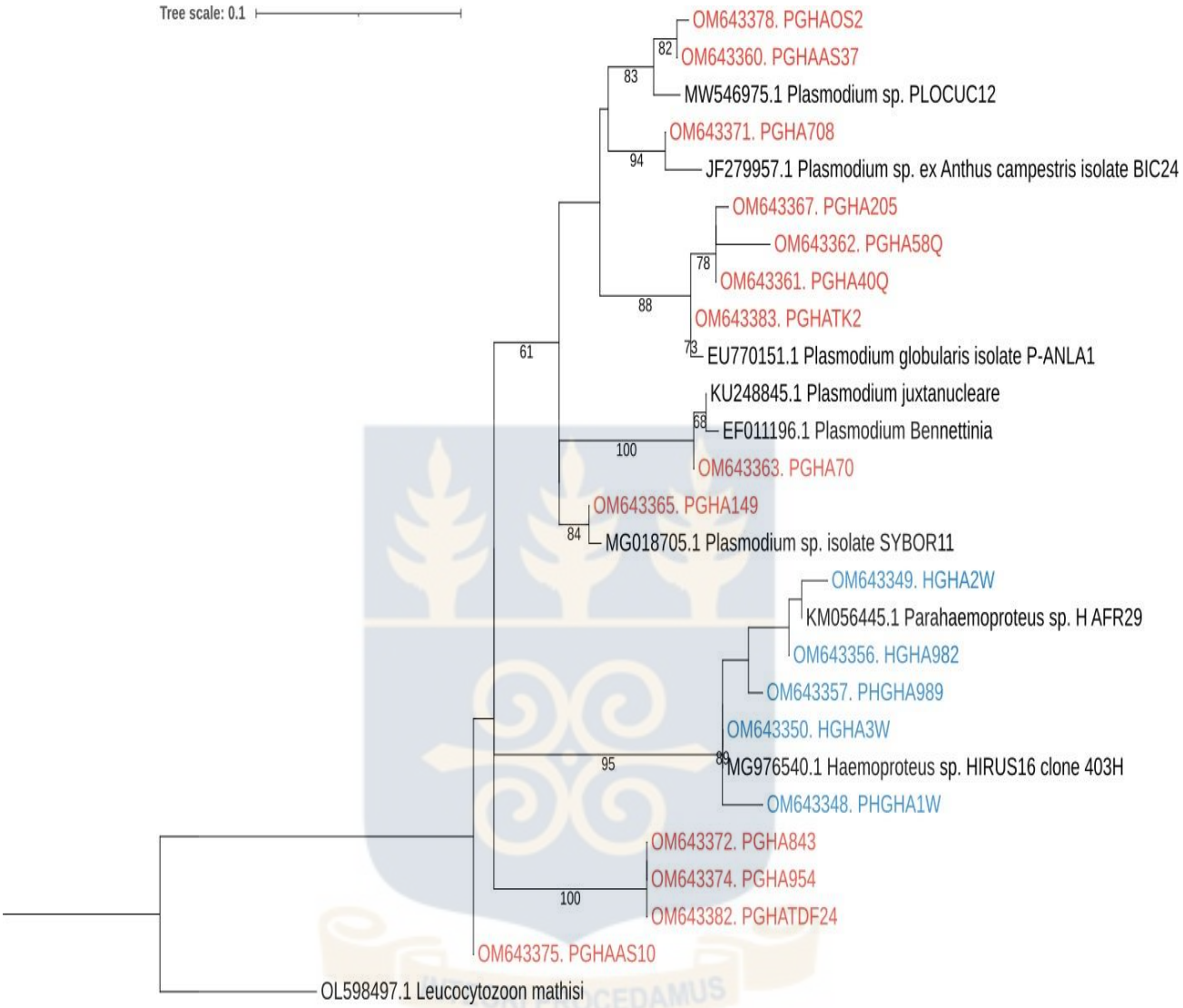

Figure 22: A Maximum likelihood tree for *Plasmodium* and *Haemoproteus*. The tree was constructed with partial mitochondrial cytochrome b gene sequences of *Plasmodium* and *Haemoproteus* parasites, with 1000 bootstraps indicating bootstraps above 50. *Plasmodium* in red, *Haemoproteus* in Blue, and Genbank sequences in black. The tree root was *Leucocytozoon mathisi*.

4.5 Whole genome enrichment

The results of the enrichment protocol showed that there was a successful depletion of host DNA. The parasite-infected avian DNA samples showed higher DNA concentrations compared to enriched parasite DNA (Figure 23).

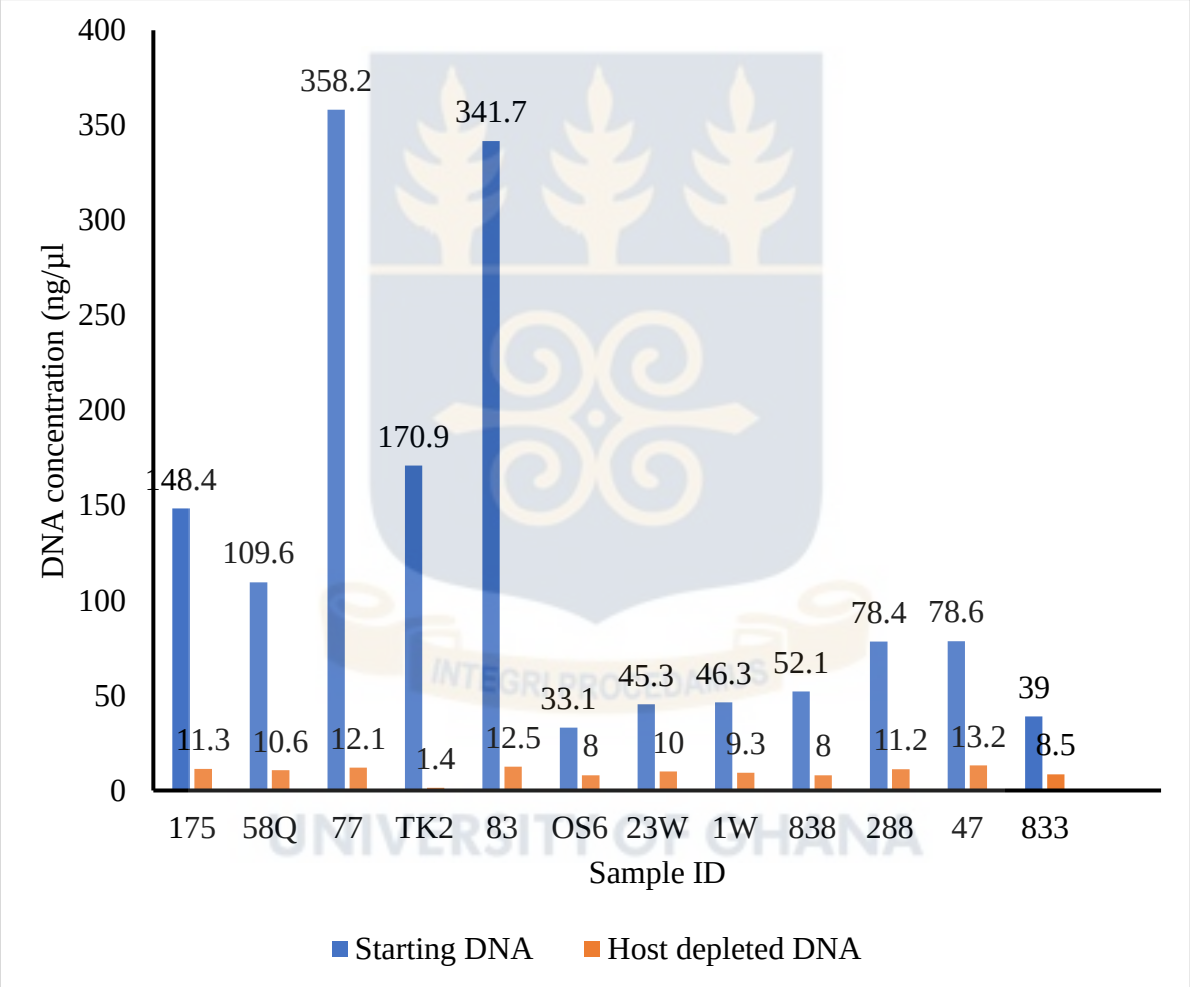

Figure 23: Considerable haemosporidian parasite DNA enrichment. Host DNA depletion using methylation shows lower DNA concentrations for host-depleted enriched DNA and higher concentration for non-depleted samples.

CHAPTER FIVE

5. DISCUSSION

The distribution of samples collected skewed towards confined chickens. Among all the domestic bird species sampled, confined chickens belonging to the order Galliformes were the most abundant. The poultry farmers mostly farmed these exotic chickens within the study areas because they form part of their major sources of income and livelihood. The study area falls under the regions of Ghana that supply poultry products, such as meat and eggs, for food all over the nation and beyond. There were other bird species raised as domesticated within the study area, but these birds were not as abundant as the confined chickens. This is because most of them are larger birds, and quite expensive to sell, and their market demand is quite low. These larger birds are mostly raised on the free range together with a few free-range chickens which are kept by backyard farmers.

Avian malaria research advancement is directly connected to the application of standard nested PCR protocols, which amplifies a partial region of the mitochondrial cytochrome b gene of the parasite (Bensch *et al*., 2000; Hellgren *et al*., 2004). Even though that is true, nested PCR also involves the use of large amounts of reagents. When processing many samples, it is necessary to find alternative methods of screening for positives in the most economical way possible. To identify malaria parasites and related haemosporidian parasites in domestic birds sampled in Ghana, this study employed three PCR methods, the qPCR method used by Bell *et al*., 2015, the existing nested PCR using Bensch et al., 2000 & Hellgren *et al*., 2004, and a modified nested PCR proposed by this study. We preferred the Bell *et al*., 2015 qPCR method in this study to the qPCR protocol described earlier by Fallon and colleague (Fallon & Ricklefs, 2008), which amplified a

portion of the same ribosomal DNA gene of *Plasmodium* and *Haemoproteus* in a single reaction but not *Leucocytozoon* (Bell *et al*., 2015). This is because the Bell *et al*. protocol amplified any of the three haemosporidian parasites in a single reaction and was successfully used to test positive and negative *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* samples from Malawi in Africa (Lutz *et al*., 2015). This gave the likelihood that this qPCR method could successfully detect parasites in our samples. We, therefore, applied the three methods to the first batch of samples collected.

The qPCR detected a significantly higher (36.4%) of infected samples compared to the 11.5% by the nested protocol with modification by this study and 1.75% by the existing nested PCR protocol. This observation agrees with the study by Bell *et al*., 2015 in which the qPCR protocol detected positive samples detected by the nested PCR as true positives. However, our study found an extremely lower parasite detection rate (1.75%) by the existing nested PCR compared to a previous report in which the existing nested protocol detected 77% positives from 693 Bell's qPCR positive samples (Bell *et al*., 2015). From the qPCR results by Bell *et al*., 2015, the melting peak for all three genera of haemosporidian parasites from the Malawi samples was between 78 to 79°C. This was not the case in our samples. Contrary to these findings, our study showed wide variations in melting curve temperatures between 76-88°C. There were three melt curves for our samples, specifically between 76-78°C, 83-85°C, and 87-88°C. This suggests that there were other products amplified in our samples, some of which could be off-target or contaminants. We confirmed this by performing agarose gel electrophoresis on the qPCR products and got two bands, which included our desired product, and was further confirmed in a singleton conventional PCR. This confirmation was necessary for us to eliminate any possibility of false positive amplifications by the comparatively high detection rate of our qPCR method.

For comparative analysis of our haemosporidian parasites with existing GenBank parasites, we needed to amplify a region of the mitochondrial cytochrome b gene. When the standard existing nested PCR was modified to amplify a shorter region of the mitochondrial cytochrome b gene which falls within the larger region amplified by the standard nested PCR protocol, the detection rate was higher (11.5%). This confirms the suggestion that low-intensity infections of parasites can be missed by molecular methods (Valkiūnas *et al*., 2008).

There is a possibility that the intensity of haemosporidian infection in the samples was lower than the threshold that could be detected by nested PCR. This is because when host DNA is higher in concentration than parasite DNA, it becomes difficult to amplify larger fragments of the parasite DNA which is usually the case of nested PCR (Bell *et al*., 2015). It was, therefore, easier to target smaller fragments, making the modification introduced in the nested protocol a better option. There is also the possibility of a mutation within the primer region, which caused the primers not to anneal to the target region of the larger fragment of the mitochondrial cytochrome b gene. The successful amplification of a shorter fragment of the cytochrome b gene region could also be because of a higher sensitivity of the primers to that region of the gene.

There were limitations introduced in our study by the screening protocols. The nested protocols used underestimated the prevalence of parasites in our samples. Extremely low parasitaemia did not allow confirmation of positive samples using microscopy as a gold standard. We assessed general haemosporidian prevalence in this study based on the new nested PCR with modifications introduced by this study.

It is important to develop new qPCR methods that will target the mitochondrial cytochrome b gene of haemosporidian parasites among the three genera *Plasmodium*, *Haemoproteus,* and

Leucocytozoon. This will make haemosporidian research in the sub-Saharan Africa region cost-effective and more accurate.

Microscopy could not detect parasites in most of the samples we screened, making the detection rate of microscopy extremely low compared to PCR. This trend is not surprising, as it has been reported in several haemosporidian studies. Even though microscopy is recognized and accepted as the gold standard for confirming haemosporidian infections and possible identification to species level, microscopy has proven to be less sensitive to molecular identification of haemosporidian parasites in previous studies (Durrant *et al*., 2006; Jarvi *et al*., 2002; Schumm *et al*., 2021). We agree with the report by Valkiunas and other researchers, which explained that sensitivity of microscopy also depends on good quality of the blood smears prepared and the expertise of the investigator (Ciloglu *et al*., 2019; Nebel *et al*., 2020; Valkiunas *et al*., 2008). Most of our blood smears did not stain properly. They could be attributed to the fact that the smears were prepared in poultry farms in dusty environments, with dust settling on the slides as they were air drying. We also observed flies feeding on the blood smear as they were air dried in the open. The stained blood films were pale, some with corpuscles stained too blue, and the parasite nuclei parasites were poorly seen or even invisible. Some of our slides from domestic birds had hemolyzed blood cells, which testify to the insufficient desiccation, or fixation of blood films, or both, in the field as described by Valkiunas about the study by Jarvi *et al*., 2002 (Valkiunas *et al*., 2008).

The natural haemosporidian infections in the birds were mostly chronic, with the possibility of light parasitaemia, so we identify in this study that if observation time is increased, we may find some stages of the parasites. Another reason which can explain the low sensitivity in our microscopy would be extremely low parasitaemia of infections. This is because our samples were

collected from wild birds caught with mist nets, and domestic birds mostly confined, which all looked healthy with no disease symptoms, suggesting that they could probably be in very early or chronic stage of disease when parasites are very few in the blood (Valkiunas, 2005). However, we have not ruled out the likelihood of abortive infections that will not allow us to find parasite morphological stages in blood smear, especially in our chronically infected birds.

Our study observations in wild birds sampled, also agree with the report by Valkiunas which suggested that both microscopic examination of blood films and nested PCR-based diagnostics show similar level of prevalence of infection of blood parasites in naturally infected birds (Valkiunas *et al*., 2008). Microscopy detected positive in all our positive wild birds sampled, though few they were. However, because of the challenges in the traditional microscopy screening in our samples, we relied mainly on PCR to estimate parasite prevalence. It is important to note that microscopy remains very important and reliable in the study of haemosporidian parasites, except that the blood smears must be of good quality and the investigator must be well trained.

Haemosporidian parasites of three genera, *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*, were recovered in domestic birds sampled within the study areas, with an overall prevalence of 21.5% in all birds. When we counted every coinfection as one infection, *Plasmodium* and *Leucocytozoon* infections were significantly higher than *Haemoproteus* in domestic birds. The *Plasmodium* prevalence (18.9%) from our study was higher than the 15% recorded in Zimbabwe (Permin *et al*., 2002) and lower than the 27% reported in Ghana (Poulsen *et al*., 2000).

We observed *Plasmodium* infections in all 7 species of domestic birds sampled. We observed the higher *Plasmodium* prevalence in turkeys (58.9%), Ostriches (50%), Duck (29.8%), and Geese (33.3%). This observation could have occurred because of the free-range nature of these birds.

These birds moved about freely and had close contact with pristine vegetation surrounding the farms, resulting in greater exposure to the risk of being fed on by vectors. The study observed rather Lower *Plasmodium* prevalence in confined birds, such as seen in quails (6.7%), guinea fowls (6.9%), and confined chickens (17.3%).

These birds were protected from predators and provided with veterinary care, which has likely boosted their immune system to fight all kinds of infections, including haemosporidian parasites. The birds were provided routinely with dietary supplements such as vitamins A, E, C and amino acids such as valine and methionine which increase feed efficiency, but when added in higher concentrations, have the added advantage of boosting the immune response of the birds against infections (Butcher & Miles, 2017). Apart from these supplements, the confined birds also obtain well formulated feed and enough food since they do not have to fend for themselves. It is known that good nourishment for the birds helps them become immunologically competent and can cope with diseases and infections better than malnourished birds (Butcher & Miles, 2017).

The *Plasmodium* prevalence of 16.7% in free-range chickens was much higher than 1.6% in chickens reported in Nigeria (Orajaka & Nweze, 1991). The 58.9% *Plasmodium* prevalence found in turkeys was higher than the 46.6% reported in turkeys in Nigeria (Orajaka v Nweze, 1991). Contrary to our findings, there were no reports of any haemosporidian parasites in ducks, geese, and guinea fowls reported in Nigeria (Orajaka & Nweze, 1991).

Our *Haemoproteus* prevalence of 2.1% in domestic birds was higher than those reported in Ethiopia and Malaysia (Gimba *et al*., 2014; Sabuni *et al*., 2011) but lower than found in Bangladesh, Uganda, and Nigeria ranging from 2.5 to 6.8% (Annorbah *et al*., 2016; Lawal *et al*., 2020; Nakayima *et al*., 2020). We observed both *Plasmodium* and *Haemoproteus* parasites in

confined chicken, guinea fowl, duck, and turkeys, suggesting that the vectors for these two parasites were present in the same locations.

A 10.4% prevalence of *Leucocytozoon* parasites was observed in the sampled birds. This prevalence was higher than 4.3% prevalence of *L. sabrazesi* reported in chicken in Zimbabwe (Poulsen *et al*., 2000), and lower than *L. schoutedeni* prevalence of 52.1% in Kenya (Sabuni *et al*., 2011), 50% in Tanzania (Fallis, 1973), 34% in Nigeria (Adene & Dipeolu, 1977) and 31% in Uganda (Sehgal *et al*., 2006) reported in domestic birds, and 15% prevalence reported in village chickens in Gombe State, Nigeria (Jallailudeen *et al*., 2022). Earlier studies reported no *Leucocytozoon* infection detected in Ghana (Poulsen *et al*., 2000). The difference in reported prevalence in various geographical locations can be because of the availability of insect vectors and the extent of sampling. We observed the highest *Leucocytozoon* prevalence in turkeys (21.1%), followed by 13.3% in free-range chickens, compared to a lower prevalence of 2.3% and 3.3% in guinea fowls and quails. Whereas guinea fowls and quails were kept in poultry sheds and were well managed, turkeys and free-range chickens were left fending for themselves. The free-range birds could have higher exposure to the vectors than the caged birds. This can explain the differences in prevalence. Exposure to vectors is a major determining factor in susceptibility to haemosporidian infections.

Our study observed an overall *Plasmodium* and *Leucocytozoon* coinfection prevalence of 9.8% in all domestic bird species. In addition, *Plasmodium*/*Leucocytozoon* co-infection was significantly higher in turkeys (21.1%) and free-range chickens (13.3%) compared to the quail (3.3%). Coinfection was similar in confined chickens (10.5% and ducks (10.6%).

Habitat characteristics (Nourani *et al*., 2020) of the farms which were surrounded by pristine vegetation could strongly influence parasite transmission by providing a conducive dwelling and

breeding place for *Culicidae* and *Simuliidae* dipteran vectors of *Plasmodium* and *Leucocytozoon* parasites. Since the free ranged turkeys and chickens had no boundary of movement, it is likely that they were more exposed to the dipteran vectors than the confined quails and chickens. Birds that were kept in confinement received better health care, including feed and vitamin supplements, which made them more resilient to parasitic infections because their immune systems were boosted. Quails had relatively lower coinfection prevalence, and we observed no coinfection in other species, like the ostriches and guinea fowls. Several factors can contribute to low levels or no haemosporidian coinfection in these birds, including unavailability of insect vectors, host resistance to parasites, host immune system, host specificity of the parasites and habitat characterization (De Aguilar *et al*., 2018; Bensch, Jönsson, & Copete, 2012; Garcia-Longoria *et al*., 2019; Gutiérrez-López *et al*., 2015; Soares *et al*., 2016).

Tropism is a leading determinant of disease severity (Brierly *et al*., 2019). From the results of this study, there was no observation of haemosporidian parasites in the various organ tissues of the bird examined. We performed histology on only one infected turkey. There were some abnormalities, such as yellowish liver, enlarged liver, and spleen observed by the physical appearance of some organs. This could be because of other unknown infections in the bird. The coinfection of haemosporidian parasites together with other infections may cause the possibility of one infection increasing the effect of the other or the combined negative effect of all the infections together. The absence of parasites observed in the tissues of the examined birds could be because of abortive infections. Abortive tissue stage infections of haemosporidian parasites have caused mortality in non-adapted avian hosts. This development occurs when a parasite invades a wrong host, develops partially, and cannot complete its full life cycle, resulting in the absence of invasive stages (Valkiunas & Iezhova, 2018). It is usually difficult to diagnose abortive

haemosporidian infections because of their unusual location and morphology in the 'wrong' host, unclear etiology, and short-term survival of parasites (Valkiunas & Iezhova, 2018). We suspect abortive infections to be responsible for the absence of blood stages of the parasites in most of the domestic birds examined.

Hematology results of the infected turkey confirmed the positive avian malaria status of the turkey as detected by PCR. Haemosporidians use haemoglobin as a nutrient source (Valkiūnas, 2005) suggesting that low haemoglobin recorded in the infected turkey was due to haemosporidians feeding on the host haemoglobin (Krams *et al*., 2013; Norte *et al*., 2009; Valkĭunas, 2005). High Leucocyte and lymphocyte counts are indicators of inflammation and infection (Campbell, 1994, 1995). Elevated leukocyte number is symptomatic of a stress syndrome, inflammatory processes, and oxidative stress (Krams *et al*., 2013). Elevated concentration of white blood cells (heterophils and/or lymphocytes) cause leukocytosis (Cerny & Rosmarin, 2012) and this condition which is the source of free oxidative radicals (ROS) has adverse effects on host tissues reducing quality of life and possibly leading to economic loss in commercial poultry activities. It is very important to conduct further studies in which we will exhaustively sample our study areas to target any opportunity of haemosporidian, especially *Plasmodium* blood stages, so we could design studies to identify tissue stage parasites.

Birds sampled in this study fall into three categories: free-range, captive/confined, and wild birds. The presence of all three genera of haemosporidian parasites (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*) was observed among all three categories of birds sampled. Prevalence of all three genera of haemosporidian parasites was higher in free-range birds and wild birds despite a small sample size than confined birds, with a significant difference in *Plasmodium* and *Leucocytozoon*

infections within the bird groups. These two categories of birds shared the same habitat and were exposed to almost the same conditions surrounding their vulnerability to infections.

These conditions include factors such as temperature, rainfall, water quality and habitat, which affect abundance of mosquito vectors that transmit *Plasmodium spp*. (Smith, Dushoff, & McKenzie, 2004). We collected the samples during a minor rainy season in Ghana and this accounts for conducive conditions for increased mosquito vectors of avian malaria. In avian malaria epidemiology, other factors also contribute to the success of infections including blood meal frequency of infected vectors, rates of transmission, and bird and vector ratio in a particular habitat (Greenwood 1997; Mbogo *et al*., 1995). The free-range birds infections, mostly occurred in areas characterized by trapped water stagnation caused by human artifacts such as abandoned cans, broken containers, and trapped rainwater among others. Mosquito larvae prefer stagnant waters to hatch, so this study suggests that there were increased mosquitoes within the study area which increased the host-vector capacity, resulting in increased *Plasmodium* prevalence in free-range birds.

We observed higher *Plasmodium* and *Leucocytozoon* prevalence in wild birds compared to confined birds. This is accounted for by the relatively high numbers of vieillot's black weavers sampled. This result is consistent with previous reports (Bennett *et al*., 1992). The vieillot's black weaver was most sampled because the sampling sites had some level of degraded forest habitats, which were cleared purposely for poultry farming. These birds usually inhabit forest clearings, wooded areas, and high grass savannahs.

Sequencing of the partial region of the mitochondrial cytochrome b gene revealed 24 new parasite lineages belonging to the three haemosporidian genera. We did not find any previously existing parasite lineages in this study.

Within the 24 lineages found, 6 (3 *Plasmodium*, 1 *Haemoproteus*, 1 *Parahaemoproteus*, 1 *Leucocytozoon*) were host generalists infecting two or more host species. *Plasmodium* lineages appeared in various hosts compared to *Haemoproteus* and *Leucocytozoon*. We detected the *Plasmodium* lineage, PGHA708, which was more generalist than the others in 5 host species within the confined, free-range, and wild bird categories. The most generalist *Leucocytozoon* lineage was LGHA146 and was found in 3 host species, which fall under the free-range and confined birds categories. *Parahaemoproteus* lineage PHGHA989 was the most generalist *Parahaemoproteus* found in 3 bird host belonging to confined and free-range bird categories. Other *Plasmodium* lineages PGHATDF24 and PGHAOS7 are also generalist parasites infecting at least two host species. PGHATDF24 infected free-range chickens and confined chickens within the same farm, likewise PGHAOS7 also infected ostriches and guinea fowls within another farm. The findings of this study reported higher prevalence of *Plasmodium* and *Leucocytozoon*, and we could attribute this to the high host generalist nature of the lineages found. The parasites were successful because of their ability to infect many host species. This is consistent with the "Niche breadth" hypothesis (Brown, 1984) which state that generalists parasites are more prevalent and can expand their distribution and host range compared to specialist parasites. The generalist *Plasmodium* PGHA708, and PGHATDF24, *Parahaemoproteus* PHGHA989 and *Leucocytozoon* LGHA146 had a broad habitat range and occurred in birds from different farms in different communities.

We found 18 host and habitat specialist parasite lineages infecting our host species.

We reported 11 *Plasmodium* lineages, 2 *Haemoproteus*, and 5 *Leucocytozoon* specialist lineages. Each of these lineages infected a single host species. The 6 *Plasmodium* lineages PGHAAS37, PGHA205, PGHAAS10, PGHA149, PGHA70, PGHA843, infected only the bird species *Gallus gallus* and did not occur in any other host species. They were not habitat specific, but they only

infected *G. gallus*. *Haemoproteus* lineages HGHA3W and HGHA1W were each specific to single wild bird species and did not occur in any free range or confined host species. Vector preference for a particular host can explain the high specificity associated with the lineages (De La Puente *et al*., 2015).

Our study found a high diversity of avian malaria and related haemosporidian parasites among domestic birds in Ghana. The results of our study suggest host and habitat generalist and specialist parasites of all three genera.

Phylogenetic analysis performed on *Plasmodium* and *Haemoproteus* lineages from our study revealed a close relationship with other existing lineages (Figure 16). Three of our *Plasmodium* lineages PGHA843, PGHA954 and PGHATDF24 cluster together on the maximum likelihood tree, suggesting a close relationship among them. These three were distantly related to the other *Plasmodium* lineages found in this study and to the existing lineages in the GenBank. Two of our *Plasmodium* lineages, PGHAOS2 and PGHAAS37 were closely related to the *Plasmodium sp*. lineage PLOCUC12. This lineage was got from South Africa in a village weaver (*Ploceus cucullatus*), a wild bird inhabiting degraded forest habitats. PGHAOS2 and PGHAAS37 were found in ostriches and chickens, respectively. Our most prevailing *Plasmodium* lineage PGHA708 shared close homology with *Plasmodium sp. ex Anthus campestris* isolate BIC24 which was found in Tawny pipit (*Anthus campestris*) bird species in Spain. PGHA708 was a generalist parasite infecting birds of different species and orders. Our *Plasmodium* lineages PGHA205, PGHA58Q, PGHA40Q and PGHATK2 are closely related to *P. globularis*, which was isolated from the blood of yellow-whiskered greenbul (*Andropadus latirostris*) (Valkiunas *et al*., 2008), a rainforest bird in Ghana. Lineage PGHA70 also shares ancestry with *P. juxtanucleare* and *P. bennetinia*. *P. juxtanucleare* is a domestic bird parasite which has reportedly spilled over to wild birds (Junior *et*

al., 2017). It was reported in chickens (*Gallus gallus*) in Brazil and in captive birds in a zoo in Japan. From the results of our study, we suspect *P. globularis*, *P. juxtanucleare* and *P. bennetinia* infecting our domestic birds within the area studied. We also identified a parasite similar to *H. nucleofascialis* in thin blood smears of vieillots black weavers sampled around the poultry farms. This *Haemoproteus* species was not confirmed in domestic birds.

Reports from farmers in the poultry communities sampled suggested that they were experiencing low egg productivity. Most farmers also reported increased mortality in birds. *P. juxtanucleare* has been reported to be responsible for severe malaria outbreaks in domestic birds (valkiunas, 2005). This parasite is known to infect chickens and other birds of the family *Phasianidae* (Massard & Massard, 1981). As observed in most of our birds, *P. juxtanucleare* rarely shows any clinical signs (Mota *et al*., 1998). Clinical signs such as weakness, difficulty in movement, slow weight loss, pale mucous membranes, severe anaemia and diarrhea occur shortly before death in immunosuppressed birds (Valkiunas, 2005). Other reports also suggest that *P. juxtanucleare* can lead to decreased productivity (Itagaki, 1970; Santos-Prezoto *et al*., 2004) or death in chicken (Al-Dabagh, 1960; Al-Dabagh, 1961; Krettli, 1972). Though we do not have any conclusive evidence, *P. juxtanucleare* in the Ghanaian poultry industry could be responsible for most of the deaths reported in chickens in the farms sampled. Further studies need to be conducted to exclude other factors and confirm these suspicions.

The *Haemoproteus* lineages found in our study are closely related to *Haemoproteus sp*. lineage HIRUS16 clone 403H which was found infecting barn swallow (*Hirundo rustica)* in Iran (NCBI), and *Parahaemoproteus sp*. H AFR29 infecting Afrotropical birds (NCBI). *Haemoproteus* species similar to *H. nucleofascialis* was morphologically identified in Vieillot's black weaver. This parasite species is characteristic of the birds of the family *Ploceidae*.

The six new *Leucocytozoon* parasite lineages LGHA146, LGHA29Q, LGHAAS2, LGHA73, LGHA111 and LGHA83, described in this study form a clade which is closely related to *L. schoutedeni*. *L. schoutedeni*, a domestic bird parasite was previously found in Uganda and Cameroon (Sehgal *et al*., 2006), Thailand (Piratae *et al*., 2021), Tanzania (Fallis, 1973), Nigeria (Lawal *et al*., 2016), Myanmar (Yee *et al*., 2020), and in Kenya (Sabuni *et al*., 2011). *L. schoutedeni* has a wide distribution but has not yet been associated with mortality. Coinfection with pathogenic strains of other haemosporidians could present severe disease. Further studies need to be carried out to establish the impact of *L. schoutedeni* infections in our poultry industry.

Various challenges exist in trying to get whole genome sequence data of avian malaria and related haemosporidian parasites. As challenging as this may seem, avian malaria researchers have shown that this is still possible. One of the best ways that has been suggested in getting host-parasite DNA ratio skewed towards the parasite is to use samples with higher parasitaemia. Our study used samples from naturally infected birds which usually showed no high parasitaemia, because birds with natural haemosporidian infection mostly show low levels of parasitaemia (Asghar, Hasselquist, & Bensch, 2011; Staffan Bensch *et al*., 2007; Valkiunas *et al*., 2004). In this study, we optimized a method for selectively enriching for haemosporidian genome for a future whole genome sequencing of the most occurring haemosporidian parasites infecting birds in Ghana. To do that from naturally infected birds, we performed a microbial enrichment protocol which employed depletion of methylated DNA (Böhme *et al*., 2018; Oyola *et al*., 2014). Our results showed a successful depletion of most of the host DNA leaving microbial DNA. When we performed AT rich selective WGA on the enriched samples, Qubit quantitation revealed high AT rich DNA concentration that was too high to quantify in 20 µL solution. A 1/10 dilution of the 20 µL DNA solution yielded ≥ 20 µL per sample. Our results show that haemosporidian DNA has

been successfully enriched and AT biased genome successfully amplified and can be used in a whole genome sequence analysis of parasites got from natural infections. This result cannot be conclusive because there is the need to further analyse the amplified, enriched sample to find out whether a complete AT-biased haemosporidian genome will be obtained. The major limitation in this procedure is that we did not get whole genome sequence data to confirm how much host DNA was successfully kept. It is very necessary to conduct future studies in which DNA from the enriched and amplified AT biased genomes will be successfully amplified and compared with existing genomes to enrich the genome biology of the haemosporidian parasites.

CONCLUSION

This PhD research was the first conducted on free range, caged and wild birds in Ghana. The findings of this study provide insights into the prevalence, diversity, and spillovers of avian malaria and related haemosporidian parasites in Ghana.

Major scientific achievements:

1. The study described a new nested PCR protocol for the screening of avian malaria and related haemosporidian parasites of the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* in domestic birds.
2. The study detected an overall prevalence of 21.5% haemosporidian infection of the three genera in domestic birds in Ghana.
3. The study detected a high diversity of avian malaria and related haemosporidian parasites in domestic birds in Ghana, with higher prevalence of *Plasmodium* (18.9%) compared to *Leucocytozoon* (10.4%) and *Haemoproteus* (2.1%).
4. The study also reported for the first time a comparison amongst haemosporidian parasite infections in free range, confined and wild birds in Ghana showing higher overall haemosporidian prevalence in free range birds (35.7%) compared to wild birds (25%) and confined birds (12.1%).
5. Our results described host and habitat generalist and specialist parasite lineages for all three genera (*Plasmodium, Leucocytozoon* and *Haemoproteus*).
6. We found 24 new haemosporidian parasites lineages comprising 13 *Plasmodium*, 5 *Haemoproteus* and 6 *Leucocytozoon*.

7. We identified the most prevailing haemosporidian parasite lineage as generalist *Plasmodium* lineage GHA708, the generalist *Leucocytozoon* LGHA146 and the generalist *Parahaemoproteus* PHGHA989.
8. We identified haemosporidian parasites in the study area to be lineages of *P. globularis*, *P. juxtanucleare*, *P. bennettinia* and *L. schoutedeni*.
9. We identified morphologically *H. nucleofascialis* in the blood of a vieillot's black weaver.
10. We successfully enriched and amplified avian malaria parasite DNA from a naturally infected bird for future whole genome sequencing.

RECOMMENDATIONS

1. Extensive sampling of both domestic and wild birds should be conducted within the study area to isolate the most prevailing haemosporidian parasites reported for further identification and experimental research.
2. Whole genome sequencing of the prevailing parasites should be performed to help in comparative evolutionary studies.
3. Dual transcriptomics analyzes should be carried out on the parasites and their hosts to assess the effect of these parasites on the birds.

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UNIVERSITY OF GHANA

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Ghana

Office Location: Department of Animal Experimentation Building, Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana

13/04/2021

ETHICAL CLEARANCE
(UG-IACUC 003/20-21)

Your protocol for an ethical clearance has been reviewed by the University of Ghana Institutional Animal Care and Use Committee and has been approved as follows:

TITLE OF PROTOCOL: IDENTIFICATION OF AVIAN MALARIA PARASITES SPECIES CAUSING DISEASE IN GHANAIAN BIRDS

PRINCIPAL INVESTIGATOR: Constance Agbemelo-Tsomafo

Please note that the final review report must be submitted to the Committee at the completion of the study. Your research records may be audited at any time during or after the implementation.

Any modification of this research project must be submitted to UG-IACUC for review and approval prior to implementation.

Please report all serious adverse events related to this study to UG-IACUC within seven days verbally and in writing within fourteen days.

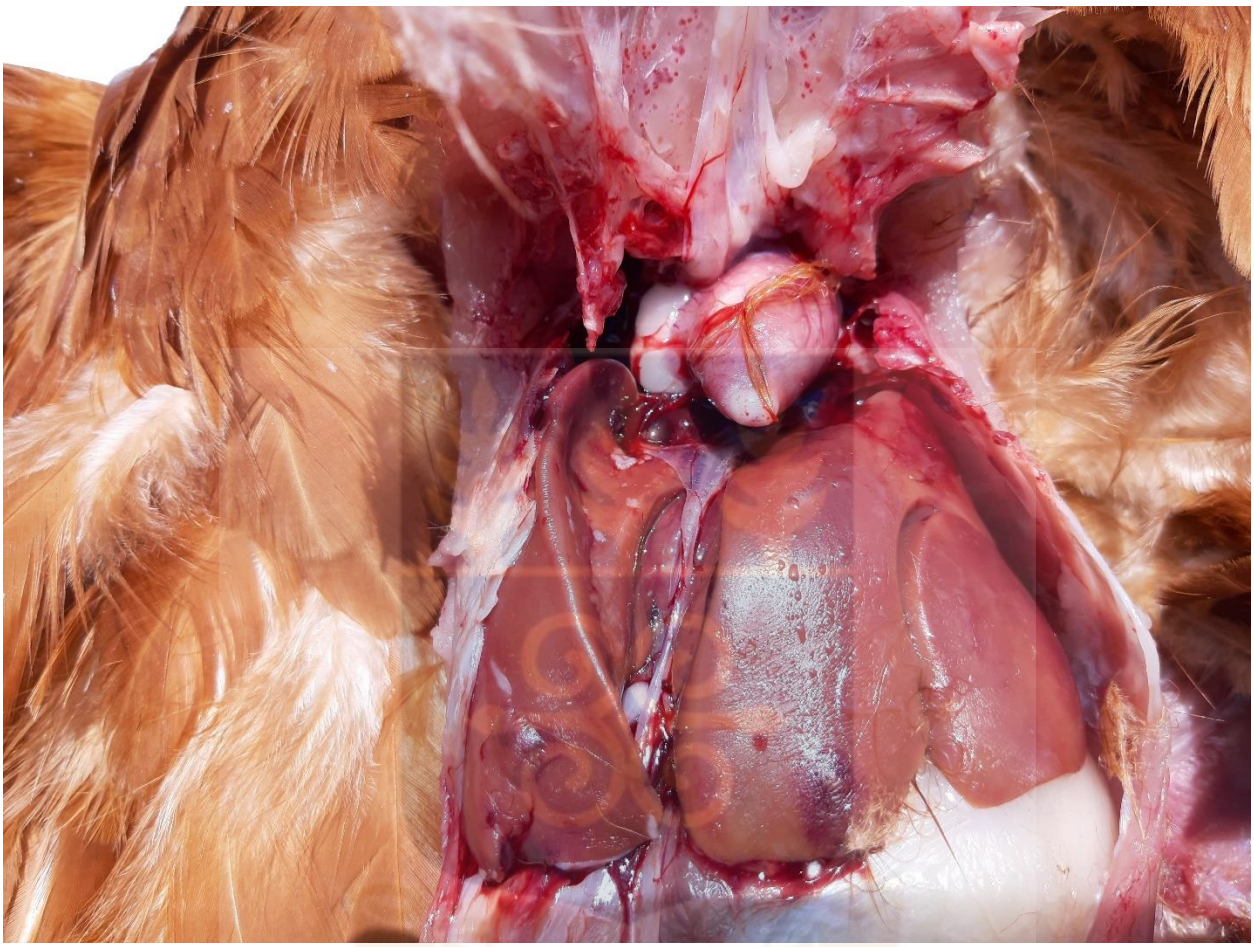
This certificate is valid till 31st December, 2021. You are to submit annual reports for continuing review.

Signature of Chairperson
Prof. Major (Rtd.) George A. Asare

Appendix 2: Blood sample collection from domestic birds.

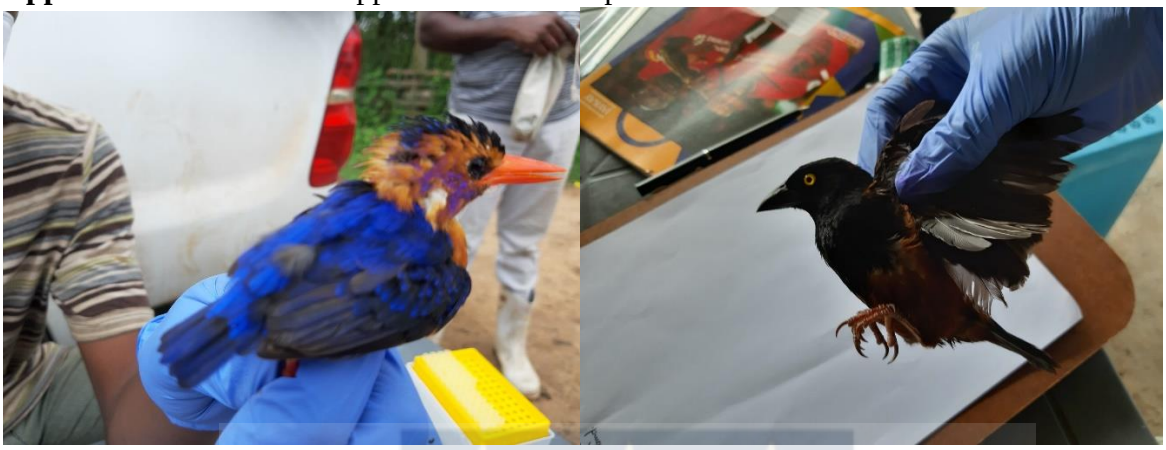
Appendix 3: Confined chickens sampled in the Brong Ahafo region.

Appendix 4: Post Mortem performed on dead chicken found on a sampling site.



Appendix 5: setting mist nets to sample wild birds.

Appendix 6: Wild birds trapped for blood sample collection.



Appendix 7: Sequences submitted to the GenBank with their accession numbers.

Sequences submitted to the NCBI GenBank

>*Leucocytozoon*_sp_LGHA29Q Accession OM643342

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>*Leucocytozoon*_sp_LGHA73 Accession OM643343

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>*Leucocytozoon*_sp_LGHA83 Accession OM643344

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>*Leucocytozoon*_sp_LGHA111 Accession OM643345

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>*Leucocytozoon*_sp_LGHA146 Accession OM643346

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>*Leucocytozoon*_sp_LGHAAS2 Accession OM643347

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>*Parahaemoproteus*_sp_PHGHA1W Accession OM643348

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>*Haemoproteus*_SP_HGHA2w OM643349

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>*Haemoproteus*_SP_HGHA3w Accession OM643350

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>*Haemoproteus*_SP_HGHA8w Accession OM643351

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>*Parahaemoproteus*_sp_PHGHA23W Accession OM643352

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>*Parahaemoproteus*_sp_PHGHA597 Accession OM643353

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>*Haemoproteus*_sp_HGHA809 Accession OM643354

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>*Parahaemoproteus*_sp_PHGHA919 Accession OM643355

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>*Haemoproteus*_sp_HGHA982 Accession OM643356

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>*Parahaemoproteus*_sp_PHGHA989 Accession OM643357

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>*Haemoproteus*_sp_PHGHAOS8 Accession OM643358

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>*Plasmodium*_sp_PGHA159 Accession OM643366

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>*Plasmodium*_sp_PGHA273 Accession OM643369

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>*Plasmodium*_sp_PGHAG26 Accession OM643377

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>*Plasmodium*_sp_PGHAOS2 Accession OM643378

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>*Plasmodium*_sp_PGHATDF24 Accession OM643382

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>*Plasmodium*_sp_PGHATK2 Accession OM643383

GGTGCAACCGTTATTACTAACTTATTATACTTTATACCTGGTCTTGTTTCATGGATTT
GTGGTGGATATCTTGTTAGCGACCCAACATTAAAAAGATTTTTTGTATTACATTTTAT
ATTTCCATTTATTGCTTTATGTATTGTATTTATACATATATTTTTTCTACATTTACAAG
GTAGCACAAAT