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PREVALENCE OF AVIAN MALARIA IN SOME PROTECTED AREAS IN GHANA

BY

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THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON, IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF M. PHIL ZOOLOGY DEGREE.

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DECLARATION

I do hereby declare that this thesis is my own work produced from research under the joint supervision of Dr. Erasmus H. Owusu and Dr. Langbong Bimi, all of the Department of Animal Biology and Conservation Sciences, University of Ghana. This work has not been previously submitted partially or wholly for the award of a degree in any University.

References to the works of other investigators have been duly acknowledged.

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DEDICATION

To God for his grace, my family and to everyone who has contributed to the success of this work.

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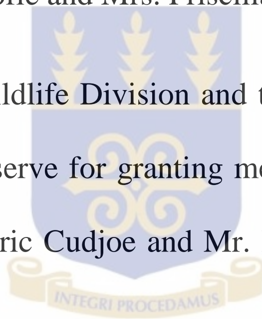

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TABLE OF CONTENTS

DECLARATION i

DEDICATION ii

ACKNOWLEDGEMENT iii

TABLE OF CONTENTS ..... **iv**

LIST OF TABLES x

LIST OF FIGURES xi

LIST OF ABBREVIATIONS xii

ABSTRACT xiii

CHAPTER ONE **1**

1.0 INTRODUCTION 1

1.1 Background 1

1.2 Justification **3**

1.3 Aim 4

1.4 Specific Objectives 4

CHAPTER TWO **5**

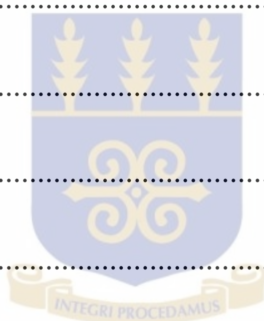
2.0 LITERATURE REVIEW 5

2.1 Brief history of avian malaria 5

2.2 Avian haemosporidian parasites 6

2.2.1 Vectors of avian malaria **7**

2.2.2 Parasite-host association 8



University of Ghana http://ugspace.ug.edu.gh

2.3 Life cycle of avian malaria parasites 8

2.4 Review of avian malaria studies **13**

2.5 Parasite diversity and global distribution 15

2.6 Effect of parasitism on bird hosts 16

2.7 Investigation of avian malaria using traditional microscopy **17**

2.8 PCR-based methods and comparison to traditional microscopy in detecting malaria. 18

2.9 Factors affecting avian malaria prevalence 21

CHAPTER THREE **22**

3.0 MATERIALS AND METHODS 22

3.1 Study design 22

3.2 Study sites and material collected 22

3.2.1 Kakum National Park 24

3.2.2 Shai Hills Resource Reserve 25

3.3 Collection of birds 26

3.3.1 Sample size 26

3.3.2 Collection of blood samples and preparation of the material for microscopic examination and DNA studies 26

3.4 Giemsa staining 28

3.5 Microscopic examination of blood films 28

3.6 Extraction of DNA from avian blood 28

University of Ghana http://ugspace.ug.edu.gh

3.7 PCR amplification 29

3.7.1Parasite identification.. 30

3.8 Research Permit 30

3.9 Statistical analysis 31

CHAPTER FOUR 32

4.0 RESULTS 32

4.1 Sample size and distribution of birds in the study areas 32

4.2 Relative Abundance of Birds in Kakum National Park 33

4.3 Relative Abundance of Birds in Shai Hills Resource Reserve 36

4.4 Microscopic investigation and PCR screening of avian blood samples 39

4.5 A comparative analysis of Microscopy and PCR for the detection of avian haemosporidians 41

4.6 Parasite lineages and habitats 45

CHAPTER FIVE 49

5.0 DISCUSSION 49

5.1 Efficiency of methods used in parasite detection and identification 49

5.2 Parasite Prevalence in Forest and Savanna Birds 50

5.3 Habitat Effect 52

CHAPTER SIX 54

University of Ghana http://ugspace.ug.edu.gh

6.0 CONCLUSION AND RECOMMENDATION 54

6.1 CONCLUSION 54

6.2 RECOMMENDATIONS 55

REFERENCES 56

APPENDICES 70

APPENDIX I 70

Sample information (Shai Hills) 70

APPENDIX II 71

Sample information (Kakum National Park) 71

APPENDIX III 72

Nucleotide Sequences 72

APPENDIX IV 75

Haemoproteus and Plasmodium lineages and their Genbank accession numbers 75

APPENDIX V 76

Sensitivity and specificity of diagnostic tests 76

APPENDIX VI 77

Protocol for DNA Extraction from avian blood 77

University of Ghana http://ugspace.ug.edu.gh

LIST OF TABLES

Table 1: Total number of birds caught in Kakum National Park and relative abundance of each bird species expressed in number of birds × 100/mist-net m × h 34

Table 2: Relative Abundance of birds trapped in Shai Hills Resource Reserve 37

Table 3: Birds tested and outcome of PCR screening and microscopic examination of blood samples 43

Table 4: Prevalence (P) and lineages of haemosporidian parasites in birds trapped in Kakum National Park (lineage names are according to NCBI database) 46

Table 5: Prevalence (P) and lineages of haemosporidian parasites in birds trapped in Shai Hills Resource Reserve 48

University of Ghana http://ugspace.ug.edu.gh

LIST OF FIGURES

Figure 1: Diagram showing the life cycle of avian malaria parasite 12

Figure 2: A schematic presentation of the study sites 23

Figure 3: Blood sample collection and preparation of blood films for microscopic examination 27

Figure 4: Distribution of sampled bird species and families in Kakum National park and Shai hills resource reserve. 32

Figure 5: Blood stages of haemosporidian parasites as seen in thin blood films 39

Figure 6: Agarose gel showing PCR results with the primers HaemF and HaemR2 on DNA samples extracted from blood samples of birds 40

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LIST OF ABBREVIATIONS

A	-	Adenine
BLAST	-	Basic Local Alignment Search Tool
Bp	-	Base pair
C	-	Cytosine
DNA	-	Deoxyribonucleic acid
G	-	Guanine
H	-	Haemoproteus
IUCN	-	International Union for the Conservation of Nature
MEGA	-	Molecular Evolutionary Genetics Analysis
NCBI	-	National Centre for Biotechnology Information
P	-	Plasmodium
PCR	-	Polymerase Chain Reaction
T	-	Thymine

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ABSTRACT

Differences in habitat types affect host-parasite interactions and can increase the risk of epizootic outbreaks in wild populations. It is thought that parasitism is a necessary factor in conservation biology and is important in understanding ecological parasitology and vertebrate conservation management. The aim of this study was to estimate the prevalence of avian malaria in forest and savanna birds. A total of 132 birds of 39 species belonging to 20 families were trapped in two wildlife protected areas in Ghana (Kakum National Park and Shai Hills Resource Reserve) and screened for the presence of the haemoparasites, *Plasmodium* and *Haemoproteus* spp. A combination of microscopy and PCR detected an overall prevalence of 45.4%. Comparatively, prevalence varied between the two sites with a higher prevalence of 69.20% in Kakum National Park compared to 12.90% in Shai Hills Resource Reserve. Sequencing of the positive PCR amplicons identified 20 mitochondrial lineages of 11 *Plasmodium* and 9 *Haemoproteus* lineages. *Plasmodium* and *Haemoproteus* prevalences varied in both sites with *Plasmodium* recording the highest prevalence in each site. The results of the study did not only confirm the presence of avian malaria in the Ghanaian wildlife protected areas, but also differences of these in the different habitats. The study also recommended that investigations should be carried out in both dry and wet seasons as well as including more study sites to make better comparison.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Haemosporidian infections are induced by a group of disease causing protozoan parasites which infect mammals, reptiles, amphibians and birds (Valkiunas, 2005). These organisms cause diseases such as avian malaria, haemoproteosis and leucocytozoonosis in their vertebrate hosts (Martinsen *et al.*, 2008).The diseases are far induced in birds by an unknown number of haematozoan species (Olias *et al.*, 2011) belonging to the order Haemosporida. There are three genera of avian haemosporidians namely *Haemoproteus*, *Plasmodium*, and *Leucocytozoon*(Beadell & Fleischer, 2005). Species of all three genera are closely related genetically but their life-history traits differ; more over they share several characteristics with human malaria parasites, and all three genera, but most often only *Plasmodium* spp. are referred to as avian malaria parasites (Hellgren *et al.*, 2004).Some of the life-history traits that are used to define these species and genera include the types of host cells that they use for schizogony, the number of daughter cells produced by each mature schizont, and the presence or absence of haemozoin pigment stored within the parasite cell which is the product of the breakdown of haemoglobin, formed by crystallization of the porphyrin (Martinsen *et al.*, 2008).The use of the genera, *Plasmodium* and *Haemoproteus*, concurrently to refer to avian malaria raised a controversy among parasitologists, ecologists and evolutionary researchers. However, due to genetic studies about the phylogeny of the group, many researchers include *Haemoproteus* spp. among malaria parasites (Perez-Tris *et al.*, 2005). In view of that, this study will refer to both genera as avian malaria parasites.

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Traditionally, the detection of avian malaria infections has been by microscopic examination of blood smears only, but most recently, the development of molecular technology has made screening for these parasites faster and more reliable (Fallon *et al*., 2003). Recent molecular studies on infection of these parasites by Polymerase Chain Reaction (PCR)-based methods indicated a high diversity of the parasites in wild bird communities (Bensch *et al*., 2007; Ricklefs *et al*., 2007; Kim &Tsuda, 2010).

Avian malaria parasites have been very useful in many studies. The frequent use of these parasites in the study of evolution and population ecology is based on the relative ease with which infected birds can be distinguished from uninfected ones and the fact that the intensity of infection can be estimated for each host using blood smears (Valkiunas, 1993; Richner *et al*., 1995; Rintamaki *et al*., 1998). This implies that the costs of infection can be examined using both quantitative and qualitative methods (Hellgren *et al*., 2004). Majority of the published works in the field of avian haemosporidian studies focused on species of the genera *Haemoproteus* and *Plasmodium*, because they are more easily detected (Atkinson and Van Riper, 1991). However, there are relatively few studies on *Leucocytozoon* spp. (Atkinson and Van Riper, 1991). The scarcity of investigations on species of the genus *Leucocytozoon* is not because the infection (Leucocytozoonosis) is itself rare, but because the life stages of the parasites are detectable in peripheral blood for only very short time periods, which makes the infection difficult to detect and accurately identified, especially using traditional ocular methods (Valkiunas, 1997). The broad understanding gained from pioneering studies of avian malaria parasites and their importance in evolutionary modeling studies of host-parasite systems and conservation biology has helped to re-establish this field of study (Braga *et al*., 2011). These parasites have also been used as model organisms for studies on many aspects of parasite-host

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interactions (Perkins and Schall, 2002; Ricklefs and Fallon, 2002), host life-history trade-offs and sexual selection (Richner *et al*., 1995; Nordling *et al*., 1998). These studies are important in understanding ecological parasitology and vertebrate conservation management (Braga *et al*., 2011).

1.2 Justification

Avian malaria is a potential threat for both domestic and wild birds (Bonneaud *et al*., 2006). It is estimated that 68% of all birds are susceptible to malaria infections (Sehgal, 2004), and the impact of the disease on bird populations is often overlooked in wildlife ornithology. However parasitism is a necessary factor in conservation biology and should therefore be considered in biodiversity preservation studies (Valkiunas, 2005; Parker *et al*., 2006). Previous studies have established that environmental conditions can impact the diversity and abundance of parasite species, either by favoring or limiting parasite numbers (Loiseau *et al*., 2010) due to sensitivity of vectors to climatic conditions (Cosgrove *et al*., 2008) and as a result, affecting the prevalence of host infection (Loiseau *et al*., 2010).

In Ghana, there are only few studies documented on avian malaria (Wink &Bennett, 1976; Loiseau *et al*., 2009). Consequently, different features of the parasites, including their prevalence and taxonomy need to be examined so as to provide information essential for future projects aiming at predicting and managing the impact of protozoan blood parasites on birds in Ghana. The results will be useful in providing vital information to resource managers about parasite distribution as well as drawing up a protection plan for protected areas against dipteran-borne diseases. This study is therefore to establish a

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baseline of malaria parasite infections of the birds and will be relevant in studying how habitat composition could affect parasite prevalence.

1.3 Aim

The goal of this study is to estimate the prevalence of avian malaria in Forest and Savanna birds.

1.4 Specific Objectives

The specific objectives of the study were:

- To detect and identify avian malaria parasites in savannah and forest birds.
- To assess the efficiency of microscopy and Polymerase Chain Reaction-based method in estimating the prevalence of the parasites.
- To compare prevalence of the disease among savannah and forest birds.
- To assess the implication of habitat types on parasite prevalence.

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CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Brief history of avian malaria

The publication of an article on blood parasites (Danilewsky, 1884) in the Russian Medical Journal marked a qualitatively new stage in the development of protozoology. Danilewsky was the first scientist to investigate avian malaria pathology where he indicated, by dissecting infected birds that the disease is accompanied by an acute anaemia, enlargement of the liver and spleen, as well as the accumulation of pigment and the presence of parasites and infected erythrocytes in the phagocytes of these organs. He associated ecological observations with seasonal dynamics of parasite infections in birds, and concluded that avian malaria parasites prevail in birds in the warm seasons; parasitemia correlates with temperature of the environment and that vectors take part in the spreading of the parasites (Valkiunas, 2005).His postulates and statements about the similarity between human malaria parasites and the parasites of birds in particular were of significant biological importance (Valkiunas, 2005).

The research on the fauna and distribution of bird malaria became more active after the founding of the organisation of the International Reference Centre for Avian Malaria Parasites in 1968 in St. John's, Canada, by M. Laird with the official support of the World Health Organization (Valkiunas, 2005). From the end of the 20th century, most of the researches on avian malaria parasites were mainly based on material collected from naturally infected birds and important data about ecology, molecular biology, distribution, prevalence, diversity and phylogeny have been accumulated (Bensch *et al.*, 2000; Beadell *et al.*, 2006; Hellgren *et al.*, 2007).

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2.2 Avian haemosporidian parasites

The most commonly known haemosporidians are the human malaria parasites from the genus *Plasmodium* (Barraclough *et al*., 2008).*Plasmodium* shares the same order or the same family with closely related parasites of the genera *Haemoproteus* and *Leucocytozoon*, depending on the author's discretion (Perkin and Schall, 2002; Valkiunas, 2005); and the three genera have an evolutionary relationship (Perkin and Schall, 2002). All three genera go through alternating cycles of sexual and asexual reproduction, however only species of *Plasmodium* go through schizogony in circulating erythrocytes which results in symptoms of malaria (Beadell and Fleischer, 2005). Among bird species haemosporidians vary over time, season, and between locations, in their prevalence and parasitaemia which could also result in different selective pressures on bird populations (Bensch and Akesson, 2003).

Comparatively, species of the genera *Plasmodium* and *Leucocytozoon* are considered to be more pathogenic than *Haemoproteus* spp. (Beadell and Fleischer, 2005). However, some *Haemoproteus* species were reported to cause disease in birds (Cardona *et al*., 2002) and to affect their ecological fitness (Marzal *et al*., 2005; Valkiunas, 2005). In many cases, infection is subclinical, (Merino *et al*., 2000; Waldenstrom *et al*., 2002) and in more severe cases, hypertension and cardiomegaly, cerebral and splenic infection, anemia, debilitation, and death can occur (Atkinson and van Riper, 1991).

The pathogenicity of avian malaria parasites in birds has been argumentative for decades (Schrenzel *et al*., 2003). In general, *Plasmodium* spp. are considered more virulent than *Haemoproteus* spp., but infection with either appears to exert a cost to the host in every case (Merino *et al*., 2000). In passerines, costs can range from marked debilitation and

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life threatening anemia to subclinical drains on physiological processes and behaviors, such as immune responsiveness, quality of parenting, and ability to cope with stress (Jarvi *et al*., 2002; Waldenstrom *et al*., 2002). In avian malaria, as in human malaria (Hill *et al*., 1991), it is the first exposure to the infection that may cause the severest fitness consequences, that is acute infection (Atkinson & van Riper 1991). On the other hand, chronic stages of malaria infections are characterized by having none or only mild fitness effects (Atkinson & van Riper 1991; Hill *et al*., 1991). Once an individual bird has been infected with malaria, the infection may persist for years or even a lifetime (Atkinson & van Riper, 1991).

The study of avian haemosporidian parasites has added to understanding emerging infectious diseases in different hosts (Kilpatrick *et al*., 2006; Jourdain *et al*., 2007). The ability of several migratory birds to travel vasts distances, makes it possible for them to transmit parasites between distant geographical locations, even between continents (Fallon *et al*., 2006; Svensson *et al*., 2007; Hellgren *et al*., 2007). In addition, many avian haemosporidian parasites are able to infect species from different bird families (Ricklefs *et al*., 2004; Krizanauskiene *et al*., 2006).

2.2.1 Vectors of avian malaria

The avian malaria parasites are transmitted exclusively by blood-sucking dipteran insects (Valkiunas, 2005). Culex mosquitoes are generally considered to be vectors of *Plasmodium* spp., while biting midges and black flies are vectors of *Haemoproteus* spp. (Beadell & Fleischer, 2005). In some cases, species belonging to other mosquito genera such as *Aedes, Culiseta, Anopheles, Mansonia* and *Aedeomyia* have also been implicated in the transmission of different species of avian *Plasmodium* (Njagbo *et al*., 2009;

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Bonneaud *et al.*, 2009). Additionally, wild mosquitoes of the genus *Coquillettidia* have been shown to be vectors of *Plasmodium* spp. in the lowland forest of Cameroon (Njagbo *et al.*, 2011).

2.2.2 Parasite-host association

Host-parasite associations seemingly reflect the physiological and immunological restrictions that are imposed by hosts, as well as ecological factors including distribution and abundance of hosts, parasites and vectors; which limit the chances for parasite transmissions between different hosts (Bensch *et al.*, 2000; Beadell *et al.*, 2008). Though host specialization has the tendency to limit resource availability and increase the risk of extinction, it could also increase contact among individuals of a parasite species restricted to a narrow host range (Beadell *et al.*, 2008). Generalist parasites (with wide host distributions) are usually thought to have low fitness in any of their hosts. However, they may have higher abundance and face reduced extinction risk relative to specialist parasites (Beadell *et al.*, 2008). Avian haemosporidians offer an important system for studying host-parasite strategies of closely related parasites since they have a high diversity as well as a diverse host fauna that is potentially available to each parasite in any particular geographical location (Beadell *et al.*, 2008).

2.3 Life cycle of avian malaria parasites

Avian malaria parasites of the genera *Plasmodium* and *Haemoproteus* are obligatory sexual organisms and require two hosts, a haematophagous dipteran vector and a vertebrate host to complete their life cycles (Barraclough *et al.*, 2008). They must undergo a round of sexual reproduction in their vectors in order to produce stages that can be transmitted to vertebrate hosts (Barraclough *et al.*, 2008). These genera have similar

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life cycles, with schizogony and gametocytogenesis within their vertebrate host leading to gametogenesis, zygote formation and sporogony within their insect vectors (Barraclough *et al*., 2008). The only difference between their life cycles is the type of vector (mosquitoes for *Plasmodium*, midges and black flies for *Haemoproteus*) because both genera parasitize the erythrocytes (Barraclough *et al*., 2008).

Fertilization is however similar among the two groups. After a period of asexual proliferation in a vertebrate host, transmission to the vector occurs through the uptake of dioecious haploid pre-sexual stages (the gametocytes) in a blood meal (Barraclough *et al*., 2008). Gametocytes are activated inside the vector in the form of exflagellation of male gametes, with several gametes arising from one male gametocyte, and the production of a single female gamete from each female gametocyte (Barraclough *et al*., 2008). Fertilisation results in a zygote in the lumen of the insect vector's midgut. This zygote undergoes multiple divisions (sporogony) to form and release haploid sporozoites that migrate into the vector's salivary glands. Infection of a bird host occurs when sporozoites are injected with saliva as the vector takes a blood meal (Barraclough *et al*., 2008).The general characteristics of the life cycle of avian malaria parasites is described below (Fig.1) in the example of *Plasmodium relictum* which is distributed worldwide in a broad range of vertebrate hosts and has been well studied (Valkiunas, 2005).

The development of the parasite in bird host can be divided into exo-erythrocytic merogony, erythrocytic merogony, and formation of gametocytes (Valkiunas, 2005). The exo-erythrocytic merogony is made up of primary (pre-erythrocytic) and secondary(post-erythrocytic) merogonies (Valkiunas, 2005). Primary exo-erythrocytic merogony consists of two generations of meronts, which are cryptozoites and metacryptozoites respectively,

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while secondary exoerythrocytic merogony includes several generations of meronts called phanerozoites (Valkiunas, 2005). Vectors inject sporozoites into birds and this gives rise to cryptozoites (Fig. 1.a). They develop predominantly in the reticular cells of many organs and tissues, including skin (Valkiunas, 2005). The second generation of primary exo-erythrocytic merogony is induced by cryptozoites which develop in macrophages in many organs and tissues (Fig. 1.b). Merozoites, develop in metacryptozoites, and infect the cells of the erythrocytic series (Fig.1.c) or induce the next generation of metacryptozoites and phanerozoites (Fig.1.d) (Valkiunas, 2005). The time from inoculation of sporozoites into birds until the maturation of the first generation of metacryptozoites is called a prepatent period of development (Valkiunas, 2005).

After merozoites penetrate into young and mature erythrocytes, they become roundish and give rise to the growing non-fissionable parasites, which are called trophozoites (Valkiunas, 2005). After the first nucleus division, the parasite develops into a stage called erythrocytic meront (Valkiunas, 2005). Due to presence of merogony in erythrocytes, the infection of vertebrate hosts can be easily achieved by sub-inoculation of infected blood (Valkiunas, 2005). Asexual division produces uninuclear merozoites in erythrocytic meronts. The cycle of erythrocytic merogony in the majority of parasite species terminates after 24 to 36 h (Valkiunas, 2005). A part of merozoites formed in the erythrocytic meronts induces the next cycles of erythrocytic merogony and gives rise to gametocytes, while the other part penetrates the endothelial cells of the capillaries of many organs including the brain, initiating secondary exo-erythrocytic merogony (Valkiunas, 2005).

Several minutes after feeding on infected birds, mature gametocytes in the midgut of mosquitoes round up and escape from the erythrocytes. Gametes are formed and

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fertilization occurs, and then motile ookinete develops (Valkiunas, 2005). Ookinetes move toward the epithelial cells of the midgut, reach the basal lamina, become round and transform into the oocysts surrounded by a capsule like wall (Valkiunas, 2005). After several germinative centers are formed many hundreds of sporozoites develop during the sporogony (Valkiunas, 2005). When mature oocysts rupture, the sporozoites get into the haemocoele and penetrate into the salivary glands (Valkiunas, 2005). The sporogony of *P. relictum* at the optimal temperature 24°C is completed in seven days after ingestion of mature gametocytes (Valkiunas, 2005). Infection of new hosts occurs by means of injection during a blood meal of infected vectors (Valkiunas, 2005).

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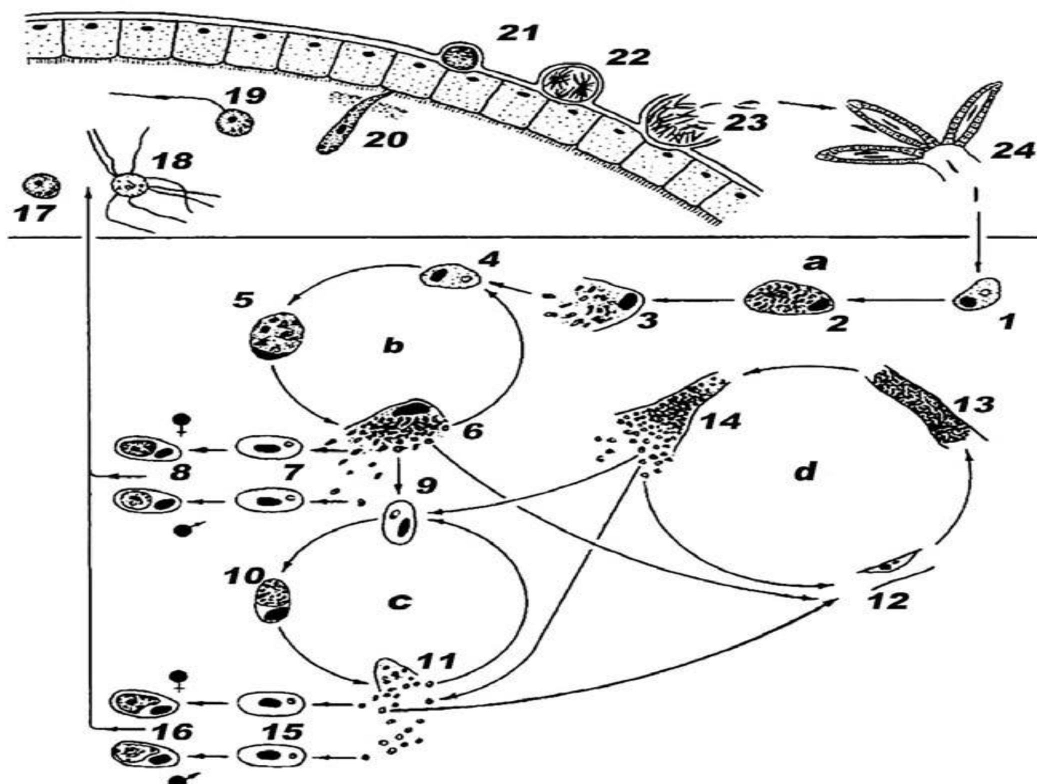

Figure 1: Diagram showing the life cycle of avian malaria parasite (Valkiunas, 2005).

Legend:

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).

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2.4 Review of avian malaria studies

Many experimental studies have been designed to be helpful in understanding the impact of malaria and other haemosporidian parasites in wildlife (Marzal *et al*., 2005; Zehtindjiev *et al*., 2008; Knowles *et al*., 2010).

A study by Chasar *et al*., (2009) indicated that host-parasite systems can be affected by habitat changes, since these systems are complex, with many biotic and abiotic variables. The study showed clearly that the dipteran insect vectors' response to environmental changes can be a major factor regulating malaria parasite distribution and prevalence. Besides habitat changes, many diseases and pathogens are known to be sensitive to climatic conditions. For instance, meteorological factors such as temperature, rainfall and humidity have been linked with the dynamics of malaria vector populations, thus the spread of the disease (Chasar *et al*., 2009). A research in Kenya associated microclimatic changes caused by deforestation to the increased survival and fecundity of the mosquito *Anopheles gambiae* and concluded that infectious disease prevalence may differ with habitat change and associated altered microclimates (Chasar *et al*., 2009).

Many researchers have addressed the relationship between avian haemosporidian parasites and various ecological factors (Valkiunas, 2005). For example, there have been extensive studies to determine the prevalence and diversity of these parasites in rainforest birds in West-Central Africa (Waldenstrom *et al*., 2002; Sehgal *et al*., 2005; Beadell *et al*., 2009). However, few studies focused on the relationship between the distribution of avian malaria infections and habitat changes (Chasar *et al*., 2009). A study which examined prevalence of infections in pristine and disturbed forests from a limited sampling over a broad geographic range in Cameroon found a higher prevalence of

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Plasmodium lineages in pristine as compared to disturbed sites (Bonneaud *et al*., 2009). However, other researchers examining prevalence of the parasites in Africa did not show clear differences in prevalence between seasons or years (Sehgal *et al*., 2005). Researchers examined how diversity and prevalence of haemosporidian parasites differ between disturbed and undisturbed habitat types and found different bird species possessing varying assemblages of parasite communities (Ricklefs *et al*.,2005; Arriero &Moller, 2008), but there is little empirical data on blood parasite diversity for individual bird species over a large scale in Africa (Chasar *et al*., 2009).

Field studies using a combination of microscopy and molecular diagnostic methods revealed mixed infections of *Plasmodium* spp. and other related haemosporidians in over 40% of infected birds (Valkiunas *et al*., 2006), and over 80% in some European bird populations (Valkiunas *et al*., 2003). Some studies reported that avian malaria parasites of the genus *Plasmodium* caused serious clinical conditions and induced fatal results to captive birds in USA, Brazil, New Zealand, and Asia (Murata, 2002; Grim *et al*., 2004; Alley *et al*., 2008; Belo *et al*., 2009).Severe illness and overwhelming occurrence of *Plasmodium elongatum* - malaria have been recorded among penguins in zoos in North America and Eurasia (Cranfield *et al*,. 1990), so this infections are of practical significance (Valkiunas, 2008).

Prevalence and diversity of haemosporidian parasites have also been studied though insufficiently, in rainforest birds in some parts of Africa (Chasar *et al*., 2009). For example, a study in Uganda showed that 76.5% of pigeons (*Columba livia*) were infected with *Haemoproteus* species and in South Africa, new species of *Haemoproteus*parasites were identified (Sehgal, 2005). However, birds in the semi-arid regions of South Africa

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lack haemosporidian parasites and this is seemingly due to the scarcity of the breeding habitats of the insect vectors (Sehgal, 2005).

A study in Cameroon reported avian malaria parasite prevalence in 220 individuals belonging to three different species of African rain-forest birds. The number of individual birds infected was 20 with thirteen verified mitochondrial lineages found (Bonneaud *et al*., 2009). A study on the effects of deforestation on the distribution of haemosporidians was conducted on large numbers of rainforest birds collected in Ghana and Cameroon during 2005-2007 (Valkiunas *et al*., 2009).In this study, three new species of *Plasmodium* were described using data on the morphology of their blood stages and sequences of the mitochondrial cytochrome b gene.

2.5 Parasite diversity and global distribution

Avian malaria parasites are distributed worldwide with a high diversity, and have been detected on every continent except Antarctica (Donovan *et al*., 2008). Apart from a few host taxa restricted to extreme arctic environments (Bennett *et al*., 1992) and several remote island taxa (Beadell *et al*., 2007), the majority of bird species are hosts to avian malaria parasites. Over 45% of bird species of the world's fauna has currently been investigated with respect to infection with haemosporidians out of which *Haemoproteus* spp. was recorded in approximately 50%, and *Plasmodium* and *Leucocytozoon* spp. in approximately 30% of the investigated bird species (Valkiunas, 2005).

The number of species of the order Haemosporida exceeds 400 species in all vertebrates, with more than 50% of them occurring in avian hosts (Valkiunas, 2005). Application of sensitive molecular techniques to the detection of haemosporidians have revealed an extremely broad diversity of parasite lineages (Durrant *et al*., 2006; Ishtiaq *et al*., 2007),

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thereby questioning previous morphological species limits (Beadell *et al*., 2006; Hellgren *et al*., 2007) and raising the possibility that haemosporidian species diversity is on the order of avian species diversity (Bensch *et al*., 2004) or even higher. Supporting this hypothesis, single host species have been shown to harbor between five and 34 distinct parasite mitochondrial lineages (Ishtiaq *et al*., 2006; Bensch *et al*., 2007).

Avian malaria parasites include approximately 40 morphologically distinct species of the genus *Plasmodium*, and 130 species of the genus *Haemoproteus* (Bensch *et al*., 2009). Understanding the diversity of these parasites is being increased by the application of molecular genetic screening techniques of blood samples collected from wild hosts (Bensch *et al*., 2004). For example, estimates of global species diversity of the order of 200 species based on microscopy, have been suggested to need revision to somewhere in the order of 10 000 species based on comparisons of nuclear and mitochondrial gene trees (Bensch *et al*., 2004).

2.6 Effect of parasitism on bird hosts

Parasites are powerful selective agents that influence almost all aspects of their hosts' life (Dronamraju, 2004). The Red Queen hypothesis proposed that hosts and parasites are involved in a constant co evolutionary arms race, in which host resistance and parasite infectivity are under strong mutual selection (Jaenike, 1978). Parasites can have a substantial influence on the demography of their host populations and thus drive ecological and evolutionary processes (Mouritsen and Poulin, 2005; Miura *et al*., 2006; Ostfeld *et al*., 2008).

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In host life history evolution, it is suggested that parasites play a significant role as mediators of fitness costs sustained as a result of reproductive effort or exaggeration of sexual ornaments (Gustafsson *et al*., 1994; Sheldon and Verhulst, 1996). Experimental researches of the importance of parasites in life history evolution have often used blood parasites of the genera *Haemoproteus*, *Plasmodium* and *Leucocytozoon* and their avian hosts as ideal systems (Desser and Bennett, 1993).

In wild animal populations, fitness of individuals can be affected by pathogens in a number of ways, such as increasing predation risk, reducing survival and reducing reproductive output (Moller and Nielsen, 2007; Johnson *et al*., 2008). These effects can be observed at higher organizational levels, with pathogens playing a critical role in host population dynamics and range distributions (Ricklefs, 2010), thereby driving genetic variation and sexual selection (Ortego *et al*., 2007; Spugin and Richardson, 2010).In wild birds, malarial infection was shown to have implications for host mate choice, parental investment, reproductive success, immune gene variability and population or species persistence (Westerdahl *et al*., 2005; Bonneaud *et al*., 2006).

2.7 Investigation of avian malaria using traditional microscopy

For several years, avian malaria parasites have been mainly studied by microscopic examination of Giemsa-stained blood films (Palinauskas, 2008). The existing information on the basic life history, geographical distribution, vertebrate hosts and vector specificity, seasonal changes of infection, and other aspects of ecology of these parasites have been gathered mainly by microscopy (Forrester and Spalding, 2003). However, researchers using traditional microscopy faced many challenges. For example, the identification of avian *Plasmodium* species from the blood smears is often impossible (Palinauskas, 2008).

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Even when recorded, most chronic infections of *Plasmodium* spp. remain unidentified to the species level, even by experts, owing to low intensities and common mixed infections (Valkiunas, 2005).

2.8 PCR-based methods and comparison to traditional microscopy in detecting avian malaria

The first PCR protocol developed for avian haemosporidian studies was designed to amplify *Plasmodium* parasites from birds in Hawaii using primers for 18SrRNA (Feldman *et al*., 1995). This not widely used because it was efficient only for a small group of *Plasmodium* species. As a means of improving upon this protocol, Bensch *et al*., (2000) published the first general PCR protocol for both avian *Plasmodium* and *Haemoproteus* parasites, where a portion of the mitochondrial cytochrome b gene was the target molecule. This protocol together with slightly modified protocols (Hellgren *et al*., 2004; Waldenstrom *et al*., 2004) is still among the most widely used protocols (Bensch *et al*., 2009). The PCR technique has become a valuable tool for the detection of haemosporidian parasites, and the use of sequencing revealed a wealth of genetic diversity (Bensch *et al*., 2000; Ricklefs and Fallon, 2002; Schrenzel *et al*., 2003).

Compared to traditional microscopy of blood smears, PCR offers increased sensitivity (Richard *et al*., 2002). However, *Haemoproteus* spp. and *Plasmodium* spp. typically are amplified generally, and identification to genus level requires sequencing (Beadell and Fleischer, 2005). A comparison of several PCR methods to microscopy for detection of avian haemosporidians revealed that PCR is faster, cheaper, and more reliable than microscopic blood smear examination for large-scale screening (Palinauskas, 2009) and that some problems associated with traditional typing of parasites can be resolved with PCR (Richard *et al*., 2002).

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Furthermore, polymerase chain reaction methods can provide sequence information that allows for the identification of the specific parasite lineage (Bensch and Akesson, 2003), which is not possible using parasite morphology alone (Hellgren *et al*., 2004).Moreover, lineage-specific data may be useful when monitoring an epidemic or measuring migratory connectivity (Webster *et al*., 2002), but ecologists, wildlife managers, and zookeepers often may benefit from genus-level knowledge of parasites in an avian community (Beadell and Fleischer, 2005).

Molecular diagnostic techniques are very sensitive in detecting blood parasites, even when there is low parasitaemia (Ricklefs *et al*., 2004; Sehgal *et al*., 2006; Hellgren *et al*., 2007).

Pérez-Tris and Bensch, (2005) also described a molecular method for detecting mixed infections of haemosporidians; they stated that the detection efficiency might vary depending on the combination of parasite lineages and the intensity of infections. However, PCR assays alone underestimate the occurrence of simultaneous infections of haemosporidian parasites in naturally infected birds (Valkiunas *et al*., 2006). In such research, the sequences obtained are presumed to represent the parasite seen under the microscope, but there may be light infections of other species that will be amplified preferentially (Valkiunas *et al*., 2007). For example, one study used a sequence obtained from a dove infected with *Haemoproteus columbae* (Escalante *et al*., 1998), but the sequence was later shown to represent a *Plasmodium* sp. lineage (Bensch *et al*., 2000). Because GenBank data have been used increasingly in investigations of phylogenetic relationships of parasites, incorrect identifications of sequence identity might be misleading, so it is important to link DNA sequences and morphospecies precisely (Sehgal *et al*., 2005). However, it is not an easy task because the great majority of natural infections of avian haemosporidian parasites are light with a few or even single parasites

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present in blood films and it is usually impossible to identify species of *Plasmodium* in such light infections (Sehgal *et al*., 2005; Valkiunas *et al*., 2005).

To determine the true species composition of the haemosporidians in each naturally infected individual host, a combination of both microscopy and PCR-based methods has been recommended (Valkiunas *et al*., 2006; Valkiunas *et al*., 2009).

Microscopy is unlikely to result in false positives, which is a major concern in large-scale PCR studies (Valkiunas *et al*., 2008). It is important that blood films, which are used for microscopic examination, should be of good quality; they should be examined properly by skilled investigators (Valkiunas *et al*., 2008). In spite of the substantial time investments associated with microscopy, such examination provides opportunities for simultaneous determination and verification of taxonomically different parasites (Valkiunas *et al*., 2008).

Studies revealed that, even though PCR methods are extremely sensitive, they also have shortcomings (Pérez-Tris & Bensch, 2005). The main problem is that the PCR methods are often selective during simultaneous infections with species of *Plasmodium* and *Haemoproteus* or both (Pérez-Tris & Bensch, 2005; Valkiunas *et al*., 2006). So, alone they are insufficient when diagnosing mixed infections of haemosporidian parasites, which are common in wildlife (Ricklefs & Fallon, 2002; Perkins & Schall, 2002). It is important to link knowledge of traditional parasitology and molecular biology because the former provides information about basic life history strategies of these organisms and the latter provides new information about their phylogenetic relationships (Bensch *et al*., 2000; Ricklefs & Fallon, 2002; Perkins &Schall, 2002; Valkiunas, 2005).

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2.9 Factors affecting avian malaria prevalence

Host factors such as age, sex and host population density may also influence host parasite infection (Wood *et al*., 2007). Prevalence may increase with age as new infections accumulate, then decrease as susceptible individuals die or tolerant individuals become immune (Wilson *et al*., 2001). Male birds tend to have a higher prevalence of infection than females (McCurdy *et al*., 1998). Population density may also influence the risk of infection, depending on how parasite transmission relates to host population density and Spatiotemporal variation in parasite infection has often been supposed to contribute to the maintenance of genetic variation in host resistance to parasites, but only rarely has it been studied in wild populations (Lively & Dybdahl, 2000; Bensch & Akesson, 2003).

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CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study design

This study was designed to involve both field and laboratory based research. Birds were sampled from two wildlife protected areas located in Greater Accra region and Central region of Ghana. All the laboratory investigations to detect malaria parasites from avian blood samples were done in the department of animal experimentation, Noguchi Memorial Institute for Medical Research (NMIMR), and in the department of animal science, college of agricultural sciences, both in the University of Ghana.

3.2 Study site and material collected

The sample collection took place between July and December, 2012, in Shai Hills Resource Reserve and Kakum National Park located in Greater Accra and central regions of Ghana respectively.

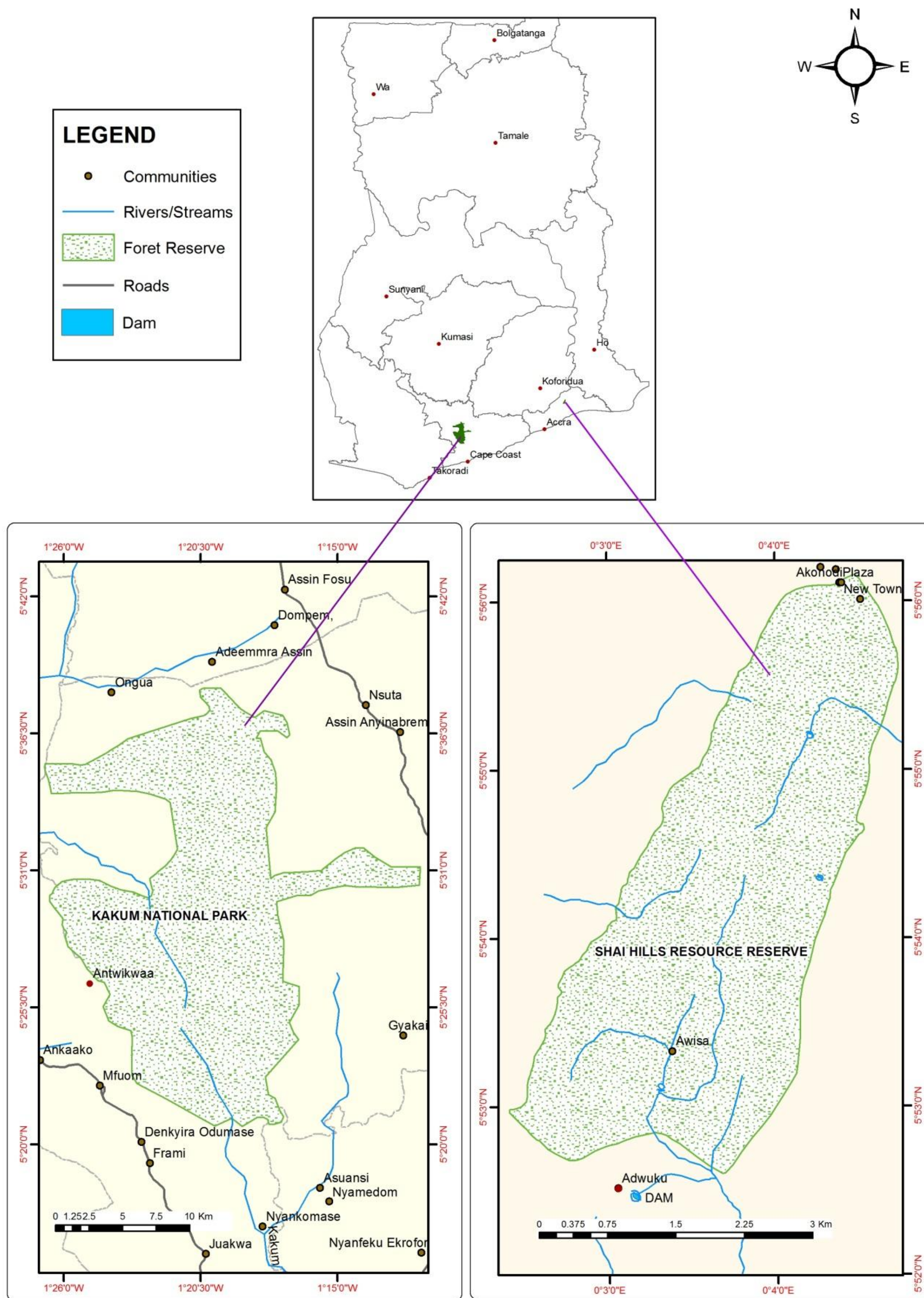

Figure 2: A schematic presentation of the study sites (not to scale)

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3.2.1 Kakum National Park

The Kakum National Park was demarcated as forest reserve between 1925 and 1926 and the reserve was established in 1931 as a source of timber and protection of the watersheds of Kakum River and other rivers which supply the water needs of Cape Coast and other surrounding areas. The park, together with the neighbouring Assin Attandanso Resource Reserve, protects 357km^2 of diverse and dense vegetation and is home to varied wildlife (IUCN, 2010).

The vegetation of Kakum National Park falls into the transition forest type and this classification was based only on the dominant emergent trees. One hundred and five species of vascular plants have so far been identified in the Kakum area (IUCN, 2010). The main features of the vegetation found in Kakum are the moist forest, swamp forest, periodic swamp forest, riverine forest and boval vegetation.

The mammals recorded in the park include potto, Demidoff's galago, grasscutter, brush-tailed porcupine, species of primates (including black and white colobus and Diana monkey), honeybadger, bongo, elephant, duikers (including the yellow-backed duiker), water chevrotain, two hogs, pangolins and squirrels and the reptiles include monitor lizard, dwarf crocodile, Home'shinged tortoise and serrated tortoise (IUCN, 2010). A total of 266 bird species have been reported, including rare species such as the white-breasted guinea fowl (*Agelastes meleagrides*) and the threatened yellow-throated olive greenbul (*Criniger olivaceus*). There is a great number and diversity of butterflies (at least 405 species) recorded in the park. The few carnivores that occur in Kakum are low in density. They include African civet, forest genet, leopard and palm civet (IUCN, 2010).

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3.2.2 Shai Hills Resource Reserve

Shai Hills Resource Reserve with an area of 52km^{2}is one of Ghana's smallest wildlife protected areas and is located in Doryumu, Dangme west district of the Greater Accra Region. The reserve is situated in Accra Plains which forms the western end of the Dahomey Gap, an area of low rainfall where the West African coastal rainforest belt is interrupted and replaced by low grass and savannah (IUCN, 2010). The Shai Hills are a series of inselbergs (mountains that have been largely worn away). The highest peak rises to 290 m. The hills are covered by a mixture of forest, thickets and grassland with unique low stature dry forest being mainly found in the intervening canyons. The hills are surrounded by savannah-covered plains, at about 60 m elevation. The reserve's vegetation is dominated by short-grass savannah with trees and shrubs on the plains, and by dry evergreen forest and thickets on the hills (IUCN, 2010).

To date, 397 plant species have been identified in the reserve, including two endemic species. The large mammals that are commonly seen in Shai Hills include olive baboons (*Papio Anubis*), kob (*Kobus kob*), green monkey (*Cercopithecus aethiops*), spot-nose monkey (*C. petaurista*) and bushbuck (*Tragelaphus scriptus*) (IUCN, 2010).There are records of Tree Hyrax *Dendrohyrax dorsalis* which are much more likely to be the Rock Hyrax (*Procavia johnstoni*). Other species are grasscutter, crested porcupine, hedgehog, Togo hares, oribi, and slender tailed mongoose (IUCN, 2010). Predators in the reserve include hyenas, leopards, civets, genets, servals and side-striped jackals. About 173 species of birds have been recorded in the reserve (IUCN, 2010).

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3.3 Collection of birds

Birds were caught using mist nets. Six mist nets comprising four 12m length and two 18m lengths were set in Kakum National park while four nets comprising two 18m nets and two 12m nets were set in Shai Hills Resource Reserve. The mist nets were opened between the hours of 05:30am and 05:00pm. Nets were checked frequently for catches. The trapped birds were carefully removed from the nets and kept in holding bags prior to processing. Processed birds were marked with a permanent marker before released so that sample was not taken from any recaptured bird. The birds were identified by using "Birds of Ghana" field guide (Borrow and Demey, 2010).

3.3.1 Sample size

Sample size depended on the relative abundance of the birds sampled from the study sites. A total of 132 birds were sampled. Out of this 78 were sampled from Kakum National Park and 54 from Shai Hills Resource Reserve.

3.3.2 Collection of blood samples and preparation of the material for microscopic examination and DNA studies

Blood samples were taken by puncturing the brachial vein of birds. Each bird was removed from the holding bag and the area around the brachial vein was sterilized by swabbing with 70% alcohol. This also moistened the surrounding feathers of the brachial vein making it more accessible. Using a sterile 26 gauge needle, the area was pricked and squeezed gently to obtain a large drop of blood (Fig. 3A). The blood was collected using a 200µl pipette tip fixed to a micropipette aid (Fig. 3B). Approximately 100 µl of whole blood was drawn from each bird, and kept for molecular analysis. The blood samples

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were kept in 300 µl EDTA micro-containers and mixed gently but thoroughly to prevent coagulation. They were held at ambient temperature in the field and later at -20 °C in the laboratory.

A blood drop of approximately 2 µl taken directly from the bird's body was used to prepare each blood film (Fig. 3C). Three thin blood films were prepared from each bird on ready-to-use glass slides; the thin film was spread using a smooth edge slide spreader. Using a grease pencil, the slides were labeled with numbers for identification. The blood films were made on slides that have frosted ends for easy labeling (Chesebrough, 2000). The smears were air-dried within 5-10 sec after their preparation, with the slides in a horizontal position after which they were placed in a separate box covered with a lid to protect them from insects and dust (Chesebrough, 2000). The slides were fixed in absolute methanol in the field for 1 min on the day of their preparation. Fixed slides were packed into slide boxes, so that they did not touch each other. All smears packed into slide boxes were brought in for processing at the department of Animal Experimentation, Noguchi Memorial Institute for Medical Research, University of Ghana.

Figure 3: Blood sample collection and preparation of blood films for microscopic examination.

A – Puncturing the brachial vein; B – taking blood sample; C – making blood smears

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3.4 Giemsa staining

Fixed smears were stained in the laboratory with 5% working solution of a commercially purchased stock solution of Giemsa's stain, pH 7.0-7.2, at 20-25°C. The methanol fixed slides were immersed in a coplin jar. The 5% Giemsa solution was poured on the slides in the jar and allowed to stand for 30 min. The stain from the jar and slides were then washed with clean water to avoid the films being covered with fine deposit stain. The back of each slide was cleaned and placed on a draining rack to air dry.

3.5 Microscopic examination of blood films

An Olympus CH30 light compound microscope was used to examine the blood slides. On each slide, a drop of oil immersion was put to the lower third of the film and examined to check the staining, morphology and distribution of the cells and to detect malaria schizonts, gametocytes and trophozoites (Chesebrough, 2000). Approximately 100 -150 fields were examined for 10-15 min at low magnification (×400), and then at least 100 fields were studied at high magnification (×1000). Pictures of the parasites were taken using a wraycam G500 (wraymer microscope ×0.5) using the software wrayview. The examination of each sample took approximately 40-50 min.

3.6 Extraction of DNA from avian blood

Deoxyribonucleic acid (DNA) was extracted from whole blood following a DNeasy kit protocol (Qiagen, Valencia, CA, and USA).

Ten microlitres (10µl) of whole blood was added into a labeled 1.5mL micro centrifuge tube. 200µl of Avian Phosphate Buffered Solution (PBS) was added to each sample. Twenty microlitres (20µl) Proteinase K was added to each sample and mixed by vortexing for 5-15 seconds. Two hundred microlitres (200µl) of Buffer AL was added and mixed by vortexing for 5-15 seconds and the samples were incubated at 50˚C for 10

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minutes. Two hundred microlitres (200µl) of cold 100% ethanol was added to each sample and mixed by vortexing for 5-15 seconds. The mixture was transferred into labeled spin columns and centrifuged at 8000 x gravity for 1 minute. The tubes containing the flow through were discarded. Five hundred microlitres (500µl) of Buffer AW1 was added to each spin column and centrifuged at 8000 x gravity for 1 minute and the tube containing the supernatant discarded. Five hundred microlitres (500µl) of Buffer AW2 was added to each spin column and centrifuged at 14,000 x gravity for 3 minutes and the tube containing the flow through discarded. Each spin column was placed in a labeled micro centrifuge tube and the tube containing the filtrate discarded. Two hundred microlitres (200µl) of Buffer AE was then added to the spin column and incubated at room temperature for 1 - 5 minutes. The sample was centrifuged at 8000 x gravity for 1 minute and the spin columns discarded. The extracted DNA samples were frozen at -20˚C for future use.

3.7 PCR amplification

For confirmation of the presence of *Plasmodium* and *Haemoproteus* spp., single polymerase chain reaction (PCR) method that amplifies a fragment of the mitochondrial cytochrome b gene was used. Positive and multiple negative controls were included to check. The positive controls were taken from infected birds, as was determined by microscopic examination of blood films, and sterile nuclease free water was used as negative controls in place of DNA template (Valkiunas *et al*., 2009). The negative controls were used to control for false amplification due to the high sensitivity of the PCR. The primers HaemF (5'ATGGTGCTTTCGATATATGCATG3') and HaemR2 (5'GCATTATCTGGATGTGATAATGGT3'), designed by Bensch *et al*., (2000) and

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derived from a relatively conserved region of several avian *Plasmodium* and *Haemoproteus* spp. previously registered in GenBank were used.

Each solution for PCR reaction contained 15 µl of 2 × Gotaq green master mix (Promega), 0.04µmol of each primer, 10.2µl of nuclease free water and 3µlof the DNA in a final volume of 30µl.

DNA amplification was done using an Applied Biosystems 2720 thermal cycler. A total of 35 cycles was carried out, consisting of denaturing at 95°C for 1minute, annealing at 55°C for 1minute, and extension at 72°C for 1minute, with an initial pre-incubation at 95°C for 5 minutes and elongation at 72°C for 10 minutes.

The amplified DNA (5µl) was then submitted to electrophoresis in 2% agarose gel and detected by ethidium bromide staining and UV trans-illumination. The expected target size was 478-bp and the band size was measured using a 50bp DNA ladder (Biolabs).

3.7.1 Parasite identification

All amplicons with band sizes between 400bp and 600bp were sent to Macrogen gene sequencing company for analysis (Macrogen, Europe). They were purified and subjected to an automatic dye-terminator cycle sequencer with Big Dye Terminator, to confirm genomic sequences. The resulting sequences were submitted to the National Center for Biotechnology Information (NCBI) BLAST nucleotide database for comparison with known sequences.

3.8 Research Permit

This work received permit from the Ghana Wildlife Division of the Forestry Commission and was done with the consent of the park managers in the two study sites

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3.9 Statistical analysis

Results were analysed using the statistical packages, SPSS (version 16), Practistat and Excel to address the objectives of the study. Kappa measurement of agreement was used to test for sensitivity and specificity of PCR and microscopy diagnostic methods in detecting avian malaria parasites. Mann-Whitney U-test was used to compare prevalences estimated in both study areas and Kruskal-wallis H-test was used to compare prevalences estimated by microscopy, PCR and a combination of the both methods in the two study areas. These tests were used because; normality test showed that the data used was not normally distributed. Sequencing results was opened using the software Bioedit. The sequences were edited and aligned using the software MEGA (version 5.2). The sequence results were analysed using the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) nucleotide database.

CHAPTER FOUR

4.0 RESULTS

4.1 Sample size and distribution of birds in the study areas

A total of 132 individual birds comprising 20 families and 39 species were trapped. Of this number, (54%) representing 21 species were recorded exclusively in Kakum National park, (41%) representing 16 species were recorded exclusively in Shai Hills resource reserve and (5%) representing 2 species, viz. *Ceyx pictus* and *Merops albicollis* were found in both sites (Figure 3). The representation of families of birds in each reserve has been shown in figure 3. Eight families occurred in only Shai Hills, seven families occurred in only Kakum and five were common to both sites and these were the families: alcedinidae, meropidae, pycnonotidae, columbidae and muscicapidae.

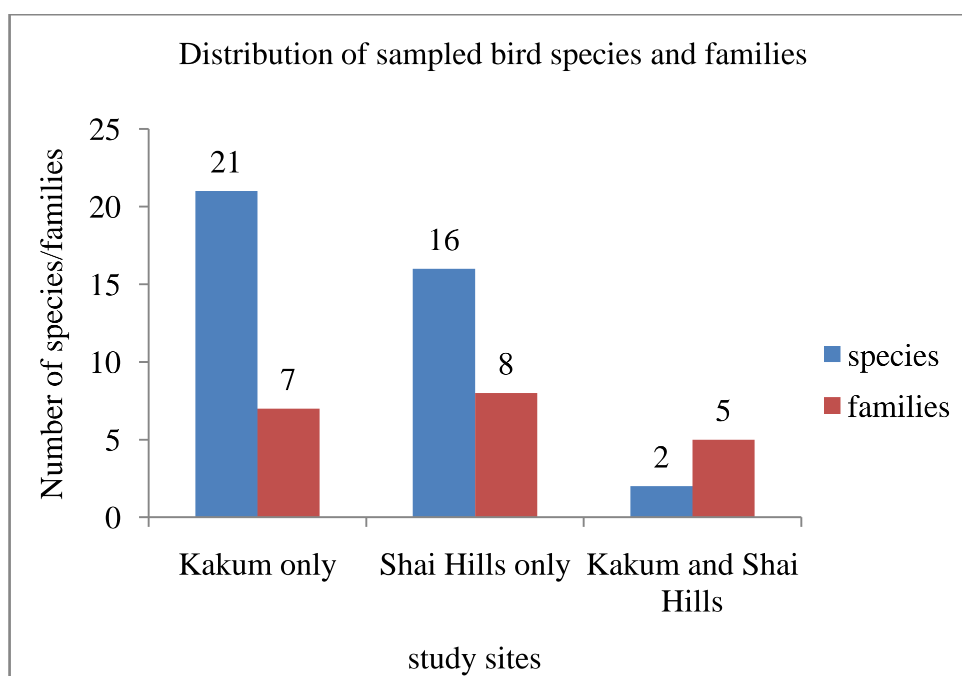

Figure 4: Distribution of sampled bird species and families in Kakum National park and Shai hills resource reserve.

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4.2 Relative Abundance of Birds in Kakum National Park

In Kakum National Park, a total of 78 individual birds of 23 species belonging to 12 families were sampled. Two species, *Ploceus nigerrimus* (0.93%) and *Andropadus virens* (0.45%) were the most abundant. The relative abundance of other species trapped is shown in Table 1.

Table 1: Total number of birds caught in Kakum National Park and relative abundance of each bird species expressed in number of birds × 100/mist-net m × h

Common Name/ Host family	Scientific Name	Number of birds caught	Relative Abundance
Nectariniidae			
Olive sunbird	*Cyanomitra olivacea*	5	0.19
Olive-bellied sunbird	*Cinnyris chloropygius*	1	0.04
Collard sunbird	*Hedydipna collaris*	1	0.04
Blue-throated brown sunbird	*Cyanomitra cyanolaema*	1	0.04
Sylviidae			
Green crombec	*Sylvietta virens*	1	0.04
Muscicapidae			
Dusky-blue flycatcher	*Muscicapa comitata*	1	0.04
Monarchidae			
Red-bellied paradise flycatcher	*Terpsiphone viridis*	1	0.04
Cisticolidae			
Grey-backed camaroptera	*Camaroptera brachyura*	2	0.07
Red-faced cisticola	*Cisticola erythrops*	1	0.04
Pycnonotidae			
Yellow whiskered greenbul	*Andropadus latirostris*	1	0.04
Red-tailed greenbul	*Criniger calurus*	1	0.04
Little greenbul	*Andropadus virens*	12	0.45
Western blue-bill	*Spermophaga haematina*	5	0.19
Estrildidae			
Chestnut-breasted negrofinch	*Nigrita bicolor*	1	0.04
Ploceidae			
Blue-billed malimbe	*Malimbus nitens*	4	0.15
Crested malimbe	*Malimbus malimbicus*	2	0.07
Vieillot's black weaver	*Ploceus nigerrimus*	25	0.93
Village weaver	*Ploceus cucullatus*	4	0.15
Black-necked weaver	*Ploceus nigricollis*	1	0.04

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Columbidae			
Tambourine dove	*Turtur tympanistria*	3	0.11
Meropidae			
White-throated bee-eater	*Merops albicollis*	3	0.11
Alcedinidae			
African pygmy kingfisher	*Ceyx pictus*	1	0.04
Zosteropidae			
Yellow white-eye	*Zosterops senegalensis*	1	0.04
Family: 12	Species:23	Individuals:78	

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4.3 Relative abundance of birds in Shai Hills Resource Reserve

A total of 54 birds of 18 species belonging to 13 families were trapped. Two species, *Turtur afer* (0.57%) and *Cossypha nivieicapilla* (0.48%) were the most abundant. The relative abundance of all other species trapped is shown in Table 2. The common and scientific names of all birds trapped are shown in APPENDIX I and II.

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Table 2: Relative Abundance of birds trapped in Shai Hills Resource Reserve

Common Name/ Host Family	Scientific Name	Number of birds caught	Relative Abundance
Alcedinidae			
African pygmy kingfisher	*Ceyx pictus*	2	0.09
Capitonidae			
Veillot's barbet	*Lybius vieilloti*	4	0.17
Columbidae			
Blue-spotted wood dove	*Turtur afer*	13	0.57
Dicruridae			
Forked-tail drongo	*Dicrurus adsimilis*	1	0.04
Lybiidae			
Yellow-fronted tinkerbird	*Pogoniulus chrysoconus*	1	0.04
Meropidae			
White-throated bee-eater	*Merops albicollis*	1	0.04
Muscicapidae			
Pied flycatcher	*Ficedula hupoleuca*	1	0.04
Pale flycatcher	*Bradornis pallidus*	1	0.04
Phasianidae			
Stone patridge	*Ptilopachus petrosus*	1	0.04
Picidae			
Buff-spotted wood pecker	*Campethera nivosa*	4	0.17
Platysteiridae			
Common wattle eye	*Platysteira cyanea*	5	0.22
Pycnonotidae			
Grey-headed bristle bill	*Bleda canicapillus*	3	0.13
Simple leaflove	*Chlorocichla simplex*	2	0.09
Yellow-throated leaflove	*Chlorocichla flavicollis*	1	0.04
Common bulbul	*Pycnonotus barbatus*	1	0.04
Sturnidae			
Violet-backed starling	*Cynnyricinclus leucogaster*	1	0.04
Turdidae			
Snowy crowned robin chat	*Cossypha niveicapilla*	11	0.48
African thrush	*Turdus pelios*	1	0.04
Family: 13	Species: 18	54	

4.4 Microscopic investigation and PCR screening of avian blood samples

Microscopic examination of blood films revealed infections with either *Plamodium* or *Haemoproteus* in 30 birds. Some infected erythrocytes are shown in figure 5.However, PCR screening of the blood samples showed that 58 birds were infected with malaria parasites. Gel electrophoresis picture showing results for some positive samples is shown in figure 6.

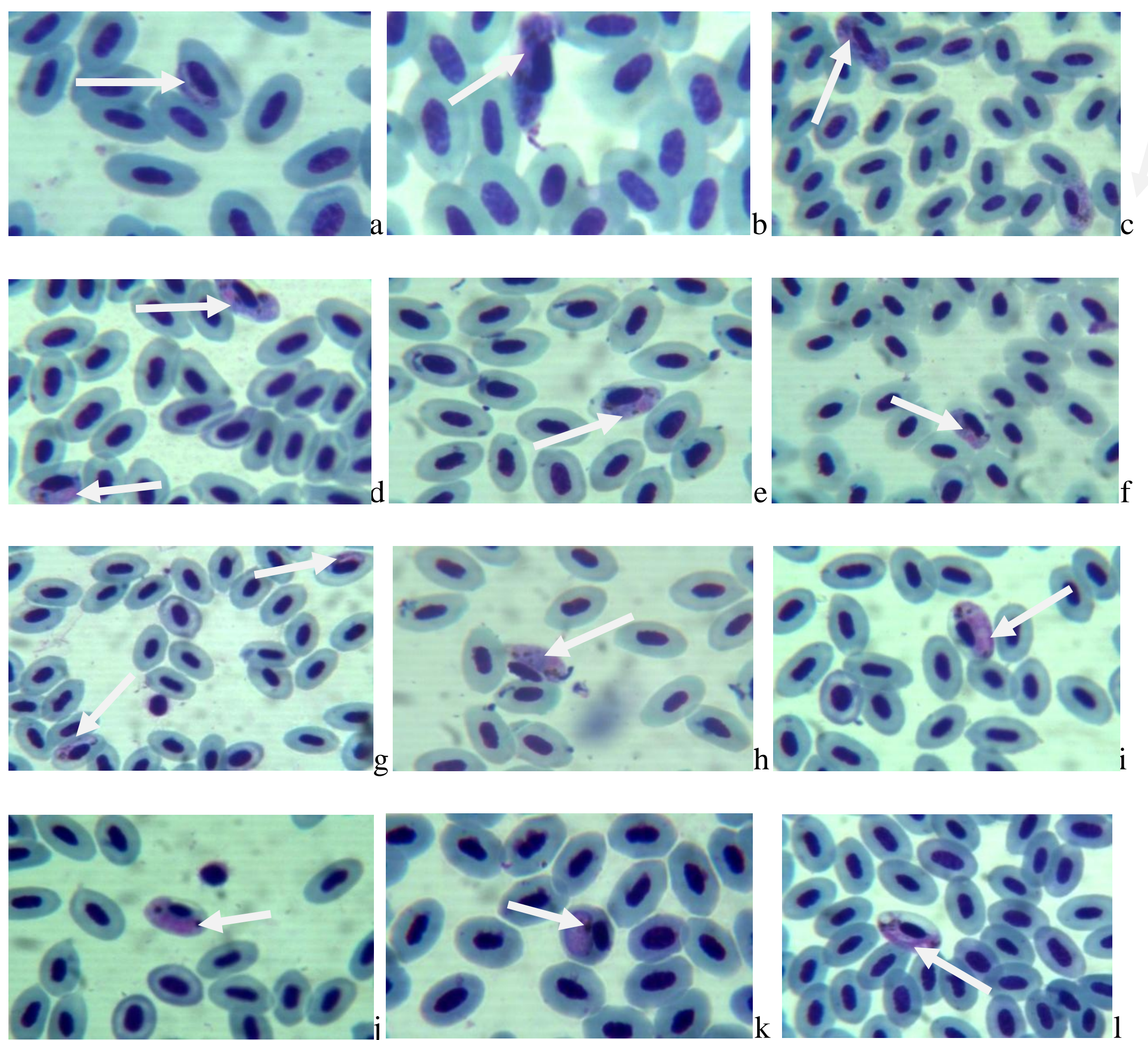

Figure 5: Blood stages of haemosporidian parasites as seen in thin blood films. a, b, c: gametocytes of *Haemoproteus* spp. in the blood of *Nigrita bicolor*; d-j: gametocytes of *Haemoproteus* spp. in the blood of *Ploceus nigerrimus*; k, l: gametocytes of *Plasmodium* spp. in the blood of *Ploceus nigerrimus*.

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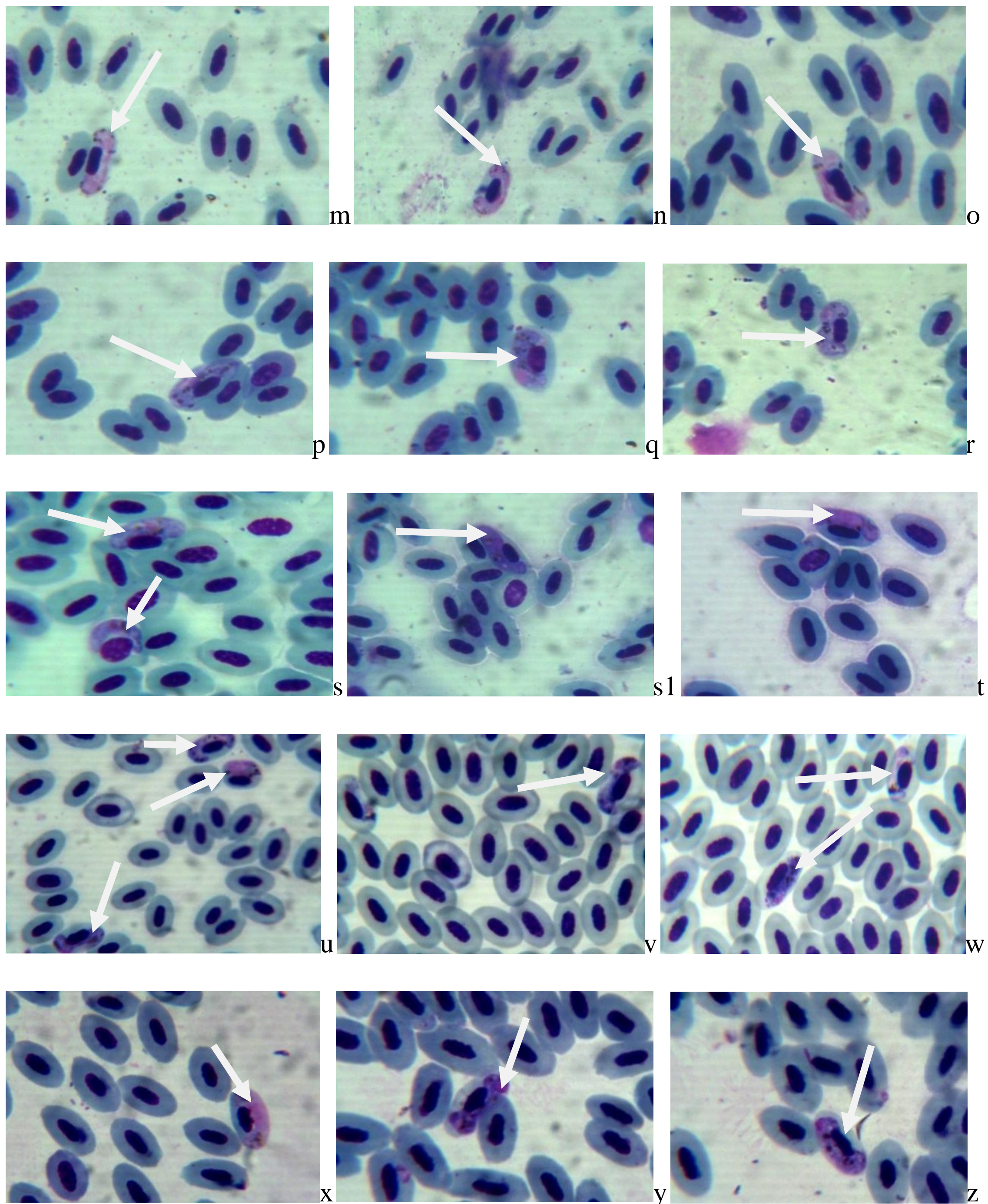

Figure 5: Blood stages of haemosporidian parasites as seen in thin blood films. m-o: gametocytes of *Plasmodium* spp. in the blood of *Ploceus nigerrimus*; p-r: gametocytes of *Haemoproteus* spp. in the blood of *Bradornis pallidus*; s, s1, t: gametocytes of *Plasmodium* spp. in the blood of *Merops albicollis*; u-w: gametocytes of *Haemoproteus* spp. in the blood of *Zosterops senegalensis*; x-z: gametocytes of *Haemoproteus* spp. in the blood of *Ficedula hupoleuca*.

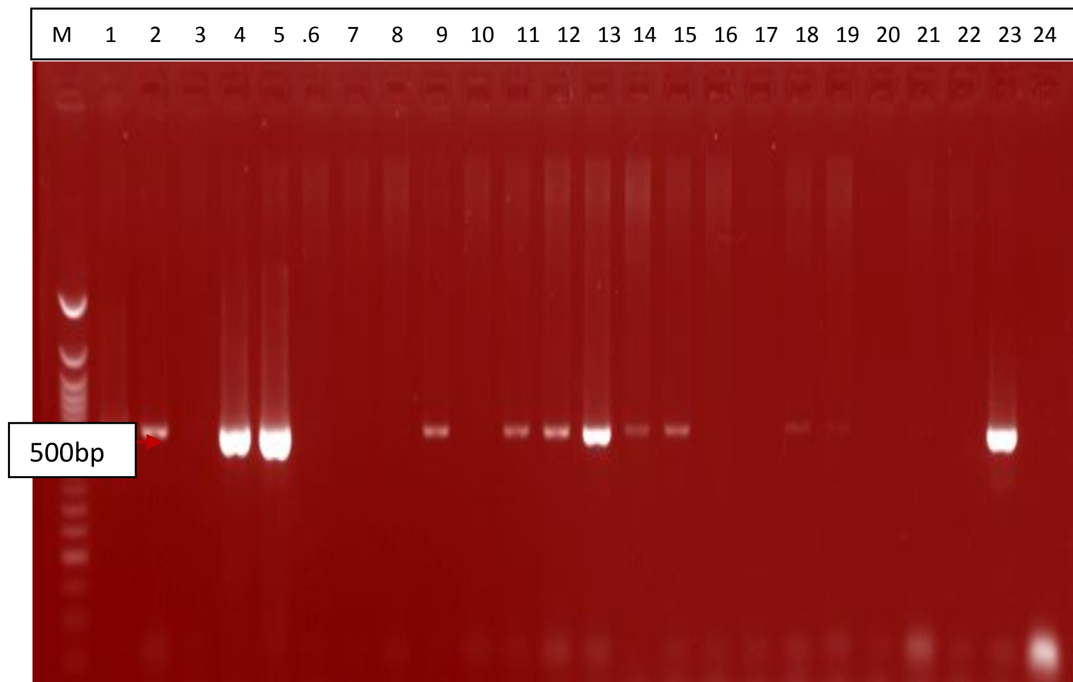

Figure 6: Agarose gel showing PCR results with the primers HaemF and HaemR2 on DNA samples extracted from blood samples of birds. Lane M- 50bp DNA ladder (Biolabs); lane 1-6, 8-16, 18-23: DNA samples extracted from avian blood; lane 23 – positive control; lanes7, 17, 24 – negative controls.

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4.5 A comparative analysis of Microscopy and PCR for the detection of avian haemosporidians

A combination of microscopy and PCR in diagnosing avian malaria revealed *Plasmodium* and *Haemoproteus* prevalence of 45.4% (Table 3). Microscopy alone detected 23% prevalence while PCR alone detected 44% prevalence. The difference in prevalence detected by the two methods was statistically significant ($p<0.05$). However, there was no significant difference ($p>0.05$) between prevalence detected by PCR alone and the combination of the two methods (Table 3).

Considering the microscopy results as the reference point, a total of 58 PCR positive samples and 30 microscopy positive samples yielded a sensitivity of 48.3%; 74 PCR negative and 102 microscopy negative samples yielded a specificity of 97.3% (APPENDIXV). Some samples showed positive by PCR (51.7%) and undetected by microscopy. Only 2.79% of detections were positive by both microscopy and PCR. Thus kappa measurement of agreement estimated a weak agreement ($k=0.48$) between PCR screening and microscopic examination as diagnostic tests for the detection of avian blood parasites.

The combination of both PCR and microscopy revealed infections of *Haemoproteus* spp. and *Plasmodium* spp. among 11 families and 18 species of avian hosts (Table 3).The species with the highest prevalence of infection were *Ploceus cucullatus* (n=4, 100%), *Camaroptera brachyura* (n=2, 100%) and *P. nigerrimus* (n=25, 92%). The prevalence of parasites in other species sampled is shown in Table 3.

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Comparatively, parasite infections varied between the two sites with a higher prevalence (69.20%) in Kakum National Park compared to (12.90%) in Shai Hills Resource Reserve. However, the difference was not statistically significant ($p>0.05$).

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Table 3: Birds tested and outcome of PCR screening and microscopic examination of blood samples

Taxa	No. screened	Microscopy H/P	PCR H/P	Both methods H/P	Prevalence (%)
Nectariniidae					
Cyanomitra olivacea	5	1	2	2	40
Cinnyris chloropygius	1	0	1	1	-
Hedydipna collaris	1	0	0	0	0
Cyanomitra cyanolaema	1	1	1	1	-
Sylviidae					
Sylvietta virens	1	1	1	1	-
Muscicapidae					
Ficedula hupoleuca	1	1	1	1	-
Bradornis pallidus	1	1	1	1	-
Muscicapa comitata	1	0	1	1	-
Monarchidae					
Terpsiphone viridis	1	0	0	0	0
Cisticolidae					
Camaroptera brachyuran	2	0	2	2	100
Cisticola erythrops	1	0	0	0	0
Pycnonotidae					
Bleda canicapillus	3	0	0	0	0
Andropadus latirostris	1	0	0	0	0
Criniger calurus	1	0	1	1	-
Andropadus virens	12	0	5	5	41.7
Spermophaga haematina	5	0	2	2	40
Estrildidae					
Nigrita bicolor	1	1	1	1	-
Ploceidae					
Malimbus nitens	4	0	3	3	75
Malimbus malimbicus	2	0	1	1	50
Ploceus nigerrimus	25	19	22	23	92

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Ploceus cucullatus	4	3	4	4	100
Ploceus nigricollis	1	0	0	0	0
Columbidae					
Turtur afer	13	0	1	1	7.7
Turtur tympanistria	3	0	0	0	0
Meropidae					
Merops albicollis	4	1	2	3	75
Alcedinidae					
Ceyx pictus	3	0	1	1	33.3
Zosteropidae					
Zosterops senegalensis	1	1	1	1	-
Capitonidae					
Lybius vieilloti	4	0	0	0	0
Dicruridae					
Dicrurus adsimilis	1	0	0	0	0
Lybiidae					
Pogoniulus chrysoconus	1	0	1	1	-
Phasianidae					
Ptilopachus petrosus	1	0	0	0	0
Picidae					
Campethera nivosa	4	0	0	0	0
Platysteiridae					
Platysteira cyanea	5	0	0	0	0
Chlorocichla simplex	2	0	0	0	0
Chlorocichla flavicollis	1	0	0	0	0
Pycnonotus barbatus	1	0	1	1	-
Sturnidae					
Cynnyricinclus leucogaster	1	0	0	0	0
Turdidae					
Cossypha niveicapilla	11	0	1	1	9.1
Turdus pelios	1	0	1	1	-

H: Haemoproteus

P: Plasmodium

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4.6 Parasite lineages and habitats

Sequencing of the mitochondrial cytochrome b gene revealed a total of 11 *Plasmodium* lineages and 9 *Haemoproteus* lineages from both Kakum National park and Shai Hills Resource reserve (Table 4, 5). However, sequences for twelve individuals were not determined due to faint bands shown on gel electrophoresis. Out of 11 *Plasmodium* lineages, three, viz. WA22, PV1L and PLASCOQ12 were common to both sites. One lineage, WA45 was recovered from Shai Hills only while seven lineages, viz. WA6, OZ45_MEX19, LIN3A, P31, PV11, COLL7 and WA16 were recovered from Kakum National Park only (Table 4, 5).

Two out of the nine *Haemoproteus* lineages, L-PFC1 and SYBOR1.SPAIN were recovered from Shai Hills while the rest NA15BRCO, WAH36, VILWE1, WAH34, LIN8A, HV42 and LIN18A were recovered from Kakum National Park (Table 4, 5).

Plasmodium and *Haemoproteus* prevalences varied in both sites with *Plasmodium* recording the highest prevalence in each site, viz. 33.3% in Kakum and 7.4% in Shai Hills compared to the lower prevalences of *Haemoproteus*, ie. 17.9% in Kakum and 3.7% in Shai Hills (Table 4, 5).

However, there was no significant difference ($p>0.05$) between *Plasmodium* and *Haemoproteus* prevalences in each site (Table 4, 5).

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Table 4: Prevalence (P) and lineages of haemosporidian parasites in birds trapped from Kakum National Park (lineage names are according to NCBI database)

	Plasmodium			Haemoproteus		Genus unknown
Taxa	N	P (%)	Lineage	P (%)	Lineage	Percentage
Cyanomitra Olivacea	5	1(20)	WA6	0		
Cinnyris chloropygius	1	0		0		
Hedydipna Collaris	1	0		0		
Cyanomitra cyanolaema	1	1(100)	WA6	0		
Sylvietta virens	1	0		1(100)	NA15BRCO	
Muscicapa Comitata	1	1(100)	PLASCOQ12	0		
Terpsiphone Viridis	1	0		0		
Camaroptera Brachyuran	2	0		0		1(50)
Cisticola erythrops	1	0		0		
Andropadus virens	12	5(41.7)	PV1L(5)	0		
Andropadus Latirostris	1	0		0		
Criniger calurus	1	1(100)	PV1L	0		
Spermophaga Haematina	5	3(60)	OZ45_MEX 19(2) PV1L(1)	0		1(20)
Nigrita bicolor	1	0		1(100)	WAH36	

Ploceus nigerrimus	25	10(40)	LIN3A(1), WA22(2) P31(1), PV11(5) COLL7(1)	10(40)	VILWE1(2) WAH34(7) LIN8A(1)	1(4)
Ploceus cucullatus	4	2(50)	PV11(1), WA22(1)	0		2(50)
Ploceus nigricollis	1	0		0		
Malimbus nitens	4	2(50)	PV1L(1), WA16(1)	1(25)	HV42	1(25)
Malimbus Malimbicus	2	0		0		1(50)
Turtur tympanistria	3	0		0		
Merops albicollis	3	0		0		
Ceyx pictus	1	0		0		
Zosterops senegalensis	1	0		1(100)	LIN18A	
		26 (33.3)		14 (17.9)		

Table 5: Prevalence (P) and lineages of haemosporidian parasites in bird species sampled in Shai Hills Resource Reserve

	Plasmodium			Haemoproteus		unknown
Taxa	N	P (%)	Lineage	P (%)	Lineage	%
Ceyx pictus	2	0		0		
Lybius vieilloti	4	1(25)	WA22	0		
Turtur afer	13	0		0		1(7.7)
Dicrurus adsimilis	1	0		0		
Pogoniulus chrysoconus	1	1(100)	PV1L	0		
Merops albicollis	1	0		0		
Ficedula Hupoleuca	1	0		1(100)	L-PFC1	
Bradornis pallidus	1	0		1(100)	SYBOR1 SPAIN	
Ptilopachus Petrosus	1	0		0		
Campethera nivosa	4	0		0		
Platysteira cyanea	5	0		0		
Bleda canicapillus	3	0		0		
Chlorocichla Simplex	2	0		0		
Chlorocichla flavicollis	1	0		0		
Pycnonotus barbatus	1	1(100)	WA45	0		
Cynnyricinclus leucogaster	1	0		0		
Cossypha niveicapilla	11	1(9.1)	PLAS COQ12	0		
Turdus pelios	1	0		0		
		4(7.4)		2(3.7)		

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CHAPTER FIVE

5.0 DISCUSSION

5.1 Efficiency of methods used in parasite detection and identification

Two diagnostic methods, Microscopy and Polymerase Chain Reaction (PCR) were used in the detection and identification of avian malaria parasites of the genera *Plasmodium* and *Haemoproteus*. The result of this study conforms to other results and conclusions regarding lower sensitivity of microscopy in comparison to the PCR-based methods in determining the prevalence of avian malaria infections (Jarvi *et al*., 2002; Richards *et al*., 2002; and Durrant *et al*., 2006). Factors that could contribute to these discrepancies include low parasitaemia and differences, the use of poor quality blood films and too few microscopic fields examined (Jarvi *et al*., 2002). Few slides from the present study contained haemolyzed blood cells, which indicate insufficient desiccation, or fixation of blood films, or both, in the field. The results also correlates with the study by Valkiunas *et al*. (2008) that microscopic examination of poor quality blood films is an unreliable method of diagnosis. In conclusion, the present study agrees with previous studies, that concluded that the reliability of PCR does not only expose the insensitivity of microscopy, but also shows that blood films prepared for microscopic examination should be of good quality.

The overall prevalence of haemosporidian blood parasites, as determined by microscopy was (23%). The difference in prevalence determined by PCR diagnostics (44%) and the combination of both methods (45.4%) was not significant. In few samples, microscopy was slightly more sensitive than PCR, and, in most samples, the opposite was true. The main shortcoming of microscopic examination of blood films is the low sensitivity in determining exceptionally light infections (1 parasite cell per 1000 microscopic fields)

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when just a few parasites are present in blood films (Valkiunas *et al*., 2008). Such light parasitaemia would be easily overlooked even with increased observation time (Valkiunas *et al*., 2008). The present study reported low levels of parasitaemia detected by microscopic examination ranging from 1-5 parasites per 1000 microscopic fields. This shortcoming coupled with the fact that, all blood stages of the parasites, important for morphospecies identification, were not available in the blood films, make it difficult to have clear identification of specific lineages.

PCR-based methods are particularly attractive because they provide sequence information for phylogenetic and epidemiologic studies of parasites and for the diagnosis of parasitic disease (Jarvi *et al*., 2002; Perkins and Schall, 2002; Bensch *et al*., 2004). A problem with PCR, but unlikely with microscopy, is the risk of false positives. The present study tried to minimize this by running multiple negative controls however, some false positives can still go undetected. Microscopy also has an advantage in providing an opportunity to determine or verify the identity and intensity of infections.

The combination of both methods revealed higher prevalence of the parasites. This agrees with the studies by Krone *et al*. (2008), which reported that the combination of diagnostic methods reveals higher prevalence of parasites in avian hosts. There is therefore the need to combine both microscopy and PCR in order to obtain proper estimates of prevalence.

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5.2 Parasite Prevalence in Forest and Savanna Birds

The prevalence of avian malaria in some protected areas in Ghana has been confirmed in this study. The overall prevalence of 45.3% of the individuals infected with the blood parasites is closer to the overall prevalence of 48.2% recorded in Balmoral, Zambia (Pierce, 1984) and relatively higher than 22.2% reported for Ghana (Wink & Bennett, 1976) and 28.2% reported for Uganda. It is also higher than the reported prevalence of 28.2% for Uganda and 28.6% in some West African rainforests (Sehgal *et al*., 2005).

A study by Wink and Bennett (1976) revealed that prevalence of blood parasites in birds from the tropical rainforest in the Bunso area in Ghana was similar to prevalence recorded in birds sampled in the savanna-urban area around Accra. However, the present study disagrees with the above results, thus reporting higher prevalence of blood parasites in the Kakum tropical rainforest than Shai Hills savanna area.

The family ploceidae was the most heavily parasitized family that occurred in Kakum forest with an overall prevalence of 23.5%. This follows the trend that has been observed in many parts of Africa including Ghana (Wink & Bennett, 1976) and can be explained by their behavioural ecology. For example, *P. nigerrimus* and *P. cucullatus*, found to have high parasite prevalence are colonial breeders and cavity nesters. Previous study showed that colonial breeders are more susceptible to parasite infection than non-colonial breeders (Tella, 2002; Merino *et al*., 1999).

The most occuring lineage recorded in this study was *Plasmodium* lineage PV1L. The fact that some lineages are more abundant than others may be due to the differential ability of the vectors to transmit some lineages better than others. Some vectors have the

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ability to transmit more than one parasite lineage, but they transmit some lineages more effectively than others (Alavi *et al*., 2003).

Generally, the variation in blood parasite prevalences observed between host species and geographic locations could be explained by differences in their exposure to the insect vectors (Valkiunas, 2005). The present study reported that bird species from the Kakum National Park had high blood parasite prevalence. This is also because they share many qualities that would increase vector exposure such as cavity nesting and habitation of forested areas (Durrant *et al*., 2006) and bright plumage coloration (Scheuerlein *et al*., 2004). For example, *P. niggerrimus* and *Zosterops senegalensis*, found to have 11-100 parasites in 100 microscopic fields are forest birds with bright plumage coloration and also cavity nesters.

Other factors that could affect parasite prevalence could include proximity to vector breeding sites, relative levels of host resistance, local temperature differences in the study sites and age of the hosts. Heavily infected individuals may also be under-sampled as observed by (Sehgal, 2005).

5.3 Habitat Effect

The effect of habitat in the ecology of haemosporidian parasites cannot be ignored in the study of host-parasite relationship. There was a higher prevalence of infection with *Plasmodium* and *Haemoproteus* spp. in birds collected from Kakum National Park forest than in Shai Hills savanna resource reserve. Several factors may be responsible for this occurrence. Such factors include differences in vegetation cover, differences in rainfall, soil moisture and species composition of the avian hosts and their insect vectors. Vegetation density and structure could influence interactions between avian hosts and

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their insect vectors, thereby playing a role in the transmission and maintenance of parasite infections (Bonneaud *et al*., 2009).

The sampling site in the Kakum National Park is forest vegetation at the edge of the conservation area and shares boundary with the Kakum National Park. This site is situated within residential and agricultural areas where human impacts may enhance the abundance of the dipteran insect vectors and dispersal into forest bird habitat. Some of the human activities provide micro-habitats that serve as larval habitat to the insect vectors. Forest disturbance such as deforestation and encroaching agriculture potentially increase vector abundance, thus, increasing disease transmission (Bonneaud *et al*., 2009). Moreover, the forested areas harbor a wide variety of vectors which implies high prevalence of the parasite (Durrant *et al*., 2006). On the contrary, Prevalence of *Plasmodium* spp. and *Haemoproteus* spp. in Shai Hills resource reserve was relatively low. One of the factors contributing to this is the low density vegetation of the savanna. This vegetation type reduces the breeding sites for dipteran insect vectors thereby reducing the level of parasite transmission to avian hosts.

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CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

The prevalence of avian malaria parasites of the genera *Plasmodium* and *Haemoproteus* was assessed in Kakum National Park and Shai Hills resource reserve using traditional microscopy and a PCR-based method. The two diagnostic methods showed some discrepancies, however the combination of both methods gave a more credible estimate of parasite prevalence in the two wildlife protected areas. Parasite prevalence was higher (69.2%) in forest birds sampled in Kakum National Park compared to (12.9%) in savanna birds sampled in Shai Hills resource reserve. Due to light infections and absence of important blood stages observed in blood films, the parasites could not be identified morphologically. However, sequencing of 478bp of the mitochondrial cytochrome b gene revealed 11 *Plasmodium* lineages and 9 *Haemoproteus* lineages throughout the study. Generally, the study has shown a higher prevalence of *Plasmodium* spp. than *Haemoproteus* spp. in both forest and savanna birds.

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6.2 RECOMMENDATIONS

The study of avian diseases is very limited in Ghana and this study can be considered as one of the baselines assessment of malaria in birds in Ghanaian protected areas. To consolidate the knowledge in this area to inform policy, the following recommendations are being made:

1. Future works on avian malaria and other haemosporidians should include a broader sampling of individual birds so as to have each species well represented as this will help in making realistic estimation of prevalence.

2. There is the need to increase the scope of the study to make conclusions more credible.

3. Seasonality should be factored into data collection so that parasite prevalence will be assessed both during the rainy and dry seasons.

4. Future works should expand the study to include intensity of parasite infection, host-specificity and genetic diversity of the lineages recovered.

5. There is also the need to carry out similar works in well-equipped laboratories for better microscopic and molecular analyses.

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APPENDICES

APPENDIX I

Sample information (Shai Hills)

Common name	Scientific name	Infection
African pygmy kingfisher	*Ceyx pictus*	-
Veillot's barbet	*Lybius vieilloti*	+
Blue-spotted wood dove	*Turtur afer*	+
Forked-tail drongo	*Dicrurus adsimilis*	-
Yellow-fronted tinkerbird	*Pogoniulus chrysoconus*	+
White-throated bee-eater	*Merops albicollis*	-
Pied flycatcher	*Ficedula hupoleuca*	+
Pale flycatcher	*Bradornis pallidus*	+
Stone patridge	*Ptilopachus petrosus*	-
Buff-spotted wood pecker	*Campethera nivosa*	+
Common wattle eye	*Platysteira cyanea*	-
Grey-headed bristle bill	*Bleda canicapillus*	-
Simple leaflove	*Chlorocichla simplex*	-
Yellow-throated leaflove	*Chlorocichla flavicollis*	-
Common bulbul	*Pycnonotus barbatus*	-
Violet-backed starling	*Cynnyricinclus leucogaster*	-
Snowy crowned robin chat	*Cossypha niveicapilla*	+
African thrush	*Turdus pelios*	-

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APPENDIX II

Sample information (Kakum National Park)

Common name	Scientific name	Infection
Olive sunbird	*Cyanomitra olivacea*	+
Olive-bellied sunbird	*Cinnyris chloropygius*	+
Collard sunbird	*Hedydipna collaris*	-
Blue-throated brown sunbird	*Cyanomitra cyanolaema*	+
Green crombec	*Sylvietta virens*	+
Dusky-blue flycatcher	*Muscicapa comitata*	+
Red-bellied paradise flycatcher	*Terpsiphone viridis*	-
Grey-backed camaroptera	*Camaroptera brachyura*	+
Red-faced cisticola	*Cisticola erythrops*	-
Little greenbul	*Andropadus virens*	+
Yellow whiskered greenbul	*Andropadus latirostris*	-
Red-tailed greenbul	*Criniger calurus*	-
Western blue-bill	*Spermophaga haematina*	+
Chestnut-breasted negrofinch	*Nigrita bicolor*	+
Vieillot's black weaver	*Ploceus nigerrimus*	+
Village weaver	*Ploceus cucullatus*	+
Black-necked weaver	*Ploceus nigricollis*	-
Blue-billed malimbe	*Malimbus nitens*	+
Crested malimbe	*Malimbus malimbicus*	+
Tambourine dove	*Turtur tympanistria*	-
White-throated bee-eater	*Merops albicollis*	+
African pygmy kingfisher	*Ceyx pictus*	+
Yellow white-eye	*Zosterops senegalensis*	+

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APPENDIX III

Nucleotide Sequences

Plasmodium sp. WA6

CAACTGGTGCTTCATTTGTATTTATTTTAACTTACTTACATATATTAAGAGGAT

TAAATTATTCATATTCATACTTACCTTTATCATGGACATCTGGATTAATTATAT

TTTTAATATCTATAGTAACAGCTTTTATGGGTTACGTATTACCTTGGGGTCAA

ATGAGTTTTTGGGGTGCTACTGTAATTACTAATTTATTATATTTTATACCTGGA

CTTGTTTCATGGATATGTGGTGGATATCTTGTAAGTGACCCAACCTTAAAAAG

ATTCTTTGTATTACATTTTACATTTCCATTTATAGCTTTATGTATTGTATTTATA

CATATATTCTTTTTACATTTACAAGGTAGCACAAATCCTTTAGGGTATGATAC

AGCTTTAAAAATACCCTTCTATCCAAATCTTTTAAGTCTTGATATCAAAGGAT

TCAATAATGTATTAGTATTATTTTTAGCACAAAGTTTATTTGGAATATT

Haemoproteus sp. – WAH36

CTACAGGTGCAACCTTTGTATTTATTTTAACTTATTTACATATATTAAGAGGAT

TAAACTATTCATATTTCTTATTTACCTTTATCATGGATAACTTGGATTAGTAAT

ATTTCTTAATATCTATTTGTTACCGCTTTTATTGGGTTATGTATTACCTTGGGG

TCAAATGAGTTTCTGGGGTGCAACTGTTATTACTAATTTATTATACTTTATACC

TGGACTTGTTTCATGGATTTGTGGTGGATATACTATTAGCGATCCAACTTTAA

AAAGATTTTTTGTATTACATTTTATATTTCCTTTTGTAGCTTTATGTATTGTATT

TATACATATATTCTTCTTACACTTACAAGGTAGCTCTAATCCTTTAGGATATGA

TACAGCTTTAAAAATACCTTTCTATCCGAAGTCTATTATGTCTAGATATCAGA

AGGATTTAATAACGTATTAGTCATATTTCTAGCACAAAGTTTATTTGGAATTC

TACCTTATCCTCCAGAAATATTGAAACNGGAANN

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Plasmodium sp. OZ45_MEX19

CAACAGGTGCTTCATTTGTTTTCATTTTAACCTATTTACATATTTTAAGAGGAT

TAAATTACTCATATTCATATTTACCTTTATCATGGATTTCAGGATTATTAATAT

TTTTAATATCCATAGTTACTGCCTTTTATGGGTTATGTATTACCATTGGGGTCA

AATGAGTTTCTGGGGTGCTACAGCTTATAACTAACTTATTATATTTTATACCA

GGACTTGTCTCATGGATTTGTGGTGGATATCTTGTAAGTGACCCAACCTTAAA

AAGATTTTTTGTATTACATTTTACATTCCCATTTATAGCTTTATGTATTGTATTC

ATACATATATTCTTCTTACATTTACAAGGTAGCACAAATCCTTTAGGGTATGA

TACAGCTTTAAAGATACCCTTCTATCCAAATTTATTAAGTCAGAGATAATAAA

GGATTTAATAATGTATTAGTTTTATTCTTATCTCAAAGTTTATTTGGAATTTTA

CCATTATCACATCAGCATAATGCACTN

Plasmodium sp. PV1L

AGAAGGAGATAACGGGATGATATATGTACTTACCTATTTACATATTTTACCAC

GAGTAAATTACTCATATTCGTATTTACCTTTATCATGGATATCAAGATTAATA

ATATTTTTAATATCAATAGTAACTGCTTTTATGGGATATGTAGTACCTTGGGG

TCAAATGAGTTTTTGGGGTGCAACCGTTATTACTAACTTATTATACTTTATACC

TGCTCTTGTTTCATGGATTTGTGGTGGATATCTTGTTAGTGACCCAACATTAAA

AAGATTTTTTGTATTACCTTTCATATTTCCACTGATTGCTACGTATATTGAATT

TATACATGAATGTTGGCTACATAGCCAAAACCGGGAGGATCGTTCAAGTGAT

GATACGCCTGCATCCAAAGCACAAGTTCCCTGAACAAGCCTGAATTCCATAG

ATGTCGGGGAGGGGGCAGGGGGGGAATATTATTACCATGACGTTTAGTTTGA

AGGAAGCTGTTACCTACAGGAGACACATAAGGCAACTACTGCCCTCAGATTC

CCAAAGAGCATTTCCTTTATCAGATACAAGGAGTACTCTCAAAATTC

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*Plasmodium*sp.Plascoq12

AAATGTGAATAACGGCAGATAAATGTACTTCACTATTTACATATTTTAACGAA

GATTAAATTACTCATATTCATATATTACCTCTTATCACGAGATATCACAGTATT

AATAATATTTTTAATATCACTAGTAACTGCGTTTATGAGATATGTATTACCTTG

TGGTCACATGAGTTTTTGGGGTGCGCCCCTGATTACTAACTTATTATACTTTAT

ACCCGGGCATGTGTCATCATGGTATGTGGGGGAGATCTCGTGAAAGAGACCC

CACTTTAAAAAGATTTTGTAGTACTACATTTTACATTCCCATATCAGATCTTTA

AGTATTAGTATTTATACATATATTCTTTCTACATTTACAAGGCAGCACTAATCC

TTTAGGGGATGATACAGCTCTAAAAACACCCTTCTATCCAAATCTTTTAAGTC

TCGATATTAAAGGATTTAATAATGTATTAGTATTATTCTTATCACAAAGTTTAT

TTGGAATATT

Haemoproteus sp. VILWE1

CTACTGGTGCTACATTTGTATTTATTCTAACTTATCTACATATTTTAAGAGGAT

TAAACTATTCATATTCTTATTTACCTCTATCATGGATATCTAGGATTATTAATA

TTCTTAATTTCTATTGTTACCGCTTTTATGGGTTATGTATTACCTTGGGGTCAA

ATGAGTTTCTGGGGTGCAACCGTTATAACTAATTTATTATATTTTATACCTGG

ACTTGTTTCATGGATTTGTGGAGGATATACTATTAGTGATCCAACTTTAAAAA

GATTTTTTGTATTACATTTTATATTTCCATTTATAGCTTTATGTATTGTATTTAT

TCATATATTCTTCTTACACTTACAAGGTAGCTCTAATCCTTTAGGATATGATAC

AGCTTTAAAAATACCTTTCTATCCAAGTCTATTATGTCTAGATATTAAAGGAT

TTAATAATGTATTAGTCCTATTTCTAGCGCATAGTTAATTTGGAATTCT

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APPENDIX IV

Haemoproteus and *Plasmodium* lineages and their Genbank accession numbers

Parasite	Lineage	GenBank number
Haemoproteus sp.	WAH36	EU810737
Haemoproteus sp.	VILWE1	DQ847181
Haemoproteus sp.	WAH34	EU810732
Haemoproteus sp.	NA15BRCO	GQ395665
Haemoproteus sp.	LIN18A	JN661945
Haemoproteus sp.	LIN8A	JN661931
Haemoproteus sp.	HV42	HQ386241
Haemoproteus sp.	L-PFC1	DQ630004
Haemoproteus sp.	SYBOR1.SPAIN	KC682871
Plasmodium sp.	WA6	EU810661
Plasmodium sp.	OZ45_MEX19	HM222481
Plasmodium sp.	PV1L	FJ404709
Plasmodium sp.	Plascoq12	HM179155
Plasmodium sp.	LIN3A	EF380115
Plasmodium sp.	WA22	EU810646
Plasmodium sp.	P31	DQ839061
Plasmodium sp.	WA16	EU810620
Plasmodium sp.	PV11	GQ150193
Plasmodium sp.	COLL7	DQ368376
Plasmodium sp.	WA45	EU810630

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APPENDIXV

Sensitivity and specificity of diagnostic tests

PCR screening * microscopic examination Cross tabulation

			microscopic examination		Total
			negative	positive	
PCR screening	negative	Count	72	2	74
		% within PCR screening	97.3%	2.7%	100.0%
		% within microscopic examination	70.6%	6.7%	56.1%
	positive	Count	30	28	58
		% within PCR screening	51.7%	48.3%	100.0%
		% within microscopic examination	29.4%	93.3%	43.9%
Total		Count	102	30	132
		% within PCR screening	77.3%	22.7%	100.0%
		% within microscopic examination	100.0%	100.0%	100.0%

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APPENDIX VI

Protocol for DNA Extraction from avian blood

(1) Add 10µl of whole blood into a labeled 1.5mL microcentrifuge tube.

(2) Add 200µl of Avian Phosphate Buffered Solution (PBS) to each sample (see below for avian PBS recipe).

(3) Add 20µl Proteinase K to each sample and mix by vortexing for 5-15 seconds.

(4) Add 200µl of Buffer AL and mix by vortexing for 5-15 seconds. Incubate the samples at 50˚C for 10 minutes.

(5) Add 200µl of cold 100% ethanol to each sample and mix by vortexing for 5-15 seconds.

(6) Transfer the mixture from Step 5 into the labeled spin columns and centrifuge at 8000 x gravity for 1 minute. Discard the tubes containing the flow through.

(7) Add 500µl of Buffer AW1 to each spin column and centrifuge at 8000 x gravity for 1 minute. Discard the tube containing the flow through.

(8) Add 500µl of Buffer AW2 to each spin column and centrifuge at 14,000 x gravity for 3 minutes. Discard the tube containing the flow through.

(9) Put each spin column in a labeled microcentrifuge tube and discard the tube containing the filtrate. Add 200µl of Buffer AE to the spin column and incubate at room temperature for 1 - 5 minutes. Centrifuge at 8000 x gravity for 1 minute and discard the spin columns. Freeze the extracted DNA samples at -20˚C for future use. Avian blood

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produces the best quality DNA and only 1-3μL of DNA is required per 15μL PCR reaction.