PREVALENCE AND MOLECULAR CHARACTERISATION OF HAEMOGREGARINES IN AFRICAN HINGE-BACK TORTOISES (*KINIXYS BELLIANA* AND *KINIXYS HOMEANA)* IN IBADAN, NIGERIA

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MATRIC No: 117521

A Thesis in the Submitted in Department of Veterinary Public Health and Preventive MedicineSubmitted to the Faculty of Veterinary Medicine

In partial fulfilment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

of the

UNIVERSITY OF IBADAN

CERTIFICATION

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LIST OF ABBREVATIONS

SCI	Straight carapace length
SPL	Straight plastron length
PW	Plastron width
BCI	Body condition Index
EDTA	Ethylenediaminetetraacetic acid
WHO	World Health Organization
PCR	Polymerase Chain Reactions
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
WBCs	White blood cells
RBCs	Red blood cells
PCV	The packed cell volume
DIC	Disseminated intravascular coagulation
BUN	Blood urea nitrogen
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ALP	Alkaline phosphatase
AHT	African hinge-back tortoise
HDL	High-density lipoprotein
TP	Total protein
ALB	Albumin
GLB	Globulin
IBD	Inflammation of the bowel
NCBI	National Center for Biotechnology Information
IITA	International Institute for Tropical Agriculture
MCL	Maximum Composite Likelihood
ICSH	International Council for Standardisation in Haematology
CLSI	Clinical and Laboratory Standard Institute

DDH_2O double distil water ddH2O

ABSTRACT

*Kinixys*tortoises are one of the most vulnerable of all vertebrates, with about 61% reportedly threatened or becoming extinct. They represent a global biodiversity and therefore require urgent conservation attention.*Kinixys* tortoises are also sentinels of ecosystem degradation. They are an endangered speciesdue to human over-exploitation and infectious diseases including those caused by haemogregarines. Information on the prevalence, health indices, biodiversity and host-specificity of haemogregarines in African Hinge-back Tortoises (AHT) in Nigeria is scarce.This study was therefore designed to determine the prevalence of and characterise haemogregarines in AHT (*Kinixys belliana* and *Kinixyshomeana*) in Ibadan, Nigeria.

One hundred and twenty AHT (*K. belliana, n*=70;*K. homeana,* n=50)captured from the wild were sourced from Bode Market in Ibadan, during the rainy (*K. belliana, n*=36;*K. homeana,* n=24) and dry (*K. belliana, n*=34;*K. homeana,* n=26) seasons between April, 2016 and December, 2017. Blood samples were collected to determine the prevalence of haemogregarines by light microscopy and confirmed using Polymerase Chain Reaction (PCR)with 18S RNA-specific primers. Purified amplicons were sequenced bi-directionally using a genetic analyser. Sequences obtained were aligned and compared with those in the GenBank. Phylogenetic analyses of the sequenced genes were performed using software. Haematology, plasma proteins and enzyme activities were evaluated in haemogregarine-positive and negative tortoises. The tortoises were examined for the presenceand quantity of vectors using standard morphological keys. Data were analysed using descriptive statistics, Student's t-test and correlation coefficient test at $\alpha_{0.05}$.

Overall, haemogregarine prevalence was 53.3% and 75.8% by light microcopy and PCR, respectively. Generally, higher prevalence of haemogregarine (82.9%) was recorded in *K. belliana* compared to *K. homeana* (66.0%). Seasonal prevalence in *K. homeana* was significantly higher during rainy (91.7%) than dry season (42.3%). However, higher prevalence
was recorded during dry (85.3%) than rainy (80.6%) seasons in *K.belliana*. Sequences (590 bp
)
generated had 100% similarity with *Hepatozoon fitzsimonsi ex zobensis* (KR069084) isolated from South African hinge back tortoise (*K. zobensis*). There was a significant difference between the counts of white blood cells in haemogregarine-positive tortoises ($7.26\pm0.99 10^9$/L) than haemogregarine-negative ($5.58\pm1.18\ 10^9$/L) as well as for eosinophils in haemogregarine-positive (40.41±2.22%) than haemogregarine-negative (29.79±3.76%). Haemogregarine-positive recorded lower values of haematocrits (22.75±2.56%), total protein (3.97±0.87 g/dL), Albumin (1.26±0.29 mg/dL) and globulin (2.71±0.58 mg/dL), when compared with 32.79±2.68%, 5.33±0.93g/dL, 1.67±0.51mg/dL and 3.66±0.42mg/dL, respectively for haemogregarine-negative. However, haemogregarine-positive had higher values for ALT (33.91±14.42U/L), ALP (179.27±92.52 U/L) than 8.21±2.21 U/L and 147.93±10.51 U/L, respectively in haemogregarine-negative. Ticks of the genus *Amblyomma* were the only vectors found on thehaemogregarine-positive tortoises. There was a moderate positive correlation (r = 0.3758) between tick infestation and parasitaemia in the haemogregarine-positive tortoises.

A high prevalence of haemogregarines in African hinge-back tortoisesin Ibadan was established, with *Amblyomma*ticks as possible vectors. The identifiedhaemogregarine, *Hepatozoonfitzsimonsi* was closely related to that of South African origin. Routine screening of *Kinixys* tortoises for haemogregarines and ticks vectors is therefore recommended to promote their conservation.

Keywords: *Haemogregarines,* African hinge-back tortoises, *Amblyomma*
Word count:492

DEDICATION

This thesis is dedicated to the Almighty God, the maker of Heaven and Earth for His immeasurable grace upon my life, if I have ten thousands of tongues that will not be enough to say thank you. I appreciate my family; my husband; Taiwo Adetunji, my children; Victor Adewale, Gideon Adebare and Deborah Adesewa. It is because of you all that I have persevered; your sacrifices, unconditional love and support have kept me going.

My sincere appreciation goes to my Parents; my late father; Pa James Adohueje ONONO, who passed on the early hours of December 25, 2018. "*Adami*" meaning "my father" you were my first teacher. I thank God for using you to set the pace for me to acquire knowledge and my lovely and dutiful mother, Mrs Rebecca Asimitu JAMES "*Avo Onyani*" meaning" thank you mother" for being there always.

I also appreciate my sister by adoption; Late Mrs Adenike Olubunmi AKINOLA, day after day, the thought of your demise is still a dream, but I am glad you are resting with your maker.

ACKNOWLEDGEMENTS

I thank the Almighty God, who is the lifter of my head and my helper of all times. His name is glorified forever.

I thank my supervisors Professor Olanike K. Adeyemo and Professor J. O. Adejinmi for allowing me to prove myself. They have been so patient with me and have demonstrated love and encouragement that I can. Some very valuable life lessons have been learnt during this study.

I thank Tetfund for financing my trip to India for the laboratory training which helped in the completion of this work. I would also like to thank Professor Latha Murali of Madras Veterinary College TamilNadu Chennai, India I want to say "thank you" in Tamil (Nandri nandri) for inviting me to the laboratory in 2015, where the idea of this project work was conceived.

My sincere appreciation to all my teachers in the Faculty of Veterinary Medicine, both at undergraduate and postgraduate. I remain eternally grateful for all that you have impacted on me. I equally appreciate and thank all other academic and non-academic staff in the Faculty of Veterinary Medicine. I want to specially thank Drs I. O. Olatoye, S.G. Olukole and H. K. Adesokan for your assistance, may God reward you all real good.

I specially thank my spiritual father, Pastor Austin Ologbese of the Breakforth Church, Bodija, Ibadan, for prayers and most importantly for your encouragement through the words of God that you preached. May God reward your labour of love.

I thank all members of staff of the Department of Veterinary Public Health and Preventive Medicine, for their immense supports and prayers. I specially thank the following People; Messers B. O. Ojomo, C. O. Omotayo, F. Abiodun, O. Okunlade, Mr Omoh and his son Godwin and Mrs Beatrice B. Kolawole for their assistance in various capacities.

I thank Dr. A. Odeseye of the Nigerian Institute of Science and Laboratory Technology, Mrs Iyabo Adetiba of the Department of Veterinary Medicine, there's no way I could have completed this work without the help of these laboratory staff.

I am grateful to my family and friends; Mr and Mrs Tunde and Mobolaji Fajimi. These people have been a great support system and have always been there for me. I appreciate the love and supports of my family by adoption, OlaOluwa, IfeOluwa and AyoOluwa Akinola; these trio have become my beloved children in whom I am well pleased.

I thank my Siblings; Mr Simeon Itopa James and Peace Ovayoza. My mother had on several occasions spent months with her grandchildren so I could complete this project. Most of all, I want to thank my husband. He supported me, reassured me, and have been the best friend, I could have ever had. Thank you so much for making sacrifices for this degree than anyone and I will always love and appreciate you for all that you stand for.

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CHAPTER ONE

INTRODUCTION

1.1 Background

Nigeria is endowed with diverse species of wildlife; ranging from reptiles, amphibians, avian, mammals and other aquatic animals. However, the rate at which the populations of these wild animals are declining is worrisome. Some of them are endangered and others are near extinction and if deliberate efforts are not tailored toward conservation, many of them will soon become extinct (Ogutu, Piepho, Said and Kifugo, 2014;Western, Waithaka and Kamanga, 2015).

There are about 14 living families and 356 species of chelonians, 61% of which are reported threatened or have disappeared, (Lovich, Ennen, Agha, and Gibbons 2018). Chelonians are most vulnerable of all vertebrates because most conservationists do not consider turtle diversity as a global focus, hence efforts at conserving them is not prioritised as we have in other species, (Roll, Feldman and Novosolov, 2017).

Most developed nations have embraced wildlife conservation as a panacea to addressing wildlife population decline, this is not currently so in some developing countries like Nigeria. Causes of wildlife population decline range from, natural disasters such as flooding, earthquake and human activities in agriculture such as; bush burning, indiscriminate hunting by poachers, and parasitic agents of diseases; such as bacteria, viruses, fungi and parasitic organisms (Ogutu, Piepho, Said and Kifugo2014).

Parasites play key roles in ecosystem functioning and structuring and are widely distributed. They can impede the growth, reproduction and have negative effects on biochemical and haematological parameters of their hosts and are capable of altering host's genetic traits and fitness thus affecting the hosts survival, (Watson, 2013;Knapp, Perez-Heydrich, Zachariah, Schnelle, Buckner, Lattin and Romero, 2019). They inhabit practically all living things; human beings, animals and arthropods, (Snorre, 2020). Studies on the prevalence of disease agents like parasites provide inception to understand the impact they have on population (Pedersen and Fenton 2015). Blood parasites affect

many species of animals both wild and domestic animals; ranging from mammalians, avian and reptiles.

The reptiles are an important group of wild animals because of their distinct physiology and anatomy. They are ectothermic in nature, their core body temperaturedepends on the environmental temperature, and the understanding of the pathogenesis of their diseases requires stringent approach. Chelonians are reptiles and represent a global biodiversity; their conservation is therefore, a necessity. They are important to humans, as food, for medicinal purposes, pets or ecological services as sentinels of ecosystem degradation and over-exploitation. Chelonians exhibit slow recovery from human exploitation leading to a sharp reduction in their population and are at the cutting edge of biodiversity decline, (Mittermeier, van Dijk, Rhodin, and Nash, 2015; Murphy, 2016). In addition, reptile families are common wild animals in the zoological gardens and therefore, require adequate protection and conservation to prevent them from extinction. The chelonians represent one of the most unique groups of animal worldwide (Lovich, *et al*. 2018). They belong to the oldest surviving reptile lineage. They are said to have been in existence for 220 million years and have witnessed the extinction of the dinosaurs (Anquetin, 2012). They are believed to have lived longest among the vertebrates, some species of tortoises are described to have lived up to two centuries. However, in captivity, inadequate care may likely cause their mortality than old age (Spencer,van Dyke, Petrov, Ferronato, McDougall, Austin, Keitel and Georges, 2018).

Haemogregarines are blood parasites found parasitising many vertebrates and blood sucking-arthropods. They belong to the Apicomplexan phylum, (Adl, Simpson, Lane, Lukes, Bass, Bowser, Brown, Burki, Dunthorn. and Hampl. 2012). They have an indirect life cycle with gamonts intra-erythrocytic, while sporogony occurs in invertebrate hosts (Barta, Ogedengbe, Martin and Smith, 2012). They are important parasites of cold-blooded vertebrates such as the tortoises, snakes, fishes and frogs.

Haemogregarines belong to these families; *Haemogregarinidae*, *Hepatozoidae* and *Karyolysidae*. The haemoparasites have been classified into six genera based on their asexual differentiation during sporogony into sporozoites in the intermediate invertebrate hosts, (Barta, *et al*. 2012). Four genera are reported to parasitise reptiles. They include; *Hepatozoon*, *Haemogregarina*,*Karyolysus*, and *Hemolivia.Hepatozoon* has been reported

to be highly spread among reptiles and other tetrapod groups, (Maia, Perera and Harris, (2012). Heamogregarines are parasites of veterinary importance. The study of wild animals' diseases has advanced in the last twenty to thirty years. This is due to the roles of parasites in host evolution, ecology and population biology which has become a major concern especially to conservationist. There is a paucity of information on host, parasite and environmental interaction with regards to wild animal diseases; this calls for further research, (Molla *et al*., 2015). Haemogregarines are said to have parasitised important groups of animals such as the dogs apart from reptiles (Hossen, Bandyopadhyay and Gurelli. 2013).

The parasite has not been confirmed to be of zoonotic importance.There is also the paucity of information on studies of haemogregarines infections in man. Besides, most researches on parasites are focused on domestic animals, leaving those affecting wild animals to be poorly researched. Hence scanty information is available on some parasites of wild and zoo animals such as haemogregarines that infect reptiles. However, with the recent increasing emergence and re-emergence of zoonotic diseases traceable to wildlife population, more attention must be focused on parasites of wild animals to bridge the gaps in knowledge.

1.2 Statement of the problem

Most *Kinixys* tortoises and several species of tortoises are at the verge of extinction, due to infectious diseases, natural disasters and human over-exploitation. Chelonians which includes tortoises, turtles and terrapins serve as sentinels of ecosystem degradations and their presence and existence serve as means of assessing the ecosystem health, Murphy, (2016)

Haemogregrarines have long been recognised as the most common haemo-parasites affecting chelonians and other reptiles. Several countries of the world have researched and reported the prevalence of haemogregarines of chelonians, with the aim of providing information on the prevalence, health indices, biodiversity and host-specificity of haemogregarines in chelonians in order to promulgate policies aimed at promoting the existence of chelonians as well as preventing their extinction, (Maia *et al*., 2012; Molla *et al*., 2015).

African hinge-back tortoises (AHT) (*K. belliana* and *K. homeana*) are commonanimal species in zoological gardensin Nigeria; next to squamata (snakes) and are common pets owned by both the rich especially traditional rulers and the lower-income owners in Nigeria. Besides, pet tortoises are as of today being regularly presented in Veterinary clinics for various health interventions in Nigeria. However, the prevalence of haemogregarines,its vectors, predisposing factors and clinical implications are largely unknownin AHT (*K. belliana* and *K. homeana*) in Ibadan, Nigeria. The study of haemogregarine in AHT is therefore very important to understand its prevalence, biodiversity, host specificity, health implications and possible ways of prevention and control, (Molla *et al*., 2015).

1.3 Justification for the study

Kinixys tortoises are one of the most vulnerable of all vertebrates, with about 61% reportedly threatened. They are at the verge of extinction due habitat loss, over-exploitations and infectious diseases including those caused by haemogregarines, human overexploitation, habitat destruction and loss.They also serve as the sentinel of ecosystem degradation and therefore, require urgent conservation attention, (Freeman, Kleypas and Miller2013).

Research on wildlife parasites is very germane because wildlife provides a nexus for which zoonotic and unknown pathogens can evolve, (Brooks, Hoberg, Boeger, Gardner, Gal-breath, Herczeg, Hejia-Madrid, Racs and Dursahinhan, 2014). This is particularly important at wildlife and domestic animal interface and this has become of public health importance.

Haemogregarines are parasites affecting reptiles especially the *Kinixys* tortoises and are capable of destroying affected RBCs, resulting in low haematocrit, RBCs counts, haemoglobin concentration and consequently low oxygen-carrying capacity in affected host, (Damas, Harris, Rosado, Tavares, Maia, Salvi and Perera, 2014). Prevalence of haemogregarines in chelonians of several countries of the world been researched and reported, to be widely spread byAbdel-Baki, Abdel-Haleem, Al-Quraishy1 and Zhang, (2014); Molla, Brandyopadhyay and Gurelli (2015). Information on the prevalence,

biodiversity and host-specificity ofhaemogregarines of*Kinixys tortoises* in Nigeria is scarce because of overt focus on parasites of domestic animals.

The chelonians (tortoise, turtle and terrapins) pose a major challenge to veterinarians in terms of husbandry and health management. This is because medical interventions in chelonians requires patience, time consuming and very often recovering period is very slow when compared to other vertebrates, (Hedley, 2014).In addition, reptiles undergo strong seasonal change in behavior, physiology, which directly influence their physiological activities, thus making their health management a difficult task, because their physiological responses are highly variable and change with age, sex, nutritional and environmental conditions, (Vitt, 2016). This study will no doubt contribute to the knowledge of their husbandry and health management.

1.4 Research Questions

1. Are haemogregarines present in *Kinixys belliana* and *Kinixys homeana* in Ibadan?
2. What are the prevalence of haemogregarine in *Kinixys belliana* and *Kinixys homeana* in Ibadan?
3. What are the species of haemogregarines infecting *Kinixys belliana* and *Kinixys homeana* in Ibadan?
4. What are thehaematology, plasma biochemistry and enzymes activities of haemogregarine positive and negative *Kinixys belliana* and *Kinixys homeana* in Ibadan?
5. Do *Kinixys belliana* and *Kinixys homeana* harbour any ectoparasites? what are the prevalence and possible role of ectoparasites in haemogregarine infection?

1.5 General and Specific Objectives

1.5.1 General Objective

To identify and characterise haemogregarine parasite in African hinge-back tortoises (*Kinixys belliana* and *Kinixys homeana*) in Ibadan, Oyo State, Nigeria

1.5.2 Specific Objectives

1. To assess the presence and prevalence of haemogregarines in *K.belliana* and *K. homeana* in Ibadan
2. To determine the prevalence of haemogregarines by microscopy and polymerase chain reaction in *K. belliana* and *K. homeana* in Ibadan
3. To characterise haemogregarines from *K. belliana* and *K. homeana* in Ibadan
4. To determine the haematological, plasma biochemistry andenzymes activities of haemogregarine positive and negative*K.belliana* and *K. homeana* in Ibadan
5. To determine the occurrence of ectoparasites associated with haemogregarine transmission in *K. belliana* and *K. homeana* in Ibadan

CHAPTER TWO

LITERATURE REVIEW

2.1 Chelonians

Tortoises and turtles represent world-wide preservation precedence. They play importance roles in the existence and sustenance of man in terms of protein supply, medicine, pets and provides ecological services as indicator of ecosystem over-exploitation, (Mittermeier *et al*., 2015). There are over 50% of the world chelonians are assumed to be in danger of extinction, (Murphy, 2016).

The order chelonian includes; tortoise, turtles and terrapins and they are in the class reptilian, (Cordero, 2017). They possess bony or cartilaginous shell or coverings, which is an outshoot of their ribs. Chelonians are the only reptiles without teeth and are found worldwide except in the Antarctica and Australia, (Ramakrishna *et al*., 2014). The word chelone was coined out from the modern Latin word "Chelonia" which was coined out of the Greek word 'khelone' which is interlocking shields or armor, (Brennessel, 2006). Chelonians are terrestrial in nature, with heavy shells and limbs without nails. They are cold-blooded ectothermic reptiles, whose body temperature is dependent on the environment and are found worldwide. (Anquetin, 2012;Ramakrishna, Jayashankar, Alexander and Avinash, 2014).

Chelonians are covered with keratinized epidermal tissues or plates referred to as the scutes which give protection to the animal. The dorsal shell or roof is called carapace and ventral shell or floor is called plastron. Both carapace and plastron are attached by a bridge which enables the retraction the head and appendages into and fro the shell, (Plates 2.1), (Cordero, 2017).

Chelonians represent one of the most unique groups of animal worldwide (Lovich, *et al*. 2018). They belong to the oldest surviving reptile lineage. They are said to have been in existence for 220 million years and have witnessed the extinction of the dinosaurs (Anquetin, 2012). They are believed to have live longest among the vertebrates, also

some species of tortoises have been reported to have lived close to two centuries. However, in captivity, inadequate care may likely cause their mortality than old age (Spencer *et al*. 2018).

2.2 The Africa tortoise

Tortoises (Testudinidae) are a group of biological taxa or species of terrestrial turtles that are found in five continents. Africa is reportedly home to 10 genera, (Fritz, Branch, Gehring, Harvey, Kindler, Meyer, Du Preez, Siroky, Vieites and Vences, 2013; Iverson, Le and Ingram, 2013). The dissemination of tortoises to Africa is reported to be poorly understood. Fossils of land tortoises found in Africa by H. J. L. Beadnel was reportedly said to be from large land-tortoise, (Fritz *et al*., 2013). The Africa land tortoise (Geochelone species) is the third largest of all tortoises. They are found in many African countries including Nigeria, (Fritz and Havas, 2007).

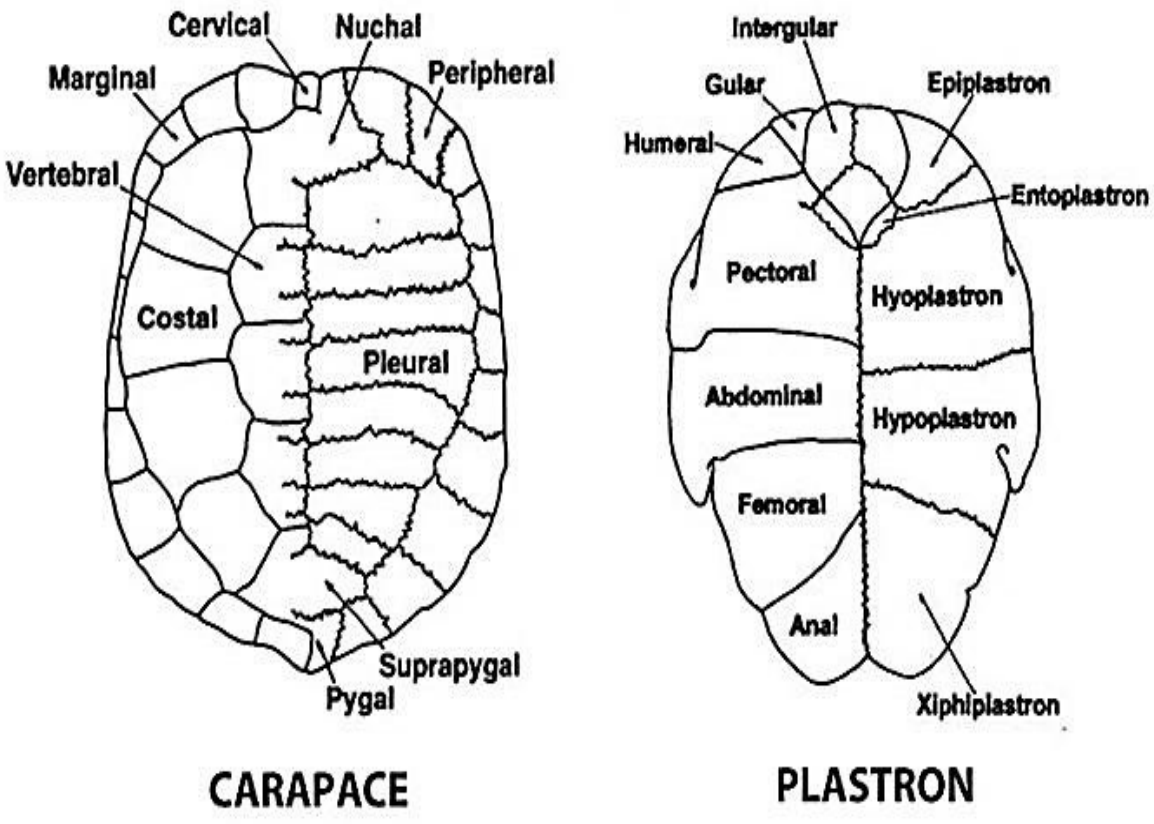


Plate 2.1 dorsal carapace and ventral plastron of the land tortoise
Source: (Schoch and Sues, 2015).

2.3 Importance of tortoises

2. 3.1 Tortoise as pet

The reptiles and amphibians are in great demand in the pet market next to the dog and cat (Thomas and Mark, 2012). The tortoise is believed by most pet's lovers as interesting to keep as pet and in keeping them, owners will learn more about their biology, conservation and welfare. The tortoises have a long lifespan and some tortoises were believed to have outlived their owners and existed for generations after the death of their owners.

Although, the world's oldest tortoise recorded was Adwaita, an Aldabra land tortoise who was said to have existed for 250 years, Adwaita belonged to Major General named Robert Clive who was a former British administrator in Bengal, before he was later transferred to work in the service of British East India Company at Madras now Chennai, India.Adwaita was kept in an Indian zoo for the rest of his life. The tortoise was reported to have died on November 22, 1774, due to liver failure in Calcutta Zoo, (Percival-spear, 2016).

In Nigeria, "Alagba, a male giant land tortoise who was believed to have lived up to 433 years and possibly the oldest land tortoises in the World, recently died after a protracted illness at the palace of Soun of Ogbomoso King in Oyo State on the 3rd of October, 2019. The legendary tortoise was popularly called "Alagba which means the elderly one"

2.3.2 Tortoise in traditional medicine

The ethno traditional medicine practitioners use plants and animals; like turtles and tortoises, for the preparations healing formulations for the treatments of different kinds of diseases and infections. The flesh and fat of tortoise and other reptiles like the lizards, the crocodiles and the snakes, are said to be very useful in ethnomedicine for the cure and the management of different types of pain, tumours and furuncle or boil (Kim and Song, 2013; Setlalekgono, 2013).

The tortoise's shell is reportedly used in Chinese divination. Chinese diviners observe the patterns of cracks that developed when a hot instrument is placed on one of its many "divination points," and theninterpret the spiritual implications of the patterns. The

prognostications and insights learned from the cracks were often written right on the shells.

2.3.3 Tortoise in folk tales

In Nigeria, the tortoise also known as “Ijapa” or “Alabahun” is generally depicted as a trickster, accomplishing heroic deeds of getting in and out of troubles in series of tales told by the Yoruba’s of Nigeria and the Benin Republic of West Africa, (Azeez, 2014).

2.4 Threat to tortoises and Implications

The chelonians are faced with the threat of over-exploitation because, they serves assources of protein are used in traditional medicine and for international trade. Habitats degradation and losses due to human encroachment into their habitats have constituted challenges to their conservation (Spencer *et al*., 2018).Although the turtles, tortoises and terrapins are represented in some zoological institutions and private collections,next to the squama-herpetofauna (lizards and snakes); they are regularly brought to veterinarians for medical intervention for various health conditions and this has remained a major challenge in terms of husbandry and health management.

2.5 Taxonomy

Kingdom: Animalia, Phylum: Chordata, Class: Sauropsida, Order: Testudines, Class: Reptilia, Order: Chelonii, Sub-order: Casichelydia, Infra-order: Cryptodira, Family: Testudinidae, Sub-family: Testudininae, Genera: Geochelone, Kinixys.(Van Dijk, Iverson, Rhodin, Schaffer and Bour, 2014). Species: *G sulcata*, *G. pardalis*, *K. belliana*, *K.homeana*, *K. erosa*, (Van Dijk *et al*., 2014).

2.5.1 Tortoises

The word tortoise is used to describe the terrestrial chelonians, (Lovich, Ennen, Agha and Gibbons, 2018). They are members of the family Testunidae; they possess heavy, high-domed shells and heavy scales on their elephant-like limbs with no movable toes. They are predominantly land dwellers. Most land tortoises are found in the tropics, sub-tropics and temperate zones of the world. Chelonians exhibit sexually dimorphism; males have larger and longer tail, a more distal vent than females; plastron of the males is concave,

while that of the female is flat. Males have a single penis protruding from the inside surface of the cloaca and two testes very close the kidney. Females are all oviparous, are larger than males or have wider shells than males except for Gopher Galapagos, (Schoch and Sues, 2015).

2.5.2 Turtles and terrapins

The word 'turtle' connotes different interpretations from place to place; In North America, chelonians are generally referred to as turtle, while in Britain and in this study, the turtle is used to denote aquatic chelonians while the terrestrial species are referred to as tortoises. They rarely come to land except to lay eggs. The turtles have streamlined bodies with webbed feet and the sea turtle possess long slippers. The turtles are omnivores. The terrapins are small species of hard-shelled turtles that are edible and are found mostly in salty waters (Lovich *et al*., 2018). The basic differences of the tortoises and turtles are shown in table 2.1 below.

Table 2.1 Basic differences between tortoises and turtles

Criteria	Tortoises	Turtles
Habitat	Mostly terrestrial	Mostly aquatic
Density and shell shape	Shells are heavy, large, dome-shaped some species with bumps	Shells are lighter, mostly flat and streamlined.
Feet	Feet are short, sturdy and covered with scales Without defined or movable toes.	Feet are webbed, with long claws
Feeds	Mostly herbivores	Mostly omnivores
Birth	Hatchlings migrate from their nest to the mothers to burrow soon after birth. Mothers provide protection for about 80 days	Hatchlings remain in their own nest for 90-120 days. Without the mother's protection
Life span	They live up to 100-250 years.	Their life span is about 20-40 years

Sources: Luiselli and Diagne (2013); Lovich *et al*., (2018)

Genera of the Testudine families

2.5.3 Geochelone

The genus Geochelone are found mostly in Africa and Asia continents and are mainly vegetarians. Storrs and Norman (2014) used the word "Chelonoidis" to describe a sub-genus of *Geochelone* from South America.

2.5.3.1 Galapagos tortoises (*Geochelone elephantopus*)

The Galapagos tortoises (*G.elelphantopus*) are perhaps the largest of all tortoises. They are widely known and they originated from the Islands of Galapagos and are mainly herbivores, (Blake,Guezou,Deem,Yackulic and Cabrera, 2015).The evolution of the Galapagos tortoises is not fully understood till date. About 15 species and subspecies have been described, although there are no clear evidences or concurrence on their classification. (Blake *et al*. 2015;Lovich, *et al*. 2018). Land tortoises are no good swimmers but are bouyant in water and can survive without food for months, this may explain the reason for their dispersion over a long distance to some Islands they inhabit today, Blake *et al*. (2015). The sea turtles and tortoises have a unique physiological adaptation to thrive for a long period of time, sometimes years without food or water. Galapagos tortoises have adapted to their native dry volcanic island habitat by storing water within their necks. Galapagos tortoises were subjected to the same over-exploitation and subsequent population losses during the Gold Rush, (Anquetin, 2012).

2.5.3.2 *Geochelone gigantea*

The Aldabra tortoises are giant tortoises found in Aldabra Islands as the name implies, the islands are located within the coast of Kenya and north of Madagascar. They are biggest land tortoises next to the Galapagos. They have a long neck and a uniformly dark grey or black carapace. The length of the carapace is about 90-120cm, while the weight can be up to 150kg-250kg. The Aldabra tortoises are characterized by a large dome-shaped shell, (Wilfredo, Richard, Samuel, Hatt, Schaepman-Strub, Arpat, Clauss and Dennis, 2018).

2.5.3.3 *Geochelone sulcata*

African spur tortoise (*Geochelone sulcata*) is believed to be the third biggest land tortoise next to Galapagos and Aldabra giant tortoises. It weighs about 84kg, (Anquetin, 2012; Blake *et al*. 2015). *Geochelone sulcata* morphologically has yellowish brown carapace with prominent annular rings on the scutes that are used in age estimation. There are two sometimes three large conical spurs on the hind limbs whose functions are not known. They have serrated anterior and posterior margins. The plastron colour is ivory and the anal scutes divided, (Rhodin, Inverson, Roger, Fritz, Georges, Shaffer. andvan Dijk, 2017).

They are found mostly in the southern border of Sahara Desert and Sahel. (Trape, Chirio and Trape, 2012; Petrozzi, Hema, Luiselli and Guenda, 2016).

2.5.3.4 *Geochelone pardalis*

The leopard tortoise (*Geochelone pardalis*)was formerly known as (*Stigmochelys pardalis*), it is one of the biggest land tortoise species found in sub-Saharan African second to *G sulcata*. This is fourth biggest tortoise next to African spur tortoise, Galapagos and Aldabra giant tortoise, (Hofmeyr, Boycott and Baard, 2014; Drabik-Hanshare and Downs, 2017). The former generic name *Stigmochely pardalis* was coined from two Greek words “Stigma and Chelone which means "mark" and tortoise respectively. The word “pardalis” was formed coined from Latin word “Pardus” which means “leopard” to describe the brown-black spots on the scutes which has a leopard-like appearance. Their carapace is pyramid-shaped with well-defined scutes that are cream and black in colour. The adult can grow up to 46cm in length and weigh18kg, (Anquetin, 2012). Larger species can grow up to 70cm or 85 cm straight shell length weigh up to 54kg, while the smaller species have a shell length of about 30 to 40 cm. (Fritz, Daniels, Hofmeyr, Gonzalez, Barrio-Amoros, Siroky and Stuckas, 2010). The leopard tortoises are found mostly in Africa savannas, Sudan to Southern Cape, Namibia and Ethiopia, (Baker, Kabigumila, Leuteritz, Hofmeyr and Ngwava, 2015).

2.5.4 Genus *Testudo*

Testudo is a word used to describe the tortoise in Latin; however, it has now been used to refer to a genus of land tortoise found around the Mediterranean regions of the North Africa, Middle East, central parts of Asia and south-western Europe. The Testudos are relatively small size tortoises and the species have been placed in this genus, however only two are found in Africa; these are *Testudo kleinmanni* also referred to as Egyptian tortoise and *Testudo graeca* also called Spur-thigh tortoise or Greek tortoise, (Highfield and Martin, 2014; Gerald, Briston, Valetin, Kostopoulos.Merceron and de Bonis. 2020). Other species of Testudo tortoises are

(i)*Testudo horsfieldii*, (ii) *Testudohermanni*, (iii) *Testudowerner*,

(vi)*Testudomarinate*

2.5.4.1 *Testudo klenmani*

The Egyptian tortoises are relatively small desert-living tortoise the longitudinal length ranges from 7cm to 35cm and they weigh between 0.7 kg to 7.0 kg. They are characterized by a high-domed golden-coloured shell. They are found in coastal areas, woodlands, arid lands and in rocky habitats. They are also found in Tripolitania and Cyrenaica regions of Libya (Rhodin *et al*., 2017). The Egyptian tortoise is classified as critically endangered.

2.5.4.2 *Testudo graeca*

Mediterranean spur-thigh tortoise *Testudo graeca* was discovered and named by Linnaeus in 1758. They are the most attractive species of tortoises found in the Mediterranean fauna, they are also found to inhabit North Africa, southern Spain and some parts of southwest Asia, (Perez, Tenza, Anadon, Martinez-Fernandez, Pedreno, and Gimenez, 2012). They have domed shaped carapace which is yellow to dark brown in colour. The male carapace and plastron are longer and wider than the female, (Rezazadeh, Alucheh and Kami, 2014). The head is blunt with symmetrical markings, and large eyes, the forelimbs have powerful thick claws with large scales. They may grow to between 7 to 11 inches and weighing as much as 5 pounds. They have a life span of 75-100 years. Several subspecies are recognised. These include:

(i) *Testudo g, ibera Ibera* Greek tortoise, (ii) *Testudo g. cyrenaica* (Libyan Greek tortoise), (iii) *Testudo g. graeca* (North African Greek tortoise), (iv) *Testudo g. subspecies* (Golden Greek tortoise), (v) *Testudo g. nabulensis* (Tunisian Greek tortoise)

2.5.5 Genus *Kinixys*

The genus *Kinixys* are unique among the chelonians, they are characterized by hinge across their posterior carapacial scutes which are serrated. The marginal scutes are pointed while the anterior scutes are upturned. There are two major species identified; *K. belliana* and *K erosa*. *Kinixys* are native to western and central Africa, (Trape, *et al*., 2012). They are omnivores; feed on edible leaves, grasses, fruits and invertebrates such as worms, insects and other smaller invertebrates. They are found majorly in forest areas, lowlands and along the river banks, the species are abundant in the Niger Delta forests and Cross rivers National park, these species are however being threatened by habitat loss due to uncontrollable activities of petrochemical industries, (Luiselli and Diagne, 2013).

2.5.5.1 Home hinge-back tortoise (*Kinixys homeana*)

The *K. homeana* belong to the *Kinixys species*. They possess a hinge on the posterior carapace that is capable of covering the entire hind limbs and the tail. They also possess nuchal scutes which is absent in *K erosa*. *K homeana* is an obligate inhabitant of the rainforest, and are found in countries like Liberia where it is said to occur in every part of the country. In addition, *K. homeana* is found in Benin Republic, Cameroon, Nigeria and several Africa countries, (Trape *et al*., 2012, Luiselli and Diagne, 2013). The African home hinge-back tortoises *Kinixys homeana* is of medium size of about 220 mm in length. The shell is typically dark with shades of brown, red, and yellow, the centres of the scutes are lighter. The serrated carapace is flattened dorsally and angled sharply downwards in the back, giving a squared appearance from the side. The plastron is yellowish in colour and has black spots on the centre of the scutes, notched in the front and projects slightly beyond the carapace. The hinge is well developed, between 7th and 8th marginal plastrons, (Luiselli and Diagne, 2013).

2.5.5.2 The African bell hinge-back *Kinixys belliana*

The bell hinge-back *Kinixys belliana* is about 230mm in length. Its carapace has convex scutes, which gives the species a characteristic domed shaped which is in contrast to the square shape appearance of *K. homeana* and *K. erosa*. The colour ranges from beige to brown with surrounding darker circles and seams that are cream or whitish. The plastron can be light or dark in colour. Colouration and patterns can vary greatly between individuals, the hinge is well developed between 7th and 8th marginal carapace, (Luiselli and Diagne, 2013). This species is common in countries such as Angola, Cameroon, Nigeria, Sudan, Ruwanda and several other Africa countries,(Luiselli and Diagne, 2013).

2.5.5.3 *Kinixys erosa* (Schweigger, 1812)

K. erosa is the largest of the *Kinixys species*. It has pointed and sharp marginal scutes with serrated shells. The male is usually larger in size than the female. It is reported to be well distributed across the globe. In Nigeria, the genus is found in several states of the country like Lagos, Oyo, Edo, Delta most states in eastern Nigeria. The tortoise is said to be abundant in hilly forests and mountains. (Luiselli and Dagne, 2014).

Table 2.2 Kinixys tortoises and their habitats

	Species	Habitation	Description
1.	***Kinixys erosa*** **Forest hinge-back tortoise)**	Tropical forests and marshes of central and West Africa Found in marshes and river banks. They are excellent swimmer and are found under logs and roots.	They can arch their backs at 90 degrees to protect their tall and hind legs from predators while asleep.
2.	***Kinixys homeana*** **Homes hinge-back tortoise Named after English Surgeon. Everald Home**	Sub-tropical wet lowland forests, swamps and in plantations, in west and central Africa	They have preference for red and pink flowers, make movement at dawn or dusk
3.	***Kinixys natalensis*** **Natal hinge-back tortoise**	Restricted to East and Southern Africa. Occur mainly in Kwazulu-Natal Province. Inhabit rocky	They are small and have long carapace of about 15.5cm in length which is slightly domed with a flat dorsal surface. Has brown to yellow head.
4.	***Kinixy belliana*** **Bell's hinge tortoise was named after English zoologist Thomas Bell. It has several sub species.**	Found in tropical and sub-tropical savannah in sub-Saharan-Africa. Also in Sudan, Tanzania, DR Congo and South Africa.	They are medium sized tortoise. up to 22 cm. they have 90-degree hinge on the posterior end, which when closed protect their hind limbs and tail from predators. The scutes are slightly domed and elongated.
5.	***Kinixys lobatsiana*** **Lobatse hinge-back tortoise**	They inhabit Southern African countries like, Botswana and are found in acacia and Combretum woodlands.	They have an elongated and rather narrow carapace up to 16.7 cm
6.	***Kinixys spekii*** **Speke's hinge-back tortoise was named after English explore John Hanning Speke**	They are found in East Africa; Kenya and inhabit savannahs and dry bush rocky areas.	They have elongated and flatten carapace up to 20cm in length carapace has a weak, disrupted medial keel and posterior marginal that are neither strongly serrated nor reverted. They have special preference for millipedes.

Sources: Luiselli and Dagne, (2014)

2.6 Feeding of the chelonians

Chelonians generally exhibit bimodal feeding habits, ability to eat on land and in aquatic environment. The terrestrial species are predominantly omnivores, while few thrive only on fruits and vegetables. Tortoises are endowed with the ability to use lingual prehension for feed uptake, (Heiss, Natchev, Schwaha, Salaberger, Lemell, Beisser and Weisgram, 2011). Besides, some terrestrial chelonians are said to be capable of completing the feeding process on land and underwater. The tongue has been reported to be the major organ of feed prehension in terrestrial species, (Natchev, Tzankov, Werneburg and Heiss, 2015)

2.7 Life cycle of chelonians

Reproductions in chelonians like other oviparous vertebrates occur after mating between the male and the female. Soon after oviposition, the embryo grows towards the dorsum of the egg with the yolk sac lying ventrally, (Cordero, 2017). Hatching occurs when the fully grown embryo uses the tooth caruncle to pip the egg, the juveniles soon migrate to live independently, (Noel, Qualls and Ennen, 2012;Cordero, 2017). Incubation periods in the different species of chelonians vary, but it has been estimated to an average of 50 days, (Cordero, 2017).

2.8 Parasites of land tortoise

Parasites inhabit practically all living organisms including, man, wide range of animals (domestic and wild) and arthropods, (Snorre, 2020). The study of parasitic infections or infestations in any animal species will facilitate the understanding of the effect of the parasitic agent on any natural host, (Pedersen and Fenton 2015). Blood parasites affect many species of animals both wild and domestic animals; ranging from mammalians, avian and reptiles. The reptiles are specifically an important group of wild animals, found in the zoological gardens, acquired as pets by individuals and are frequently being presented on regular basis for various medical intervention, they, therefore, require adequate health care and protection to conserve and prevent their extinction, (Anquetin, 2012).

2.8.1 Haemoparasites of chelonians

Heamoparasites of reptiles can be categorised into two groups depending on site of infection, intra-erythrocytic or extra-erythrocytic; extra-erythrocytic parasites include *Trypanosoma*, *Leptomonas*, and *Leishmania*s, while the intra-erythrocytic haemoparasites include *Karyolysus*, *Hepatozoon* and *Haemogregarina* species. Because haemogregarines are morphologically undifferentiated in blood smear, the term haemogregarine is commonly used to describe their presence in the blood of affected host. *Hepatozoons* are common haemoparasites of land and water snakes; their sporozoites are often transmitted by infected arthropods and leeches, (Karadjian, Chavatte and Landau, 2015).

Haemoparasites in reptiles, especially chelonians are discovered during a clinical screening and are mostly apicomplexan. The genera that have been reported to commonly affect chelonians are *Haemogregarina* and *Hepatozoon*. Their life cycle has tissue schizonts and can lead to tissue reactions, also, severe parasitaemia can consequently lead to anaemia, and loss of condition and host morbidity, (Harris, Damas-Moreira, Maia. and Perera, 2014). Mihalca, Racka, Gherman and Ionescu (2008), reported 100% prevalence with *Haemogregarina spp*. in all eight European pond turtles (*Emys orbicularis*) examined in Romania. They reported intensity of infection to have varied from 0.08 and 2.20%, and this was higher in juveniles than adults

2.8.2 Haemogregarines

Haemogregarines are apicomplexan haemoparasites found in many vertebrates and blood-sucking invertebrates, (Van As, Davies and Smit, 2013). They belong to a group of intra-erythrocytic protozoan which parasitises cold-blooded lower vertebrates (reptiles and fishes) as an intermediate host, while leeches, ticks and other ectoparasites serve as definitive hosts, (Van As *et al*., 2013; O'Donoghue, 2017).

Apicomplexan group of pathogens are of veterinary importance. The *Eimeria species* causes a variety of intestinal diseases in poultry and cattle; also the genus *Plasmodium* parasites cause malaria and are of major public health significance, (O'Donoghue, 2017). Haemogregarinesbelong to the order Eucoccidiorida. Some members of the apicomplexan are microscopic, single-celled and sporulated parasites and can infect man, domestic and wild animals. The example includes; *Toxoplasma gondii and Isospora belli*, O'Donoghue, (2017). All apicomplexans are parasitic, a wide range of parasitic protists of medical and veterinary importance belong to the phylum apicomplexan.

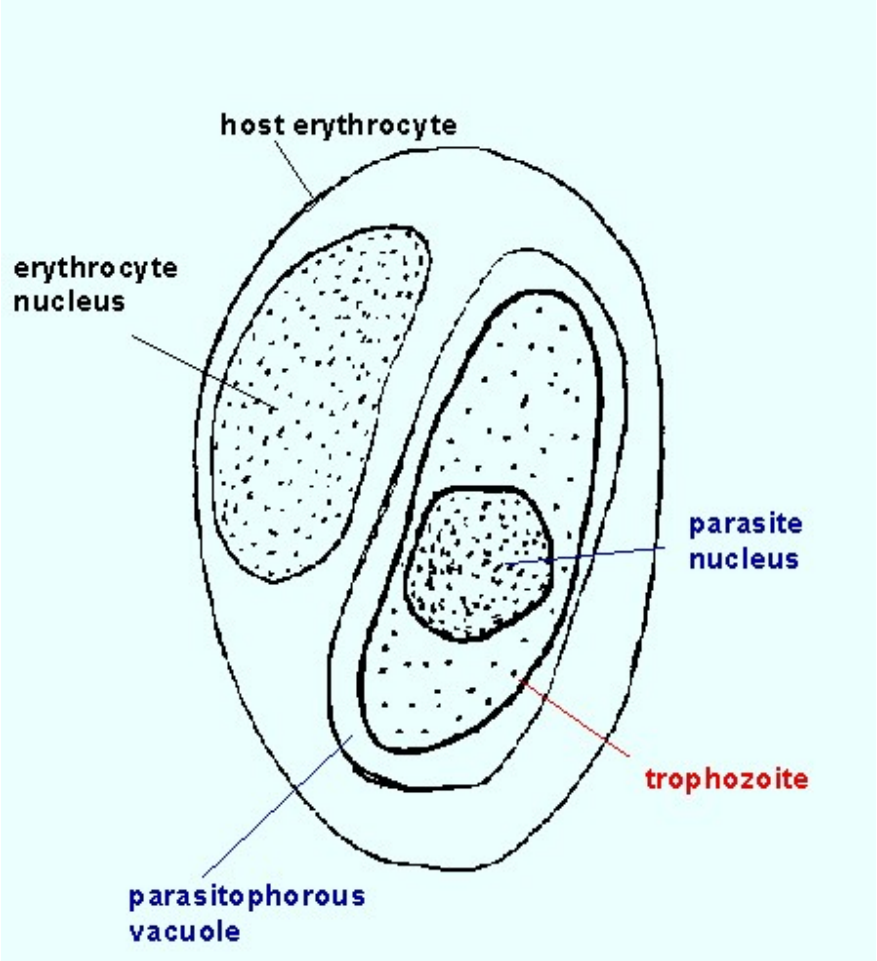


Figure 2.1 Haemogregarines in frog erythrocyte (Redrawn after Baker 1969)

2.8.3 Classification of haemogregarines

Haemogregarines are classified based on their lifecycle within their invertebrate hosts, although some studies have argued that these criteria for classification may not depict the phylogeny of the parasite, (Barta, Ogedengbe, Martin and Smith, 2012).

Haemogregarines belong to the phylum Apicomplexan; a unicellular, spore-forming obligate parasite of all species of animals except Nephromyces; an asymbiotic marine parasite (Paight, Slamovits, Saffo, and Lane, 2019). The sporozoite is the infective stage of the parasites and it possesses three distinct organelles distinguishable only by electron microscopy and is commonly referred to as the "apical complex" from which the parasites derive its name; Apicomplexan, (Shen and Sibley, 2012). Levine completed a comprehensive survey on the phylum and identified 4516 species of the parasites, out of which 339 genera have been named, (Adl *et al*., 2012).

The phylum contains two classes: *Conoidasida* whose sporozoites have conoids, gregarines and coccidians are examples, while the *Aconoidasida* lack conoids; malaria parasites and piroplasms are examples of this class. They are in the order- eucoccidiorida and sub-order: adelerina. According to Maia *et al*. (2012), six suborders of haemogregarines have been reported as parasites found in the erythrocytes of the intermediate vertebrate hosts such as chelonians (tortoises, turtles and terrapins), while the haematophagus invertebrates are reported to be the definitive hosts. Four species have identified to parasitise reptiles and amphibians mostly. These four include; *Hepatozoon*, *Haemogregarina*, *Karyolysus* and *Hemolivia*, (Cook *et al*., 2014). The genera, *Desseria* and *Cyrilla* parasitise fishes and other aquatic organisms.

Furthermore, other taxonomic classifications of chelonians haemogregarines have categorized them as*Haemogregarina* and *Hemolivia* species, (Maia *et al*. 2012; Cook *et al*. 2014). Furthermore, Barta *et al*. (2012) reclassified the phylum into five suborders,

(a) Gregarinasina, (b) Coccidiasina, (c)Adeleorina, (d) Eimeriorina, (e) Haemospororina

The above classification is the basis for following groups identified within the phylum:

1. Gregarines: one-host parasites infecting invertebrates.
2. Adeleorins: one or two-host parasites of both invertebrates and vertebrates hosts.
3. Eimeriorins: one or two-host parasites of both invertebrates as well as vertebrates.

4. Haemospororins: one or two-host haemoparasites of blood sucking arthropods and some four-legged vertebrates. They are the common malaria parasites.

5. Piroplasms: are two-host haemoparasites of vertebrates (Barta *et al*. 2012).

Over 300 described genera and an estimated 6,000 named species; several order species probably existed more than have been described to date (Perkins, Barta, Clopton, Peirce, and Upton, 2000;Snorre, 2020). According to Adl, Simpson and Farmer, (2005), about 0.1% of extant protists species have been formally described to date.

In summary, the classification of Haemogregarines has been given in line with Barta *et al*. (2012) and Pineda-Catalan, Perkins, Peirce, Engstrand, Garcia-Davila, Pinedo-Vasquez and Aguirre, A.A. (2013).

Taxonomic classification of Haemogregarines

Kingdom: Protozoa, Clade: Chromalvelata, Infra-kingdom: Alveolata, Phylum: Apicomplexan, Infraphylum: Sporozoa, Class: Coccidia, Order: Eucoccidia, Family: Haemogregarinidae, Genus: Haemogregarina, Family: Karyolysidae, Genera: Karyolysus, Hemolivia,Family: Hepatozoidae, Genus: Hepatozoon

2.8.3.1 *Hepatozoon*

The genus *Hepatozoon* is highly diverse and has been described from many tetrapod vertebrates and blood-sucking arthropods, where they serve as vectors and definitive hosts respectively. About 300 species of the parasites have been identified. The genus is the most abundant of all haemogregarines and is broadly spread in reptiles as well as amphibians, (Maia *et al*. 2012). *Hepatozoon* is of veterinary importance with several studies that have reported apparently mild infection in natural hosts to life-threatening illness in aberrant hosts,Damas *et al*. (2014). Although the pathogenesis of Hepatozoonosis in reptiles is not well understood as most of such previous studies on the parasites were carried out in domestic animals and common symptoms manifested were reported as low haematocrit values, general body weakness, loss of body weight and wasting body condition. The pathogenic effects of the parasites on wildlife have not been clearly stated, (Allen, Yabsley, Johnson, Reichard, Panciera, Ewing and Little, 2011; Hossen *et al*., 2013). The epidemiology and spread of this genus are reportedly characterized by the absence of salivary transmission between hosts; which is a common feature of other apicomplexans. Hepatozoonosis is said to occur when an animal feeds on

an infected tick or other infected arthropods; and not by the bites of infected arthropods. *Hepatozoon* is present in different species of snakes and lizards, (Maia *et al*. 2012). Some common species of the parasites include *Hepatozoon muris* and *Hepatozoon canis*, which commonly infect mice and dogs, respectively. *Hepatozoon atticorae* is a haemoparasites of birds.

2.8.3.2 *Haemogregarina* (Danilewsky, 1885)

Haemogregarina was first described by Danilewsky in 1885 in *Emysorbicularis* (European pond turtle), according to Ozvegy*et al*.(2015). Morphologically, the parasite is elongated in form and is found intra-erythrocytic. It stains basophilic in Giemsa with a surrounding clear zone. It is a parasite of cold-blooded vertebrates, which commonly parasitises fishes and reptiles as intermediate hosts, while the blood sucking worms called leeches serve as final hosts. The parasite is considered benign and has been found infecting the red blood cells of desert tortoises, (Ozvegy *et al*., 2015).

2.8.3.3 *Karyolysus* (Labbe, 1894)

The genus *Karyolysus* was described by Labbé in 1894. The haemoparasite is found in many species of lizards and the gamasid mites (*Ophionyssus)* which are believed to be the vectors (Haklova-Kocikova, Adriana, Igor, Karol, David, Gabor, Piotr, Natalia, Beat and Majlathova, 2014). *Karyolysus* is reportedly found parasitising lizards mainly from Asia and Europe, (Hassl, 2012; Ozvegy *et al*., 2015).

Karyolysus has an indirect life cycle like other haemogregarines, merogonic differentiation occur in the intermediate (vertebrate) host, both gametogony and sporogony occurs in alimentary tract in definitive hosts which are invertebrate arthropods, (Maia *et al*., 2012; Haklova-Kocikova *et al*., 2014; Ozvegy *et al*., 2015). *Karyolysus* differs from other haemogregarines in the apicomplexan group because their life cycle consists of motile sporokinetes formed in the oocyst which becomes encysted to form sporocysts in the host. On the contrary, *Hepatozoon* has a large poly-sporocysts oocyst, while in *Hemolivia* there is intra-erythrocytic merogonic stage. In addition, gamonts of *Karyolysus*appearance in peripheral blood reveals a vacuole which is very characteristic (Haklova-Kocikova *et al*., 2014).

2.8.3.4 *Hemolivia*

Hemolivia was first described by Sergent and Sergent in 1904, whileBrumpt in 1938, described the sporogonic stages of the parasites in the tick of the *Hyalomma aegyptium L* found attached to *Testudo graeca* in an Algerian specimen according to Karadjian, Chavatteand Landau(2015). Furthermore, *Hemolivia* from *Bufo marinus L* a cane toad from Brazil was described by the workd of Landau and Paperna as reported by Cook *et al*. 2015. Karadjian, Chavatte and Irene (2015) elucidated the molecular characterization of the parasites from 25 years archived smear of crushed tick *Amblyomma rotondatum*. The sporogonic stage of the parasite was reported in tick, *Amblyomma limbatum*, attached to an Australian skunk and *Hyalomma aegyptium* detached from the land tortoise *Testudo gracea* (Dvorakova *et al*., 2015). The life cycle of the parasite differs from other haemogregarines; in that, the progeny of sporocysts in *Hepatozoon* often remained within the wall of parent oocyst, while in contrast, *Hemolivia* sporokinetes retain a characteristic structure called the apical complex, (Karadjian, *et al*., 2015).

2.8.3.5 Morphology of haemogregarines

Haemogregarines appear microscopically as banana-shape occurring with little deviation during developmental stages in the different hosts, in addition, it possesses certain apparatus called an apical complex which is an organelle for organism' penetration into the host cell. Haemogregarines are unicellular and spore-forming. Some genus possess locomotive structures such as flagella or pseudopodia at the gametogonic stages, this is distinguishable only by electron microscopy. (Shen and Sibley, 2012; Haklova-Kocikova *et al*., 2014). Haemogregarines have a heteroxenous life cycle, the gamont occurs intra-erythrocytic Barta *et al*. (2012), while sporogony takes place in the invertebrate hosts (Dvorakova, Kvicerova, Hostovsky and Siroky, 2015).

Meronts: Thisis spherical with foamy cytoplasm and loosely arranged chromatin within the nucleus, which stains lightly acidophilic. The meronts of haemogregarines are rarely detected in the peripheral blood, but could appear as cysts with or without pigment surrounded by inflammatory cells in organs of the body. (Dvorakova *et al*., 2015).

Merozoites: This is an elongated shape tapering possibly towards the anterior pole while the posterior pole has a rounded edge, they are either free-living or intra-erythrocytic, the

cytoplasm is vacuolated, and stains lightly basophilic, (Soares, Brito, Paiva, Pavan and Viana, 2014).

Gametocytes: These are in two forms the micro-gametocytes and macro-gametocytes.

The microgametocyte has two morphological types (a) organism appears folded, vacuolated and the nucleus is centrally located, affected erythrocytes show no morphologic changes. (b) The second morphological type appears like the first with the exception of the nucleus which occupies three-quarters of the cell.

The macrogametocytes also has two morphological type (a) affected erythrocytes have displaced nuclei and the cytoplasm of organism appear deeply basophilic within the erythrocytes. (b) The second morphological type has a lightly stained basophilic cytoplasm with fragmented nuclei within the erythrocytes, (Soares *et al*., 2014).

2.8.3.6 Life cycle

Hepatozoon is reported to inhabit many arthropod invertebrate hosts, such as lice, fleas, tsetse flies, ticks and mites, (Van As *et al*. 2013). In reptiles and chelonians, the mode of spreadof haemogregarines parasites is thought to be through haematophagus arthropods by consumption of an infected final host. In addition, a congenital or vertical transmission has been reported in some snakes, Kauffman*et al*.(2016).

Life-cycle is indirect; merogonyoccurs in the peripheral circulatory as well as in visceral organs of vertebrate host, while gametogonyas well as sporogony take place in the alimentary tract of arthropods, (Maia *et al*. 2012). The parasites have been reported in circulating erythrocytes and often less in leukocytes, (Maia *et al*. 2012). Oocysts and sporozoites formation occurs within the gut epithelium of the tick *Hyalomma aegyptium* to form the sporocysts which contain sporozoites (Karadjian*et al*. 2015).

Ticks, leeches, fleas and other haematophagus arthropods could act as definitive hosts. Transmission of parasites is said to take place by the ingestion of infected invertebrate intermediate hosts, tick. (Maia *et al*. 2012). Sporozoites are released after ingestion and they invade the erythrocytes to undergo schizogony in various tissues of the body. The circulating merozoites and gametocytes found within the erythrocyte and are ingested by haematophagus insects in preparation for subsequent transmission. Following transmission by bites of insects or consumption of infected arthropods by the vertebrate

host, merogonic differentiation takes place in RBCs and reticuloendothelial system and cystic forms are found in organs such as kidney, spleen and liver. (Karadjian*et al*., 2015).

2.8.3.7 Host range

Haemogregarines affect many vertebrates' hosts such as; fishes, reptiles and amphibians. They have been reported to parasitize important groups of animals such as the dogs apart from reptiles (Hossen *et al*., 2013). They have also parasitized haematophagus invertebrates, (Van As *et al*., 2013). These cold-blooded vertebrates' serves as intermediate hosts, while tick and blood sucking worms called leeches and other ectoparasites serve as final host. According to Hossen *et al*. (2013), haemogregarines have infected dogs. *Hepatozoon* has a wider distribution and host range, Maia, *et al*.(2012). Further reviews carried out by Maia, *et al*.(2012), in their research findings reported a higher infection of haemogregarines in lizards by microscopy. Cook *et al*., (2009) recounted that land tortoises captured from four South Africa provinces were parasitised with *Hepatozoonfitzsimoni* which was earlier reported as *Haemogregarina fitzsimoni* in land tortoises from Mozambique, this being a clear indication of the breed, species and geographical and host range of parasite

2.8.3.8 Prevalence of haemogregarine infection

2.3.3.8.1 Haemogregarine infection in fish

Three species of Haemogregarines; *Cyrilia spp*, *Haemogregarina spp*. (*sensu lato*) and *Desseria spp* has been reported in fishes according to Esteves-Silva, da Silva, O'Dwyer, Tavares-Dias and Viana, (2019). *Cyrilia spp*. and *Haemogregarina spp*. (*sensu lato*) and are characterised by the appearance of intraerythrocytic merogonic stage of parasites within RBCs of the fish host. This stage is absent in *Desseria spp*. In addition, it undergoes sporogony in leeches. In *Cyrilia spp*., sporogonic development produces numerous sporozoites and two life cycles were reported by Hayes and Smit (2019). *Haemogregarine bigemina* has been reported in over 90 species of fishes, belonging to about 70 genera and 34 families. In these hosts, the parasite is capable of undergoing binary fission within the erythrocyte to form mature paired gamonts. In addition, intraleukocytic development has also been reported in other hosts aside fishes.

They are commonly present in marine fishes, where they are found in circulating RBCs and cells of the leukocyes, (Magro, de Oliveira and O'Dwyer, 2016).

2.8.3.8.2 Haemogregarine infection in amphibians

Haemoparasitic protozoans have been widely reported in amphibians, some of which includes *Haemogregarines*, *Plasmodium*, *Aegyptianella*, *Haemoproteus* and *Lankesterell a,* (Adl, *et al*., 2012; Barta*et al*., 2012). In South Africa, an overall prevalence of 30.1% (16/53 haemogregarine infections was recorded in three species of frogs. Furthermore, in a similar work by Netherlands, Cook, Smitand du Preez, 2014) on 20 wild-caught frog species of *Amietia quecketti*, they reported a haemogregarine prevalence of 3/10 (30%) and 2/10 (20%), which varied over spring and winter of the parasitized frog species respectively.

Foronda, Santana-Morales, Oros, Abreu-Costa, Ortega-Rivas, Lorenzo Morales and Valladares, (2007) reported that atovaquone-proguanil was very efficacious in treating haemogregarine infection in *Gallotia caesaris* lizards. Two *Hepatozoon*species *Hepatozoon ixoxo* and *Hepatozoon theileri* were said to possess similar characteristic of the Apicomplexan but they however appear dissimilar in outward cellular structure. These parasites were discovered in South African toads. *H. ixoxo* was described in *Amietophrynus species* toads, while *H. theileri* was found in *Amietia quecketti* a river frog.The information was obtained from the morphological and molecular characteristics of the PCR amplified 18S RNA gene fragments, (Conradie, Cook and du Preez, 2016; Netherlands, Cook and Smit, 2017).

2.8.3.8.3 Haemogregarine infection in reptiles

The haemoparasites of reptiles include haemogregarines, trypanosomes, microfilaria, piroplasmids and Plasmodium. The haemogregarines infecting reptiles are of three genera *Haemogregarina* is present in water aquatic turtles, *Hepatozoon* is present in some snakes and lizards, while *Karyolysus* is present in some lizards and tree snakes, (Sajjadi and Javanbakht, 2017). Haemogregarines often infect erythrocytes and rarely the leukocytes, (Stacy *et al*., 2011). Tome, Rato, Harris and Ana in (2016), elucidated the ultrastructures of

Haemogregarina tarentannulari in wall gecko (*Tarentola annulari*), the parasite was reported to have invaded the erythrocytes and the lungs of heavily infected gecko. In addition, Maia *et al*. (2014), reported an overall prevalence of 7% (5/73) and intensity of haemogregarines infection of 73 reptile specimens which comprised of 51 chameleons, 5 lizards and 13 snakes. An overall prevalence of 13% was reported from smears of blood samples obtained from sand snakes (*Psammophis schokari*) in Saudi Arabia. Average parasitaemia in infected snakes was 5%. This report is in agreement with the prey-predator transmission hypothesis with regards to infection in snakes, (Abdel-Baki *et al*., 2014).

2.8.3.8.4 Haemogregarine infection in crocodiles

The research carried out in Brazil on haemogregarine parasites of Caiman crocodiles reported a prevalence of 71.4% (20/28) of *Hepatozoon caiman* infection in 28 Caiman crocodiles yacare. In addition, leeches and mosquitoes were observed inhabiting the bodies of these animals. Morphology appearance of the parasites suggested they are *H caiman* (Viana, Paiva, Coutinho and Lourenco-de-Oliveira, 2010).

2.8.3.8.5 Haemogregarine infection in lizards

The works of Haklova-Kocikova,*et al*. (2014), evaluated the occurrence of *Haemogregarina mariae* on the health of *Tiliqua rugosa* an Australian sleepy lizard in their natural environment. The result showed lizards that have a greater home range were more active and has greater exposure to parasitic infection. This evolutionary trend eventually leads to reduced activity and the consequently reduced home range.

2.8.3.8.6 Haemogregarine infection in snakes

The vertical transmission of *Hepatozoon* reported by Kauffman, Sparkman, Bronikowski and Palacios (2017), of naturally infected nine viviparous western terrestrial garter snake (*Thamnophis elegans*), captured at parturition. Infected offspring were born to four infected mothers and highest parasitaemia were found in neonates at two months. Although sex-skew parasite prevalence did not vary significantly in offspring's, females generally exhibit a greater prevalence than males at two months old. This mode of transmission of parasites might be played down in wildlife disease transmission.

Furthermore, Abdel-Baki *et al*., (2014), reported an overall prevalence of 13% in *Psammophis schokari* (sand snakes) from Riyadh in Saudi Arabia.

2.8.3.8.7 Prevalence of haemogregarines in chelonians

Four haemogregarines genera parasitising the reptiles include; *Haemogregarina*, *Karyolysus*, *Hepatozoon*, and *Hemolivia*, (Cook, Lawton, Davies and Smit, 2014). *Hemolivia mauritanica* has been reported to parasitize tortoises as intermediate host, while ticks are final hosts. Transmission is said to occur via ingestion of infected tick by the tortoise, (Dvorakova *et al*. 2015; Karadjian *et al*. 2015).

Overall prevalence of 52.2% haemogregarine infection was reported in a population of turtle in North America. In addition, it was also reported that 70% of turtles in North America harbour haemogregarines, (Dvorakova *et al*. 2015). Studies by Hossen *et al*., (2013) and Dvorakova *et al*.,(2015) submitted that female turtles habour more leeches because of their bigger body sizes; however, this phenomenon was not implicated to have resulted to higher parasitism in female turtles.

Cook *et al*., (2009), reported an overall prevalence of 25% (38/154) of two haemogregarines species (*Hepatozoon fitzsimonsi* and *Haemogregarina parvula*) found in five species of chelonians from four provinces of South Africa. 84 tortoises were wild-caught, while 70 were captive-bred. They reported a significantly higher prevalence of 35% (13/37) infection with *H. fitzsimonsi* in captive *S. pardalis* at the National Zoological Garden (NZG) Pretoria, compared with 5% (2/41) in the wild-caught species of the same tortoise species. Similarly, Ozvegy, Marinkovic, Vucicevic, Gajic, Stevanovic, Krnjaic and Kovacevic, (2015), reported an overall prevalence of 100% (30/30) haemogregarines infection in European pond turtles using both microscopy and PCR methods. This report is in agreement with similar research works of Mihalca *et al*., in (2008) on pond turtles from Romania, where 100% prevalence was reported. Furthermore, Dvorakova, et al. (2014) reported 86.7% prevalence in freshwater turtle (*Emys mauremys*). The works ofMolla *et al*., (2015), reported 33.3% prevalence of new species of haemogregarines in common mud turtle, *Lissemys punctata punctate* sourced

from Canning, Sundarbans region of West Bengal, India. The haemogregarine species were reported to have differed morphologically and in other characteristics of haemogregarines previously described and because of its novelty, the haemoparasite was named *Haemogregarina sundarbanensis sp. n*. after the locality from where the mud tortoises were sourced.

*Haemogregarines*have been mostly reported in Africabellhinge-back tortoises *Kinixys belliana*, in the most recent times, (Cook *et al*., 2014).

2.9 Standard measurements in chelonians tortoise

Body condition assessment is a quick method of evaluating health conditions in chelonians. It has been a difficult procedure to undertake, because the chelonian's body is enclosed in a bony box; comprise of carapace; which forms the dorsal aspect of the roof, the ventral aspect or the floor is called plastron, In addition, experts in chelonian's husbandry have hesitated to give an opinion other than 'light for size' or 'average for size' (Adkins, 2012).

Non-invasive methods have been widely used in many studies involving animal's ecology and management to evaluate the body physiology of various animal species. This is achieved by taking morphometric measurements of animal's body to obtain an empirical value known as the Body Condition Index (BCI) described by Carlson, Woodman, Bracewell, Granger, Buhlmann, Finney, Garrett and Segura, (2018). Such methods have become common and largely used in calculating BCI in many species of mammals, (Schulte-Hostedde, Zinner, Millar and Hickling, 2005), reptiles, birds (Labocha and Hayes, 2012), fish and amphibians (MacCraken and Stebbings, 2012).

According to Adkins, (2012), there are various methods of determining body condition in animals, the commonest being a comparism of body mass with size.

2.9.1 Straight carapace length (SCL)

The SCL is a straight line measurement taken from the cranial aspect of the nuchal scutes to caudal scutes. Measurement can be taken using a flexible measuring tape, meter rule and a pair of a mechanical caliper. SCL is measured to the nearest 1mm on a flat surface, (Adkins, 2012;Ljubisavljevic, Dzukic, Vukov and Kalezic, 2012)

2.9.2 Straight plastron length (SPL)

This is the horizontal measurement of the plastron taken from the tip of gulars scutes to end of the anal plastron. This measurement may extend to the intergular and the ventro-caudal aspect of the neck of the plastron, (Ljubisavljevic *et al*., and 2012).

2.9.3 Plastron width (PW).

This is the widest part of the plastron. The tape is held vertical and at right angle to the horizontal length of the plastron at its widest points, (Adkins, 2012; Rawskiand Jozefiak 2014).

2.9.4 Height (H)

The body depth also called height is the maximum vertical length from the roof of carapace to the plastron. This is calculated as the maximum vertical length to the ground, (Ljubisavljevic *et al*., 2012).

2.9.5 Weight (W)

The weights of animals are determined to the nearest gramme using a weighing balance, (Rawskiand Jozefiak 2014; Durakic and Milankov 2019).

2.9.6 Body condition index

The BCI is calculated as the observed mass M of a chelonian compared to an expected or predicted mass (Mi). In addition, The Mi is calculated from a linear relationship between mass and the linear body measurements, (Carlson *et al*. 2018; Durakic and Milankov 2019). In addition,Labocha, Schutz and Hayes, (2014) also reported that, LogM/LogMi has been the best measurement for BCI in fishes and chelonians, when LogM/Log Mi is equal to 1 or 100% it indicates an apparent healthy state and if less than 1 or it means the health may have been compromised, when it is greater than 1 it may also connote obesity in the chelonians.

2.10 Diagnostic techniques

2.10.1 Blood collection in chelonians

The total blood volume of chelonian is considered to be made up of 4-8% gross body weight. Only one- tenth total blood volume is considered safe for collection in any healthy tortoise for analysis. This implies that for a 1kg tortoise only 4-8 ml of blood on the maximum can be safely bled for blood work without impairing the health of the tortoise (Perpian, 2013).

2.10.2 Anticoagulant

The anticoagulant considered most suitable for chelonians blood work is lithium heparin. Ethylenediaminetetraacetic acid (EDTA) causes haemolysis in several species of turtles and tortoises. Furthermore, a lithium heparinized blood sample of less than 0.5 ml is considered adequate to do haematology and biochemical analysis, (Perpinan, 2017).

2.10.3 Needle gauge and size

The erythrocytes of chelonians are large and can be up to 20 μm in diameter when compared to the mammalian erythrocytes which are about 7μm in diameter, (Perpian, 2013). Therefore needle gauge sizes for blood collection in chelonians should be carefully selected to prevent lysing of erythrocytes which can lead to inaccurate evaluation of haematologic and biochemical values. In addition, small sizes-needle should not be used to prevent imposition of too much pressure on the plunger which might compromise blood sample integrity. In most chelonian species, 23 guage to 25 guage hypodermic needle mounted on a 5cm syringe is recommended, (Perpian, 2017).

2.10.4 Venipuncture sites in chelonians:

There are several anatomical locations indicated for blood collection in tortoises and turtles. The choice of any site depends on the size, sex and species of turtle/tortoise, preference and personal experience of the researcher in chelonian blood collection and to avoid haemodilution. Such anatomical sites as the sub-carapacial sinuses are exposed to a high risk of infection when blood collection is not properly carried out, (Perpian, 2017). The routes for blood collection in chelonians are dorsal coccygeal vein (this site is said to be easier to access in males due to their longer and thicker tails) and sub-carapacial veins or sinuses.

2.10.4.1 The sub-carapacial vein or sinus

Thissite is easy to access in many chelonians, especially in those species which retract their heads (Cryptodiran hidden-neck tortoise), into the carapace. The venous access is carried out by inserting the needle into the junction of the skin and the ventral carapace, dorsal to the neck; the needle is directed dorso-caudally towards the junction of the cervical vertebrae and the ventral carapace. Blood is obtained from the post-occipital venous plexus situated dorsal to the cervical vertebrae and caudal to the occipital protrusion,(Perpian, 2013; 2017). This method, however, may result in haemodilution with extracellular fluids and secretions if procedure is not properly done. (Perpian, 2017))

2.10.4.2 Jugular vein

This jugular vein is difficult to access in most chelonians species, although it is a more appropriate route for blood sample collection, and is closely associated with the carotid artery. The route fills syringe very quickly with bright red blood when the needle is inserted, (Perpain, 2013).

2.10.4.3 Occipital sinus

The occipital sinus is situated below the skull. Some species of tortoises with long occipital process has to be tilted, while the needle is directed caudo-ventrally towards the tip of the occipital process to collect blood, (Perpain, 2013).

2.10.4.4 Cephalic vein

The cephalic vein is not easy to access, in chelonians < 1kg in weight and also in cryptodirans. The cephalic vein is situated between the elbow joint and the tendon which passes below the joint, (Hernandez-Divers *et al*., 2006; Perpain 2013)

2.10.5 Light microscopy

The evaluation of peripheral blood films using light microscopy is an important investigative tool in human and veterinary medicine, (Stacy, Alleman and Sayler, 2011). Evaluation of haemogregarine parasites is carried out by the examination of thin blood smear prepared with Romanowsky or Giemsa stains and examined with light microscope. To validate the occurrence of haemo-parasite; thin blood smears are examined by placing a drop of immersion oil on the feathered edge of the thin film and moving from the 10x objectives lens to the 100x oil immersion lens, the examination of the feathery end or

edge of the thin film where the red cells lay side by side is done, ensuring minimal overlap of blood cells. This pattern of movement is followed by movement along the edge of the film, the movement ofthe slide outwards by one field, inwards by one field and returning in a lateral movement and so on. This is continued until all the haemogregarine parasites on the slide have been identified and enumerated.

Figure 2.2 Thin blood film examination
World Health Organization (WHO), 2016

2.10.5.1 Morphology of haemogregarines

Haemogregarines haveuniform morphologic appearances within the cytoplasm of affected erythrocytes. They are sausage or banana-like in appearance with nucleus situated centrally or eccentrically. In heavy infections, the RBC of affected animals may contain two or more parasites enclosed intra-cytoplasmic. Besides, there may be mild to severe morphologic changes in the RBC, such as a hypertrophied or macrocytic appearance and the displacement of the nucleus due to the presence of parasites. Evaluation of stained thin blood smear appears to be the easiest and fastest method of making a haemogregarine diagnosis; this method lack the ability to differentiate the different haemogregarines genera, as the parasites have similar morphologic appearances, (Glenda, 2016).

2.10.6 Molecular diagnosis of haemogregarine infection

A molecular diagnosis is a powerful epidemiology tool, which has potential for disease trace back, origins and spread of new pathogens such as haemogregarine, Mycobacterium tuberculosis (TB) and other disease agents. This technique relies on information relating to contact with potential vector or reservoir hosts and genetic database relating to the phylogeny of the pathogen in question, (Snow, 2011).Identification of the various species of apicomplexan by morphology in blood smears is difficult to attain, thus the routine laboratory methods have been supported with molecular techniques of diagnosis such as Polymerase Chain Reactions(PCR). The use of molecular methods facilitates the discovery and identification of parasites in samples, as well as the characterization of haemogregarine within the apicomplexan group, (Barta *et al*., 2012; Dvorakova *et al*.,2014). Polymerase Chain Reactions (PCR) method entails duplication of DNA or RNA template to which specific primers; both forward and reverse primers have been added in a reaction mixture set inside a thermocycler using the Taq DNA polymerase in 30 to 45 PCR cycles to produces 2n numbers of molecule of template on an n¼ number of reactions to yield billions DNA molecules per cycle. The PCR method requires speedy procedure of genomic DNA extraction from different mammalian tissues and blood to generatecontaminant-free product to prevent unspecific amplification of genomic DNA (Kenah, Britton, Halloran and Longini. 2016).

2.10.6.1 Genomic DNA extractions

There are many commercial and conventional methods for the extraction of mammalian DNA from peripheral blood. The protocol for DNA extraction from biological samples can be very laborious and time-consuming. The main goal of nucleic acid separation is de-proteinisation followed by separation of nucleic acid from protein. The charged phosphate backbone of the nucleic acids makes its composition rather hydrophilic when compared to the protein. Isolation of the nucleic acid entails cell breaking, enzymatic degradation, differential solubility and precipitation (Radmeshyan, Brijesh and Shyyam, 2013).

Extraction protocols that yield high-quality DNA from little quantity of tissue require the use of organic solvents such as phenol or enzymatic digestion using proteinase k in specific buffer solutions. The disadvantage ofDNA extraction by conventional methods is the undesirable effect of organic solvent like phenol required for protein digestion and the several items of washing and centrifugations which increase the possibilities of sample contamination, (Fernandes, Meissner, Fernande and Rocha, 2004). The proliferations of non-conventional DNA extraction kits have provided a good substitute for the usage of phenol. The kits methods are rapid than the conventional methods. Although the quantity of DNA generated through the kits method may be flexible, also the cost of DNA extraction kit may be considered very expensive when compared to the conventional or standard methods (Kusec, Radisic, Komlenic and Kusec. 2015).

2.11.0 Haematology of tortoises

Haematology is generally employed for three main purposes in medicine and clinical practices as reported by Abramowicz, Łukasz and Lutnicki, (2019); (a) Differentiating normal from abnormal blood (b) Diagnosis of disease or abnormality (c) Detailed haematological studies. (Zhang, Li, Gu and Ye, 2011). The evaluation of blood parameters in chelonians has been reported to be very few when compared to other animals species generally. Although, the keeping of chelonians as pets and for breeding in captivity has advanced and also gained tremendous attention in recent times. Haematologic investigation of chelonians in free-range and in captivity will go a long

way in facilitating early disease diagnosis and prompt treatment, thus enhancing adequate health management of chelonians, (Zhang *et al*., 2011).

Studies on reptile's haematology have existed many years ago, (Arikan and Cicek, 2014; Dissanayake *et al*., 2017). Besides, further researches on the ultrastructure studies of chelonian's blood cells have been reported by several authors, whereas studies on their haematopoiesis and blood cells development, biochemical profile and characterization have been reportedly very few. Although, some works on chelonian haematology and biochemistry reported in China, this is still not adequate when compared to other animal species, (Zhang *et al*., 2011;Dissanayake *et al*., 2017).

Diseases occurrence are linked generally with changes in haematology in all animals and such changes have formed the basis for disease diagnosis and useful in health assessment of chelonians and other animals and for post-treatment assessment, (Joyner, Shreve, Spahr, Fountain and Sleeman, 2006; Giori, Stacy, Ogle, Nelson, Fecteau and Cushing, 2020). Haematological profiles in chelonians are influence by age, sex, season, geographic location, physiology and reproduction of animal. (Hofmeyr, Henen and Walton, 2017; Nollens, Haney, Stacy and Robeck, 2020). Therefore the evaluation of the blood and its biochemical constituents and responses to physiology and environmental factor coupled with comparative studies of healthy and unhealthy chelonians is of great help in conservation, especially for critically endangered species (Dissanayake *et al*., 2017).

2.11.1 Blood cell morphology

Chelonians blood cells includes; RBCs, WBCs and platelets. The WBC known as leukocytes is further divided into granulocytic leukocytes and agranulocytic leukocytes. Granulocytic leukocytes consist of heterophils, eosinophils and basophils, while agranulocytes are lymphocytes and monocytes (Stacy, *et al*., 2011; Zhang *et al*., 2011; Arikan and Cicek, 2014). The azurophils have been described as a type of leucocytes found commonly in reptile's species like snakes, lizards and crocodiles but seldom in chelonians. The heterophils and eosinophils both possess acidophilic granules and are not too distinct microscopically, (Stacy *et al*., 2011; Arikan and Cicek, 2014).

Some researchers have established the fact that leucocytes are not easily identified morphologically. For instance, the small lymphocytes have similar morphological appearance as the thrombocytes, while some authors are also of the opinion that reptiles do not possess neutrophils, but possess only heterophils and eosinophils, whichhave acidophilic granules. Some studies have further identified and classified acidophils as blood cell type undergoing various stages of cell maturation (Nardinin, Leopardi and Bielli, 2013; Arizza, Russo, Marrone, Sacco and Arculeo, 2014).

2.11.1.1 Erythrocytes

The chelonian erythrocytes are ellipsoidal in shape and the nucleus is centrally positioned with deeply basophilic granules. The cytoplasm appears light yellowish pink with Wright stain,(Zhang *et al*., 2011; Perpinan, 2017). Mature erythrocytes have no organelles but some possess small basophilic inclusion bodies. Immature erythrocytes can be observed sometimes in chelonians blood, morphologically, they are more rounded, with the nucleus slightly stain, (Dissanayake *et al*., 2017; Perpinan, 2017). The packed cell volume (PCV) is an index for estimates the proportion of RBCs in the peripheral blood circulation. Low PCV can indicate blood loss, various types of anaemia, nutritional deficiencies, Erythrocytes in tortoises are larger than those in snakes and lizards and occupy more space in the blood; thereby total erythrocyte counts in tortoises tend to be lower than other reptiles,(Dilrukshi, Jayasooriya and Prathapasinghe, 2019).

2.11.1.2 Heterophils

Heterophils are granulocytic leukocytes, they are large and round in shape and have been reported to be the most abundant granulocytes cells in reptiles blood, (Stacy *et al*., 2011). The cytoplasm contains pinkish-red granules, the granules could be rounded or dumbbell shape, heterophils granules are usually smaller and fusiform in appearance than the granules of eosinophils. The nuclei could be round or oval or bilobed, the unlobed nucleus is eccentrically located (Nardinin, *et al*., 2013; Perpinan, 2017). The heterophils function as the mammalian neutrophils in phagocytosis (Oros, Calabuig, Arencibia, Camacho and Jensen, 2011). Cytoplasmic organelles have been reported at ultrastructure studies (Zhang *et al*., 2011; Nardinin, *et al*., 2013).

2.11.1.3 Eosinophils

Eosinophils are round, having different sizes in chelonians. They resemble heterophils morphologically, and the nuclei are positioned eccentrically and could also be found lobed at times (Zhang *et al*., 2011). They have eosinophilic spherical granules that are usually heavily stained; this distinguishes it from heterophils which have eosinophilic fusiform granules. The cytoplasm is weakly basophilic and contains pinkish-red round granules with Wright's stain (Zhang *et al*., 2011; Stacy *et al*., 2011; Perpinan, 2017). Elevated values of eosinophil have been associated with parasitosis,

2.11.1.4 Basophils

Basophils in reptiles aresmall in size and round. They contain many small round dark-purple granules with Wright's stain and the metachromatic granules are usually found obscuring the nucleus, (Stacy *et al*., 2011; Perpinan, 2017). The nucleus is round to oval and is usually positioned centrally or eccentrically (Zhang *et al*., 2011;Perpinan, 2017; Dissanayake *et al*., 2017). In chelonians basophils counts tend to increase with certain haemoparasitosis such as haemogregarinosis and trypanosomosis (Perpinan, 2017).

2.11.1.5 Lymphocytes

Lymphocytes are medium or small round nucleus centrally or eccentrically positioned within scanty cytoplasm. The lymphocytes of reptiles resemble those of the mammalian and birds morphologically (Perpinan, 2017), hence it is usually a difficult task to separate small lymphocytes and thrombocytes when enumerating the complete white blood cells count using haemocytometer (Stacy *et al*., 2011). Lymphocytes in reptiles are classified into B and T lymphocytes and they function in immunological responses. In most reptiles, lymphocytes are predominant up to 80% in proportion (Stacy *et al*., 2011).

2.11.1.6 Monocytes

The monocytes have variable morphological appearances from round to oval or amoeboid shapes; they are usually larger than the lymphocytes. The nucleus could be oval to round or indented and stains bluish purple. The cytoplasm is weakly basophilic, reactive monocytes; possess vacuoles (Arikan and Cicek, 2014; Perpinan, 2017). Monocytes comprised 0-10% and up to 20% leucocytes in reptiles. They usually transform to macrophages when they exit the peripheral circulation into the tissue. There

is increase monocytosis during chronic inflammation as well as in bacterial and parasitic diseases (Stacy *et al*. 2011).

2.11.1.7 Thrombocytes

The thrombocytes of reptiles, birds, fishes and amphibians are usually nucleated, unlike the mammalian platelets (Stacy *et al*., 2011), but like the mammalian platelets, they function in maintaining haemostasis, wound healing and also phagocytosis of foreign invaders. Thrombocytes are oval-shaped cells, with scanty cytoplasm; the nucleus is oval or angular with clump chromatin. They usually aggregate in blood smears when blood samples are collected using lithium heparin as an anticoagulant, this features helps in distinguishing thrombocytes from small lymphocytes. They stain only in Periodic acid Schiff (PAS) stain (Stacy *et al*., 2011; Dissanayake *et al*., 2017). There is also a superficial canalicular system in thrombocytes of many reptiles this helps to amplify the plasmalemma thus facilitating efficient movement of metabolites across cells and extracellular space(Dissanayake *et al*., 2017).

2.11.1.8 Azurophils

Azurophils are round with distinct cytoplasm, which contains purple granules. The nucleus of azurophil is oval to round and is position eccentrically. They are abundant in snakes and could account for up to 35% of total peripheral leukocytes, (Stacy *et al*., 2011).The azurophils are commonly found in some reptile's families; the squamatas and crocodilians, but rarely in chelonians (Stacy *et al*., 2011; Martins, Alevi, Azeredo-Oliveira and Bonini-Domingos, 2016). The azurophils of snakes are similar morphologically to neutrophils in mammalians, while the azurophils in lizards are similar to mammalians monocytes. They function in inflammatory reactions and acute state bacterial diseases (Stacy *et al*., 2011).

2.12.0 Development and function of reptiles blood cells

2.12.1 Haematopoiesis in reptiles

There are very few studies on the haemopoiesis of reptiles (Harvey, 2012; Arikan and Cicek, 2014). The blood islets are believed to be the sites of erythropoiesis at embryonic stage in reptiles. The liver and spleen play some roles in erythropoiesis at this early stage of growth. Thereafter, blood cells production in reptiles takes place after birth majorly in the bone marrow (Arikan and Cicek, 2014). Haematopoiesis has been described in the turtles; *Pelodiscus sinensis* and *M. mutica*. in China, while further studies carried out on the ontogeny of haematopoietic organs in *P. sinensis* by some other researchers reported that the primitive haematopoietic organ in *Pelodiscus sinensis*is the yolk sac blood islands, while it was agreed with previous authors that haematopoiesis is to the bone marrow after birth (Guedes, Oliveira, Manso, Caputo, Cotta-Pereira and Pelajo-Machado, 2014

; Kodera, 2019). Similarly the spleens function as major sites for granulopoiesis (Kulkeaw. and Sugiyama, 2012). Research reports further ascertained that sparse blood islets were discovered at postnatal in a snake *Bothrops jararaca*, when the kidney and liver did not showed any obvious blood cells synthesis, (Guedes *et al*., 2014). Erythrocytic development has also been divided into three phases; the primitive phase, the immature phase and the mature phase. Similarly, the development of leukocytes has also been divided into promyelocyte, myelocyte and metamyelocyte (Zhang *et al*., 2011, Arikan and Cicek, 2014).

2.12.2 Functions of blood cells b

2.12.2.1 Erythrocytes

The erythrocytes contain haemoglobin which is function in oxygen transportation from the lungs to other parts of the body and conversely returns deoxygenated blood from other body tissues back to the lungs (Arikan and Cicek, 2014).

2.12.2.2Heterophils

The heterophils of reptiles are similar to the avian's heterophils and they function primarily in phagocytosis and are associated with inflammatory diseases and tissue injury. The heterophils of *P. sinensis* have been reported to have killed about 50% bacterial organisms in about 180minutes (Nardinin *et al*., 2013).

2.12.2.3 Eosinophils

The eosinophils function in parasitic infections as phagocytes and also help in the stimulation of the immune system; they have been reported to participate in the immunological response of chelonians where they phagocytose immune complexes (Perpinan, 2014).

2.12.2.4 Basophils

The basophils in reptiles and birds function in early inflammatory and immediate hypersensitivity reactions and not in delayed hypersensitivity like in the mammals (Stacy *et al*., 2011). Severe stress has been linked to increasing basophilic response in birds. Chelonians are reported to have a higher circulating basophils when compare to other reptiles. Some can have up to 60% differentials. It has not been elucidated the reasons for the high levels of circulating basophils. Some researchers have reported the degranulation of basophils and correlated it with the release of histamine from the basophils (Hofmeyr *et al*., 2017).

2.12.2.5 Lymphocytes

Lymphocytes are many in several reptiles. They function mostly in immunological responses, which include antibodies production and phagocytosis of foreign bodies in reptiles. The lymphocyte counts have been reported to be a function of the immunological state of an organism. The band T-lymphocytes in reptiles are the same as that of the avian's functionally. Seasonal variations of lymphocytes values in reptiles have been reported, lymphocytosishas been associated with the winter, (Hernandez, Castro, Saavedra, Ramirez and Oros, 2017). Similarly, lymphocytosis has been associated with inflammation, wound recovery, parasitosis and viral infections. Some researchers have recorded lymphopenia in malnutrition and stress while, (Zhang *et al*., 2011; Dissanayake *et al*., 2017).

2.12.2.6 Monocytes

The monocytes perform phagocytic functions and also migrate to the tissues, where they replace tissue macrophages. Monocytosis of highly vacuolated monocytes may cause granulomatous diseases and increased phagocytosis in response to systemic antigen (Arikan and Cicek, 2014). Monocytes and heterophils are found in phagocytic activities.

Researchers have reported heterophilia and monocytosis in turtles suffering from fibropapillomatosis and phaeohyphomycosis (Stacy *et al*., 2011).

2.12.2.7 Thrombocytes

Thrombocytes are important in maintaining haemostasis at the site of vascular injury by forming the haemostatic plug. Also, their phagocytic abilities may have a function in nonspecific immunity (Di Ianni, Merli, Burtini, Conti, Pelizzone, Di Lecce, Parmigiani, Squassino, Del Bue, Lucarelli, Ramoni. and Grolli, 2015).

2.12.3 Hematologicalparameters

The hematological profiles in reptiles can be affected by such factors as species, seasons, physiological state of the animals; hibernation and reproductive state, nutrition, health status, gender and age. Because the morphology and blood cell counts vary extensively among over 8000 reptiles. Some internal and external factors are capable of making the evaluation of haematological parameters in the reptiles very difficult. Therefore published reference can only provide baseline for evaluation and interpretation, while veterinarians need to consider these factors critically to be able to interpret correctly the haematological profiles and clinical observations in reptile (Stacy *et al*., 2011, Zhang *et al*., 2011). Assessment of blood cell morphology using stained smears andestimation of haematological parameterss; haematocrit value, haemoglobin concentration, RBCs counts, WBCs and the differentials counts provides useful and rapid information for clinical diagnosis in reptile's health management (Zhang *et al*., 2011; Hofmeyr*et al*., 2017). The differences observed in the physiology of the reptile's blood when compared to mammalians and domestic animal as well as attaining accuracy of their blood cell counts is most challenging because their RBCs are nucleated, hence conventional methods are used to evaluate the leukocytes counts. Therefore analytic haematological assessment in reptiles begins with careful sample collection, handling, methods, and laboratory protocols to ensure thoroughness (Perpinan, 2014).

The haematocrit values of most apparently healthy reptiles ranges from 25% to 40%, this has been reportedly low when compared to the mammal's species and birds, which

signifies low oxygen-carrying ability in reptiles. In addition, haemoglobin concentrations in reptiles have been considered low (5.5–12 g/dL), when compared to other animals (Stacy *et al*., 2011; Perpinan, 2017).

The reptile's erythrocyte has an average lifespan which range from 1½ – 2 ½ years and this is considered very slow when compared to the relative human erythrocytes turnover of 120 days, this occurence has also been linked with the slow rateof metabolism in reptiles. Also, RBCs count in reptiles is lower because of large - size erythrocyte (Stacy *et al*., 2011; Perpinan, 2017).

The white blood cell counts are affected by some internal and external factors; age, sex, environment, season as previously mentioned. Thus it may be difficult to properly interpret these parameters without taken into cognizance these factors that can affect the reptile's response to infections and diseases. The proportion of each kind of leukocytes type differs among different species.

Heterophils counts increases in disease of reptiles, the severity of such disease affects the degree of toxic changes such as increase cytoplasmic basophilia, vacuolation and degranulation. There are also increased numbers of immature heterophils released into the circulations. Heterophils are said to be the most abundant leukocytes in chelonians, and can be as high as 50% (Nardinin *et al*., 2013; Dissanayake *et al*., 2017).

There is an increase in lymphocytes activity especially in viral diseases; this is often observed as the development of a basophilic cytoplasm with or without viral inclusion bodies. Lymphocytes may also transform to plasma cells; these are round structures called "Russell bodies" containing immunoglobulin. Lymphocytes are the most abundant in peripheral circulation. Some chelonians such as *C. mydas* have recorded as high as 80%. On the contrary, some researchers reported that lymphocytes are the second most abundant leukocytes in peripheral blood circulation (Deem, Norton, Mitchell, Segars, Alleman, Cray, Poppenga, Dodd and Karesh, 2009; Dissanayake *et al*., 2017).

Monocytes have the phagocytic ability and can engulf erythrocytes, leukocytes in response to anaemia and infectious diseases. An increase in monocytes count is an indication of an ongoing chronic or granulomatous inflammation. Monocytes are very few in circulation, maybe between 0-10percent of differential (Hofmeyr *et al*., 2017).

Eosinophils presence are rare in the peripheral circulation of most chelonians, however, researches have reported that they are found in large numbers in some species of tortoises such as *L. kempii*, *Mauremys leprosa* and *C. mydas*, (Stacyand Raskin, 2015;Dissanayake *et al*., 2017). Some wild chelonians are said to yield easily to parasitic infections and diseases and can lead to increase eosinophil counts and the consequent stimulation of immunological response (Dissanayake *et al*., 2017).

Basophils are the least abundant of all leukocytes, but their counts in the peripheral circulation are reported to increase in parasitic and viral infections, wounds and respiratory disease. Basophils count varies in chelonians depending on species; they are high in some freshwater turtles according to Hofmeyr *et al*. (2017), who reported 63% prevalence in *Chelydra serpentine*. On the contrary, basophils were reportedly rare in marine turtles and loggerhead turtle (Perpinan, 2017).

The primary function of the thrombocyte is haemostasis. The thrombocytes counts in peripheral circulation range between 25-35 thrombocytes per 100 leukocytes in a blood smear. Increased in immature thrombocytes counts can be due to a regenerative response to excessive utilization, while a decrease in the level of thrombocytes is suggestive of either a reduced bone marrow production or excessive peripheral utilization; which may be due to septicemia or disseminated intravascular coagulation (DIC), (Zhang *et al*., 2011; Nardinin *et al*., 2013). Activated thrombocytes usually appear as aggregated clusters of cells (Stacy *et al*., 2011).

2.13 Blood biochemistry

2.13.1 Blood biochemical analysis

The evaluation of blood parameters is useful in disease surveillance and evaluation in animals (Giori *et al*., 2020). Clinical biochemistry uses chemical and biochemical assay techniques in health evaluation. Clinical biochemistry remains a useful diagnostic tool for theevaluation of health in free-living wild animals and those in a captive state (Paltrinieri, Ibba and Rossi, 2014; Stacy and Raskin, 2015). Haematological and biochemical parameters analysis in chelonians is germane to conservations (Omonona, Olukole and Fushe, 2011). It involves the evaluation of the different substances and their levels in body fluids such as blood and urine; the assessment of enzymes profile and activities

using methods such as immunoassays, spectrophotometry and electrophoresis for diseases diagnosis. Plasma and serum biochemical evaluations are important in reptile health assessment (Arikan and Cicek, 2014).

Disease occurrences are due to the malfunctioning of molecules, chemical reactions or biochemical processes in the body. Therefore biochemical tests results are of great diagnostic value in disease surveillance, prognosis and clinical trials. The determination of haematological profile and biochemical parameters can help in the evaluation of the physiological and pathological conditions of animals,(Etim, 2014;Onasanya, Obadire, Sanni and Ibrahim, 2015).

This is because; substances measured in the serum are products of internal organs, which serve as a measure of the integrity of the organ or tissue and /or damage and organ dysfunction, such internal organs as the liver, kidney, pancreas and thyroid gland. According to Etim, (2014) and Onasanya*et al*. (2015), the changes in serum biochemical indices are indicative of ill-health and they aid in the diagnosis, severity and prognosis of disease conditions.

Table 2.3 Some Biochemical tests and their specificity

Organ functions	Enzymes
Kidney function test	Blood urea nitrogen (BUN) and creatinine
Serum electrolytes	Chloride, potassium, phosphorus, calcium, magnesium
Liver function and enzyme tests	Alanine aminotransferase (ALT)
	Aspartate aminotransferase (AST)
	Alkaline phosphatase
	Gammma-glutamyltransferase (GGT)
	Total and direct bilirubin, albumin
Pancrease	Amylase, lipase,, glucose
Serum protein test	Total protein, albumin,, alpha, beta and gamma globulins via protein electrophoresis
Muscle damage / Injury	Creatine kinase (CK), lactate dehydrogenase
Lipids profile analysis	Cholesterol, triglycerides, high-density lipoprotein (HDL), low-density lipoprotein, very low-density lipoprotein

Sources: Hamooda *et al*., (2014); Andreani *et al*., (2014)

Note: Biochemical parameters values vary from one animal species to another and may be affected by climate, nutrition and sub-clinical infections, although Sex seems not to have effects.

2.13.2 Plasma/serum enzymes

Biochemical analysis in animals is a very significant technique in veterinary medicine.This has also become the basis for early disease diagnosis in all species of animals,

including man (Hetenyi, Satorhelyi, Kovacsn and Hullar, 2016; Dissanayake, Thewarage, Manel, Rathnayake, Kularatne, Ranasinghe and Jayantha,Rajapakse, 2017). The chemical constituents of chelonians body fluids especially blood have been explored by various researchers. Some of the parameters being evaluated include; include enzyme assay, total protein, albumin, globulin, and bilirubin and so on (Hamooda, El-Mansoury, and Meha, 2014).

2.13.2.1 Alanine aminotransferase (ALT)

Alanine aminotransferaseis present in the liver. An elevated level of ALT is an indication of liver trauma or injury, toxin ingestion and metabolic disorders. Decreased levels may indicate malnutrition or starvation. Andreani, Carpene, Cannavacciuolo, Di Girolamo and Isani, (2014), reported significantly higher activities of ALT and AST in male's chelonians when compared to the females. Also, ALT was reported to have spiked up in the July to August, while decrease enzymes activities were recorded in April to June (Scope, Schwendenwein and Schauberger, 2013).

2.13.2.2 Aspartate aminotransferase (AST)

Aspartate aminotransferase (AST) was formally referred to as serum glutamic oxaloacetic transaminase (SGOT) and it is present in different body parts such as the heart, liver, lungs, pancreases and muscles. It is not a liver-specific enzyme; however, it is usually evaluated concurrently with ALT in diagnosing liver diseases or injuries. AST functions in the metabolism and elimination of nitrogen. AST levels in the blood are usually very low. An increased level may be suggestive of muscle damage, heart and liver damage, inflammation, toxin ingestion and metabolic disorders. Decreased levels of AST could be an indication of starvation or malnutrition. The AST levels in blood are directly proportional to the degree of tissue injury. It has been reported that AST levels have risen 6 to 10 hours; and has maintain same high level for 4 days, Scope *et al*., (2013), after severe tissue damage. The ratio of ALT-to-AST is important for diagnosis. Andreani *et al*., (2014), reported that, AST was higher in males than females and the reasons may be

due to aggressive behaviour associated with mating in the males. Furthermore, Hetenyi *et al*., (2016), reported a range of (30.0-280.0 IU/L of AST in male Hermann tortoises *Testudo hermanni*. Similarly a range of (10.05-346.5 IU/L) was reported by Labrade-Martagon, Mendez-Rodriguez, Gardner, Lopez- Castro and Zentero-Savin, 2010) in healthy *Chelonia mydas*

2.13.2.3 Alkaline phosphatase (ALP)

The alkaline phosphatases (ALP) are grossly spread and are linked with cell membranes. They are found in bones, bile ducts and the liver (Kido, Itagaki, Kiryu, Omiya and Ono, 2017). High levels of ALP may be suggestive of increased osteoblasts activities and this is the reason why Juveniles tend to have elevated levels of ALP because their bones are growing. Increased levels could indicate hepatic injury, occlusion of the bile ducts, or bone infection, (Xie, Xu and Liu, 2013.). Furthermore, ALP is said to play very important roles in the mammalians bone mineralisation (Price, Toroian and Chan, 2009). The ALP test may also be useful in cancer diagnosis that has metastasised into the bones. The test could also be useful in diagnosing bone malformation or to monitor treatment of bone conditions, such as avitaminosis D. (Kido *et al*., 2017).

2.13.3 Plasma/serum electrolytes

Reports of increased levels of plasma/serum albumin, calcium, phosphorus, AST, and creatinine have been recorded in healthy *Chelonia mydas* (sea turtle), Alvarez-Varas, Contardo, Heidemeyer, Forero-Rozo, Brito, Cortes, Brain, Pereira and Vianna, (2017). Similarly, evaluation of blood sera in healthy African hinge-tortoises (*Kinixys erosa*) for their blood constituents and enzyme activities hasrecordedincreased levels of plasma/serum constituents (Anderson, Socha, Gardner, Byrd and Manire, 2013; Adamovicz*et al*., 2015). Furthermore, evaluation of blood serum of sea turtles for enzyme activities reported variations in ALT, AST, lactate dehydrogenase (LDH) and creatinine kinase (CK) at rehabilitation (Anderson*et al*., 2013;Petrosky, Knoll and Innis, 2013)

2.13.3.1 Calcium

Calcium ions, total calcium and phosphorus levels in female chelonians are reported to be significantly higher than male. Also, females in active reproduction have elevated calcium levels. Calcium is also said to increase in osteolytic changes, (Falcon, Baxter, Furrer, Bauert, Hatt, Schaepman-Strub, Ozgul, Bunbury, Clauss and Hansen, 2018), it has been reported that ionised calcium-phosphorus ratios and solubility indexes can help in the diagnosis of renal diseases. Elevated solubility index of (>9) is an indication of soft tissue mineralization and hyperuricaemia (Hetenyi *et al*., 2016; Cordero,2017). The calcium and phosphorus index parameter is not significantly influenced by sex and season, (Hetenyi *et al*., 2016).

2.13.3.2 Potassium

Electrolyte imbalance is a common disease in reptiles and amphibians, which is associated with renal diseases. Diseases of the renal tubules can lead to an elevation in plasma potassium with corresponding reduction in sodium level in reptiles and amphibians. Also, hyperkalaemia and hyperphosphatemia have been reported in blood loss in reptiles (Arikan and Cicek, 2014). Furthermore, it has been reported also that fluid administration in severely dehydrated reptiles can result in a fatal metabolic anomaly called "re-feeding syndrome". This condition is characterized by decreased potassium and phosphorus levels (Arikan and Cicek, 2014; Spencer *et al*., 2018).

2.13.3.3 Sodium

The concentration of sodium is higher in sea turtles than in other chelonians or reptiles. They possess salt glands which help to moderate the amount of Na, K, and chloride in circulation (Arikan and Cicek, 2014). Besides, sodium levels may increase when water intake is reduced or following condition of severe water loss like diarrhoea, vomiting (Arikan and Cicek, 2014; Hetenyi *et al*., 2016; Kido *et al*., 2017).

2.13.3.4 Chlorine

The level of Sodium chloride and its bicarbonate account for 80% of osmotic constituents of reptiles' plasma. Snake and lizards have been reported to have chloride level of about

115mmol/L, while in chelonians plasma sodium and chloride attain highest value in winter (Yu, Yang, Chiu and Chi, 2013).

2.13.3.5 Phosphorus

Research conducted on concentrations of phosphorus in chelonians, reported that elevated levels of phosphorus have been found in metabolic bone diseases(MBD) of reptiles, such as osteodystrophy and osteoporosis, also in chronic kidney disease, while reducing levels have been associated with anorexia or starvation (Hetenyi *et al*., 2016; Cordero, 2017). Also, phosphorus levels in Mediterranean species of tortoises have is said not to exceed 1.7mmol/l in healthy species of chelonians (Arikan and Cicek, 2014; Hetenyi *et al*., 2016).

2.13.3.6 Total protein

Evaluation of total protein (TP) in animals is a common laboratory procedure in veterinary practices, (Tothova, Oskar and Kovac, 2016). The total protein is attributed to the highest proportion of the total solutes of the blood of any animal. Fibrinogen one of the plasma proteins is usually missing in the serum and it constitutes 3-6% of the total protein. Adamovicz, Bronson, Kelvin and Deem, (2015), reported that the increase values of TP in female turtles examined in Tennessee, Indiana, was related to the high vitellogenic activities of female turtles in summer. Similarly, total protein (2.4 to 6.1 g/dL) value recorded by Andreani *et al*., (2014) in their study is similar to those reported (2.2 to 5.5 g/dL) in the desert tortoise *G radiata* by Zaias, Norton, Fickel, Spratt, Altman and Cray, (2006). The total plasma proteins values in reptiles, especially chelonians are reported to be generally lower other mammals, (Drake, Bowen, Lewison, Esque, Nussear, Braun, Waters and Miles, 2017). Base on solubility properties, serum proteins can be categorized into two groups; albumin and globulin.

2.13.3.7Albumin

Albumin is a major component of the plasma TP, making up about 60% and has a low molecular weight of about 69,000. Albumin is synthesized mainly by the liver. It functions as ions carrier and other water-soluble substances like bilirubin, vitamins, hormones etc. Turtles sampled from Tabasquillo recorded decrease total protein as well as albumin; increase bilirubin as well as calcium at rainy season, while in

contrast,Paltrinieri *et al*.(2014) reported higher values of total protein, albumin, bilirubin as well as calcium at winter in certainAmerican river turtles (*Dermatemys mawii*) sampled in Tabasco, Mexico. Albumin value tends to decrease during dehydration, hepatopathy; which can cause decrease protein synthesis. Biochemical indices for loss of body fluid may manifest as increased uric acid and /or PCV and total protein, (Arizza*et al*., 2014).

2.13.3.8 Globulin

Globulin is one of plasma protein produced by the liver, which function in regulating the circulatory system. It functions in proteins transportation across the lipoproteins cell membranes, also in blood clotting. Globulin can transform to plasma cells when antibodies are a deficiency in the circulatory system. Globulin levels are measured against the levels of albumin in the blood. The proportion of globulin to albumin may decrease or increase. Low values of globulin could be a sign of disease conditions such as kidney disease, liver malfunction, abdominal disease, inflammation of the bowel (IBD), acute blood loss, agammaglobulinemia and hypogammaglobulinemia. In other words, elevated level of globulin may indicate leukaemia, osteoblastic diseases, autoimmunity disorders, renal diseases or a chronic viral or bacterial disease. Omonona *et al*., (2011), reported serum globulin concentration range of (2.63-3.36 g/dL) in the African Side neck turtle (*Pelusios sinuatus*), their report agrees with the studies of Macrelli, Marcello and Letizia, (2013),
on Hermann's Tortoises (*Testudo hermanni*).

Table.2.4 Plasma Biochemical values of some land tortoises

	Radiated tortoise	**Red-footed tortoise**	**India star tortoise**	**Desert tortoise**	**African hinged-backed**
	Astrochelys radiate	***Chelonoidis carbonaria***	***Geochelone elegans***	***Gopherus agassizzi***	***Kinixys erosa***
ALP (U/I	72-392	84(39-173)	174(36-379)	43-176	
AST	25-392	230(97-616	87(12-296)	41-106	N.A
ALT (U/L)	18(4-63)	18(4-63)	5(0-15)	21(0-66)	
Ca+(mg/dL	8.6-18	1.7(9.5-15.8)	12.7(9.5-15.8)	9.3-14.7	
Cl (mEq/L	91-112	100(89-119)	104(90-112)	94-112	98(±0.19
Phosphorus	2.5-7	n3.8(1.8-5.8)	4.1(2.7-5.7)	1.0-6.3	N.A
Potassium	3.1-5.8	5.4(3.7-6.8)	5.2(3.9-5.9)	3.5-4.7	4.87(±0.06)
Sodium	121-146	131(116-155)	128(122-133)	122-139	122(±0.7)
Total Protein	3-6.6	5.2(3.3-7.4)	4.7(3.9-5.9)	3.0-4.6	
Albumin	0.6-2.4	1.9(1.3-3.4)	2.1(1.5-3.1)	1.2-2.2	
Globulin	1.4-.3.2	3.1(2-5.3)	2.7(2.3-3.1)	1.2-2.6	

Sources: Gibbons, Klaphake and Carpenter, (2013).

CHAPTER THREE
MATERIALS AND METHODS

3.1 STUDY ONE

3.1.1 Prevalence of Haemogregarines in *Kinixys belliana a*nd *Kinixys homeana* in Ibadan, Oyo State

3.1.2 Equipment, materials and consumable Reagents

Equipment included a digital microscope, autoclave, biosafety and cabinet, -40^{o}C deep freezer. Materials were glass slides and cover slides, blotting paper, markers, pencils, bottles, glass slides, face mask, boots, ice pack, transport flask, Reagents were Giemsa/Wright stains, methanol.

3.1.3 Sample size and Ethical approval

The sample size was determined using the formula;

$$n = \frac{1.96^2 Pexp\ (1 - Pexp)}{d^2}$$

(Thrusfield, 2007). Where,

n = Sample size

Pep = expected prevalence (this was taken as 25%) the prevalence of haemogregarine infection reported in *Geochelone pardalis* (leopard tortoises) by Cook *et al*., (2009) d= absolute precision 8.0%.

1.962 x 0.25 (1- 0.75)/0.08x0.08 = 112.56

Percentage attrition 7.0% = 7.88 (112.56+7.88) = 120. 44

The total sample of 120

Clinically healthy animals maintained in a temperature-controlled, nutrition-controlled laboratory setting). At the minimum, 40 individuals are suggested for statistically robust results (Thrall, Dale, Baker and Lassen, 2004;Geffre, Friedrichs, Harr, Concordet, Trumel and Braun, 2009).

The ethical approval for this study was obtained from the University of Ibadan Animal Care and Use Research Ethics Committee (UI-ACUREC), with approval number: UI-ACUREC/App/10/2016/01

3.1.4 Study type and experimental animals

This was a cross-sectional study conducted on *K. belliana* and *K. homeana* available at the central herb/wildlife market at Bode, Molete in Ibadan, Oyo State (Plates 3.1). Bode market was selected for the study, Oduntan, Akinyemi, Ojo, Ogunyode and Adesina, (2012), about 72% of the traders in the markets were engaged in the wildlife trade. In addition, the market is a major hub which attracts traders, marketers of traditional medicines and wild animals from within and across states in western Nigeria into the capital city of Oyo state. The sampled tortoises captured from the wild were sourced from Bode Market in Ibadan. They were supplied by the traders in batches of ten (10) every month. The sampling was done over a period of one year to cover both rainy and dry seasons.

3.1.5 Eligibility criteria for the selection of animals

Animals were selected based on the length of their longitudinal axis- straight carapace length (SCL), a straight line measurement is taken from the cranial aspect of the nuchal scutes to caudal scutes. Tortoises with SCL of less than 10cm were excluded for ease of sex determination.

3.1.6 Tortoise housing and feeding

The tortoiseswere housed upon arrival in a wooden vivarium constructed for housing the tortoises. The vivarium dimensions: height 250cm, width 80cm and length 350cm. The vivarium was further demarcated into smaller compartments; dimensions; length 80cm breadth 80cm and width 80cm (Plate 3.2). Each animal was housed in each compartment to avoid the spread of ectoparasites. The tortoises were fed vegetables; water leaves *Talinum triangulare*, fruits; mango, pawpaw and pineapples. Water was given *ad libidum.*

Plate 3.1 BODE MARKET Inset arrowed Researcher and the focal person

3.1.7 Identification of sexes of tortoises

The identification number was assigned to each animal using an indelible marker and nail varnish, the numbers were written on both carapace and plastron (Plates 3.3). They were assigned identification numbers inscribed as GC-1 to GC-120. Sex identification was based on the concavity of the plastron, the length of the tail, position and location of the vent to the margin of both carapace and plastron caudally, as described by Stuart, Chanson, Cox, Young, Rodrigues, Fischmannand Waller, (2004). The tortoise's species were determined using field guides by (Boycott and Bourquin, 2000; Rose and Judd, 2014). Morphometric parameters such as: straight carapace length, width (W), straight plastron length, height (H), of animals were determined according to Willemsen *et al*. (2002. Besides, the body weight of the tortoises were evaluated with a weighing scale (Camry Emperor^{R}China) and recorded

3.1.8 Procedure for blood collection

Blood collection was done via the sub-carapacial sinus, 3ml of blood was withdrawn (Plate 3.2) from each tortoise according to Perpinanm(2017), depending on the tortoise size, using a 25 guage needle and syringe. 1ml of the blood was withdrawn each into three lithium heparin tubes; 1ml for haematology and plasma biochemical studies another 1ml for enzyme analysis and another 1ml of blood samples collected and stored at -20^{o}C for molecular work.

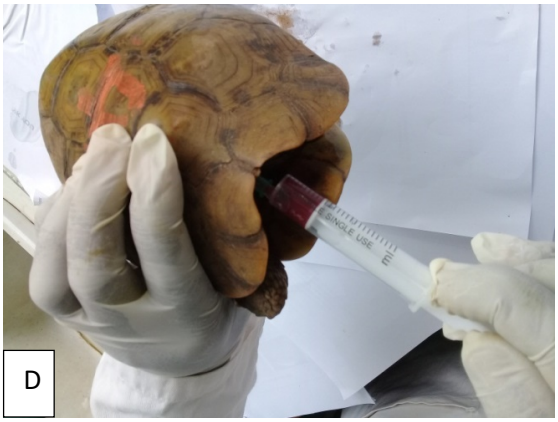


Plate 3.2 (A) Wooden vivarium, (B) and (C) Labeling Left to right Carapace and Plastron right of *Kinixys belliana* with an indelible marker (D) Blood collection via the sub-carapacial sinus of a *Kinixys belliana*

3.1.9 Slides preparation and blood examination

Thin smears were prepared at the vivarium according to standard method as described by Houwen (2000) and Cook *et al*. 2014; Cook, Netherlands and Smit 2015). Slides were first air-dried, labeled with an HB pencil and moved to the laboratory, where they were stained by first fixing for 10 minutes in methanol and stained with Giemsa's stain (Sigma-Aldrich, Steinheim, Germany) for 30 minutes.Slides were rinsed under a running tap, drained, and arranged vertically on a slide rack to dry naturally. They were initially screened with oil immersion at x100 using Olympus BX41 microscope. Thereafter images captured were captured using a digital camera. Parasites prevalence and abundance (no of RBCs affected and number of parasites affecting each RBC) were estimated for each tortoise and the level of parasitaemia was enumerated as numbers of parasitised RBCs per 100 RBCs. Four slides were prepared per tortoise. 10^4 RBCs were enumerated per slides according to the standard method of Garcia (2001) described by Cook, Smit and Davies(2010) and Cook *et al*. 2015).

3.1.10 Data analysis

Data were enumerated using an online statistical tool https://www.socscistatistics.com/tests/Default.aspx. Results were presented using descriptive statistics. A statistical difference in proportion for sex, species and seasons was done usingPearson's chi-square test (X^2). Statistical differences in the means for sex, species and seasons were done using the Studentisedt-test. The level of significance was taken as 5 %.

3.2 STUDY TWO

3.2.1 Molecular detection of haemogregarines in *Kinixys belliana* and *Kinixys homeana* using Polymerase chain reaction (PCR)

3.2.2 Equipment, materials and consumable Reagents

ZR Genomic DNATM – Tissues Mini-Prep Zymo Research®, PCR Master Mix (2X) (One Tag Quick –Load^{R}, Cat. M0486S), 0.05 U/µL*Taq*DNA polymerase, Buffer, 0.4nM MgCl2, Buffer, 4mM MgCl2, 0.4 mM each of dNTP; (dATP, dCTP, dGTP and dTTP), Nuclease-free water (Biolabs), Positive control (This is ths positive sample obtained using the HEP primers during the pilot study), Negative control (Nuclease-free water), Primers sets, Ethanol (96–100%), Benchtop Centrifuge Centurion Scientific United Kingdom , Adjustable pipettes, Sterile, DNase, RNase-free pipette tips, Vortex (VMR Mixer Touch Vortex Henry Troemnar USA), Microcentrifuge tubes, Thermocycler (Thermo Fisher Scientific Vantag Finland), Water bath (Uniscope water bath Surgifriend Medicals), Electrophoretic Machine (SciePlasHu 25-Max-Plus Standard England), UV transilluminator (Spectrmoline Vision, USA).

3.2.3 Sample size

The sample size is similar to the design in section 3.1.3

3.2.4 Blood sample collection

1ml of whole blood collected from the sub-carapacial sinus of each tortoise into lithium heparin tube and stored at -20^{o}C in study one as described by Perpinan, (2017) was used.

3.2.5 DNA extraction

DNA extraction was accomplished using commercial Kit (ZR Genomic DNA TM Tissue Mini-Prep Kit) by Zymo Research Corporation, following manufacturer's instruction except for some slight modifications to facilitate proper dissolution of the nucleated RBCs of chelonians.

3.2.5.1 DNA extraction protocol

The DNA extraction was performed by pipetting 100 µl of blood each into Eppendorf tubes, followed by the 95 µl digestion buffer, 5 µl of proteinase K the mixture was thoroughly mixed by vortexing with VMR mixer Touch Vortex. Henry Troemnar, (USA). Mixtures were incubated at 55^{o}C for 20 minutes in water bath (Uniscope Water Bath

Surgifriend, Medicals). The later was followed by the addition of 700 µl of Genomic Lysis buffer; mixture was mixed thoroughly by vortexing. The slight modification became necessary due to the nucleated nature of chelonians RBCs which did not dissolve properly, protocol was slightly modified by a further incubation of 55^{o}C for another 40 minutes, until the blood cells were properly dissolved and vortexed. The mixture was pipetted each separately into the Zymo-SpinTM IIC column placed into a centrifuge (Centurion Scientific CSD-XT5 Chef Centrifuge, West Sussex P018 9JL, United Kingdom) and spun for 1 minute at 10,000 g. Filtrates were discarded, while the spin columns were moved each into another clean tube each, 200 µl DNA Pre-wash buffer was added into each tube and centrifuged at10, 000 g for 1 minute. Filtrate was discarded and 400 µl gDNA wash buffer was pipetted into each tube and again centrifuged at 10,000 g for 1 minute. The filtrate was again discarded and spin column assembled without any addition and again spun at 8,000 g for 1 minute to remove any residual gDNA buffer. Filtrate was discarded and the spin column each was placed over new sterile and labeled Eppendorf tube of (1.5 ml capacity). Then 50 µl DNA elution buffer which has been pre-equilibrated by heating for at 60^{o}C for 5 minutes was pipetted into the spin column placed over Eppendorf tubes to increase concentration and yield of eluted DNA. The eluted DNA each was further incubated for another 5 minutes at room temperature. Finally tubes containing extracted DNA in spin columns were centrifuged at 14,000 g for 30 seconds for the elution of the parasite DNA. Eluted DNA amplicons were stored at -20^{o}C for further molecular work.

3.2.6The Polymerase chain reaction

Polymerase chain reaction (PCR) was undertaken in a thermocycler (Bio-RadC1000 Touch™ Hempstead, UK). The PCR reactions volume of 50 µl was prepared each, using the following cocktails; 25 µl (2x Mastermix containing 0.05 U/µl Taq DNA/PCR buffer, 0.4 mM of each dNTP, and 4 mMMgCl2), 2.5 µl of each forward and reverse primer of concentrations 10 pmols, and at 4 µl DNA and 16 µl PCR grade nuclease-free water (Thermo Scientific).

PCR was carried out using three pairs of primers, this is facilitate the isolation and charcterisation of the different species of haemogregarines that may be circulating in African hinge-back tortoises.

(a) Apicomplexan specific parasite primer set 4558F: 5^{1}GCTAATACATGAGCAAAATC TCAA$3^{1}$and 2733R: 5^{1} CGGAATTAACCAGACAAAT 3^{1} according to Mathew, vanden Bussche, Ewing, Malayer, Latha and Panciera, (2000), this primer sets is targeted towards a long DNA fragment of approximately 1120 bp of the18S RNA gene of haemogrerines. PCR conditions were as follow: Initial denaturation at 94°C for 3 minutes followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 2 minutes extension at 72°C for 2 minutes then final extension at 72°C for 10 minutes.

(b) Hemo primer set: HEMO1:5^{1}TATTTGGTTTTAAGAACTAACTAATTTTATGATT G 3^{1} and HEMO2 5^{1} CTTCTCCTTCCTTTAAGTGATAAGGTTCAC3^{1}, (Perkin and Keller, 2001).This primer targets approximately 900 bp of the 18S RNA genes fragment of the haemogregarines. PCR protocols were as follows; Initial denaturation at 94°C for 4 minutes followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 52°C for 30 seconds extension at 72°C for 1 minute then final extension at 72°C for 10 minutes.

(c) Hepatozoon specific primer set: HepF 300 5^{1} GTTTCTGACCTATCAGCTTTC 31 and Hep R 900 51CAAATCTAAGAATTTCCACCT 3^{1} according toUjvari, Madsen and Olsson, (2004). The primer targets approximately 600 bp of 18S RNA genes haemogregarines. The PCR conditions were as follows; Initial denaturation at 94°C for 4 minutes followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 45°C for 30 seconds extension at 72°C for 1 minute then final extension at 72°C for 10 minutes.

3.2.7 PCR products gel electrophoresis

Amplified PCR products were run on agarose gel following the PCR. The gel casting tray was sealed with paper tape on each side. 1.2 g of molecular grade agarose powder (1.2 %) (CSL-AG 100 by CS Cleaver Scientific Ltd, United Kingdom) was weighed using a digital balance (Scout-Pro, Chaus Cooperation, NJ, USA) and 100 ml of 1xTAE buffer (50x stock solution of TAE containing 242 g Tris base dissolved in 750 mL deionized acetic acid and 100 ml of 0.5 M EDTA at P^{H} of 8.0 and the final solution was made up to 1 litre by the addition of distil water). The agarose solution was microwaved in a domestic microwave (Haier Thermocool Microwave oven, HTMO-2380MG, Quingdao Shandong, China) for about 3 minutes, while the mixture was being swirled intermittently every 15 seconds until agarose powder melted completely. Mixture was cooled and 4 µl ethidium bromide was added to gel, swirled and poured into the gel tray to which gel combs have

been inserted (to make wells on the gel) allowed to set by solidification. After setting, the paper tapes and gel combs were removed from the solid gel formed inside the gel caster, while the caster containing the solidified gel with its wells were immersed into an Electrophoretic tank (SCIE-PLAS MAXI PLUS STANDARD, Cambridge Science Park, England) which has been filled with 1x TAE buffer to the level indicated on the tank. For each PCR product, 5 µl was added to 5 µl loading dye and pipetted into the wells. The molecular ladder was added to one of the free wells. And two other wells one containing negative control (PCR product which contained no parasite DNA but nuclease-free water) and the other a positive control (PCR product containing the parasite DNA). These were loaded into their respective wells and electrophoresed for 1 hour at 110 V. The gel was then visualized using UV transilluminator (Spectrmoline Vision, West Ruby, USA), and captured with digital camera. Clear and sharp gel products were cut and purified for sequencing in subsequent studies.

3.2.8 Sequencing of PCR products containing amplified DNA

Purified PCR products that which have the best band amplicons of approximately 600 bp usable DNA HepF 300 and HepR 900 primers were purified and sequenced at the International Institute for Tropical Agriculture (IITA) Bioscience Laboratory in Ibadan, Oyo state, Nigeria. The Sequencing reaction was carried out with Hep primers using standard methods (Applied Biosystems^{TM}). The procedure was accomplished with ABI PRISM 377 Sequencer. Ambiguities were clarified visually with corresponding ABI chromatograms. The sequences generated from study were blasted and aligned with published 18Sribosomal sequences downloaded from the National Center for Biotechnology Information (NCBI). Table 5.1. Eighteen *Hepatozoon species* belonging to chelonians, other reptiles, amphibians, mammals and ticks were used in this study. The resulting sequences were compared with related 18S RNA available from GeneBank using the BLAST programme for confirmation.

3.2.9 Phylogenetic tree construction

Phylogenetic analyses were performed using MEGA7 computer package according to Kumar,Stecher and Tamura (2016). Published sequences derived from apicomplexan DNA with high similarity to the present study species downloaded from the NCBI website (2019) along with sequences originating from the current project's PCR products. These were blasted into BioEdit Sequence Alignment (2019). All retrieved sequences excluding the gaps were positioned using the evolutionary records. They were then extrapolated with Maximum Composite Likelihood (MCL) techniques described by Tamura and Nei mode (1993). The tree with greatest log likelihood (-4906.15) was displayed close to the branches. Evolutionary tree(s) for root search was achieved using the BioNeighbour Joining algorithms to a matrix of pairwise distances. Tree configuration with highest log likelihood value was selected. The final breakdown involved 20 sequences. Codons positions 1st+2nd+3rd+Noncoding were utilised. 1760 positions were present on the concluding datase.

3.2.10 Data analysis

Data were analysed using an online statistical tool https://www.socscistatistics.com/tests/Default.aspx. Results were presented using descriptive statistics. Parasites abundance and intensities per sex, species and seasons were determined with the Studentised t-test. Sex, seasons and species prevalence were evaluated using the Pearson's chi-square test (X^2). The level of significance was taken as 5 %

3.3 STUDY THREE

3.3.1 Determination of haematology, plasma biochemistry and enzyme activities of haemogregarine positive African hinge-back tortoises (K*inixys belliana* and *Kinixys homeana*)

3.3.2 Equipment, materials and consumable Reagents

Refrigerator, Micro haematocrit centrifuge, Haematocrit reader Haematocytometer with improved Neubauer ruling, Capillary tubes, Capillary tube reader, blood sample, set of micropipettes, glass Pasteur pipette, plasterseal, lithium heparin, test tubes and racks, beaker, centrifuge, biosafety and cabinet, -40^{o}C, deep freezer, Natt- Herrick's solution, Whatman paper, RBCs Dilution Pipette, Microscope slides, Coverslips, Giemsa stain, Mounting fluid, set of micropipettes, colorimetercuvette.

Other materials used were forceps, blotting paper, markers, pencils, bottles, face mask, boots, ice pack and transport flask. Reagent Kits for plasma/serum biochemistry and enzyme assay, Randox Laboratories Limited, Country Antrim, BT29 4QY the United Kingdom and TECO diagnostics 1268 N. Lakeview AVE, Anaheim, U.S.A) calculator and writing materials.

3.3.3 Blood samples collection

The sample size is similar to sections 3.1.3

3.3.4 Method for PCV determination

The Packed Cell Volume (PCV) was evaluated using standard method according to International Council for Standardisation in Haematology (ICSH), (2001). Heparinized whole blood was suctioned into microhaematocrit tube (NRIS Vitrex Medical A/S Herlev, Denmark) with one end sealed with plasticine and centrifuged at 12,000 g for 5 minutes using a haematocrit centrifuge (Surgifield Instrument SH120-1, England) The haematocrit was read on a micro-capillary reader to measure packed cell volume, two measurements were taken and the average used for data analysis.

Haemoglobin concentration was evaluated for each sample using standard methods of Clinical and Laboratory Standard Institute (CLSI) 2001.

The remaining blood samples were stored in lithium heparinized microtubes (2-mL Axiom Equator Medics, China), and stored at 4^{o}C. Aliquot was used for RBCs counts, leukocytes

as well as thrombocytes counts. Analysis was performed within 24 hours after blood collection.

3.3.5 Blood cells evaluation

Total erythrocytes, and leukocytes counts were evaluated within 24 hours by the direct method using Natt -Herrick's stain (Natt-Henrrick-TICR 1:200 Plus) Germany and a Neubauer haemocytometer (Canemco Inc., Quebec, Canada) as described by Arikan and Cicek, (2011). Conventional technique was used to evaluate total leukocyte and differential leukocytes counts using previously established techniques, (Stacy *et al*. 2011). Automated cell counts procedure was ruled out due to the nucleated structure of reptile's leucocytes and erythrocytes, (Nardinin *et al*., 2013).

3.3.5.1 Natt-Herricks method

This method allows for direct counting of the total erythrocyte and total leukocyte counts simultaneously from the same charged haemocytometer because same dilution 1:200 ratio is used for both cell types.

The Natt-Herrick's stain solution was filtered using a Whatman filter paper to remove any precipitate. Using a micropipette 5 µl of the lithium-heparinised blood was mixed with 995µl Natt-Herrick's solution to give a 1:200, as directed by manufacturer. The diluted blood was allowed to mix for two minutes before discharging into the haemocytometer counting chamber. This was allowed to stand for 3 minutes to enable the blood cells to settle and charge.

3.3.5.2 Total erythrocyte count

The haemocytometer was mounted under a digital microscope at 40X magnification, total erythrocytes were counted on the four edges and middle squares of the large centre cell. Cells overlaying top and left border were enumerated, while those overlapping below or right borders were excluded. Total number of erythrocytes counted was multiplied by 10,000 to attain the gross or total erythrocyte counts per/µl.

3.3.5.3 Leukocyte count

Total leukocyte count was enumerated by counting every leukocyte seen on nine large ruled squares on the haemocytometer. Cells that overlaid upper and left corner were enumerated, while those overlaying below or right corner were excluded from counted.

Total leukocytes counted were evaluated thus:

Total leucocytes/µl = (total leukocytes counted on 9 squares + 10% of total leucocytes) X 200.

3.3.5.4 Differential white blood cell counts

Differential leucocytes counts were enumerated by counting 200 different leucocytes on peripheral smear proportionately.

3.3.6 Plasma biochemical analysis

3.3.6.1 Blood plasma constituents

Plasma constituents were measured following the separation of plasma from the whole blood sample collected into lithium heparin tube by centrifugation at 10,000g for 5 minutes using (Bench top centrifuge Centurion Scientific ESD72A, United Kingdom). Plasma was used for biochemical and enzymes activities work, because clot formation in reptile blood is variable, besides the time required for clot formation can cause alteration in plasma constituents, (Bolten, *et al*., 1992). A standard biochemical profile was determined including total protein, albumin, globulin, calcium, phosphate, sodium, potassium and chloride. Activities of enzymes such Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and Alkaline phosphatase (ALP) were determined. The selected analytes were evaluated using commercial kits (Randox Laboratories Limited, Country Antrim, BT29 4QY United Kingdom and TECO diagnostics Anaheim, USA). The end products were later analysed with a spectrophotometer (Spectrumlab 23A, Zhejiang, China).

3.3.6.2 Total protein (TP)

The TP kit contained Biuret reagent (R1), Blank reagent (R2) and CAL standard. The R1 and R2 reagents were diluted each with 400ml of double distil water (ddH_2O) and stored at +2 to +25^oC before use. The Spectrophotometer was recalibrated with fresh 0.05 ml ddH_2O in a cuvette. The TP button was selected and the blank test was run.

Procedure: The blank reagent was made by pipetting 0.01 ml ddH2O into a cuvette and 0.5ml of R1 and mixed properly. The standard reagent was made by pipetting 0.01ml of Standard CAL in a cuvette and 0.5 ml of R1 and mixed properly. The test sample was also made by pipetting 0.01ml plasma into a cuvette and 0.5ml of R2 and mixed properly.

Lastly, the sample blank was prepared by pipetting 0.01 ml plasma into a cuvette and 0.5 ml of R2 and mixed properly. The mixtures were incubated at +20^{o}C to +25^{o}C for 30 minutes before reading results on the spectrophotometer, at a wavelength of 546 nm.

3.3.6.3 Albumin and Globulin

The Albumin (ALB) kit contained bromocresol (BCG) concentrate (R1) and CAL standard. The R1 reagent was diluted with 87ml distil water dH2O and stored at +15^{o}C to +25^{o}C before use. The CAL standard was supplied as ready to use. The Spectrophotometer was recalibrated with fresh 0.05ml ddH_2O in a cuvette. The ALB button was selected and the blank test was run.

Procedure: The Reagent blank was prepared by pipetting 3 µl ddH2O into a cuvette and 1000 µl of R1, mixture was mixed properly. The standard was prepared by pipetting 3 µl of the standard into a cuvette and 1000 µl of R1 and mixture mixed properly. The test sample was prepared by pipetting 3 µl test sample plasma into a cuvette and 1000 µl of R1 and mixed properly. The reaction mixtures were incubated for 10 minutes at 37^{o}C before reading results on the spectrophotometer, at a wavelength of 630 nm. The evaluation of total protein made the determination of globulin indirectly possible, through the subtraction of albumin value from total protein.

3.3.6.4 Calcium

The Ca kit contained standard calcium of concentration 2.5 mmol/l labeled (R1), Buffer made up of 2-amino-2-methyl-propan-1-ol 3.5 mmol/l and labeled (R2), chromogen containing O-cresolphthlein complexon 0.16mmol/l, 8-hydroxyquinoline 6-89 mmol/l, hydrochloric acid 60 mmol/l labeled (R3) and EDTA 150 mmol/l labeled (R4), all solutions were supplied as ready to use.

Procedure: The working mixture was prepared by mixing equal volume of buffer and chromogen depending on the number of samples to assay and kept at +25^{o}C before use. Blank reagent was made by pipetting 25 µl distilled water into a cuvette, 25 µl of the standard into a second cuvette and 25 µl of test sample into the third cuvette. 1.0 ml working sample was pipetted into each tube and mixed properly. Mixtures were incubated at 37^{o}C for 30 minutes. Sample absorbance, standard absorbance were later determined using the spectrophotometer at wavelength 570 nm. Calcium in mg/dl was calculated for

samples by dividing sample absorbance by standard absorbance and multiplying the result by a factor of 10.

3.3.6.5 Potassium

The K kit contained precipitating reagent trichloroacetic acid 0.3 mol/l (R1), sodium tetraphenylboron 0.2 mol/l (R2), sodium hydroxide 2.0 mol/l (R3) and standard solution 5.0 mol/l, supplied as ready to use.

Procedure: Into two test tubes labeled macro and micro were pipetted 100 µl of test sample into macro and 50 µl into micro tubes, thereafter 1000 µl and 500 µl of precipitating reagent into macro and micro tubes respectively. Mixtures were mixed properly and centrifuged at 10,000 g for 10 minutes. Supernatants were separated into clean tubes and labeled accordingly. Working reagent was prepared by the addition of equal volume of R2 and R3 and kept at +25^{o}C before use. Into four cuvettes labeled standard1, macro sample, standard 2 and micro sample, were pipetted volumes of working solution; 200 µl, 200 µl, 100 µl, 100 µl respectively. Followed by 20 µl and 10 µl of standard solution added into the middle of cuvettes labeled standard 1 and 2 respectively to make the blank reagents. Then 20 µl, 10 µl of supernatants pipetted from cuvettes labeled macro and micro were added into the middle of cuvettes labeled macro and micro respectively. The mixtures were incubated for 5 minutes at 25^{o}C. Thereafter samples absorbance as well as standard absorbance was read against the blank reagent on the spectrophotometer, at 578 nm a wavelength. Concentrations of Potassium in mg/dl were calculated for samples by dividing sample absorbance by standard absorbance and multiplying the result by a factor of 5.

3.3.6.6 Sodium

The Na+ kit contained filtrate reagent (R1) containing uranyl acetate 2.1 mM and magnesium acetate 20 mM ethyl alcohol, acid reagent (R2) contains dilute acetic acid. Sodium colour reagent (R3) containing potassium ferrocyanide, non-reactive stabilizer, filters, then a standard sodium chloride solution 150 mEq/L (R4). Reagents were supplied ready for use and stored at 25^{o}C.

Procedure: Filtration of reagent was performed into tubes that were labeled; blank, standard, control and test sample. Then, 1.0 ml R1 was pipetted into tube respectively, followed by the addition of 50 µl test sample into each tube except blank to which 50 µl

distilled water was added. Solutions were mixed properly by shaking for 3 minutes, centrifuged at 10,000 g for 10 minutes. Supernatants were separated and labeled accordingly. To each corresponding labeled cuvettes, 1.0 ml R2, and 50 µl supernatants was added and mixed properly, followed by the addition of 50 µl R3 and mixing of mixtures. Spectrophotometer was recalibrated with distil-water at 550 nm and the absorbance of each cuvette was read and recorded. Concentrations of sodium in mEq/L were calculated for samples using the formula below.

Conc. of Sample (mEq/L) =

(Absorbence of Blank- Absorbence of Sample X Concentration of standard (mEq/L)

(Absorbence of Blank - Absorbence of Standard.

3.3.6.7 Chloride

The Cl^- kit contained chlorine reagent with active ingredients; mercuric nitrate 0.058 mM, mercuric thiocyanate 1.75 mM, mercuric chloride 0.74 mM, and ferric nitrate 22.3 mM and non-reactive ingredients in dilute acid and methanol (R1). Chloride calibrator (R2) which contains sodium chloride 100 mEq/L. All reagents were supplied as ready to use and were stored at 25^oC protected from direct sunlight.

Procedure: Three tubes labeled; blank, calibrator, and test were added respectively. 1.5 ml of R1, followed by the addition of 10 µl R2 into the tube labeled calibrator. Test sample of 10 µl was added into test sample and calibrator respectively, followed by the addition of 10 µl distil- water into blank. Mixtures were incubated for 5 minutes at 25^oC. Spectrophotometer was recalibrated using the blank reagent. At a wavelength of 520 nm the absorbance of each sample was read and recorded. Concentrations of Sodium in mEq/L were calculated for samples using the formula below.

(Abs of test sample X Conc of Calibrator) = Conc. of Chloride (mEq/L)

(Abs of the calibrator.

3.3.6.8 Phosphorus

The P^- kit contained blank reagent (R1) made of sulphuric acid 0.36 mol/l, sodium chloride 154 mmol/l. The molybdate reagent (R2) containing ammonium molybdate concentration 3.5 mmol/l, sulphuric acid concentration 0.6 mol/l, sodium chloride of concentration 154 mmol/l. Cal Standard (R3) which contains potassium phosphate.

Protocol: Working Reagent was prepared by the addition of one bottle of R1 with another bottle of R2 to make a volume of 300 ml working reagent, depending on the number of samples to assay. The mixture was stored at +25^{o}C. Three test tubes labeled; blank, standard and sample were used, into each corresponding tube, 10 µl distilled water; 10 µl standard and 1 µl of sample were pipetted. Thereafter, 1.0 ml working reagent was added to each labeled test tube and mixed properly. The mixtures were incubated at 37^{o}C for 5 minutes. Finally, sample and standard absorbance were evaluated against the blank reagent in the spectrophotometer, at 540 nm. The concentration of phosphorus in mg/dl was calculated by dividing sample absorbance by standard absorbance.

3.3.7 Plasma enzymes activities

3.3.7.1 Aspartate aminotransferase (AST)

The AST kit contained buffer (R1) made of phosphate buffer, L - aspartate and (R2) containing 2,4dinitrophenyihydrazine, α oxoglutarate and 0.4 mol/l sodium hydroxide was supplied. The R1 and R2 were supplied as ready to use.

Procedure: The sample blank was made by adding 0.5 ml R1 into a cuvette (A), followed by the addition of 0.1 ml sample and 0.5 ml R1 into another cuvette (B) both mixtures were mixed properly and allowed to incubate at 37^{o}C for 30 minutes. Then into tube (A), 0.5 ml of R2 and 0.1 ml of the test sample were pipetted and thoroughly mixed. Into B 0.5 ml of R2 was added and mixed properly. Thereafter, A and B were incubated for 20 minutes at 25^{o}C and 5.0 ml sodium hydroxide was pipetted into each of the tube A and B and mixed properly. The absorbance of B (sample test) was read against A (sample blank) after 5 minutes at a wavelength of 546 nm and values recorded.

3.3.7.2 Alanine aminotransferase (ALT)

The ALT kit contain buffer (R1) (phosphate buffer, L-aspartate, and 2, 4-dinitrophenyihydrazine, α-oxoglutarate (R2) and 0.4 mol/l sodium hydroxide and distil water were supplied. R1 and R2 were supplied as ready to use.

Procedure: The reagent blank was prepared by pipetting 0.5 ml of R1 and 0.1 ml of distil water into a cuvette (A) and mixture was mixed properly. Into another cuvette (B) 0.1 ml of sample and 0.5 ml of R1 were added and mixed properly. Tubes were allowed to incubate at 37^{o}C for 30 minutes. Thereafter into A and B, 0.5 ml of R2 was added each, mixed properly and incubated at 25^{o}C for 20 minutes. Finally, 5.0 ml sodium hydroxide

was pipetted into each tube and mixed properly. Sample test (B) absorbance was read against blank reagent (A) after 5 minutes at a wavelength of 546 nm and values were recorded.

3.3.7.3 Alkaline phosphatase (ALP)

The ALP kit contain buffer (R1a) diethanolamine 1mol/l, MgCl2 0.5 mmol/l supplied as ready to use and p-nitrophenylphosphate 10 mmol/l (R1b) supplied as 3 vials of the substrate.

Procedure: The working solution was prepared by mixing appropriate volume of R1a pipetted into each vial of the substrate R1b according to the manufacturer's instructions. Mixtures were mixed very well and kept at three different temperatures; 25^{o}C, 30^{o}C and 37^{o}C. Afterwards, 3.00 ml, 1.00 ml and 0.50 ml of test sample was pipetted into three cuvettes labeled macro, semi-micro and micro respectively. Mixtures were also kept at temperatures 25^{o}C, 30^{o}C and 37^{o}C respectively. The mixtures were mixed properly and the initial absorbance was read and timer started simultaneously. Mixture absorbance was determined after 1, 2 and 3 minutes respectively inside spectrophotometer, at a wavelength of 405 nm. ALP activities were calculated as different temperature and the mean values were recorded.

3.3.8 Data analysis

Data were analysed using an online statistical tool https://www.socscistatistics.com/tests/Default.aspx. Results were presented using descriptive statistics. Haematogical parameters, plasma biochemical parameters and enzymes activities per sex, species and seasons were determined with the Studentised t-test. The level of significance was taken as 5 %.

3.4 STUDY FOUR

3.4.1 Determination of occurrence of ecto-parasites infecting on African hinge-back tortoises (K*inixys belliana* and *Kinixys homeana*)

3.4.2 Equipment, materials and consumable reagents

Equipment used included a dissecting microscope, biosafety cabinet, hand lens, petri dish, forceps Whatman filter paper, pair of forceps, markers, pencils, bottles, face mask, boots, ice pack, transport flask, a preservative solution prepared by adding 80ml of 90% ethanol with 5ml of 5% glycerol and 15ml distil water. The solution was to preserve ticks from dehydration.

3.4.3 Methods

3.4.3.1 Ectoparasites collection from tortoises

The tortoises were observed systematically starting from the anterior body to the posterior for the presence of various types of ectoparasites. Ectoparasites seenwere carefully detached using a pair of forceps from infested tortoises into labeled bijou bottles with preservative solution, they were kept over ice being careful to prevent freezing.

3.4.3.2 Ectoparasites enumeration

The detached ectoparasites were counted and recorded for each tortoise. The mean for each tortoises group was evaluated and recorded. The parasites load or preponderance was calculated and compared with the level of parasitaemia in study one to determine the level of significance of ectoparasites loads and haemogregarine parasitaemia in the tortoises. Factors such as sex, species and seasons were considered

3.4.3.3 Ectoparasites identification

Ticks were identification carried out using field guide of Estrada-Pena, Mihalca and Petney, (2017). Ticks were identified at the first level with naked eyes, followed by the use of a simple hand lens of x 10 magnification. They were removed from the preserving solution, blotted with a Whatman filter paper and cleaned with a very fine artist brush, sorted into a petri dish placed on a white surface. Ticks were viewed dorsally and ventrallyfrom the anterior to the posterior end for proper evaluation, under a dissecting microscope (Optika St-40-2L SN 20611 Ponteranica Italy) at x10 and x20 and image recorded with a digital camera. The identification keys used were (a) mouth part (b) presence of the eyes (c) presence of scutum in male and coscutum in females (d) presence of festoon (e) presence or absence or vestigial adanal plate (f) banded legs.

3.4.4 Data analysis

Data were analysed using an online statistical tool https://www.socscistatistics.com/tests/Default.aspx. Parasites abundance and intensities per sex, species and seasons were determined with *Mann U Whitney* Test.Sex, seasons and species prevalence were evaluated using the Pearson's chi-square test (X^2). Correlation coefficient test was used in the qualification of data. The level of significance was taken as 5 %.

CHAPTER FOUR

RESULTS

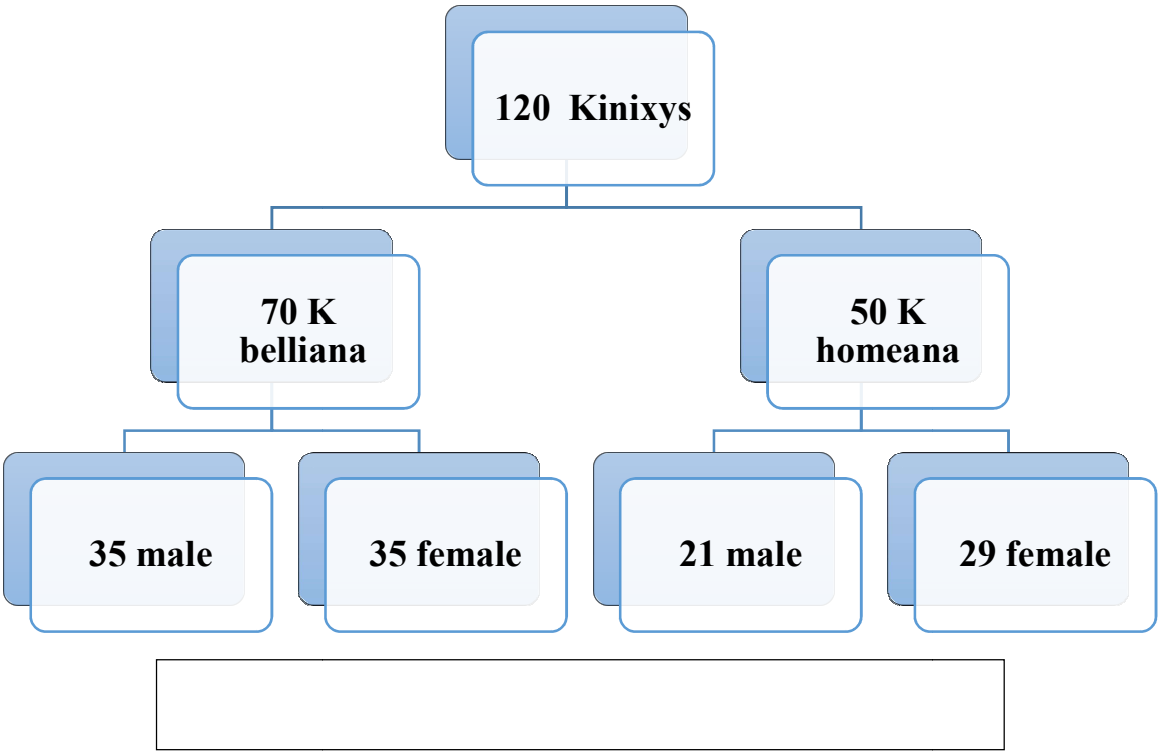
4.0 Total animal acquired

A total of 135 tortoises were sourced, while 15 were excluded from the study based on the lengths of their straight carapace length that were less than 10cm. A total of 120 tortoises were selected for sampling: 60 were sampled at rainy season and 60 at dry season, for the determination of seasonal prevalence. *Kinixy belliana* were 70 (35 males and 35 females) while *Kinixys homeana* were 50 (21 males and 29 females). On the overall, 56 male and 64 female*Kinixys* tortoises were selected for sampling (Figure 4.1).

4.1 Prevalence of haemogregarines in *Kinixys belliana a*nd *Kinixys homeana* in Ibadan, Oyo State

4.1.1 Morphology of haemogregarine infected erythrocytes

The infected erythrocytes were observed containing intraerythrocytic sausage or banana-shaped elongated parasites gametocytes which were tilted toward the nucleus of the infected erythrocytes and lied eccentrically within in the cytoplasm. Affected red blood cells appearedhypertrophied with their nuclei atrophied and marginalised as shown in Plates 4.2A & B.


120 Kinixys
70 K belliana
50 K homeana
35 male
35 female
21 male
29 female

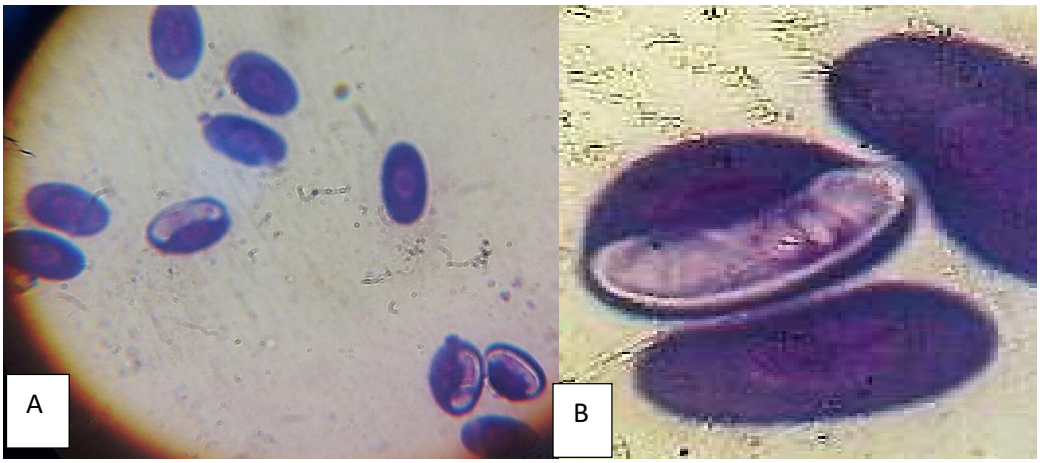


Plate4.1 Giemsa stained gametocytes of parasites observed as sausage or banana-shaped organism lying within the cytoplasm of infected erythrocytes.Magnification: A = x 100; B = x 1000

4.1.2 The overall prevalence of haemogregarine in *K. belliana* and *K. homeana*

The overall prevalence of haemogregarine was 53.3% (64/120) in the hinge-back tortoises (Figure4.3). The prevalence level was higher in *Kinixys belliana* (57.1%) than*Kinixys homeana* (48.0%). Haemogregarine parasite intensity was also higher in *K. belliana* (0.23±0.23) than *K. homeana* (0.21±0.23), (Table 4.4).

4.1.3 Sex prevalence of haemogregarine in *K belliana* and *K. homeana*

Overall sex prevalence in the African hinge-back tortoises recorded, slightly higher infection rates in the males 53.6% (30/56) than females 53.1% (34/64), (Figure 4.3). Whereasprevalence rate was higher in female *K. belliana* (60.6%) than males (54.3%), the males have a higher prevalence (52.4%) than the females (44.8%) in *K. homeana* (Table 4.1).

The overall mean haemogregarine parasite intensity in females (0.24±0.22) of *K. belliana* was higher than that of the males (0.22+0.24).In contrast, to *K. homeana*, in which parasite intensity was higher in males (0.23 ± 0.24) than females (0.19 ± 0.23),Table 4.4.

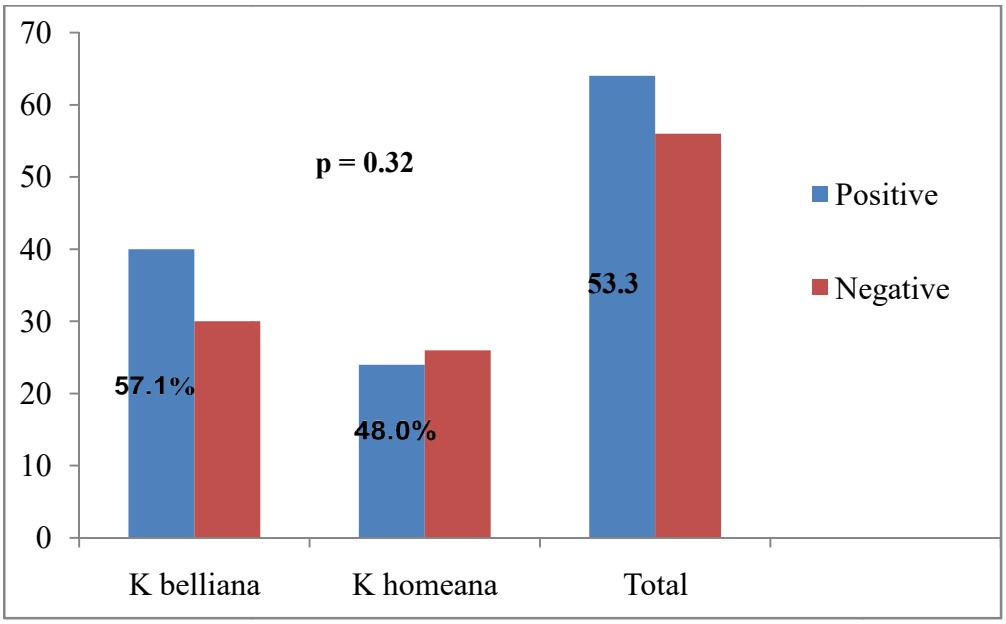


Figure 4.2 Prevalence of haemogregarine in *Kinixys belliana* and *K. homeana*

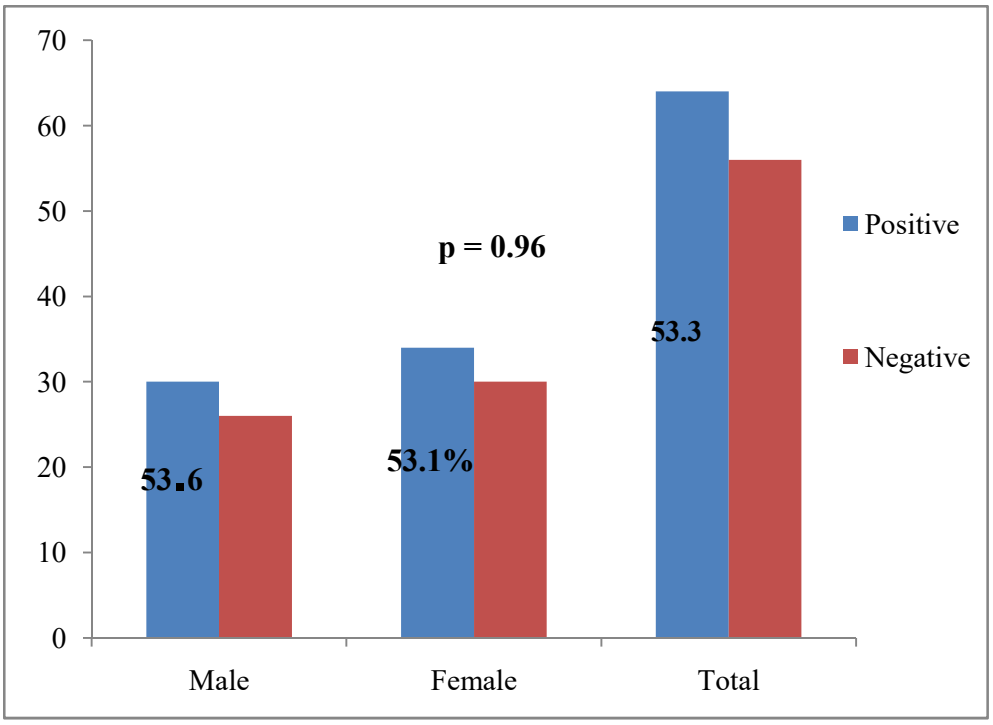


Figure 4.3 Sex prevalence of haemogregarine in *Kinixys belliana* and *K. homeana*

4.1.4 Overall seasonal prevalence of haemogregarine in *K. belliana* and *K. homeana*

The overall prevalence of haemogregarine recorded during the rainy season was 76.7% in both African hinge-back tortoioses (*K. belliana* and *K homeana*), compared to 30.0% prevalence recorded during the dry season. This was statistically significant (X^2= 26.25; p= 0.000001) as shown, (Figure4.4).

Furthermore, 80.6% (29/36) of sampled *Kinixys belliana* and 70.8% (17/24) *Kinixys homeana* were infected during the rainy season and result was statistically significant (X^2= 16.59; p= 0.00005, X^2= 9.64; p= 0.000019), respectively. Table 4.2

In addition, 32.4% (11/34) of *K belliana* tortoises and 26.9% (7/26) *K. homeana*sampled during the dry season were haemogregarine positive.

4.1.5 Species and sex parasite prevalence

The overall mean haemogregarine parasite intensity in *K. belliana* and *K. homeana* was 0.22 $\pm$ 0.23, while the overall mean parasite intensity in males was greater than females, (0.23 $\pm$ 0.24 vs 0.22 $\pm$ 0.22 t= -0.12, p= 0.45, respectively). Similarly, the overall mean parasite intensity in *K. belliana* (0.23 $\pm$ 0.23) was higher than *K homeana* ((0.21 $\pm$ 0.23), t= -0.49, p= -0.31. Furthermore, the overall mean intensity of parasites in female *K. belliana* was higher (0.24 $\pm$ 0.22) male *K. belliana* (0.22 $\pm$ 0.24), t= -0.39, p= 0.34.

On the contrary, the overall mean haemogregarine intensity in male *K. homeana* was higher (0.22 $\pm$ 0.24) than that of the female *K. homeana* (0.19 $\pm$ 0.23), t= 0.57, p= 0.29. Also, the overall mean parasite intensity in male *K. homeana* was higher (0.23 $\pm$ 0.24) than that of male *K. belliana* (0.22 $\pm$ 0.24), t= -0.18, p= 0.43. On the contrary, the overall mean parasite intensity in female *K. belliana* was higher (0.24 $\pm$ 0.22) than that of female *K. homeana* (0.19 $\pm$ 0.23), t= -0.86, p= 0.20. The results described above were not statistically significant Table 4.4.

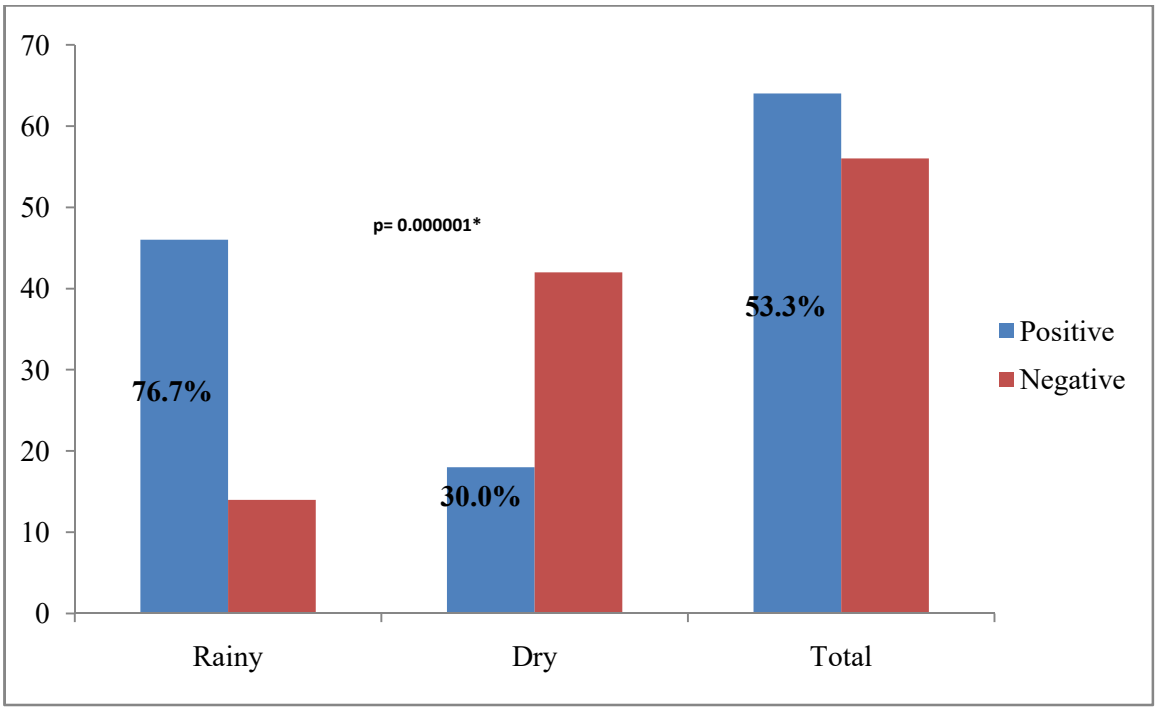


Figure 4.4Overall seasonal prevalence of haemogregarine in *Kinixys belliana* and *K. homeana*

Table 4.1Species and sex skew prevalence of haemogregarines parasites of*Kinixys belliana* and *K. homeana*

Species		***Kinixys belliana***			***Kinixys homeana***	
Sex	**No tested**	**No. positive**	**% Positive**	**No tested**	**No Positive**	**% Positive**
Male	35	19	54.3	21	11	52.4
Female	35	21	60.6	29	13	44.8
Total	70	40	57.3	50	24	48.0
		$X^2 = 0.28$; P = 0.60			$X^2 = 0.23$; P = 0.63	

Table 4.2 Species-skew seasonal prevalence of haemogregarine parasites of *Kinixys belliana* and *Kinixys homeana*

Species	***Kinixys belliana***			***Kinixys homeana***		
	No tested	**No. positive**	**% Positive**	**No tested**	**No. positive**	**% Positive**
Rainy season	36	29	80.6	24	17	70.8
Dry season	34	11	32.4	26	7	26.9
Total	70	40		50	24	
	$X^2 = 16.59$; p = 0.00005			$X^2 = 9.64$; p = 0.00019		

Table 4.3 Sex seasonal prevalence of *K belliana* and *K homeana* species

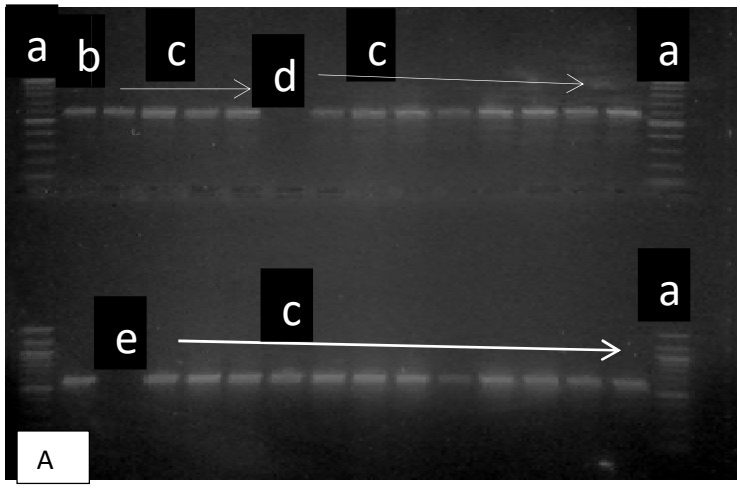
	K. belliana		***K. homeana***	
	Season		**Season**	
Sex	Rainy	Dry	Rainy	Dry
Male	13	6	7	4
Female	16	5	10	3
Total	29	11	17	7
	$X^2 = 0.30$; p = 0.58		$X^2 = 0.51$; p =0.48	

Table 4.4 Parasites intensity across sex species and seasons in *K. belliana* and *K. homeana*

Comparism of sex species and seasons variation of parasites intensities	Mean ± SD	p values
Overall male vs female (*K. belliana* and *K. homeana*)	0.23 ± 0.24 vs 0.22 ± 0.22	0.45
Overall *K. belliana* Vs *K. homeana*)	0.23 ± 0.23 vs0.21 ± 0.23	-0.31
Overall male vs female (*K. belliana*)	0.22 ± 0.24 vs 0.24 ± 0.22	0.34
Overall male vs female (*K. homeana*)	0.23 ± 0.24 vs 0.19 ± 0.23	0.29
Male *K. belliana* vs Male *K. homeana*	0.22 ± 0.24 vs 0.23 ± 0.24	0.43
Female *K. belliana* vs Female *K. homeana*	0.24 ± 0.22 vs 0.19 ± 0.23	0.20
Overall rainy vs dry (*K. belliana* and *K. homeana*)	0.34 ± 0.22 vs 0.10 ± 0.17	0.00001
***K. belliana* Rainy vs Dry**	0.36 ± 0.22 vs 0.10 ± 0.15	0.00001
***K. homeana* rainy vs Dry**	0.32 ± 0.22 vs 0.11 ± 0.19	0.0006
***K. belliana* rainy vs *K. homeana* rainy**	0.36 ± 0.22 vs 0.32 ± 0.22	0.23
***K. belliana* dry vs *K. homeana* dry**	0.10 ± 0.15 vs 0.11 ± 0.19	0.35

4.1.6 Seasonal parasites intensity

The overall mean seasonal haemogregarine intensity in *K. belliana* and *K. homeana*, during the rainy season (0.34±0.22) was significantly higher than dry season, (0.10 ± 0.17), t= 6.66, p = 0.00001. Furthermore, the overall mean parasite intensity in *K. belliana* was also significantly higher during rainy(0.36 ± 0.22) than dry season, (0.10 ± 0.15), t= 5.86, p = 0.00001). Similarly, the overall mean haemogregarine intensity in *K.homeana* was significantly higher at rainy (0.34 ± 0.22) than dry season (0.10± 0.17), t= 6.66, p = 0.0006). Thus seasonal variations of haemogregarine intensity in the African hinge-back tortoise were statistically significant. Besides, the overall mean haemogregarine intensity in *K. belliana* was higher than *K. homeana* during rainy season, (0.34 ± 0.22 vs 0.32 ± 0.22 t= 0.74, p= 0.23), on the contrary, overall mean parasite intensity in *K. homeana* was slightly higher than *K. belliana* during the dry season, (0.11 ± 0.19 vs 0.10 ± 0.15 t= 0.38, p= 0.357), Table 4.4. These observations were not signicant statistically.



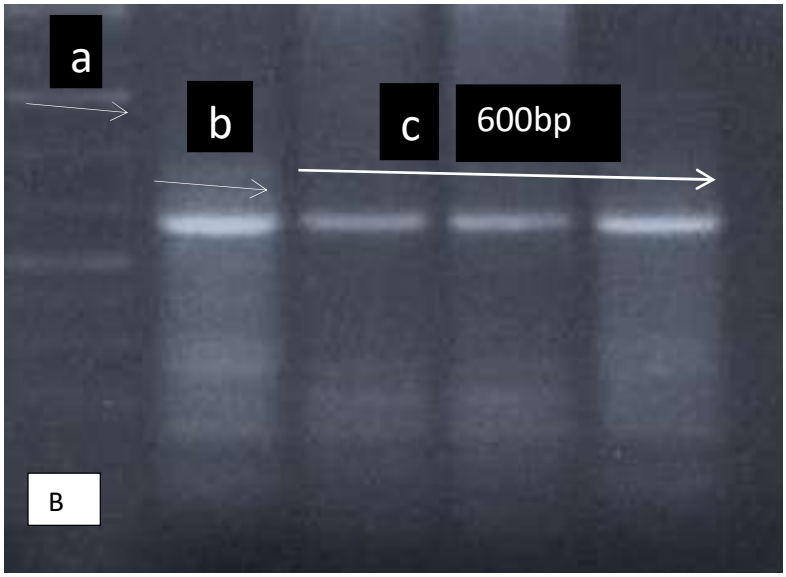


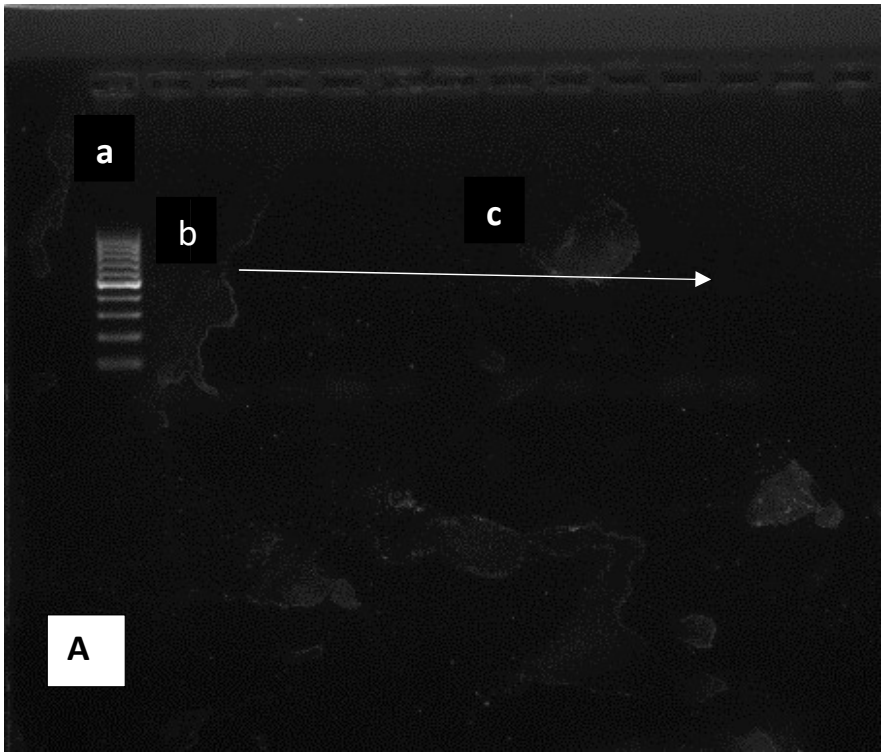
Plate 4.2 (A) DNA amplification with HEPF300 and HEPR900 primers of 600 bp fragment from blood (a) DNA ladder 100bp (b) Positive control (c) Positive samples (d) Negative control (e) Negative sample

(B) DNA amplification with HEPF300 and HEPR900 primers of 600 bp fragment from blood (a) DNA ladder 100bp (b) positive control (c) positive samples

4.2 Molecular detection of haemogregarines in *Kinixys belliana* and *Kinixys homeana* using Polymerase chain reaction (PCR)

4.2.1 Polymerase chain reaction PCR result with three pairs of primers

The results of the PCR with Apicomplexan-specific parasite primer set 4558F 5′-GCTAATACATGAGCAAAATCTCAA 3′ and 2733R: 5′CGGAATTAACCAGACAAA T-3` of expected product size of 1120bp, according to Matthew *et al* (2000) and Netherland *et al* (2014) and Hemo primers: HEMO1 5`TATTTGGTTTTAAGAACTAAC TAATTTTATGATTG3`HEMO2 5`CTTCTCCTTCCTTTAAGTGATAAGGTTCAC3`wi th an expected product size of 900bp according Perkins and Keller (2001) did not yield any positive amplifications. The Hep primers HepF300 (5′ GTTTCTGACCTATCA GCTTTCGACG3′) and HepR 900(5′- CAAATCTAAGAATTTCACCTCTGAC 3′) of an expected product size of 600bp according to Ujvari *et al*., (2004) amplified 75.8% (91/120) of the DNA products and the gel documentation resultsare shown in the Plates 4.2 A & B, 4.3 below.



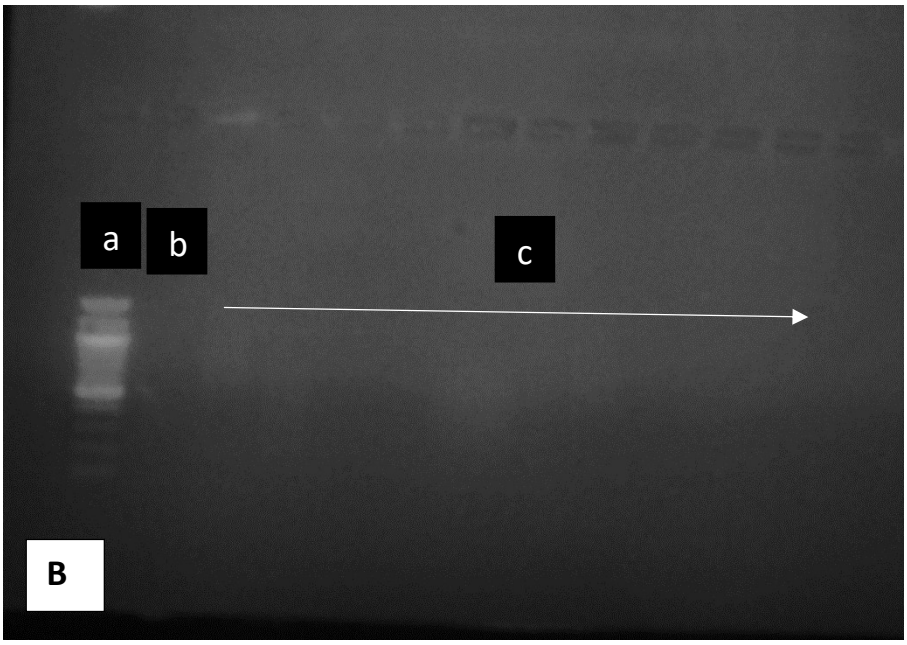


Plate 4.3 No amplification of 900bp expected DNA product fragment from blood with HEMO1 and HEMO 2 Primers (a) DNA ladder 100bp (b) negative control (c) negative samples

Plate 4.3 B No amplification of 1120 bp expected DNA product fragment from blood with Primers 2733R and 4558F (a) DNA ladder 100bp (b) negative control (c) negative samples

4.2.2 PCR and DNA amplification

The HEP primers set yielded amplifying useable DNA amplicons with an overall prevalence of 75.8% (91/120). (Table 4.5).The HEMO1 and HEMO2,4558F and 2733R primers yielded no positive amplification, (Plates 4.3 A and B).

The prevalence of *Haemogregarine* infection was signifivantly higher in *K. belliana*- (58/70- 82.9%) than *K. homeana* (33/50- 66.0%), (X^2= 7.73; p= 0.005). Furthermore, there were no statistical differences in the prevalence of haemogregarine parasites between sexes using the Pearson's Chi-square test method. Similarly, the overall seasonal prevalence was significantly higher during the rainy season (51/60-85.0%) than the dry season (40/60- 66.7%), (X^2= 4.44; p= 0.04). Besides, the overall seasonal prevalence in *K homeana* was higher in the rainy season (22/24-91.7%) than the dry season (11/26-42.3%), result was also statistically significant (X^2= 8.92; p= 0.003). Although the overall haemogregarine occurrence in *K. belliana* was slightly higher at rainy it was notstatistically significant. (Table 4.5)

The comparison of both microscopy and PCR methods showed that, PCR method (91/120-75.8%) detected signifivantly higher positive results than light microscopy method (64/120-53.3%), (X^2= 12.25; p= 0.0005),(Table. 4.6).

4.2.3 Molecular Characterisation of haemogregarines in *Kinixys belliana* and *Kinixys homeana* and Phylogenetic analysis

The sequence results showed that only DNA amplicons obtained with HepF300 and HepR900 primers produced useful DNA of approximately 590 bp for phylogenetic analysis. The DNA sequences of 590bp were aligned with the downloaded 18S RNA genes, which had the closest similarity of 100% and belonging to *Hepatozoon fitzsimonsi ex zombensis*. This was followed by several others speices such as the ticks, mammalian and amphibian which were positive for *Hepatozoon species*. The phylogenetic tree drawn from this study by all the applied methods as stated above had a similar topology with different support values. The phylogenetic analysis (Figure 4.5) showed that *Hepatozoon fitzsimonsi ex zombensis* KR 069084 and the *Hepatozoon* species from this study form sister clade with *Hepatozoon species* (MK918613.1) from ticks in Ankara, Turkey according to Orkun, Karaer, Cakmak and Nalbantoglu(2014) Unpublished). The sister clade also clustered with other *Hepatozoon species* from Vulture, Jackals, Wild cat *Felis silvers*, Wildebeast *Sus scrofa leucomystx*, Hodzic, Alic, Prasovic, Otranto, Baneth and Duscher (2015).

Table 4.5 Species and seasonal prevalence study by PCR using HEP primer sets

Overall species prevalence			
Tortoises species	**sample size (male/female)**	**no. positive (male/female)**	**Prevalence (%) (male/female)**
Kinixys belliana	70 (35 / 35)	58 (30 /28)	82.9 (85.7 / 80.0)
Kinixys homeana	50 (21 /29)	33 (14 / 19)	66.0 (70.0 /65.5)
Total	120 (56 /64)	91 (44 /47)	75.8 (78.6 /73.4)

Seasonal prevalence within and across species			
Seasons	**Sample size OVR (*Kinixys belliana* / *Kinixys homeana*)**	**No. positive OVR (*Kinixys belliana*/ *Kinixys homeana*)**	**Prevalence (%) OVR(*Kinixys belliana* / *Kinixys homeana*)**
Rainy	60 (36 / 24)	51 (29 / 22)	85.0 (80.6 / 91.7)
Dry	60 (34 / 26)	40 (29 /11)	66.7 (85.3 /42.3)
Total	120 (70 / 50)	91 (58 /33)	75.8 (82.9 /66.0)

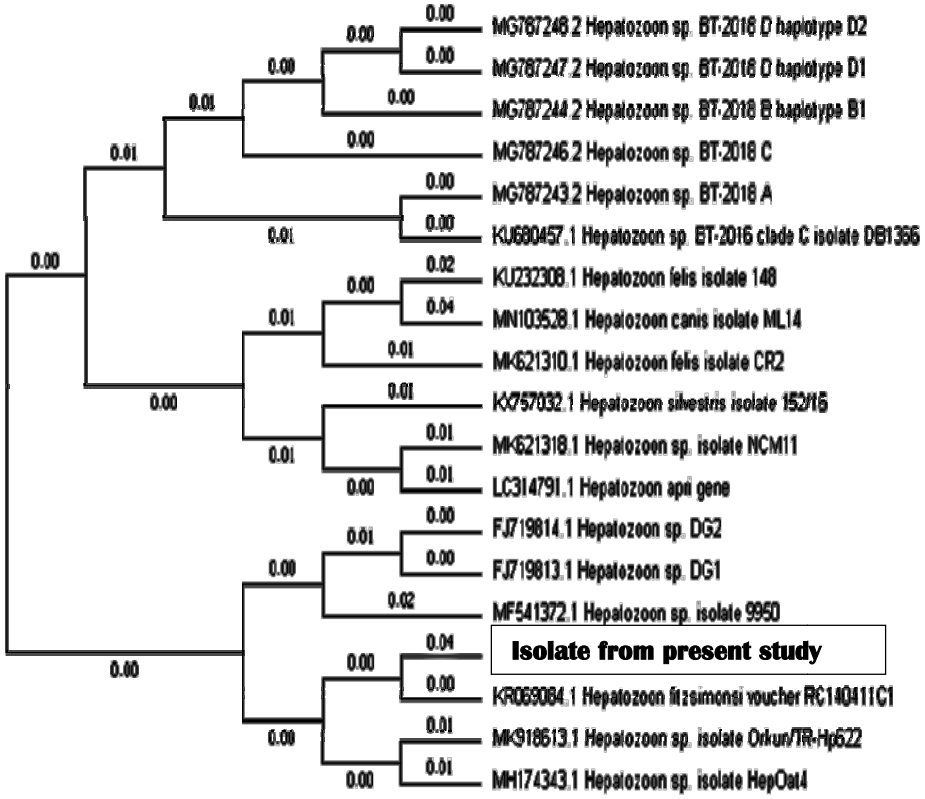
***OVR Overall Prevalence**

Table 4.6 Comparism of Prevalence of Haemogregarine infection by Microscopy and Polymerase chain reaction (PCR)

Prevalence method	Total animal tested	Overall Positive	Overall Negative
Microscopy	120	64	56
PCR	120	91	29
$X^2 = 12.25$	p = 0.0005		

Table 4.7 List of Isolates and sequence results associated GenBank accession numbers and references

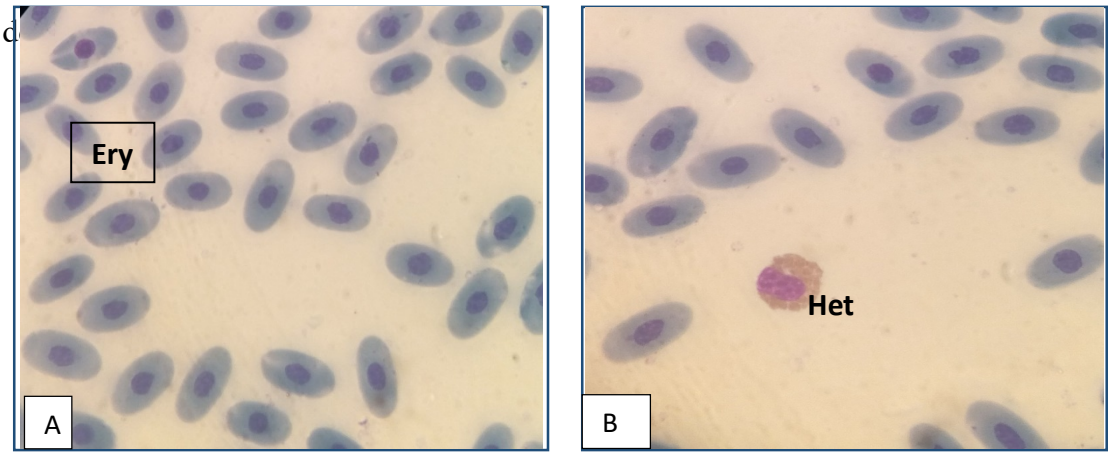
Organisms	Accession numbers	References
Hepatozoon sp. BT-2018 D haplotype D2	MG787248.2	Tome *et al*., 2018
Hepatozoon sp. BT-2018 D haplotype D1	MG787247.2	Tome *et al*., 2018
Hepatozoon sp. BT-2018 B haplotype B1	MG787244.2	Tome *et al*., 2018
Hepatozoon sp. BT-2018 C	MG787246.2	Tome *et al*., 2018
Hepatozoon sp. BT-2018 A	MG787243.2	Tome *et al*., 2018
Hepatozoon sp. BT-2016 clade C isolate DB1366	KU680457.1	Tome *et al*., 2018
Hepatozoon apri gene	LC314791.1	Yamamoto *et al*., 2017
Hepatozoon silvestris isolate 152/16	KX757032.1	Hodzic *et al*., 2015
Hepatozoon sp. isolate NCM11	MK621318.1	Vilijoen *et al*., 2018
Hepatozoon felis isolate CR2	MK621310.1	Vilijoen *et al*., 2018
Hepatozoon felis isolate 148	KU232308.1	Furtado *et al*., 2018
Hepatozoon canis isolate ML14	MN103528.1	Franic *et al*., 2019
Hepatozoon species DG2	FJ719814.1	Merino *et al*., 2009
Hepatozoon species DG1	FJ719813.1	Merino *et al*., 2009
Hepatozoon species isolate 9950	MF541372.1	Nadler *et al*., 2017
Hepatozoon species isolate HepOat4	MH174343.1	Muno-Leal *et al*., 2018
Hepatozoon species isolate Orkun/TR-Hp 622	MK918613.1	Orkun *et al*., 2014
Hepatozoon isolate from Present study		**Isolate from Present study**
Hepatozoon fitzsimonsi voucher RC140411C1	KR069084.1	Cook *et al*., 2015


MG787248.2 Hepatozoon sp. BT-2016 D haplotype D2
MG787247.2 Hepatozoon sp. BT-2016 D haplotype D1
MG787244.2 Hepatozoon sp. BT-2016 B haplotype B1
MG787246.2 Hepatozoon sp. BT-2016 C
MG787243.2 Hepatozoon sp. BT-2016 A
KU680457.1 Hepatozoon sp. BT-2016 clade C isolate DB1366
KU232308.1 Hepatozoon felis isolate 148
MN103528.1 Hepatozoon canis isolate ML14
MK621310.1 Hepatozoon felis isolate CR2
KX757032.1 Hepatozoon silvestris isolate 152/16
MK621318.1 Hepatozoon sp. isolate NCM11
LC314791.1 Hepatozoon apri gene
FJ719814.1 Hepatozoon sp. DG2
FJ719813.1 Hepatozoon sp. DG1
MF541372.1 Hepatozoon sp. isolate 9950
Isolate from present study
KR069084.1 Hepatozoon fitzsimonsi voucher RC140411C1
MK918613.1 Hepatozoon sp. isolate Orkun/TR-Hp622
MH174343.1 Hepatozoon sp. isolate HepOat4

4.3 Determination of haematology, plasma biochemistry and enzyme activities of haemogregarine positive African hinge-back tortoises (K*inixys belliana* and *Kinixys homeana*)

4.3.1 Haematological parameters of haemogregarine-positive and haemogregarine negative *Kinixys belliana and Kinixys homeana*

Summary statistics are shown in (table 4.8). Haemogregarine-positive *Kinixys belliana* and *Kinixys homeana* recorded statistically significant lower values for haematocrit (22.75 ± 2.56 %), red blood cells counts (1.13± 0.21 10^{12}/L), heterophils (20.58±3.03%) and monocytes(1.05± 0.38 %) than the haemogregarine-negative *Kinixys belliana* and *Kinixys homeana* (32.79± 2.68%, 1.99±0.13 10^{12}/L, 29.62 ±5.35%) and 1.45+0.83% respectively. There was statistically significant difference between the counts of heterophils (7.26±0.99 10^{9}/L) in haemogregarine-positive *Kinixys* tortoises than haemogregarine-negative (5.58±1.18 10^{9}/L), similarly values for eosinophils (40.41±2.22%), in haemogregarine-positive was statistically significant higher than haemogregarine-negative (29.79±3.76%).



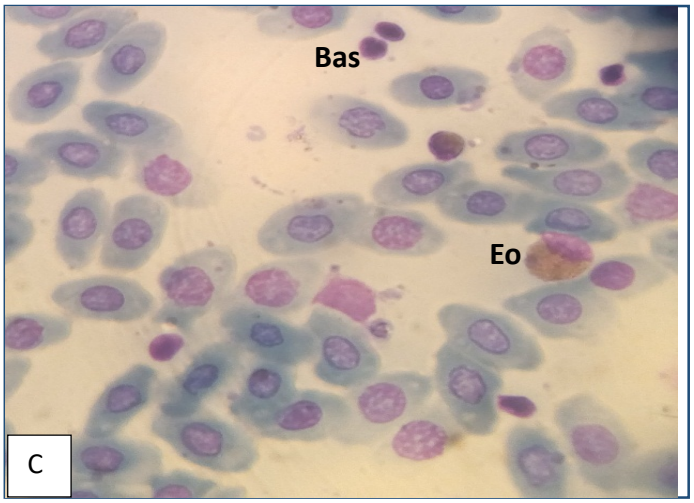
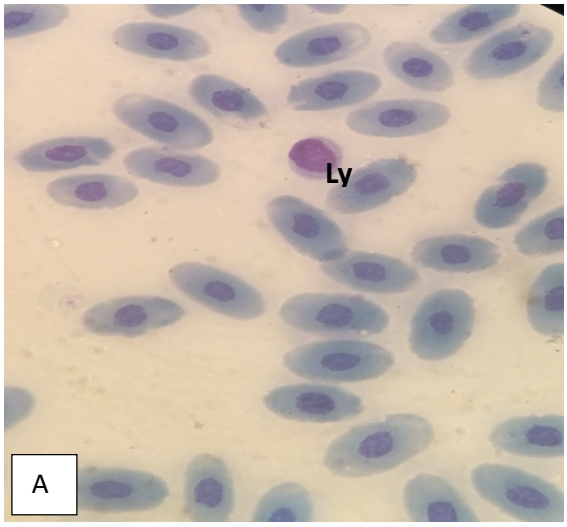


Plate 4.4 (A)*Kinixys belliana* Ery-erythrocyte Giemsa staining method x100(B)*Kinixys belliana*Het-Heterophils Giemsa stain method x100,(C)*Kinixys homeana*Bas-Basophils, Eo-Eosinophil Giemsa staining

Table 4.8 Comparison of Haematological parameters between haemogregarine positive and negative*Kinixys belliana* and *Kinixys homeana*

Parameter	*Kinixys belliana* and *Kinixys homeana*		P-value
	Positive (n=91) Mean ± SD	Negative (n=29) Mean ± SD	
Haematocrit (%)	22.75 ± 2.56	32.79 ± 2.68	0.00001 [a]
Haemoglobin g/dl	7.04 ± 0.96	7.05 ± 0.88	0.49
RBC (10^{12}/L)	1.13 ± 0.21	1.99 ± 0.13	0.00003 [a]
WBC (10^{9}/L)	7.26 ± 0.99	5.58 ± 1.18	0.00001 [a]
Lymphocytes (%)	37.31 ± 2.34	38.0 ± 5.91	0.18
Eosinophils (%)	40.41 ±2.22	29.79 ± 3.76	0.00001 [a]
Monocytes (%)	1.05 ± 0.38	1.45 ± 0.83	0.0003 [a]
Heterophils (%)	20.58 ± 3.03	29.62 ± 5.35	0.00001 [a]
Basophils (%)	0.53 ± 0.60	0.76 ± 0.58	0.09

N.A.=Not available
[a]= values recorded were statistically significant at $p<0.05$



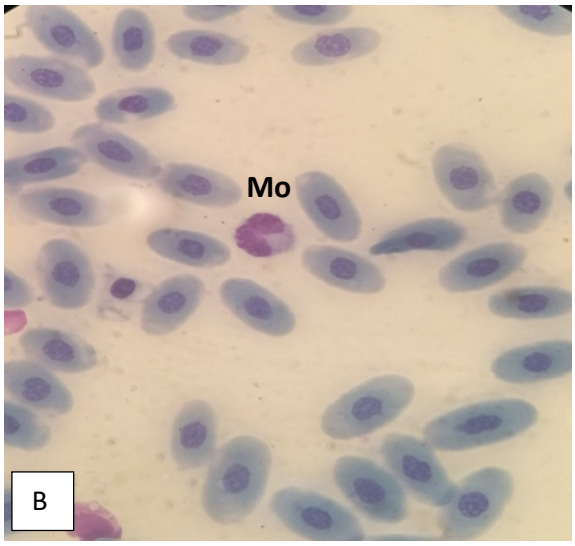


Plate 4.5*Kinixys belliana (*A) Ly-lymphocyte Giemsa staining method X 1004.9*Kinixys homeana*(B) Mo-monocyte Giemsa staining method X 100

4.3.2 Plasma biochemical parameters of Haemogregarine positive and negative *Kinixys belliana and Kinixys homeana*

Summary statistics in (Table 4.9), recorded values for the following plasma biochemical constituents; total protein (3.97±0.87 g/dL), albumin (1.26±0.29 mg/dL), globulin (2.71±0.58 mg/dL), calcium (7.97+ 1.09 mg/dL) and chlorine (94.40±15.51, showed that h aemogregarinepositive *Kinixys belliana* and *Kinixys belliana* recorded statistically significant lower values 5.33±0.93 g/dL, 1.67±0.51 mg/dL, 3.66±0.42 mg/dL, 8.41±0.19 mg/dL and 102.79± 8.50, than haemogregarine negative *Kinixys belliana* and *Kinixys belliana* respectively.

4.3.3 Plasma enzymes activities parameter of Haemogregarine positive and negative *Kinixys belliana and Kinixys homeana*

Haemogregarine-positive recorded statistically significant higher values for ALT (33.91±14.42 U/L), ALP (179.27±92.52 U/L) than 8.21±2.21 U/L and 147.93±10.51 U/L, respectively in haemogregarine-negative. Furthermore, haemogregarine-positive had statistically signicant lower values for AST ((46.47±14.56 U/L) than 64.38±23.17 U/L recorded in haemogregarine-negative*Kinixys belliana* and *Kinixys homenana*.

Table 4.9Comparison of biochemical parameters and enzyme activities of haemogregarine positive and negative*Kinixys belliana* and *Kinixys homeana*

Plasma analytes	*Kinixys belliana* *K homeana*		p values
	Infected N=91 (Mean±SD)	**Non-infected N=29 (Mean±SD)**	
Albumin (mg/dl)	1.26± 0.29	1.67± 0.51	0.00001 [a]
Globulin (mg/dl)	2.71± 0.58	3.66±0.42	0.00001 [a]
Total protein (g/dl)	3.97± 0.87	5.33±0.93	0.00001 [a]
Calcium (mg/dl)	7.97± 1.09	8.41±0.19	0.02 [a]
Potassium (mg/dl)	5.16± 1.07	4.94±0.13	0.14
Phosphorus (mg/dl)	6.89± 2.30	6.51±1.05	0.19
Sodium (mg/dl)	125.44±23.91	125.64 ± 2.64	0.48
Chloride (mg/dl)	94.40 ± 15.51	102.79±8.50	0.003 [a]
Aspartate aminotransferase (AST) (U/L)	46.47±14.56	64.38± 23.17	0.00001 [a]
Alanine transaminase (ALT) (U/L)	33.91± 14.42	8.21±2.21	0.00001 [a]
Alkaline phosphatase (ALP) (U/L)	179.27± 92.52	147.93+10.51	0.004 [a]

N.A.=Not available

[a] =values recorded were statistically significant at $p<0.05$

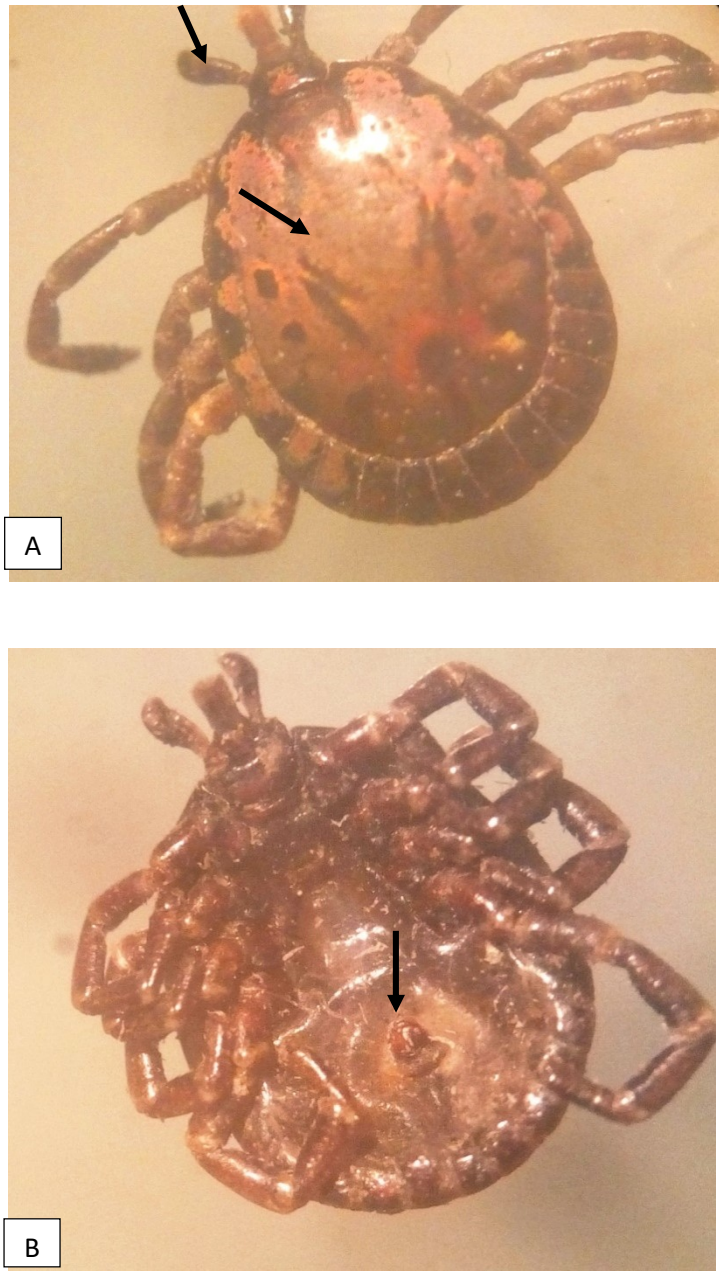
4.4 Determination of Ecto-parasites and their roles in the transmission of Haemogregarine inAfrican hinge-back tortoises (K*inixys belliana* and *Kinixys homeana*)

4.4.1 Ectoparasitesfound on the tortoises

Hard ticks were the only ectoparasites found on 65 out of the 120 African hinge-back tortoises examined in this study. The ticks were found attached to various body part of tortoises; the head, neck, shoulder, legs, the tail area and sometimes on the fissure of the carapace and plastron.

4.4.2 Tick identification

The ticks found on the tortoises were identified to genus level by comparing the mouth part and the coxa. The tick of the genus *Amblyomma is* longirostate (long rostrum) which is made up of basic capitulum and pedipalps, has a dorsal shield called the scutum. The capitulum or mouthpart protrudes forward and this can be easily viewed dorsally. The palps are long and have three coxae; the second coxa is twice as long as the width. The eyes and festoon are well developed in the female. The male is smaller in size and is ornate. Plate4.6 A & B.



Plates4.6 (A) Dorsal view of a male *Amblyomma* tick Note palp article 2 longer than 1 and 3 and the elaborate ornamentation on the dorsum (orange enamel) arrowed

(B) Ventral view of a male Amblyomma tick Note the arrowed anal and reduce adanal plate.

4.4.3 Prevalence of tick infestation in *K. belliana* and *K. homeana*

The overall prevalence with ticks of the *Amblyomma species*, recorded was 54.0% (65/120) (Figure 4.6). The prevalence was slightly higher in *Kinixys belliana* (55.7%) than *Kinixys homeana* (52.0%).

4.4.4 Sex skew tick prevalence in *Kinixys belliana* and *K. homeana*

Overall sex prevalence of tick infestation in the African hinge-back tortoises, showed slightly higher tick prevalence of 60.7% (34/56) in the males tortoise than 48.4% (31/64) in the females, (Table 4.11). Also, the prevalence was slightly higher in the males of both *K. belliana* and *K. homeana* (60.7) than the females of both species (48.4%) although, these variations are not statistically significant. (Figure4.7).

4.4.5 Season-skew tick infestation in *K. belliana* and *K. homeana*

The overall prevalence of tick infestation recorded at rainy season was 55.3% (33/60) in *K. belliana* and *K homeana* while 53.0% (32/60) was reported at dry season as shown in (Table 12). Prevalence of tick infestation recorded during the rainy season was higher but not statistically significant, (Figure 4.8). Furthermore, 58.3% (21/36) of *Kinixys belliana* and 50.0% (12/24) of *Kinixys homeana* were infested during the rainy season. Besides, 52.9% (18/34) of *K belliana* and 53.9% (14/26) of *K. homeana* sampled during the dry season were infested with ticks, (Table 12).

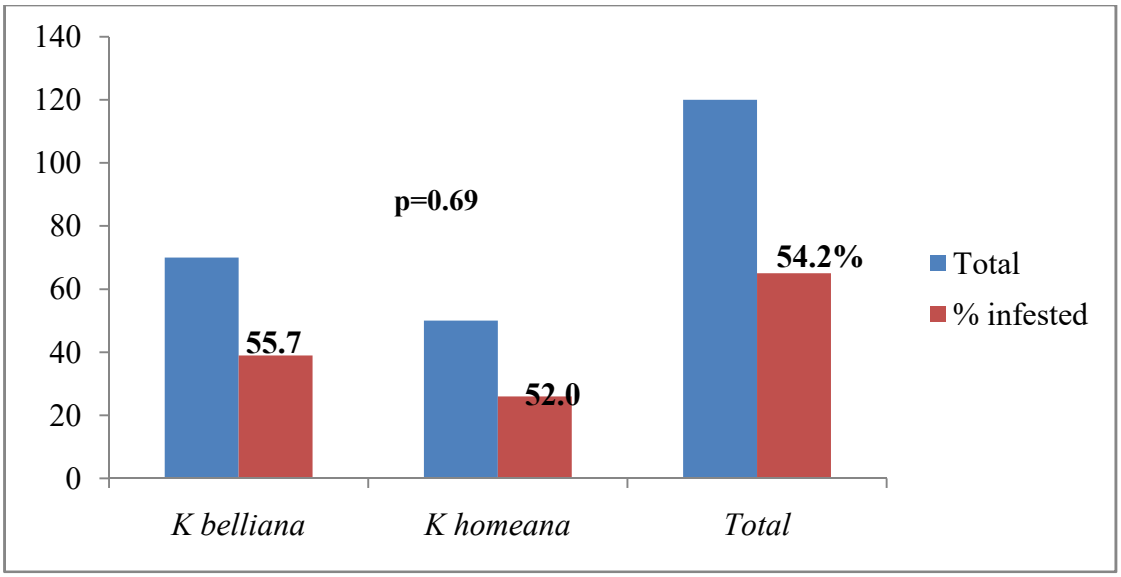


Figure 4.6 Prevalence of ectoparasites in *K. belliana* and *K. homeana*

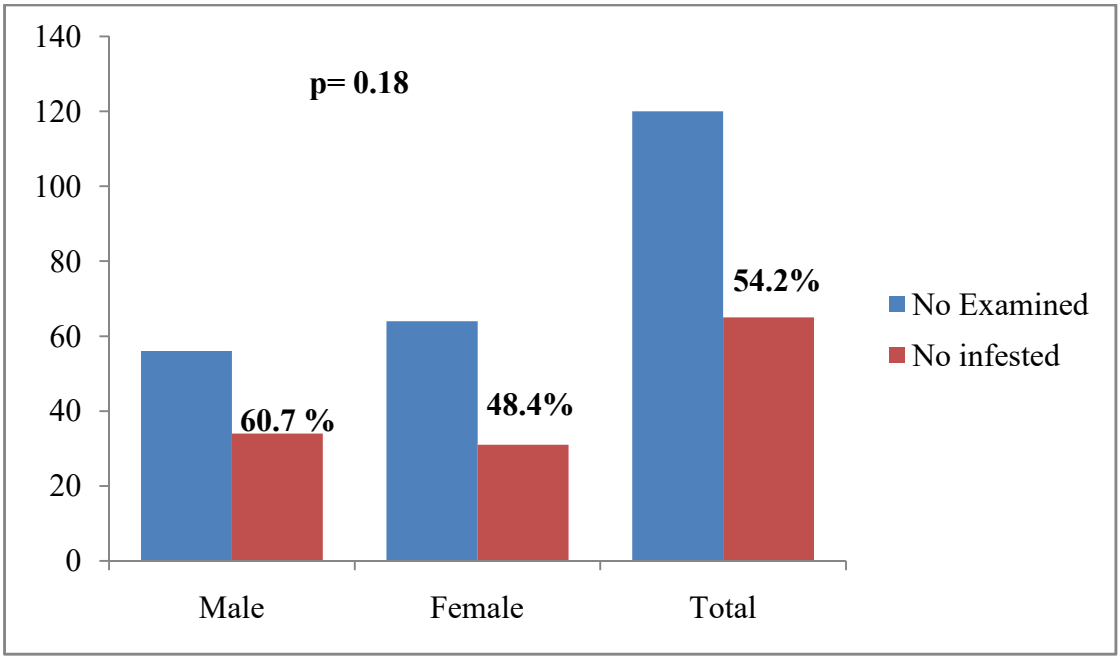


Figure 4.7 Prevalence of ectoparasites in male and female *Kinixys* tortoises

Table 4.10 Prevalence of ectoparasites infestation in Africa hinge-back tortoises

Species	No of tortoise examined	No. infested ectoparasites	% infested
K. belliana	70	39	55.7
K. homeana	50	26	52.0
Total	120	65	54.2
		$X^2 = 0.16$ P = 0.69	

Table 4.11 Sex prevalence of ectoparasites infestation in Africa hinge-back tortoises

Sex	No. of tortoise examined	No. infested ectoparasites	% infested
Male	56	34	60.7
Female	64	31	48.4
Total	120	65	54.2
		$X^2 = 1.81$; p = 0.18	

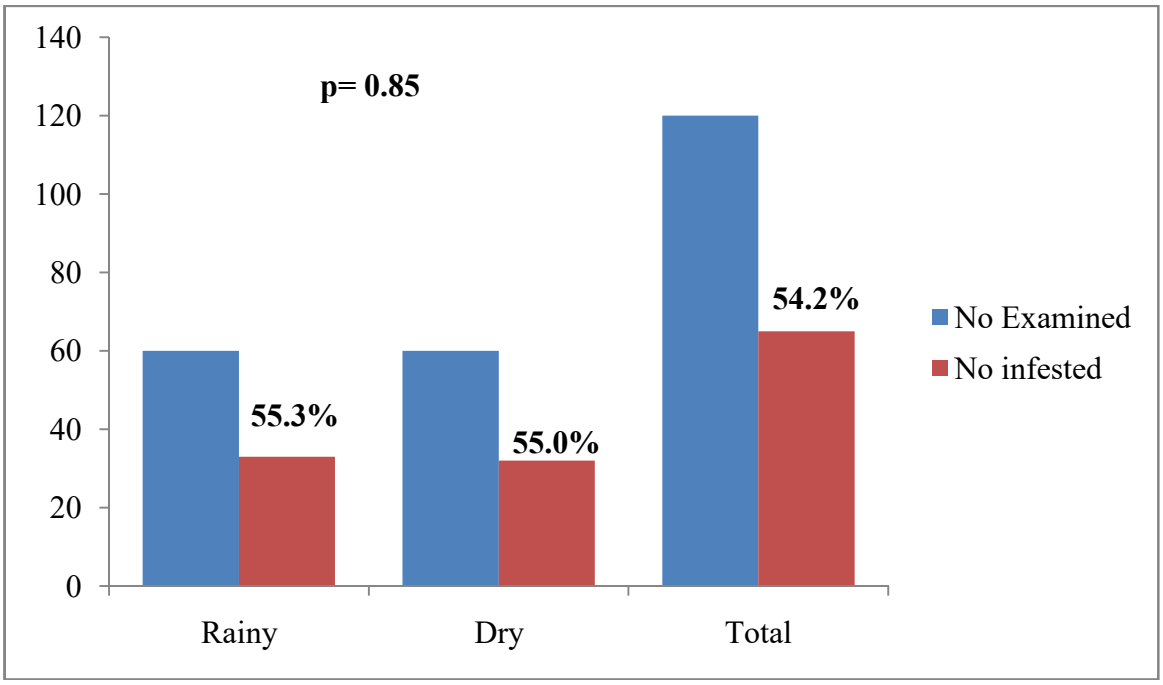


Figure 4.8 Prevalence of ectoparasites during rainyand dry seasons

Table 4.12 Overall seasonal ectoparasites infestation in the two African hinge-backs tortoises

Species	No tested	No. positive	% Positive
Rainy season	60	33	55.0
Dry season	60	32	53.3
Total	120	65	54.2
		$x^2 = 0.03$; P = 0.85	

Table 4.13 Season-species skew ectoparasites infestation in *Kinixys belliana* and *Kinixys homeana*

Species	*Kinixys belliana*			*Kinixys homeana*		
	No tested	**No. infested**	**% infestation**	**No tested**	**No. infested**	**% infestation**
Rainy season	36	21	58.3	24	12	50.0
Dry season	34	18	52.9	26	14	53.9
Total	70	39		50	26	
		$x^2 = 0.21$; p = 0.65			$x^2 = 0.07$; p = 0.79	

4.4.6 Association between tick infestation and level of Haemogregarine parasitaemia intensity on infested *K. belliana* and *K. homeana*

The association of the tick abundance and level of haemogregarine intensity on tick infested tortoises was evaluated with Pearson's correlation coefficient and the frequency polygon was used to depict the level of association as shown in (Figure 4.9). The result displayed a moderate positive correlation between tick infestation and the level of haemogregarine intensity (Correlation coefficient ($r = 0.3758$, $p=0.002$).

Table 4.14 Ectoparasites intensity across sex-species and seasons in *K. belliana* and *K. homeana*

Comparison of sex-species and seasonal variation of parasites abundance	**Mann U Witney U scores**	**Z scores**	**P values**
Overall infested (*K. belliana* Vs. *K. homeana*)	1637.5	0.59619	0.5485
Overall male vs. female (*K. belliana* and *K. homeana*)	1487.5	-1.59914	0.1096.
Overall rainy vs. dry (*K. belliana* and *K. homeana*)	1758.5	0.21519	0.82588
K. belliana Rainy vs. Dry	569.5	0.49354	0.62414
K. homeana rainy vs. Dry	297	0.28157	0.77948
K. belliana rainy vs. *K. homeana* rainy	378.5	0.79973	0.42372
K. belliana dry vs. *K. homeana* dry	440	0.02238	0.98404

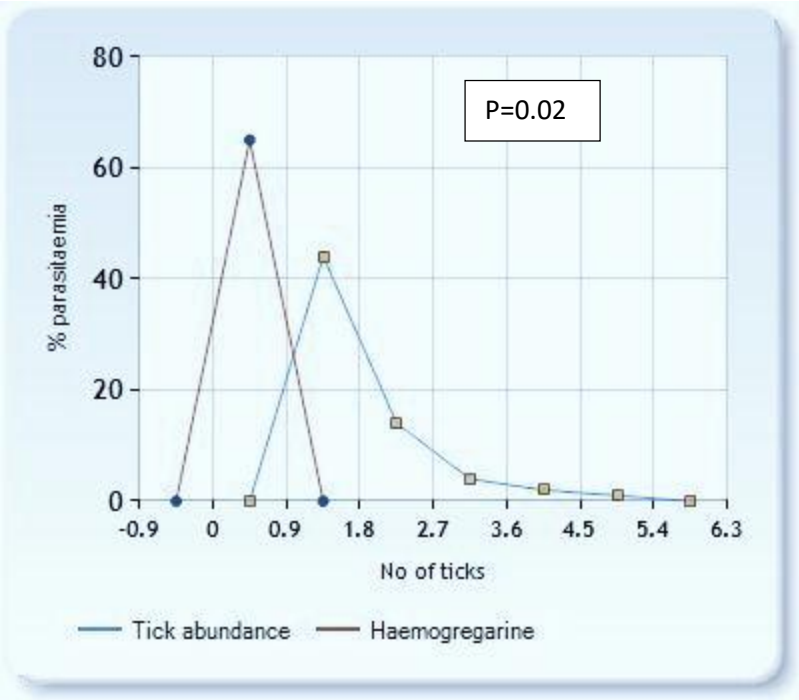


Figure 4.9 Tick infestation and Haemogregarine parasitaemia

CHAPTER FIVE

5.1 DISCUSSION

The occurrence of high prevalence of haemogregarines in *K. belliana* and *K. homeana* in Ibadan, Nigeria has confirmed the presence of haemogregarines in chelonians in Nigeria. The appearance of haemogregarine infected erythrocytes in this study showed hypertrophy of the erythrocytes, atrophied and marginalised nuclei. This observation has been described in similar studies in tortoises and turtles infected with haemogregarines (Cook *et al*. 2015; Molla *et al*. 2015). The intraerythrocytic haemogregarines within the RBCs of the intermediate hosts (*Kinixys* tortoises) is a confirmation of the parasites' ability to destroy erythrocytes and thus leading to a decrease in haematocrit and haemoglobin concentration levels. This causes a clinical condition of decrease in oxygen-carrying capacity in the affected hosts (Arikan Cand Cicek, 2014; Ozvegy *et al*., 2015). Reduced oxygen supply to body organs and tissue can consequently affect different physiologic activities of the affected host, causing loss of fitness and body condition, (Martinez-Silvestre, Lavin and Cuenca, 2011; Ozvegy *et al*., 2015).

The higher prevalence of haemogregarine infection recorded by microscopy in *Kinixys belliana* (57.1%) against the (48.0%) in *Kinixys homeana* could be attributed to the anatomical features of *K. homeana* which possibly endow the species the ability to retract its whole appendages fully into its shell, concealing every part of its and thus reducing the surface area available for vector or ectoparasites attachment. Consequently, higher tick infestation was recorded in *Kinixys belliana* (55.7%) against the (52.0%) observed in *Kinixys homeana.* This is most probably responsible for the lower haemogregarine prevalence recorded in *K. homeana* species. Although ectoparasites usually attach when the hostare in motion and body parts are retract when they are motionless. It could also be as a result of fewer numbers of *K. homeana* examined as compared to *K. bellana*. The overall sex prevalence of haemogregaines in the African hinge-back tortoises (*K. homeana* and *K. belliana*) showed that, infection rates were higher in the males (60.7%) (34/56) than the females (48.4%) (31/64). This is linked to higher tick infestation in the males than

females. Kao, Hill, Burnett and Deane (2014), reported higher occurrence of ticks amongst male than female opossums. Similar work on stray and pet dogs in Bhubaneswar, India also reported higher tick infestation in the males of both stray and pet dogs. The higher overall prevalence recorded in the males *K homeana* is attributable to the high levels of testosterone in sexually mature male animals which makes them more active sexually as they search for the female partners which consequently predispose males to greater tick infestation (Sahu, Mohanty, Panda, Sardar and Dehuri 2013). This is in additionto their sexual activities, which could promote the spread of ectoparasites as they mate multiple female partners which expose them to more ectoparasite loads (Kao *et al*. 2014). This is in contrast to observations of Karadjian *et al*. (2019) who submitted that there were no statistical differences in the occurrence of haemogregarines among female, male and juvenile of *Testudo marginata* sampled in Greece. Whereas more males were positive in *K. homeana,* more females were haemogregarine-positive in *K. belliana.* No reason can be adduced for this latter observation although Cook *et al.* (2015) attributed it to females roaming about in the wild in search of male and consequent increase in arthropod vector contacts as they migrate from one location to another.

The result from this work has also established, a fact, that haemogregarines in *Kinixys* tortoises is endemic, occurring throughout all seasons in Nigeria, with significantly higher prevalence during rainy season, (X^2 = 4.44, p = 0.004). The high prevalence of haemogregarine infection observed in the *Kinixys* tortoises (*K. belliana* and *K. homeana*) in this study both at rainy and dry seasons; is suggestive of the fact that haemogregarine infection can persist in its host over an extended period (Siroky *et al.*, 2009; Cook *et al.,* 2009) recorded a high occurrence of haemogregarines in all seasons in South African tortoises. The significantly higher prevalence (X^2=4.44, P=004) of haemogregarines during the raining season in the present study may be attributed to the higher tick infestation during the rainy season (55%) than dry season (53%) due to increase in abundance of the definite ectoparasite hosts. Salkeld and Schwarzkopf (2005) have associated seasonality with differences in prevalence and intensity of haemogregarines with more infection occurring during rainy season. In addition, Turner, Versfeld, Kilian and Getz, (2012), in their works have associated seasonal variations of parasites intensity to factors, such as rainfall, temperature, and relative humidity, these may affect the occurrence, abundance

and intensity of haemogregarines within a reptile community, which may ultimately result in seasonal shift of parasite burden. Studies on social mole-rats (*Cryptomys hottentotus hottentotus*) reported, that ectoparasite occurrence,abundance and intensity increase in wet season (Viljoen, Bennett, Ueckermann and Lutermann, 2011).

Sahu *et al*. (2013) also recorded a significantly higher prevalence of tick infestation in dogs at Bhubaneswar, India during rainy season, this was attributed to the warm and humid weather during rainy season, which favour breeding of hard ticks. Nigeria has a humid and warm rainy season which is possibly responsible for the high prevalence of tick infestation recorded in this study.

The overall tick prevalence recorded in this study 54.2% (65/120), is higher than previous reports of 43.8% (14/31) in *Testudo graeca* sampled in Iran (Tavassoli, Asiabi and Tavassoli, 2007), but lower than the 71.4% prevalence recorded in a similar work on *Testudo graeca* in Turkey (Kirecci, Ozer, Balkaya,Tanis and Deveci, 2013), 77.8% (49/63) recorded in *Stigmochely pardalis* and 100% (25) in *Kinixys zobensis* sampled in South Africa. It is therefore confirmed that *Amblyomma species* are the commonest ixodid ticks infesting *Kinixys* in Ibadan, Nigeria. Ticks pose a health risk to animals as well as humans because of the roles they play in the transmission of diseases. Results from several studies have shown that ticks gained entry into other countries via imported animals including reptiles, and these ticks have been transmitted from importers to breeders, zoos, wildlife parks and other animals holding facilities such as pet stores and private hobbyists (Tavassoli *et al*., 2007). Besides, many hard ticks' species have been implicated as vectors of haemogregarines in reptiles (Tavassoli *et al*., 2007; Maia *et al*., 2012). Therefore the reports from this study support their purported role in the transmission of haemogregarines in *Kinixys* tortoises in Ibadan.

The result of Pearson's Correlation Coefficient analysis showed a moderate positive correlation between tick abundance and the level of haemogregarine parasitaemia ($r = 0.3758$; $p = 0.002$). Maia *et al*. (2012) reported that, the transmission *Hepatozoon* in terrestrial tortoises occur, when they feed on infected tick which serves as a definite host of the haemogregarine species; *Hepatozoon* and not by the bites of infected ticks. This implies that the more abundant the tick population on the intermediate vertebrate host (tortoise), the more the animal is would groom itself and in the process ingests

haemogregarine-positive ticks. Besides, haemogregarines have heteroxenous life cycle; with merogony occurring in intermediate vertebrate host (land tortoises), while gametogony and sporogony take place in the gut of invertebrate final host (ticks) (Haklova-Kocikova *et al*., 2014;Ozvegy *et al*. 2015). Barta *et al*. (2012) hypothesised that haemogregarines have co-evolved with their definite hosts. By implication, the high occurrence of tick infestation observed in this work is suggestive of the probable roles, ticks play in the transmission dynamics of haemogregarines in *Kinixys* tortoises. The positive correlation between tick abundance and haemogregarine parasitaemia and reports from previous studies corroborated the roles of ticks in haemogregarine transmission. In addition the presence of ticks (*Amblyomma species*) on most of the hinge-back tortoises is suggestive of frequent contact between the ticks and the tortoises, (Goes, Brito, Valadao, Gutierrez, Picelli and Viana, 2018). Also, the warm humid weather in Ibadan, Oyo state where this study was carried supported the breeding of ixodid ticks throughout the two seasons, thereby making tick infestation and haemogregarines infection to be present at any time of the year in Africa hinge-back tortoises (*K. belliana* and *K homeana*).

The results obtained from this present study and prior research works on haemogregarines using light microscopy indicates some level of sensitivity in the use of microscopy as a first-line in the diagnosis of haemogregarine infection (Dvorakova *et al*., 2014). The higher overall prevalence of 75.8% (91/120) recorded in this study by PCR method, against the prevalence of 53.5% (64/120) recorded by microscopy confirmed the sensitivity of PCR method at detecting positive infection than the routine laboratory method. In addition, identification of various haemogregarines species by morphology in blood smears is very difficult to attain, but the use of PCR methods with sensitive and species-specific primers makes identification of parasites in sample possible (Bartha *et al.* 2012;Ogedengbe, Ogedengbe, Hafeez and Barta 2015).

This study successfully elaborated the partial fragment of 18S RNA genes and constructed an evolutionary tree using the 18S RNA molecular marker. Results obtained supported the fact that *Hepatozoon spp*. could be categorised into many sister clades. In addition, the close evolutionary relationship of *Hepatozoon species* from vulture, jackals, wildcats, wildebeest (*Suis scrofa*), geckos and the result from this study could be attributed to increased pet trade among several countries of the world and the fact that

Hepatozoon species have a world-wide host range distribution of all *Haemogregarine species* Cook *et al*. (2014). The occurrence of *Hepatozoon species* in *Kinixys* tortoises in Ibadan, Nigeria, supported the wide host range distribution. Furthermore, several possibilities could be adduced to 18S RNA genes sequence-based evolutionary relationship of *Hepatozoon species* as reported by Tome *et al*. (2013) and Abdel- Baki *et al*. (2014). This result further explained the prey-predator and oral transmission assertion of *Hepatozoon* in the terrestrial chelonians, where parasites transmission is believed to occur when the intermediate hosts (tortoises) feed on *Hepatozoon* positive definite invertebrate hosts which are mostly ticks during grooming and become infected in the process. Haemogregarines are believed to undergo division in peripheral RBCs of the intermediate hosts and vectors such as leeches who would possibly transmit the parasites to the definitive host when they feed. Besides, *Hepatozoon fitzsimonsi* seldomly has peripheral blood phase multiplication; therefore, the probability of terrestrial chelonian becoming parasitised by leeches is very minimal due to the arid nature of the land tortoises' habitats. Also, vectors that commonly parasitise the land tortoises are mostly ticks, rather than leeches. In addition, several haemoparasites belonging to the apicomplexa are believed to have been spread by arthropods vectors (Maia, *et al*., 2012; Cook *et al*., 2014).

The phylogenetic analysis showed that *Hepatozoon species* sequenced from this study was identical to *Hepatozoon fitzsimonsi* discovered in *Kinixys zobensis* from South Africa. This report is inconsistent with the assertion that interspecies *Hepatozoon* were associated more to hosts geographic spread than species, (Barta *et al*. 2012; Abdel- Baki *et al*. 2014). It is therefore, necessary in further studies to provide information on the reassortment of *Hepatozoon* species from invertebrate hosts (ectoparasites) of land tortoises, in Nigeria. Moreover, molecular information has proven to be a practicable technique required in grouping apicomplexan. Therefore, other molecular markers aside 18S RNA genes, such as mitochondrial cytochrome c oxidase subunit I (COI), should be explored in future studies to appraise the rationale of the current 18S RNA genes -based evolutionary relationship of *Hepatozoon species* parasitising reptiles and chelonians in Nigeria. The haematological parameters reported in haemogregarine-positive and negative *K. belliana* and *K. homeana* closely mirrored published studies on

haemogregarines of tortoises. The low haematocrit values and red blood cells counts recorded in haemogregarine-positive of both *Kinixys species* is suggestive of decreased oxygen capacity through the invasion of haemogregarine into affected RBCs thus imparing the red blood cells activities. Furthermore, significantly high values of white blood cells and eosinophils recorded in haemogregarine- positive tortoises can be attributed to the presence of intraerythrocytic haemogregarines. Stacy *et al*. (2011) and Dilrukshi *et al*. (2019), have also submitted that haematological values in reptiles infected with *Haemogregarine stepnowi* have lower hematocrit, haemoglobin concentration and RBCs counts, when compared to previously reported ranges. In addition, haemoparasites infection in reptiles are said to be generally associated with anaemia. Perpinan, (2017) reported that intra-erythrocytic haemogregarines were linked to anaemia, basophilia and eosinophilia in affected reptiles. Furthermore, values of haematocrit, haemoglobin and red blood cells reported in haemogregarine-negative *K. belliana and K. homeana* closely mirrored the results of Arizza *et al*., (2014) in pond turtle(*Emys trinacris*), who reported similar reference ranges. These values are however significantly higher than the findings in Gopher tortoise (*Gopherus polyphemus*) and Testudo graeca, Hamooda, El-Mansoury and Mehdi, (2014). This implies higher oxygen-carrying capacity in African hinge-back tortoise than Gopher tortoise and Testudo graeca, a variation that may be as a result of species differences.

The values recorded for albumin (ALB), globulin (GLB), total protein (TP) and chlorine (C^-) respectively in both haemogregarine-positive *K. belliana* and *K. homeana* were within previously reported ranges. Perpinan, (2017), reported that, the values of plasma electrolytes and protein recorded did not show any significant differences in a group of haemogregarine infected and non-infected Russian tortoises. Furthermore, study conducted in Austria reported no significant changes in the plasma biochemical analytes of haemogregarine infected and uninfected snakes (Martinez-Silvestre*et al*., 2011). Although, literature is scarce on the influence of haemogregarines on blood chemistry of haemogregarine-positive tortoises or turtles. Besides, no interspecies differences were observed for Na^+, K^+, Ca^{2+}, Cl^- and P^-, values recorded were comparable to previously reported ranges from several other species of tortoises. Although elevated level of phosphorus was reported in haemogregarines-positive *Kinixys* tortoises in this study, non-

postprandial hyperphosphataemia is reportedly a dependable index of renal insufficiency in reptiles and this is used in the early diagnosing of renal disease, (Drake *et al*., 2017). The plasma electrolyte concentrations are said to be unchangeable due to strict control of body fluid concentration which is connected to basal biochemical activities in low and high vertebrates. It is therefore, suggested that in agreement with Perpinan, (2017), haematological and biochemical values recorded in haemogregarine-negative *Kinixys* tortoises in this study could be useful as reference for clinical health assessment in African hinge-back tortoises. However, differences in age and reproductive seasons should be taken into consideration.

Statistically significant high values recorded for ALT (33.91 $\pm$ 14.41U/L) in haemogregari ne-positive *K. bellana* and *K. homeana* showed that high values of ALT is associated with haemogregarine-positive *Kiniyxs*tortoises. This observation is suggestive of the impact of haemogregarines on the liver cells as a result of increased erythrophagocytosis of haemogregarine infected RBCs by the hepatocytes (Stacy *et al.*, 2011; Dvorakova*et al*., 2015; Nobuhide *et al.*, 2017), reported the negative impact of haemogregarines on the general wellbeing of affected hosts. It can, therefore, be inferred that haemogregarines have an impact on blood enzymes values (Ozvegy *et al*., 2015). Although, values of ALP, ALT and AST have been reported to rise during liver disease, elevated ALT is pathognomonic in the diagnosis of liver disease in most reptiles (Zaias *et al*., 2006; Andreani *et al*., 2014).

Besides the plasma activities of ALP and AST in both haemogregarine positive and h aemogregarine-negative *Kinixys* are within previously reported ranges for healthy tortoises. In higher vertebrates however, ALP is involved in bone formation, while in reptiles it is present in several tissues and organs; including bones and reproductive tract. Diseases affecting any of these organs can lead to increase in serum/plasma ALP levels in the body (Price *et al*., 2009; Kido *et al*., 2017). Consequent to this present research findings and reports of previous researches, it is evident that making a definitive diagnosis of the different liver diseases in reptiles is very complicated, thus making intravital diagnosis a huge challenge for clinicians who is left with no option than to combine different diagnostic methods. Therefore, biochemical assays is best compared with other methods such as diagnostic imaging, histopathology examinations of liver biopsy to arrive

at a definitive diagnosis in liver diseases of *Kinixys* tortoises (Dvorakova*et al*., 2015; Abousafeey, Mohamadain, Abdel-Gaber and Emara, 2019).

CHAPTER SIX

SUMMARY AND RECOMMENDATIONS

6.1 SUMMARY

This study has reported a high occurrence of 75.8% by PCR, 53.3% by light microscopy) of haemogregarines in *K. belliana* and *K. homeana*. It has also established the endemic nature of haemogregarines in chelonians during rainy and dry seasons in *K. belliana* and *K. homeana* with higher prevalence during rainy season in Ibadan, Nigeria. The study has further corroborated the higher sensitivity of molecular methods such as PCR in disease diagnosis. The molecular characterization of haemogregarine species sequenced from this study has a similarity of 100% and is identical to the isolate of *Hepatozoon fitzsimonsi* by Cook *et al*. (2015) from *Kinixys zombensis* in South Africa. Thus, it was submitted that the haemogregarine species isolated from *K. belliana* and *K. homeana* belonged to the *Hepatozoon species*

The presence of intra-erythrocytic haemogregarines in the RBCs of affected host causes destruction of the RBCs and the consequent low haematocrit level and reduced haemoglobin concentration affecting the general well- being of *Kinixys* tortoises if left untreated. Results of enzymes activities showed that haemogregarines have negative influences. High values of ALT obtained in haemogregarines-positive *Kinixys species* is attributed to liver damage or malfunctioning of the liver as a result of increased erythrophagocytosis of affected RBCs by the liver hepatocytes. ALT is pathognomonic for liver diseases in most reptiles (Andreani *et al.*, 2014; Perpinan, (2017).

The possible roles of the ticks *Amblyomma species* have been established. Furthermore, the study has supported the proposition and hypothesis that haemogregarines have co-evolved with their final hosts, as no other *Hepatozoon species* have so far been described in terrestrial chelonians Barta *et al*. (2012).

This study is first to the best of my knowledge to report the occurrence of haemogregarines in Africa hinge-back tortoises in Ibadan, Nigeria. It has also reiterated the high prevalence and the persistent nature of haemogregarine parasites in chelonians and by implication the deleterious effects on the general well-being of affected hosts if left untreated, as it is capable of reducing oxygen-carrying capacity to tissues and body organs through massive destruction of affected erythrocytes.

6.2 RECOMMENDATIONS

The following recommendation becomes imperative as a result of this project works:

- A comprehensive preventive health programme should be aimed at promoting the health of tortoises in *in-situ* and private facilities. Also, a routine health check is advocated throughout the seasons to promote their conservation.
- Chelonian enclosures; vivarium should be regularly treated against ectoparasites invasion.
- The application of the molecular techniques in diagnosing haemo-parasite in reptile is highly imperative to be able to make a definite diagnosis that will help in the treatments of various haemo-parasitic diseases affecting reptiles
- Moreso, extensive study in other states of Nigeria will be applicable to have a better understanding of the distribution as well as control of haemogregarines in chelonians.

6.3 CONTRIBUTIONS TO KNOWLEDGE

1. To date, no haemogregarine parasite species have been elucidated from *Kinixys* or any chelonian species in Nigeria, whereas several works have been carried out on haemogregarines of chelonians and other reptiles and amphibians in several countries across the globe. This research work was first to report the occurrence of haemogregarine (*Hepatozoon species*) in African hinge-back tortoises in Ibadan, Nigeria; this is particularly novel and a contribution to knowledge.

2. This study was first to characterise *Hepatozoon fitzsimonsi* in African hinge-back tortoises (*Kinixys species*) in Ibadan, Nigeria based on morphological and molecular characterisation.

3. This study has also contributed to establishment of values for haematology, plasma biochemistry and plasma enzymes activities ranges in *K. belliana* and *K. homeana* which could be useful in the health management of the Knixys tortoises.

4. This study is probably the first to elucidate the prevalence of *Amblyomma ticks* on African hinge-back tortoises in Ibadan, Nigeria, thus reiterating the high occurrence of tick infestation and the persistent nature of haemogregarine infection in the chelonians.

5. This study has also improvised a modified method for DNA extraction in chelonians blood with commercial kit through the modification of the heating time.

6.4 FURTHER RESEARCH

Further researches shall be based on providing information on the heterogeneity of *Hepatozoon* species using other molecular markers aside 18S RNA genes, such as mitochondrial cytochrome C oxidase (COI), to authenticate the current 18S RNA gene-based phylogenetic relationship of *Hepatozoon species* in Kinixys and other chelonians of Nigeria. In addition research on possible production of oral vaccine for the control of haemogregarines of reptiles in captive state and those on free range.

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Appendix 1. Reference range ofhaematological values from apparently healthy parameters of *Kinixys* tortoises

Parameter	Reference ranges
Haematocrit (%)	25.0 -38.4
Haemoglobin	5.7 - 11.55
RBC (10^{12}L)	0.46 -1.06
WBC (10^{9}/L)	3.3 -7.4
Lymphocytes (%)	26- 44
Eosinophils (%)	25- 30
Monocytes (%)	0 – 4
Heterophils (%)	N.A.
Basophils (%)	0 -30

Sources: McArthur,(2004); Gibbon and Carpenter, (2013)

Appendix 2. Reference range of blood biochemical values from apparently healthy parameters of *Kinixys* tortoises

Analytes	Reference Range
Albumin (mg/dl)	1.3-2.0
Globulin (mg/dl)	1.9-4.7
Total protein (g/dl)	3.3-6.14
Calcium (mg/dl)	8.2-8.6
Potassium (mg/dl)	4.8-5.0
Phosphorus (mg/dl)	5.0-8.0
Sodium (mg/dl)	121.3-130
Chloride (mg/dl)	90-120
Aspartate aminotransferase (AST) (U/L)	0-359
Alanine transaminase (ALT) (U/L)	N.R.
Alkaline phosphatase (ALP) (U/L)	122-606

Source: McArthur, S. 2004.