EVALUATION OF PATHOLOGICAL CHANGES IN THE PANCREAS OF BUCKS EXPERIMENTALLY INFECTED WITH *TRYPANOSOMA BRUCEI BRUCEI*

BY

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DEPARTMENT OF VETERINARY PATHOLOGY,
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ZARIA

SEPTEMBER, 2012

DECLARATION

I hereby declare that the work in this thesis titled "Evaluation of Pathological Changes in the Pancreas of Bucks Experimentally Infected with *Trypanosoma brucei brucei*" was performed by me in the Department of Veterinary Pathology, under the supervision of Prof. N.D.G. Ibrahim, Prof. M.Y. Fatihu and Prof. S.B. Oladele.

The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this work has been presented for another degree or diploma at any institution.

_____ _____

Mka'ave Peter NENSHI Date

CERTIFICATION

This thesis titled: Evaluation of Pathological Changes in the Pancreas of Bucks Experimentally Infected with *Trypanosoma brucei brucei* meets the regulations governing the award of the degree of Master of Science (M. Sc.) of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

I dedicate this work to the Almighty God, and to all men who love Him and are earnestly waiting for the coming of our Lord and Saviour Jesus Christ.

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ABSTRACT

In this study, the pathological changes in the pancreas of bucks experimentally infected with *Trypanosoma brucei brucei* Federe strain were evaluated, based on the appearance of pancreatic lesions, serum glucose and insulin profiles in a 40-day experimental study. Fifteen Savannah Brown bucks, aged between 12 and 15 months were used for the study. They were randomized into three groups: A, B and C of five bucks each. Each buck in groups A and B was inoculated via the jugular vein with 1×10^6 and 2×10^6 *T. b. brucei* respectively, while the bucks in the control group C, were each administered 1 ml of normal saline via the jugular vein. Parasitaemia in groups A and B appeared one day after infection. There was no significant difference ($p > 0.05$) in the mean parasitaemia levels of the two groups at the end of the experiment. The mean packed cell volume (PCV) at the end of the experiment in groups A, B and C were: 19.5 ± 1.9%, 19.8 ± 3.0%, and 30.4 ± 2.3%, respectively. There was a statistical significant difference ($p < 0.05$) in the mean PCV among the groups at the end of the experiment. The clinical signs observed in the infected groups included intermittent pyrexia, pale ocular mucous membranes indicative of anaemia, lethargy, lymphadenopathy and death. The cumulative mean serum glucose concentrations of the bucks in groups A, B and C at the end of the experiment were: 2.90 ± 0.07 mmol/L, 2.88 ± 0.08 mmol/L and 2.62 ± 0.06 mmol/L, respectively. There was statistical significant increase ($p < 0.05$) in the cumulative mean serum glucose concentrations of the infected groups A and B when compared to the control group C. The cumulative mean serum insulin concentrations in groups A, B and C, at the end of the experiment were: 0.3642 ± 0.0395 µg/L, 0.3131 ± 0.0306 µg/L and 0.2192 ± 0.0128 µg/L, respectively. There was statistically significant increase ($p < 0.01$) in the cumulative mean serum insulin concentrations of the bucks in group A when compared with that

of the bucks in the control group C. The gross lesions observed in the *T. b. brucei* - infected bucks in groups A and B were similar. They included pale carcasses indicative of anaemia, pale pancreas, lymphadenopathy, splenomegaly and hepatomegaly. No observable gross lesions were found in the organs from bucks of the control group C. The microscopic lesions observed in the pancreas of the *T. b. brucei* - infected bucks were dissimilar. In the infected group A bucks, focal degenerative changes were observed in the acinar cells and the islets of Langerhans, while in the infected group B bucks, diffused degenerative changes were observed in the acinar cells and the islets of Langerhans. There were no observable microscopic lesions in the pancreases of the control bucks. The study showed that experimental *T. b. brucei* infection in bucks produced clinical disease, degenerative changes in the pancreatic acinar cells and the islets of Langerhans, and a concurrent increase in serum glucose and insulin concentrations. Further research into evaluation of pathological changes in the pancreas of other animals such as dogs, experimentally infected with *T. b. brucei* is recommended.

TABLE OF CONTENTS

TITLE **PAGE**

TITLE PAGE ---------- i

DECLARATION ---------- ii

CERTIFICATION ---------- iii

DEDICATION ---------- iv

ACKNOWLEDGEMENT ---------- v

ABSTRACT ---------- vii

TABLE OF CONTENT ---------- ix

LIST OF FIGURES ---------- xiv

LIST OF PLATES ---------- xv

LIST OF APPENDICES ---------- xvi

LIST OF ABBREVIATINS ---------- xviii

CHAPTER 1: INTRODUTION ----------1

1.1 BACKGROUND OF THE STUDY ----------1

1.2 STATEMENT OF THE RESEARCH PROBLEM ---------- 3

1.3 JUSTIFICATION OF THE RESEARCH ---------- 4

1.4 AIM OF THE RESEARCH ---------- 5

1.5 OBJECTIVE OF THE RESEARCH ---------- 5

1.6 STATEMENT OF THE RESEARCH HYPOTHESIS ---------- 6

1.6.1 Null Hypothesis ---------- 6

CHAPTER 2: LITERATURE REVIEW ---------- 7

2.1 TRYPANOSOMOSIS ---------- 7

2.2 THE TRYPANOSOMES ---------- 10

2.2.1 Classification of Trypanosomes ---------- 10

2.2.2 General Morphology of the Family Trypanosomatidae ---------- 10

2.2.3 General Life Cycle of Trypanosomatidae ---------- 10

2.2.3.1 Developmental Stages in the Life Cycle of Trypanosomatidae -------- 11

2.2.4 Salivarian Trypanosomes ---------- 12

2.2.4.1 Sub-genus *Dutonella* ---------- 12

2.2.4.2 Sub-genus *Pycnomonas* ---------- 12

2.2.4.3 Sub-genus *Nannomonas* ---------- 13

2.2.4.4 Sub-genus *Trypanozoon* ---------- 13

2.2.5 Stercorarian Trypanosomes ---------- 14

2.2.5.1 Sub-genus *Herpetosoma* ---------- 14

2.2.5.2 Sub- genus *Megatrypanum* ---------- 15

2.2.5.3 Sub- genus *Schizotrypanum* ---------- 15

2.3 GEOGRAPHICAL DISTRIBUTION OF TRYPANOSOMOSIS ---------- 16

2.4 PATHOGENESIS OF TRYPANOSOMOSIS ---------- 16

2.5 PHASES OF ANIMAL TRYPANOSOMOSIS ---------- 18

2.5.1 Per Acute Phase ---------- 19

2.5.2 Acute Phase ---------- 19

2.5.3 Chronic Phase ---------- 19

2.5.4 Post-crisis or Recovery Phase ---------- 20

2.6 Diseases caused by *Trypanososma brucei* ---------- 20

2.7 Immunosuppression in Animal Trypanosomosis ---------- 22

2.8 Trypanotolerance in Animal Trypanosomosis ---------- 25

2.9 DIAGNOSIS OF ANIMAL TRYPANOSOMOSIS ---------- 28

2.10 CONTROL OF ANIMAL TRYPANOSOMOSIS ---------- 29

2.11 THE PANCREAS ---------- 31

2.12 INSULIN ---------- 34

2.13 GLUCOSE ---------- 36

CHAPTER 3: MATERIALS AND METHODS ---------- 39

3.1 EXPERIMENTAL ANIMALS ---------- 39

3.2 THE TRYPANOSOME USED ---------- 40

3.3 EXPERIMENTAL DESIGN ---------- 40

3.3.1 Grouping of Experimental Animals ---------- 40

3.3.2 Infection of Experimental Animals ---------- 40

3.3.3 Clinical Examination ---------- 41

3.3.4 Determination of the Rectal Temperature ---------- 41

3.3.5 Sample Collection and Processing ---------- 41

3.3.5.1 *Blood* ---------- 41

3.3.5.2 *Tissue* ---------- 42

3.3.6 Laboratory Analysis---------- 42

3.3.6.1 *Determination of Parasitaemia* ---------- 42

3.3.6.2 *Thin Blood Smear for Trypanosome Parasite Identification* ---------- 43

3.3.6.3 *Packed Cell Volume* ---------- 43

3.3.6.4 *Serum Glucose* ---------- 43

3.3.6.5 *Serum Insulin* ---------- 43

3.3.7 Histopathological Analysis ---------- 44

3.3.8 Statistical Analysis ---------- 44

CHAPTER 4: RESULTS ---------- 45

4.1 CLINICAL SIGNS ---------- 45

4.2 PARASITAEMIA ---------- 45

4.3 MEAN RECTAL TEMPERATURE ---------- 47

4.4 MEAN PACKED CELL VOLUME ---------- 49

4.5 MEAN SERUM GLUCOSE CONCENTRATIONS ---------- 51

4.6 MEAN SERUM INSULIN CONCENTRATIONS ---------- 54

4.7 GROSS PATHOLOGICAL OBSERVATIONS ---------- 57

4. 8 HISTOPATHOLOGICAL OBSERVATIONS ---------- 57

CHAPTER 5: DISCUSSION ---------- 61

CHAPTER 6: SUMMARY, CONCLUSIONS AND RECOMMENDATIONS-67

6.1 Summary ---------- 67

6.2 Conclusions ---------- 68

6.3 Recommendations ---------- 68

REFERENCES ---------- 69

LIST OF FIGURES

FIGURE	TITLE	PAGE
Figure 4.1:	Mean parasitaemia (+) of *T. b. brucei* - infected groups A and B bucks	46
Figure 4.2:	Mean rectal temperature (^{O}C) of control and *T. b. brucei* - infected groups A and B bucks	48
Figure 4.3:	Mean PCV (%) of control and *T. b. brucei*- infected groups A and B bucks	50
Figure 4.4:	Mean glucose concentration (mmol/L) of control and *T. b. brucei* - infected groups A and B bucks	52
Figure 4.5:	Cumulative mean glucose concentration of the control and the *T. b. brucei* - infected groups A and B bucks	53
Figure 4.6:	Mean insulin concentration (µg/L) of the control and *T. b. brucei* - infected groups A and B bucks	55
Figure 4.7:	Cumulative mean insulin concentration of the control and *T. b. brucei* – infected group A and B bucks	56

LIST OF PLATES

PLATE	TITLE	PAGE
Plate I:	Photomicrograph of H& E stained section of a pancreas from a *T. b. brucei* – infected group A buck	58
Plate II:	Photomicrograph of H & E stained section of a pancreas from a *T. b. brucei* – infected group B buck	59
Plate III:	Photomicrograph of H&E stained section of a pancreas from a control buck	60

LIST OF APPENDICES

APPENDIX	TITLE	PAGE
Appendix I:	Mean parasitaemia (+) of *T.brucei* - infected group A bucks	90
Appendix II:	Mean parasitaemia (+) of *T.brucei* - infected group B bucks	91
Appendix III:	Mean temperature values (^{O}C) of *T.brucei* infected group A bucks	92
Appendix IV:	Mean temperature values (^{O}C) of *T. b. brucei* - infected group B bucks	93
Appendix V:	Mean temperature values (^{O}C) of control group C bucks	94
Appendix VI:	Mean PCV (%) of *T.b.brucei* - infected group A bucks	95
Appendix VII:	Mean PCV (%) of *T. b. brucei* – infected group B bucks	96
Appendix VIII:	Mean PCV (%) of control group C bucks	97
Appendix IX:	Mean serum glucose concentrations (mmol/L) of *T. b. brucei* infected group A bucks	98

Appendix X: Mean serum glucose concentrations (mmol/L) of *T. b. brucei* - infected group B bucks ---------- 99

Appendix XI: Mean serum glucose concentrations (mmol/L) of control group C bucks ---------- 100

Appendix XII: Mean serum insulin concentrations (µg/L) of *T. b. brucei* - infected group A bucks ---------- 101

Appendix XIII: Mean serum insulin concentrations (µg/L) of *T. b. brucei* infected group B bucks ----------102

Appendix XIV: Mean serum insulin concentrations (µg/L) of control group C bucks ---------- 103

Appendix XV: The assay of serum glucose ---------- 104

Appendix XVI: The assay of serum insulin---------- 106

Appendix XVII: Standard curve ---------- 108

LIST OF ABBREVIATIONS

A = absorbance

g = Relative centrifugal force

^{o}C = degree centigrade

T^{o} = temperature

% = percent

PCV = packed cell volume

SEM = standard error of mean

p.i. = post infection

H&E = haematoxylin and eosin

A.M. = ante meridian

G = gauge

Ltd = limited

mins = minute (s)

nm = nanometre

µm = micrometre

cm =centimetre

µg = microgram

mg = milligram

kg = kilogram

mmol = millimole

µl = microlitre

ml = millilitre

dl = decilitre

L = litre

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Trypanosomosis (trypanosomiasis) is a protozoan infection of animals and man. It is of economic importance and caused by different species of trypanosomes*; Trypanosoma vivax, T. congolense, T. equiperdium, T. evansi, T. simiae, T. brucei brucei* which are found in animals, and *T. brucei gambiense* and *T. brucei rhodesiense* which are found in man, are the most pathogenic species (Maudlin *et al*., 2004; WHO, 2005). These parasites are either haematic, such as *T. vivax and T. congolense,* which are found in the plasma or tissue invasive, such as *T.brucei brucei, T. brucei gambiense, T. brucei rhodesiense, T. equiperdium* and *T. evansi* which are found intravascularly *or* extravascularly (Ikede and Losos, 1972; Soulsby, 1986).

These protozoan parasites e.g. *T. vivax, T. brucei and T. congolense* are biologically transmitted by tsetse fly of the genus *Glosina*. Others e.g. *T. evansi, T. vivax* are transmitted mechanically by biting flies such as *Tabanus* and *Stomoxys* species and *T. equiperdium* is transmitted venereally in equidae (Antony and Ron, 2004). The risk of transmission is primarily linked to intensity of encounters between vectors and hosts (Michael *et al.,* 2002).

African animal trypanosomosis is most important in cattle but can cause serious losses in pigs, camels, goats and sheep (Maudlin *et al*., 2004).). The course of the disease can be per acute, acute and chronic depending on the host and parasite virulence (Masake, 1980; Ikede, 1981; Adejinmi and Akinboade, 2000). Clinical signs of the disease include pyrexia, progressive anaemia, pale mucous membrane,

lacrimation, emaciation, nasal discharges and either diarrhoea or dark hard faeces. Other signs are oedematous swelling of the external genitalia, lower abdomen and thorax, ventral region of the neck and lymphadenitis in the supraorbital fossa and limbs in per acute cases, and lumbar paralysis in chronic cases (Ikede, 1981; Adeiza *et al.*, 2008). *Trypanosoma vivax* causes sudden tear and haemorrhage on the skin of the infected animal mostly seen in bovine species (Usman *et al.,* 2008). The disease terminates in death, if not treated (Taylor and Authie, 2004).

The pathogenesis and pathology of African trypanosomosis has for long remained the subject of intense investigation (Saror, 1980). The disease caused by protozoan of the genus *Trypanosoma* is characterized by serum biochemical and haematological changes, which drastically influence the pathogenesis and pathology of the disease (Anosa, 1988 a, b; Ngure *et al.,* 2008). It is also known that pathophysiological alterations occur in the cellular and plasma components of blood during trypanosome infection (Esievo, 1979; Takeet and Fagbemi, 2009). The disease is associated with a rapid decline in red blood cell (RBC) counts, haemoglobin (Hb) concentration and packed cell volume (PCV) and pallor of mucous membranes in the infected hosts, confirming that anaemia is a critical feature in the pathogenesis of African trypanosomosis (Esievo *et al.,* 1982). Changes in serum glucose profiles have also been the consistent observations in trypanosome infections (Anosa, 1988 a, b; Ngure *et al*., 2008; Sazmand *et al*; 2011).

Gross and histopathological lesions in various tissues and organs of animal, including the liver, lung, spleen, lymph nodes, kidneys, heart, thymus, testicles, gall bladder, brain and gastro-intestinal tract in cattle, sheep and goats have been reported

in animal trypanosomosis (Ikede and Losos, 1972; Anosa, 1991; Ibrahim *et al*., 2005; Audu *et al*., 2008; Fatihu *et al*., 2009).

The endocrine dysfunctions have also been studied in both animal and human trypanosomosis (Sekoni, 1994; Omeke and Igboeli, 2000; Allam *et al*., 2006; Blum *et al*., 2007). Trypanosome infections caused adrenal insufficiency, hypopituitarism, hypogonadism and hypothyroidism in human and animals, and polyglandular endocrine failure due to local inflammation of the pituitary, thyroid, adrenal and gonadal glands were reported (Anosa, 1991; Ogwu *et al*., 1992; Kennedy, 2006; Fatihu *et al*., 2009; Blum *et al*., 2011).

1.2 STATEMENT OF RESEARCH PROBLEM

Animal trypanosomosis occurs throughout the Tropical regions of Africa, the Middle East, Asia and Latin America. In Tropical Africa, the disease is found between latitude 14ºN and 29ºS covering 10 Million km² and stretching across over 37 countries in the region (Seifert, 1996; WHO, 1998; Mulumba, 2003). The disease has long been recognised as the major cause of poor production and losses in cattle, sheep and goats in Tropical Africa (Edward *et al.*, 1956). African trypanosomosis is responsible for 3 million livestock and 55,000 human deaths annually (Abenga *et al*., 2003; ILRAD, 1994; Mulumba, 2003; Samdi *et al*., 2010 a).

In Nigeria, tsetse flies which are the vectors for trypanosomosis still infest 80% of the nation land mass between latitude 13^0N and 4^0S (NITR, 1989; Onyiah *et al*., 1983). This includes the highlands of Jos, Mambilla and Obudu plateaux, which were before known to be tsetse-free (Onyiah, 1997). Thus, the disease has rendered about 80% of Nigerian landscape unprofitable for livestock production.

The pathogenesis and pathology of animal trypanosomosis has over the years received much attention. Consequently, gross and histopathological lesions in several tissues and organs in different animal species due to the disease have been reported (Ikede and Losos, 1972; Ibrahim *et al*., 2005; Audu *et al.,* 2008; Fatihu *et al*., 2009). These reports notwithstanding, there is dearth of information on the pathology of the pancreas due to trypanosomosis.

1.3 JUSTIFICATION OF THE RESEARCH

Trypanosomosis remains a major constraint to human and animal health, and increased livestock production in most countries of Tropical Africa (FAO, 2002; Ijagbone *et al*., 2004; Bourn *et al.,* 2005; WHO, 2005; Osanya *et al*., 2008). The prevalence of the disease in Nigeria and perhaps, in most African countries appears to be on the increase (Halid, 2001; Odoya *et al.,* 2003). It is reported that over 587, 273 km^2 (63.4%) of the Nigerian total land mass of 926,488 km^2 is affected by trypanosomosis (Balak *et al*., 2003; Yanan *et al*., 2004; Usman *et al.*, 2008).

The disease is responsible for about 3 million livestock and 55,000 human deaths annually in the sub-Saharan Africa (ILRAD, 1990; Abenga *et al*., 2003; Mulumba, 2003; Abenga *et al*., 2010). About 25.5 million goats and 14.5 million sheep equitably distributed in Nigeria are endangered by the disease (Ikede and Losos, 1987; FAO/OIE/WHO, 1995; Swallow, 2000).

About 35 million doses of trypanocidal drugs are used for the management of the disease in Africa (Geerts and Holmes, 1998). The cost of these drugs and other control measures used for the management of animal trypanosomosis annually in

Africa is of major financial implication to livestock owners and the government (Geerts and Holmes, 1998, Shaw, 2004).

Although, there are many reports on gross and histopathological lesions of several tissues and organs in both domestic and wild animals due to animal trypanosomosis (Ikede and Losos, 1972; Anosa, 1991; Ibrahim *et al*., 2005; Bissalla *et al.,* 2007; Audu *et al.,* 2008; Fatihu *et al*., 2009), reports on pancreatic dysfunction due to the disease are perhaps, currently scanty in the available literature. A study to evaluate the pathological changes in the pancreas and its functional status in bucks experimentally infected with *T. brucei brucei* becomes imperative.

1.4 GENERAL AIM OF THE RESEARCH

The general aim of the research was to evaluate the pathological changes in the pancreas and some serum biochemical profiles of bucks experimentally infected with *T. b. brucei*.

1.5 SPECIFIC OBJECTIVES OF THE RESEARCH

The specific objectives of the research were to determine:

1. The serum glucose concentrations of bucks experimentally infected with *T.b. brucei*.
2. The serum insulin concentrations of bucks experimentally infected with *T. b. brucei*.
3. The gross and histopathological lesions of the pancreas of bucks experimentally infected with *T. b. brucei*.

1.6 STATEMENT OF RESEARCH HYPOTHESIS

1.6.1 Null Hypotheses (Ho):

1. There are no biochemical changes in the pancreatic functions of bucks experimentally infected with *T. b. brucei*.

2. Trypanosomosis produces no pathological changes in the pancreas of bucks experimentally infected with *T. b. brucei*.

CHAPTER 2

LITERATURE REVIEW

2.1 TRYPANOSOMOSIS

Trypanosomosis is a disease complex in man and animals caused by species of protozoan parasites, belonging to the genus *Trypanosoma* (Maudlin *et al*., 2004). *Trypanosoma vivax, T. congolense* and *T. brucei* are the three most pathogenic species and are responsible for considerable production losses and livestock mortality where they occur (Kristjanson *et al.*, 1999; Maudlin *et al*., 2004). The severity of the infection with these trypanosome parasites depends on a range of factors such as the strain, infective dose and virulence of the parasite, and the susceptibility of the host to the infection, among others (Maudlin *et al*., 2004).

The transmission of the trypanosome parasite from an infected animal or man to a susceptible animal or man is mostly via the bite of tsetse fly of the genus *Glossina* (Losos, 1986). *Haematophagus* insects such as *Tabanus, Stomoxys, Haematopota, Chrysops and Hippobosca* species are the major biting flies that are also involved especially in the mechanical transmission of the parasite (Davies, 1997). Triatome bugs were incriminated in the transmission of Chagas' disease in human (Ikede and Losos, 1972; Losos, 1986).

Trypanosomosis is endemic in Africa and it causes chronic anaemia (Taylor and Authie, 2004), enlarged lymph nodes, starry hair coat, weakness or depression, and general loss of productivity and overall condition, including reduced milk yield and impaired fertility (Connor, 1994). *Trypanosoma brucei*, which is perhaps, the least pathogenic of the three species in cattle (Kristjanson *et al*., 1999), has three sub-species

namely, *T. brucei brucei, T. brucei gambiense* and *T. brucei rhodesiense* (Maudlin *et al*., 2004).

The gross and histopathological lesions in the various organs including the liver, lung, spleen, lymph nodes, kidneys, heart, thymus, testicles, gall bladder, brain and gastro-intestinal tract in cattle, sheep and goats have been reported (Ikede and Losos, 1972; Van Den Ingh *et al.*, 1976; Valli and Farsberg, 1979; Ikede, 1981; Anosa and Isoun, 1980; Anosa, 1991; Ibrahim, 2005; Umar *et al.*, 1998; Audu *et al*., 2008; Fatihu *et al*., 2009).

The pathology of the endocrine glands due to trypanosomosis in both man and animal has been investigated by quite a number of researchers (Sekoni, 1994; Omeke and Igboeli, 2000; Blum *et al*., 2007; Fatihu *et al*., 2009). *Trypanosoma brucei* infection in human has been reported to produce adrenal insufficiency, hypopituitarism, hypogonadism and hypothyroidism (Omeke and Igboeli, 2000; Kennedy, 2006; Blum *et al*., 2011). The infection in animals induced polyglandular endocrine failure due to local inflammation of the pituitary, thyroid, adrenal and gonadal glands (Reincke *et al*., 1993; 1998). Mutayoba *et al*. (1988a, b) reported ovarian, pituitary, thyroid and adrenal dysfunctions in female goats experimentally infected with *T. congolense.* Ogwu *et al*. (1992) observed adrenal and thyroid disorders in experimental *T. congolense* - infected cattle. Disorders of the pituitary and thyroid gland have been reported in *T. congolense* - infected Boran cattle (Abebe and Roberts, 1992; Abebe *et al.*, 1993).

The endocrine disorders have also been observed in experimental *T. brucei* infection in gilts (Allam *et al*., 2006). Testicular pathologic changes in sheep and goats, and white Fulani bulls experimentally infected with *T. vivax* have also been observed

(Anosa and Isoun, 1980; Adamu *et al.*, 2006). Experimental infections with *T. congolense* in Yankassa sheep resulted in thyroid malfunctions (Bissalla *et al*., 2007). Similarly, Fatihu *et al.* (2009) reported dysfunction of the thyroid in experimental *T. vivax* infection in Zebu cattle. Chronic pancreatitis with acinar atrophy, fibrosis and mononuclear cells infiltrations were also observed in mice, cats, guinea pigs, rabbits, hamsters, monkeys and dogs infected with *Trypanosoma cruzi* (Santos, 2004). The above review clearly indicates that little attention was given to the investigation of the status of the pancreas in trypanosome infection. Information on the pathology of the pancreas in trypanosomosis is therefore, undoubtfully scanty and demands for a more thorough investigation.

Tsetse flies, the main vectors for animal trypanosome parasites infest about 10 million km^2 of fertile land spread across 37 countries on the African continent, from Senegal in the North to South Africa in the South. Many areas that are infested with tsetse flies are the most suitable areas for livestock and crop production (Shaw, 2004). It has been estimated that about 46 million cattle and millions of other species of domestic animals are exposed to the menace of trypanosomosis in Africa (Kristjanson *et al*., 1999). About 25.5 million goats and 14.5 millions sheep equitably distributed in Nigeria are endangered by the disease (Ikede, 1987; FAO/OIE/WHO, 1995). The cattle per capita ratio, that is the average number of cattle per head of the population, is almost as high in Africa as in the developed world and the small ruminant per capita ratio is considerably higher, but productivity is much lower in Africa mainly due to the disease (Shaw, 2004).

2.2 THE TRYPANOSOMES

Trypanosomes are unicellular haemoflagellated protozoan parasites that cause trypanosomosis in animals and man. These are obligate parasites that usually have two hosts: the vertebrate and the invertebrate (Soulsby, 1986; Seifert, 1996).

2.2.1 Classification of the Trypanosomes

The pathogenic trypanosomes in the genus *Trypanosoma* belong to the order Kinetoplastida and family Trypanosomatidae (Soulsby, 1986; Stephens, 2008).The genus *Trypanosoma* is divided into two groups: the Salivarian and the Stercorarian, based on their sites of development in the vector. Transmission in the Salivarian and the Stercorarian trypanosomes occur through the saliva and fecal contamination of the wound caused by the bite of the vector respectively (Losos, 1986).

2.2.2 General Morphology of the Family Trypanosomatidae

Members of the family are leaf-like in shape, with a single flagellum attached to the body by an undulating membrane. The trypomastigotes or the bloodstream forms are lanceolate in shape. The flagellum which represents the locomotor apparatus is a single structure under light microscope while under electron microscope; the flagellum is seen to be convoluted with a distal end flagella sheath. The kinetoplast represents mitochondrium (Hoare, 1972). Members of the genus *Trypanosoma* are covered by a single cell membrane but the developmental stages are covered by an additional 10-15 mm thick surface coat which apparently protects the parasite against the resistance mechanisms of the host (Seifert, 1996).

2.2.3 General Life Cycle of Trypanosomatidae

The majority of this family group of trypanosomes undergoes a cyclical development. Haematogenous invertebrates are vectors, while the vertebrate animals serve as the definitive hosts. The vectors take the blood forms of the trypanosomes while feeding on an infected host. A cycle of development takes place in the intermediate host producing metacyclic trypanosomes or metatrypanosomes which are infective to a new mammalian host. The intermediate stages in the vector are non-infective to the final vertebrate host, hence the occurrence of indirect or cyclical transmission. Therefore, the life cycle of trypanosome parasite starts when the infective metatrypanosomes are injected into the mammalian host either by active inoculation through a bite as can be seen in the salivarian species e.g. *T. brucei,* or by passive contamination of the skin or mucous membrane as may be the case in the stercorarian species e.g. *T.cruzi* (Soulsby, 1986).

2.2.3.1 *Developmental Stages in the Life Cycle of Trypanosomatidae*

The following stages have been recognized in the life cycle of trypanosomes (Soulsby, 1986):

1. Trypomastigote stage: This is an infective stage for the definitive host, previously referred to as trypanosome stage. It is seen mostly in the vertebrate host and occasionally in the invertebrate vector.
2. Epimastigote stage: Hitherto referred to as crithidial stage seen mostly in the vector. It can occur in the vertebrate host as a developmental stage.
3. Promastigote stage: A stage formerly known as leptomonal stage. It is found in arthropods or plants.

4. Amastigote stage: A stage that before now was referred to as leishmania stage. It occurs both in the vertebrate and invertebrate hosts.

2.2.4 The Salivarian Trypanosomes

The salivarian group is divided into four subgenera: *Duttonella*, *Pycnomonas*, *Nannomonas* and *Trypanozoon* (Hoare, 1972; Longan-Henfrey *et al*., 1992). Members of these subgenera are transmitted by *Glossina* species.

2.2.4.1 *Sub-genus Duttonella*

Generally, members of this sub-genus are monomorphic with a large sub-terminal kinetoplast and a short, free flagellum. Their movement on wet blood mounts of fresh blood is highly rapid and distinctive. The site of development of these trypanosomes is in the proboscis (Hoare, 1972; Ijagbone *et al*., 2004). The sub-genus has two species *Trypanosoma (Duttonella) vivax* and *T. (D.) uniforme*. The later species is smaller but morphologically similar to the former. *Trypanosoma vivax* is the most important species of the sub-genus and widespread throughout the tsetse infested regions of Tropical Africa. *Trypanosoma vivax* infects cattle, sheep, goats, equines, camels and water buffaloes. Pigs, dogs, cats and most laboratory animals are known to be the resistant species (Ikede and Losos, 1972; Maudlin *et al*., 2004). However, few strains of *T. vivax* had been found to adapt in laboratory animals (Leeflang, *et al*., 1976). *Trypanosoma uniforme* is known to cause mild disease in cattle, sheep and goats in Central and East Africa.

2.2.4.2 *Sub-genus Pycnomonas*

This subgenus contains one specie, *Trypanosoma (Pycnomonas) suis*. It is known to infect pigs. *Trypanosoma suis* has short, stout monomorphic structure with a

free flagellum. Its site of development in the vector comprises the gut, salivary glands and the proboscis (Hoare, 1972., Stephen, 1986).

2.2.4.3 *Sub-genus Nannomonas*

The sub-genus has three identifiable species which are: *Trypanosoma (Nannomonas) congolense, T. (N.) simiae* and *T. godfreyi.* Generally, their developmental cycle in the vector is confined to the midgut and proboscis. The trypanosomes in this group have medium-sized marginal kinetoplast with no free flagellum. They could be monomorphic or pleomorphic and with a moderately developed undulating membrane. The vectors are tsetse flies (Hoare, 1972; Mc Namara *et al.,* 1994). *Trypanosoma congolense* is widely distributed in East Africa and its prevalence is on the increase in West Africa. Cattle, sheep, goats, equine, camels, domestic carnivores and pigs are susceptible hosts (Soulsby, 1986; Itard, 1989*). Trypanosoma simiae* was originally found in monkeys. It is a parasite of pigs and can cause severe disease in camels. It fails to infect laboratory rodents unlike *T. congolense*. The parasite is most prevalent in east and central Africa. *Trypanosoma godfreyi* infects pigs (Dukes *et al.,* 1991; Maudlin *et al*., 2004).

2.2.4.4 *Sub-genus Trypanozoon*

This sub-genus comprises three species, *Trypanosoma (Trypanozoon) brucei, T. evansi* and *T. equiperdum* which are morphologically indistinguishable. Variations within the species exist on the basis of distribution, pathogenicity and host range (Hoare, 1972; Itard, 1989). Man, cattle, sheep, goats, equine, camels, donkeys, pigs, dogs, cats and water buffaloes are susceptible natural hosts (Losos, 1986).

Trypanosoma evansi has two sub-species*: T. evansi evansi* and *T. evansi equinum*. It is distributed in many regions of the world such as South-East Asia, Middle East, North and East Africa (Dargantes *et al*., 2005). It is transmitted mainly by biting flies such as *Tabanus, Stomoxys and Lyperosia*. The absence of kinetoplast in *T. equinum* distinguishes it from *T. evansi* (Hoare, 1972; Soulsby, 1986). *Trypanosoma equiperdum* is similar morphologically to *T. evansi*. It is venereally transmitted and causes dourine in horses (Soulsby, 1986).

Three sub-species exist under the species *T. brucei* and are morphologically indistinguishable. *T. brucei brucei* causes Nagana (African trypanosomosis) in domesticated and various wild ungulates; *T. brucei gambiense* causes Gambian sleeping sickness of man and *T. brucei rhodesiense*, causes Rhodesian trypanosomosis or East African sleeping sickness of human (Soulsby, 1986; Maudlin *et al.,* 2004).

2.2.5 Stercorarian Trypanosomes

The trypanosomes of this group are commonly referred to as posterior station, lewisi group and group A. The stercorarian trypanosomes are divided into three sub-genera: *Herpetosoma, Megatrypanum* and *Schizotrypanum*. The group has large non-terminal kinetoplast with the posterior end tapering to form a flagellum. Members of the group have an inconspicuous undulating membrane (Hoare, 1972). Their development in the vector is confined to the midgut and transmission is by contamination from the posterior end. Trypanosomes of this group comprise of the non, mildly and highly pathogenic species e.g. *T. theileri*. Stercorarian trypanosomes have been reported in Africa (Soulsby, 1986).

2.2.5.1 *Sub-genus Herpetosoma*

The trypanosomes of this sub-genus are mostly parasites of rodents. They are monomorphic measuring between 21-37 µm in length, have inconspicuous undulating membrane and well developed free flagellum (Hoare, 1972). Members of the sub-genus are non-pathogenic and include: *Trypanosoma* (*Herpetosoma*) *lewisi* in rats, *T. musculi* in mouse, *T. nabiasi* in rabbits and *T. duttoni* in rats, monkeys and man. The vectors are the rat fleas (*Xenopsylla cheopis*) (Hoare, 1972; Stephens *et al*., 2001).

2.2.5.2. *Sub-genus Megatrypanum*

Trypanosoma (Megatrypanum) theileri is the typical specie of the sub-genus. It was discovered by Theiler in 1903 in the blood of cattle in South Africa and is world-wide in distribution. It is a large trypanosome measuring between 60-100 µm in length. It has a pointed posterior end and the kinetoplast is large and marginally placed farthest away at the posterior end (Hoare, 1972). It is a non-pathogenic, common parasite of domestic and wild ruminants and *Tabanus* flies are the major vectors (Hoare, 1972; Itard, 1989).

2.2.5.3 *Sub-genus Schizotrypanum*

A typical member of this sub-genus is *Trypanosoma (Schizotrypanum) cruzi*. The trypanosomes of this sub-genus are small and indistinguishable morphologically with a mean length measuring between 10-24 µm (Hoare, 1972). It is monomorphic, crescent shaped with a pointed posterior end. It has large, sub-terminal kinetoplast which fills the body of the parasite at a point. The undulating membrane is well developed and there is a free flagellum (Soulsby, 1986). *Trypanosoma cruzi* is highly pathogenic and it causes American human trypanosomosis or Chagas' disease. A wide

variety of animals such as dogs, cats, foxes, pigs and monkeys serve as reservoir hosts. *Triatomid* bugs are its vectors (Stephens *et al*., 2001).

2.3 GEOGRAPHICAL DISTRIBUTION OF TRYPANOSOMOSIS

Trypanosomes can be found wherever the tsetse fly exists. Tsetse flies are the vectors and they transmit the salivarian species which cause the most important forms of trypanosomosis in Africa. The disease occurs in most regions of the Middle East, Asia, Latin America and Tropical Africa. In the Tropical Africa, trypanosomosis is endemic in the regions between latitude 14ºN and 29ºS. These areas total about 10 million square kilometers (Losos, 1986; Seifert, 1996).

In Nigeria, the tsetse vectors are encountered in approximately 80% of the total landmass between latitude 13ºN and 4ºS (NITR, 1989; Onyiah *et al*., 1983).The plateau and the Sahel Savannah areas which were often listed as 'tsetse – free' because of their reclamation by various tsetse eradication operations (Jordan,1986). These areas are the plateau of Mambilla, Obudu and Jos (Davies, 1977 and Jordan, 1986), and the Sahel vegetation zone (Iwuala and Ajezie, 1980). However, these reclaimed areas have been reinfested by tsetse flies from the neighbouring Cameroun and other belts which were not covered by the control programme due to some administrative and or financial constraints (Jordan, 1986; Anene *et al*., 1991; Kalu, 1996; Dadah *et al*., 1997; Yanan *et al*., 2004).

2.4 PATHOGENESIS OF ANIMAL TRYPANOSOMOSIS

The pathogenesis of trypanosomosis generally starts at the site of the tsetse bite, where a hard, painful, red nodule known as chancre appears in the skin. Chancre may be several centimetres in diameter (usually 2-3cm) and it arises 4-14 days after a bite

(Apted, 1970). Morphologically, the chancre is a localized inflammatory site with oedema, infiltrations of macrophages and lymphocytes, and the multiplying parasites (Maudlin *et al*., 2004). The trypanosome parasites spread from the lesion into the lymphatics and the blood system. They continue to multiply and cause various types of tissue damage and disorders (Esievo *et al*., 1986; Donelson, 2003; Faye *et al*., 2005). Antibody is developed to the glycoprotein coat of the trypanosome parasite to kill them. This leads to the development of immune complexes. The aggregation of immune complexes as the infection continues, interferes with and or causes organ dysfunction (Williams *et al*., 1991).

The clinical and pathological manifestations of animal trypanosomosis are common to domestic animals, irrespective of the species of the trypanosomes involved (Maudlin *et al*., 2004). The severity of these pathological changes are influenced by several factors including the virulence of the parasite, the susceptibility of the host, the period of the infection during which samples are taken, among others. The prepatent period is generally between 1-3 weeks (Maudlin *et al*., 2004).

One most significant feature in the pathogenesis of clinical trypanosomosis in both animals and man that is consistently observed is anaemia (Saror, 1980; Goossens *et al*., 1998; Ogunsanmi and Taiwo, 2001; Cadiolli *et al*., 2006; Fatihu *et al*., 2009). It is defined as the absolute decrease in the total number of erythrocytes or haemoglobin per unit volume of blood and is responsible for the development of most tissue degenerative changes and disorders seen in trypanosome - infected animals (Logan-Henfrey *et al*., 1992). The onset of anaemia is related to the first wave of parasitaemia and its severity is associated with the level and initial peaks of parasitaemia (Esievo *et al*., 1982; Seifert, 1996).

The pathophysiological mechanism of anaemia is multifactorial (Anosa and Isoun, 1980) and therefore not fully established (Suliman *et al*., 1997; Cadiolli *et al*., 2006). However, some possible factors responsible for its occurrence were postulated among them are: hydraemia (haemodilution) (Fiennes, 1954; Maxie and Valli, 1978), haemolytic factors such as haemolysin, protease, phospholipase, lysolecithin and free fatty acids (Huan *et al*., 1975; Tizard *et al*., 1977; Seifert, 1996), coagulation disorders (Boreham and Facer, 1974; Valli *et al*., 1978) and the cleavage of erythrocyte surface glycoproyeins by trypanosomal sialidases (Esievo *et al*., 1982). Other factors also considered include, the depression of erythrogenesis (Igbokwe, 1989), immunologic mechanisms (Esievo and Saror, 1991; Logan- Henfrey *et al*., 1992; Watson, 2004), oxidative damage to erythrocytes (Igbokwe *et al*., 1994), the possible roles of a blunted erythropoietin response (Suliman *et al*., 1997) and erythrophagocytosis by macrophages including kupffer cell (Ibrahim, 1997).

The diverse nature of the reports notwithstanding, the general consensus is that the anaemia in both man and animal trypanosomosis is predominantly the result of haemolytic crisis, in which the erythrocytes are being destroyed by an expanded mononuclear phagocytic system (Taylor and Authie, 2004; Adamu *et al*., 2009). Thus, following infection with trypanosomes, an intense erythrophagocytosis, which is mainly extravascular, due to the activated and expanded mononuclear phagocytic system is elicited, in which the half-life of the erythrocytes is significantly reduced and destroyed (Witola and Lovelace, 1997; Taylor and Authie, 2004; Adamu *et al*., 2009).

2.5 PHASES OF ANIMAL TRYPANOSOMOSIS

The pattern of a trypanosome infection is quite variable due to the variety of physiological and environmental factors such as age, sex, pregnancy, nutrition, extreme

exercise, previous exposure to trypanosome infections and intercurrent diseases. Thus, per acute, acute, sub-acute and chronic forms of the disease have been described (Losos, 1986; Anosa, 1988a).

2.5.1 Per Acute Phase

This phase is noticed mostly in rodents when they are infected with *T. congolense* and *T. brucei*. The parasitaemia in these animals is uncontrollably high and the affected animals die within a week (Maudlin, 2004).

2.5.2 Acute Phase

The beginning of this phase is marked by the first appearance of the trypanosomes in the blood after inoculation and variable period of incubation depending on the immune status of the host and the pathogenicity of the trypanosome. The parasitaemia is usually high and fluctuates leading to anaemia. Anaemia is the most prominent feature of trypanosomosis and may be observed as pallor of the mucous membranes. Most of the affected animals die or pass into chronic phase of the disease and some may occasionally recover (Nantulya, 1990; Anosa, 1988a; Maudlin *et al*., 2004).

2.5.3 Chronic Phase

This phase is characterized by intermittent parasitaemia to aparasitaemia (Maudlin *et al.*, 2004). The temperature shows wide fluctuations and there is marked pancytopenia and alterations in the blood chemistry. This phase of the infection appears to be the period during which the infected animals have adequately mobilized its defense mechanism to depress the multiplication of the parasite, but is inadequate to stop the

infection or reverse the pathological changes that were established in the acute phase. It also permits extra vascular establishment of tissue – invasive trypanosomes thus becoming less numerous vascularly (Anosa, 1988a, b). The animals that suffer from the chronic form of trypanosomosis are extremely weak; however, they continue to eat. The chronic phase may last for months or years and is most often terminated by death, self cure or a persistent carrier state (Murray and Dexter, 1988).

2.5.4 Post-crisis and the Recovery Phase

This is the period in which the affected animal recovers from the disease. In this phase, there is slow reversion of cellular and other pathological changes to normal pre-infection state (Anosa, 1988a, b).

2.6 DISEASES CAUSED BY *TRYPANOSOMA BRUCEI*

Three sub- species of *Trypanosoma brucei* have been recognized, namely *T. brucei brucei, T. brucei gambiense* and *T. brucei rhodesiense.* Among these, *T. b. brucei* is considered most virulent in domestic animals (Ikede, 1981). Human trypanosomosis is caused by the other two sub-species. Human infection caused by *T. b. rhodesiense* manifest in form of an acute inflammatory disease while the *T. b. gambiense* variant causes a chronic autoimmune disease (Maudlin *et al*., 2004).

Trypanosoma b. brucei has relatively short incubation period and causes infections in horses, camels, cattle, sheep, goats, pigs and dogs. Varying prepatent periods of the disease have been reported ranging from 2 – 6 days p.i. (Omeke and Onuora, 1992; Allam *et al*., 2006; Adeiza *et al*., 2008; Mbaya *et al*., 2009). The specie is generally considered as being of little clinical importance in cattle, but may be responsible for acute and chronic infections in goats and sheep. The severity of the

infections with *T. b. brucei* is dependent on a range of factors. Diseases of *T.b. brucei* however, seem less prevalent and pathogenic than those of *T. vivax* and *T. congolense* (Maudlin *et al*., 2004).

The pathology caused by *T. b. brucei* is more widely distributed in the host when compared to other trypanosome species. The parasite has more affinity for tissues than for blood and can cause severe lesions in the tissues it invades. The myocardium is more commonly affected with degenerative changes and focal areas of necrosis of the myocytes and fibrosis. The lesions due to long standing infections have also been observed in various organs including the pituitary, adrenal, kidney and gonads (Seifert, 1996). The pathology in cattle was dominated by three features namely anemia, tissue lesions and immunosuppression (Anosa and Kaneko, 1983).

Cattle, sheep and goats experimentally infected with *T. b. brucei* developed an acute disease that lasted for few days. Chronic phase follows with the involvement of central nervous system and may terminate in death (Ikede and Losos, 1972; Moulton, 1986). The disease in dogs is acute with high parasitaemia, moderate to severe anaemia and central nervous system disorders which often lead to death (Adamu *et al.,* 2009). *Trypanosoma b. brucei* infection in horses and camels is characterized by high fever, anaemia, anorexia, subcutaneous oedema, emaciation and often death (Ikede *et al*., 1977). Inco-ordination and spinal paralysis may occur in horses. *Trypanosoma b. brucei* can develop in many sites including the eye producing lacrimation, photophobia and keratoconjuctivitis (Adamu *et al*., 2009). High prevalence of *T. brucei* infections was reported in Nigeria (Omotainse *et al.,* 2000). The Clinical signs manifested in pigs include intermittent fever and parasitaemia, anaemia, loss of weight and body condition, dehydration, anorexia, paresis and wobbling of hind legs, ascites, petechial

hemorrhages and death (Otesile *et al.*, 1992; Allam *et al*., 2006). Rats and mice are common laboratory animals used in *T. brucei* experimental studies.

2.7 IMMUNOSUPPRESSION IN TRYPANOSOMOSIS

The trypanosome - induced suppression of the host's immune responses is termed immunosuppression. The trypanosome parasites present in the bloodstream of the mammalian host develop very sophiscated mechanisms to evade the host's immune defences (Donelson *et al*., 1998; Mansfield and Paulnock, 2005). It is considered as one of the major mechanisms the parasite employs to evade the immune defences of the host (Donelson *et al*., 1998). Immunosuppression reduces the infected animal's resistance to other infections leading to secondary diseases (Darji *et al.*, 1996; Sileghem *et al.*, 1994; Uzonna *et al*., 1998).

It is a vital factor that complicates the clinical and pathological effects of trypanosomosis (Moulton and Sollod, 1976). Ekanem *et al*. (1994) stated that, active biological substances derived from trypanosomes or those induced by trypanosomes from the host or their interplay may be the basis for the pathology in trypanosome infections. Major modifications of immune system that have been observed in African trypanosomosis included lymphadenopathy, splenomegaly with destruction of lymphatic tissue architecture and hypergamaglobulinemia. This immune reaction does not lead to protection but are involved in immunopathology disorders (Vincendeau and Bouteille, 2006).

One of the major characteristics of trypanosomes is the presence of the variant surface glycoprotein (VSG) which covers nearly all the membrane of trypanosomes in mammals and is the predominant surface antigen of African trypanosomes. The variant

surface glycoprotein constitutes an important molecular interface between trypanosomes and the host immune system. It prevents trypanosome lysis by complement alternative pathway and above all enables them to avoid the specific immune response via the phenomenon of antigenic variation (Vincendeau and Bouteille, 2006). The variant surface glycoprotein is also released in the host fluid. The variant surface glycoprotein has several effects on immune elements such as induction of auto antibodies and cytokines, in particular tumor necrosis factors (TNF) –α (Tachado and Schofield, 1994; Okomo- Assoumou *et al*, 1995; Magez *et al*, 2002). Other trypanosome components and soluble factors, such as a trypanosome – released triggering factor (TLTF) which triggers interferon (IFN) –γ production by T-cells, are also involved in the modulation of the immune system by acting on the synthesis of immune elements (Olsson *et al*, 1991; Vaidya *et al*., 1997).

Furthermore, increased levels of circulating endotoxins are features of human and experimental trypanosomosis. These endotoxins are potent immunomodulatory molecules which precipitate the immune disorders observed in the disease (Nyakundi *et al*., 2002). Another escape mechanism discovered later is host arginine induction. Trypanosomes decrease immune response efficacy and increase the production of L-ornithine, an essential growth factor (Gobert *et al*., 2000; Vincendeau *et al*., 2003; Duleu *et al*., 2004).

Immunosuppression has been described in both natural and experimental infections of domestic mammals as well as laboratory rodents. It is suggested to be responsible for the poor responsiveness of trypanosome – bearing host to vaccination (Greenwood *et al*., 1973). Immunosuppression due to trypanosome infection was first reported in studies on the response of mice and rabbits infected with *Trypanosoma*

brucei to heterologous red cells (Goodwin, 1970; Goodwin *et al*., 1972). It has been reported that antibody response to *T. brucei* infected sheep red cells is impaired when assessed by the production of haemaglutinating antibody and by the plague technique (Goodwin *et al*., 1972; Freeman *et al*., 1974). Murray *et al*. (1974 b) observed a decreased antibody response to liposacaride, a thymus dependent antigen. A decreased IgG and IgE antibody responses to worm infestation was also observed in mice infected with *T. brucei* and *Nippostrongylus brasiliensis* (Urquhart, 1977). Immunosuppression has also been observed and reported in cattle infected with *T. congolense* (Ilemobade *et al.*, 1982; Rurangirwa *et al*., 1983) and in sheep infected with *T. evansi* (Onah *et al*., 1997).

Immunointervention by use of non specific immunostimulants such as Calmette Guerin Bacilli (BCG) and *Propionibacterium acnes* were used to activate and increase mice resistance to trypanosome infection (Murray and Morrison, 1979; Black *et al.*, 1989), BCG and *Corynebacterium pavum* (Clark *et al*., 1977; Murray and Morrison, 1979), endotoxins (Singer *et al*., 1964) and polyribonucleotide (Herman and Baron, 1971). Non-specific immunostimulants increase survival time of the host, prolong prepatent period, a delay in the time taken to reach parasitamia peak and a reduction in the level of parasitaemia (Bissalla *et al*., 2007).

More advances on the study of immunosuppresssion in trypanosomosis has led to the discovery of T and B lymphocyte subpopulations especially, the T helper (Th) subsets and the cytokines produced by each Th1 and Th2 subset. Vincendeau and Bouteille (2006) stated that these factors influence diverse aspects of the immune responses particularly the synthesis of nitric oxide (NO) known to be involved in several steps in the immune mechanisms.

2.8 TRYPANOTOLERANCE IN TRYPANOSOMOSIS

Trypanotolerance has been described as a relative capacity of an animal to control the development of the trypanosome parasite and to limit its pathological effects (Naessens, *et al.*, 2002; Abenga and Vuza, 2005). Natural resistance to trypanosomosis has been known to occur in certain livestock breeds (Murray *et al.*, 1982; Naessens, 2006). These breeds of livestock show remarkable resistance to the effects of African trypanosomosis and are said to be trypanotolerant. Unlike trypanosensitive animals, trypanotolerant animals control parasitaemia and do not show anaemia or production losses without recourse to trypanocidal drugs (Nganga *et al.*, 2007).

Some breeds of cattle such as the humpless long-horn N'dama, Baule, Laguna, Samba, Dahomey, short-horn Muturu in West Africa and Orma Boran in East Africa, do exhibit trypanotolerance (Murray *et al.*, 1982; Naessens, *et al.*, 2002). Trypanotolerance was demonstrated in small ruminants and some species of wild life animals (Griffin and Allonby, 1979; Murray *et al.*, 1981; Itard, 1989; Black *et al.*, 2001). The main breed of sheep and goats in the Gambia, the Djallonke sheep and the West African dwarf goats have been shown to possess an increased resistance to trypanosomosis (Osaer *et al.*, 1999., Geerts *et al.*, 2009). The West African dwarf goats found in the tsetse endemic zones are known to have more resistance to the trypanosome infections than the exotic breeds found in tsetse free regions (Adah *et al.*, 1993). However, the West African dwarf goats are found to possess lesser degree of trypanotolerance than the Djallonke sheep (Leak *et al.*, 2002; Geerts *et al.*, 2009). Trypanotolerance in small ruminants is less pronounced than in cattle. It should therefore, be considered as a resilience rather than resistance (D'leteren *et al.*, 1999;

Geerts *et al.*, 2009). Studies have shown that there is an important introgression of genes of trypanosusceptible breeds into West African goat population which may possibly explain the loss of trypanotolerance in this specie (Geerts *et al.*, 2009). There is need to take appropriate measures to safeguard and upgrade the genetic purity of trypanotolerant goats and sheep breeds in Africa.

Trypanotolerance is a complex mechanism which involves some factors that are yet to be fully understood. It is manifested by the higher degree of the efficiency of the animal to control the development of parasitamia and anemia (Esievo *et al*., 1986; Wang *et al.*, 1999; Abenga and Vuza, 2005). Trypanotolerant trait has been described in *T. congolense* infected Nigerian puppies (Abenga *et al.*, 2005 a, b). Trypanotolerance has been described as a relative rather than an absolute trait. It may be affected by nutritional status, stress of work and concurrent infections (Seck *et al.*, 2002, Faye *et al.*, 2005). No single inherited genetic controlling factor for the innate resistance has been found yet, but trypanotolerance appeared to be inherited as a polygenic trait (Tabel *et al.*, 2000; Hanotte *et al.*, 2003).

Molecular based studies have identified genomic regions controlling trypanotolerance in mice and cattle (Iraqi *et al*., 2001; Hanotte *et al*., 2003). These biotechnologies however, have not been able to identify the complete pool of genes involved in trypanotolerance (Abenga and Vuza, 2005). Brett *et al.* (2004) reported that innate immunity to trypanosome infections exhibited by trypanotolerant animals was as a result of natural selection following exposure to the disease over the years. Teale (1991) however, demonstrated that previous exposure to trypanosomosis does not influence the expression of the trypanotolerant trait.

Some factors determining trypanosomes control in trypanotolerance were outlined. These are complement-dependent and clone-specific lytic activities, and complement-independent trypanocidal activities that are not confined to trypanosome clones and species (Wang *et al.*, 1999). Antitrypanosomal activities in the sera of trypanotolerant Cape buffalo, giraffe and greater kudu which inhibit the replication of *T. brucei* have been observed (Black *et al*., 1999). A higher rate of occurrence of spleen cells that produced Variant Surface Glycoprotein – specific immunoglobulin G (IgG) was reported in trypanotolerant cattle than trypanosusceptible ones. Trypanosusceptible cattle however, produced greater quantities of polyspecific IgM that deplete compliment and render these animals unable to control parasitamia (Taylor *et al*., 1998). The role of superior antibody mediated destruction of trypanosomes in trypanotolerance remains doubtful (Williams *et al.*, 1996; Abenga and Vuza, 2005).

In addition to their resistance to trypanosome infections, trypanotolerant breeds of animals have been reported to be resistant to several other important infectious diseases (Murray *et al*.,1981) including ectoparasitisms, helminthoses and a number of tick-borne infections such as heartwater, bovine anaplasmosis and bovine babesiosis (Mattioli *et al*., 2000). Trypanotolerance has been identified as a more sustainable approach to controlling animal trypanosomosis (Gbodjo *et al.*, 2001; Maichomo *et al*., 2001).

2.9 DIAGNOSIS OF ANIMAL TRYPANOSOMOSIS

The clinical diagnosis of animal trypanosomosis is difficult because the clinical signs are non-pathognomonic. The disease may also run a subclinical course and a variety of diseases have similar clinical manifestations. Generally, for field diagnosis in rural settings, microscopy-based techniques using direct observation of wet blood

film, or concentration techniques such as the buffy coat technique (BCT) (Murray *et al*.,1977) and the haematocrit centrifugation technique (HCT) (Woo, 1970) are the most common methods of parasites detection, and have historically been considered the gold standard techniques. Picozzi *et al. (*2002), illustrated however, that microscopy has a very poor sensitivity compared to the molecular tools, highlighting that previous studies using these methods were likely to have significantly underestimated both animal and herd-level prevalence of the pathogens.

The molecular diagnostic tools, and in particular polymerase chain reaction (PCR), has vastly improved the detection of trypanosome infections over standard parasitological techniques, by lowering the parasitaemia limit by several orders of magnitude. The analytical sensitivity of concentration techniques, such as the HCT or the BCT ranges between detectable parasitaemia levels of 2.5×10^2 to 5×10^3 parasites /ml of blood depending on the trypanosome species (Massake *et al.*, 2002), whereas PCR can detect the presence of the parasite DNA equivalent to one trypanosome in 10 ml of host blood (Eisler *et al.,* 1997).The analytical detection limit of trypanosomes especially *Trypanosoma brucei* specific PCR has been shown to be as low as 1/10 of the genetic material of a single trypanosome per PCR (Moser *et al.,* 1989). The internal transcribed spacer (ITS)-PCR has been shown to detect trypanosomal DNA at detection limit equivalent to less than one parasite/ml of host blood (Cox *et al*., 2005). Furthermore, primer design targeting precise DNA sequences ensures high specificity of PCR, making it independent of morphological differences required for speciation by microscopy. The polymerase chain reaction was incorporated as the diagnostic tool of choice into a wide number of studies investigating the epidemiology of trypanosomosis.

The PCR-based diagnosis of trypanosome infections in livestock has now been used in a number of studies across Africa (Cox *et al*., 2005; Ng'ayo *et al*., 2005; Pinchbeck *et al.,* 2008), using different protocols and methods (Cox *et al*., 2005; Picoozzi *et al.,* 2008). The field applications of PCR include estimating prevalence for monitoring of control programs. However, due to the cost and level of laboratory equipment involved, PCR is not suitable for diagnostic testing of individual animals for treatments at the local level (Bronsvoort *et al.,* 2010).

2.10 CONTROL OF ANIMAL TRYPANOSOMOSIS

The control of animal trypanosomosis in Africa principally involves three approaches: Vector control, management of the disease by use of drugs and or vaccines and the breeding of trypanotolerant breed of livestock (Vale *et al*., 1985; Barret, 1997).

In the past, bush clearing to decrease tsetse natural habitat, elimination of game animals to decrease the natural reservoirs and aerial spraying of insecticides to kill arthropod vectors were the major control measures employed in the control of African trypanosomosis. These measures however, were faced with a lot of problems, for instance, bush clearing was destructive to our natural environment whereas, game elimination and aerial spraying were detrimental to the ecosystem (Watson, 2004).

The control of the vectors is considered the most favoured approach because tsetse is the most vulnerable component of the host, parasite and vector relationship in terms of habitat requirements. More so, the biology and behavior of tsetse flies have made these arthropods readily susceptible to insecticide-based techniques (Vale *et al*., 1985). Vector control on a large scale is dependent on the use of insecticides. The application of the insecticides could either be from the ground or by aerial spraying

using helicopters or aero planes in the infested areas (Vale, 1993; Bauer *et al.,* 1995). Aerial spraying has however, been reported to enhance the development of insecticide resistance (Guisanc *et al.,* 1980; Van der Vloedt *et al.,* 1980).

Trypanocides are now widely used in the management of trypanosome infections in domestic animals. The three major trypanocidal drugs in use are isometamidium chloride, homidium chloride, and homidium bromide and diminazene aceturate. The first two possess both prophylactic and therapeutic effects, while the later has only therapeutic properties. These drugs have been in use for half a century, and about 35 million doses of which are being used each year in Africa for control of about 50-70 million animals at risk of trypanosomosis (Geerts and Holmes, 1998). Drug resistance has emerged due to their long use. This notwithstanding, the International pharmaceutical industries, the sole producers and suppliers, have shown little interest in the development of new trypanocides possibly due to the relatively poor and limited market demand in Africa (Geerts *et al.*, 2001).

Due to drug resistance, toxicity, environmental pollution and chemical production costs (Eisler *et al.,* 1997), the search for alternative means for control of tsetse flies and trypanosomosis becomes imperative. The use of trypanotolerant livestock and the use of pyrethroids in the form of dip or spray to protect individual animals is a viable alternative. However, these have not been effective control measures on a larger scale (Aksoy *et al*., 2003).

The need for trypanotolerant animals has led to intensified researches into trypanotolerant animal breeding programs, but this has been more on cattle than other species of animals. Trypanotolerant breeds have lower mortalities and higher calving rates than trypanosusceptible breeds (FAO, 2000). Jordan (1995) suggested

chemoprophylaxis with trypanocides for trypanotolerant breeds of livestock located in areas with high vector density, high level of trypanosomosis and challenge of other stress factors such as ploughing and malnutrition.

Other methods of tsetse control such as the use of biconic, monoconic or pyramidal, bipyramidal traps often combined with odour baits to attract tsetse flies (Gouteux, 1991; Seifert, 1996), the application of sterile male technique (as used in screwworm eradication in United States of America) (Sarmiento and Jawonisi, 1981) and catching of the tsetse flies by hand are also important.

2.11 THE PANCREAS

The pancreas of domestic animals is a bilobed gland located adjacent to the proximal part of the duodenum and the proximal part of the jejunum where it appears as an elongated gland of loosely connected aggregated nodules. It is a dual functional gland composed of the exocrine and endocrine components (Kaneko *et al*., 2008).

The exocrine component of the pancreas produces enzymatic secretions which are delivered into the lumen of the duodenum by one or two ducts such as in dogs. The main pancreatic duct enters the first part of the duodenum close to the common bile duct, which comes from the liver. In sheep and goats, a single pancreatic duct empties directly into the common bile duct, such that a mixture of bile and pancreatic juice enters the duodenum and the enzymes help in digestion (Frandson *et al*., 2009).

The endocrine component has cell aggregates that form small masses of the endocrine gland scattered throughout the substance of the pancreas. They produce and release secretions directly into the blood stream (Frandson *et al*., 2009).

Microscopically, stained sections of the pancreas reveal two different types of parenchymal tissues, the pancreatic acini and pancreatic islets. The pancreatic acini are dark stained, small and berry-like clusters of cells. They produce and secrete pancreatic juice which contains digestive enzymes (amylase, chymotrypsin, elastase, lipase and phospholipase) in an alkaline medium that pass to the small intestine. These enzymes play important role in the digestion of carbohydrates, proteins and lipids (fats) in the chyme (Frandson *et al.*, 2009).

The pancreatic islets, formerly referred to as islets of Langerhans are lightly stained, large and spherical clusters of cells. These cells produce and secrete hormones. Approximately a million pancreatic islets form the endocrine pancreas (Reece, 1987).

Four main cell types exist in the islets which are relatively difficult to distinguish by standard staining techniques, but can be classified by their secretions. The α cells secrete glucagon which function to increase glucose level in the blood, the β cells secrete insulin which function to decrease glucose level in the blood, the δ cells secrete somatostatin which regulates and or stops the action of α and β cells, and pp cells secrete pancreatic polypeptide (Harmon, 1992).

Generally, the pancreatic islets play an imperative role in glucose metabolism and regulation of blood glucose concentration (Croom *et al*., 1992). The pancreatic hormones, insulin and glucagon interact together to maintain the proper level of glucose in the blood. Glucose is used by the body for energy (Croom *et al*., 1992).

The secretory activity of the pancreas is regulated directly via the effect of hormones in the blood on the pancreatic islets and indirectly through the effect of the autonomic nervous system on the blood flow (Frandson *et al*., 2009).

Endocrine diseases of the pancreas include diabetes mellitus and tumours. Tumours of the endocrine pancreas include insulinoma, glucagonoma, gastrinoma, somatostatinoma and vasoactive intestinal polypeptide tumour (VIPoma). The tumours of the endocrine pancreas may also be part of the multiple endocrine neoplasia type1 (MEN1), denoted as Werner syndrome (Zlatoš, 1996; King *et al.*, 1998; Kim *et al.*, 2006).

Diabetes mellitus is the most common endocrine disorder. It is a chronic disorder of carbohydrate metabolism due to relative or absolute deficiency of insulin production by the β cells or its insufficient effect in target tissue, usually in the presence of a relative or absolute excess of glucagon. The common and the most permanent symptom of the disorder is hyperglycemia ((Zlatoš, 1996; Mbaya and Ramiaya, 2006).

In animal species, diabetes mellitus has been reported in virtually all laboratory animals (gerbils, guinea pigs, hamster, mice, rats, non-human primates) and in horses, cattle, sheep and pigs (Gould, 1981; Tajima *et al*., 1999., Clark, 2003). However, the disease occurs most commonly in middle-aged dogs and cats. In dogs, more cases have been reported in small breeds and females than males. Breed nor sex disposition in cats has not been observed (Ettinger and Feldman, 2005; Kim *et al.*, 2006; Kaneko *et al*., 2008).

The World Health Organisation (WHO) defines diabetes mellitus as a state of chronic hyperglycemia caused by simultaneous effect of genetic and environmental factors including nutrition. This definition indicates that diabetes has multifactorial genesis. Genetic, immunological, viral, toxico-chemical and nutritional factors participate in the origin of the disease. Besides that, diabetes mellitus is associated with

other primary diseases or syndromes of the pancreas (WHO, 2003; Lenzen, 2008; Gwarzo *et al*., 2010).

Chronic pancreatitis with acinar atrophy, fibrosis and mononuclear cells infiltrations have been reported in mice, cats, guinea pigs, rabbits, hamsters, monkeys and dogs were infected with *Trypanosoma cruzi* (Santos, 2004).

2.12 INSULIN

Insulin is a peptide hormone. Its precursor, proinsulin, is single-chain looped polypeptide linked by disulfide bridges. It varies in length from 78 amino acids residues in dogs to 86 amino acids in human, horses and rats. Its molecular weight is 9 x 10^3 daltons. Proinsulin is biosynthesized in the pancreatic β cells on the rough endoplasmic reticulum. It is transported and stored in the secretory granules on the Golgi apparatus. There, the central connecting polypeptide or C-peptide is cleaved from the chain by proteolytic enzymes, producing two linked-end fragments, the monomeric insulin molecules. C-peptide has a molecular weight of 3.6 x 10^2 daltons, and is devoid of biological activity (Sanger, 1958).

Increase in the concentration of glucose in the extracellular fluid initiates a physiologic stimulus which activates the release of insulin from pancreatic β cells. It was reported that plasma insulin concentration increased after feeding in ruminant animals as in other mammalian species, reflecting an augmented release of insulin by the pancreas (Bassett 1975). Glucose and, possibly, short-chain fatty acids produced in the rumen are involved in maintaining elevated plasma insulin for about 6 hours after feeding (Sano *et al*., 1999; Udum, 2008). On the other hand, it seems unlikely that amino acids are important in the regulation of plasma insulin in ruminants (Allen,

1972; Sasaki, 2002). Insulin stimulates anabolic reactions of carbohydrates, fats, proteins and nucleic acids. It also decreases the rate of lipolysis, proteolysis, ketogenesis and gluconeogenesis. Therefore, insulin is known to be in the centre of metabolism in ruminants (Basset, 1975., Hart *et al.*, 1980).

The plasma insulin in ruminants, as measured by radioimmunoassay, varies between 0.2275 – 1.82 µg/L. Ruminants are less sensitive to insulin. The plasma insulin reaches 0.91 – 1.82 µg/L within 4 hours postprandial, and then gradually declines to less than 0.455 µg/L, at 48 to 72 hours. Principal target organs for insulin are liver, adipose cells and skeletal muscles (Allen, 1972; Sacher *et al*., 2000)

Lack of insulin due to destruction of pancreatic β cells causes insulin-dependent diabetes (type I diabetes) whereas, less insulin release due to defects in β cells or decrease in insulin ability to stimulate uptake of glucose in tissues which is referred to as insulin resistance, causes non-insulin dependent diabetes (type 2 diabetes) (Zlatoš, 1996., Sasaki, 2002).

It has been reported that experimental infections in hamsters with *T. cruzi* caused pancreatitis and erratic blood decreased glucose levels, and tendency to hypoglycemia (Santos, 2004).

2.13 GLUCOSE

Glucose is a carbohydrate compound of much importance in biology. It is derived from digestion of dietary carbohydrate, breakdown of glycogen in the liver (glycogenolysis), and production of glucose from amino acid precursors in the liver (gluconeogenesis) (Brockman, 1993; Udum, 2008)**.** The dietary sources of supply of carbohydrate are however, variable especially among various animal species. In

ruminants, the main source of glucose supply is gluconeogenesis from volatile fatty acids (propionates). This is because the dietary carbohydrate (including cellulose) undergoes microbial fermentation in the rumen to volatile fatty acids (acetate, propionates and butyrate), and only small amount of glucose are absorbed. However, glucose is equally an important metabolite for all mammals (Houtert, 1993; Frandson *et al*., 2009).

Glucose plays a key role in the metabolism and provision of essential source of energy needed for the maintenance of the physiological functions of the animal body. It also serves as a precursor for the biosynthesis of essential mammalian cell components (Kaneko *et al.* 2008)**.** It has long been reported that pathogenic as well as saprophytic trypanosomes utilize glucose and probably obtain from it the energy required for life and active motility**.** Glucose is decomposed by trypanosomes aerobically as well as anaerobically **(**Bakker *et al*., 1999; April, 2011).

The blood glucose concentration depends on a variety of factors; its concentration at any time is the net result of equilibrium between the rate of its entry and removal from the circulation. As such, all the factors that exert influence on the entry or removal become important in the regulation of blood glucose concentration. Among these factors are the liver, extra hepatic tissues, several hormones including insulin, glucagon, epinephrine, glucocorticoids and thyroid hormones (Bassett, 1975; Harmon, 1992, Udum, 2008). The most important hormone involved in glucose metabolism is insulin. However, hypo- or hyperglycemic activity of plasma is not a direct reflection of insulin concentration (Allen, 1972; Udum, 2008).

The blood glucose levels in mature ruminants are considerably less than those in non-ruminants and the ruminants are relatively insensitive to insulin. The blood

glucose level in young ruminants is the same with that of non-ruminants. However, it decreases sharply after first few weeks of life as rumen functions develop (Reece, 1987; Udum, 2008). The concentration of blood glucose levels in various animal species ranges from 3.3 - 7.0 mmol/L (60 – 125 mg/dl) every time of the day. When it is greater than 80 mg/dl, it is detected by the β cells of the pancreas and insulin is then released. Blood glucose levels are best established in ruminants and non-ruminants by a standard overnight (12 – 16 hrs) fasting before sampling. However, this is not necessary in matured ruminants, because feeding elicits no blood glucose response (Kaneko *et al.,* 2008; Frandson *et al*., 2009).

The glucose level in serum or plasma is stable for 24 hours when stored at 2 - 8 ^{o}C. It decreases at the rate of 7% per hour in whole blood. Glucose breakdown (glycolysis) by erythrocytes takes place very rapidly, at about 10% per hour at room temperature. This is even more rapid if there is microbial contamination of the sample. This is averted by quick separation of serum or plasma from red blood cells, through refrigeration and by use of sodium fluoride-potassium oxalate at a concentration of 10 mg/ml (Allen, 1972; Sacher *et al.*, 2000*).* Increase in the blood glucose level is referred to as hyperglycaemia whereas a decrease is termed hypoglycaemia.

The serum biochemical changes due to Trypanosomosis in general and particularly glucose have remained an area of intense investigation by many researchers in the last few decades. Hypoglycaemia was reported in *T. vivax* infected cattle (Kadima *et al*., 2000), in *T.brucei* infected sheep (Taiwo *et al*., 2003), in vervet monkeys experimentally infected with *T. brucei* (Ngure *et al*., 2008) and in *T. evansi* infected camels (Sazmand *et al.,* 2011). Excessive utilization of the blood glucose by trypanosomes for their metabolism has been thought to account for the hypoglycaemia

observed during trypanosomosis (Anosa, 1988b). Hilali *et al*. (2006) however, reported no significant changes in the mean serum glucose of *T. evansi* infected buffalo calves. Similarly, Ukwueze and Anene (2011) reported no effect on the glycaemic status of either normal or diabetic rats infected with *T. brucei and T. congolense*. Increase in the blood glucose levels were correlated with degenerative lesions which were regularly found in the pancreatic islets of guinea pigs infected with *T. cruzi* (Santos, 2004).

CHAPTER 3

MATERIALS AND METHODS

3.1 EXPERIMENTAL ANIMALS

Fifteen adult Savannah Brown bucks, aged between 12 and 15 months were used for the study. They were purchased from Kafur market in Katsina State of Nigeria, which is an area that is apparently tsetse fly - free. The bucks were housed in an insect- proof animal pen of the Department of Veterinary Pathology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. They were acclimatized for 2 months and neck-tagged for the purpose of identification. The bucks were fed with corn bran, ground-nut hay, cowpea hay and salt licks. Water was given to them *ad libitum*. They were subjected to routine handlings, such as physical examination, determination of the body weight and rectal temperature, collection of blood samples for screening of haemoparasites and determination of baseline haematological indices. They were also screened for endo- and ectoparasites, and treated with oxytetracycline (Tridax®) intramuscularly, at a dose rate of 20 mg/kg body weight and albendazole (Sambezole®, Sam pharmaceutical Ltd., Animal Health Care, Nig. Ltd) orally, at a dose rate of 7.5 mg/kg body weight. The bucks were sprayed against ectoparasites with diazinon (Diaznol®, Animal care, Nig. Ltd.), at concentration of 2 ml/litre of water (162 mg/ml concentration). Before commencement of the experiment, the bucks were certified free of trypanosomes in their blood using centrifugation technique (Woo, 1970).

3.2 THE TRYPANOSOME USED

The *Trypanosoma brucei brucei* used in this study was obtained from the Nigerian Institute for Trypanosomiasis Research (NITR), Kaduna, Nigeria. It was originally isolated from a pure natural infection in cattle in Federe, Kaduna state in 1995.The parasites were inoculated intraperitoneally into two albino rats and transported to the Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. The parasites were sub-inoculated intraperitoneally into five albino rats and kept in separate cages in the Department of Veterinary Parasitology and Entomology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. They were fed with commercial pelleted feed. Blood samples were collected daily from each of the rats to determine parasitaemia using the method described by Woo (1970).

3.3 EXPERIMENTAL DESIGN

3.3.1 Grouping of the Experimental Animals

The bucks were randomized into three experimental groups: A, B and C, based on their mean haematocrit index values. The bucks in groups A (27.6%) and group B (27.2%) were infected while the bucks in group C (27.2%) were the control. Each group consisted of five bucks.

3.3.2 Infection of Experimental Animals

When the trypanosome parasites were detected in the blood of the inoculated rats, it was monitored to its peak value. The rats were euthanized with an overdose of diethyl ether in a jar and bled through cardiac puncture to collect sufficient blood into Bijou bottles, containing 2 mg ethylene diamine tetraacetic acid (EDTA) for the

infection of the bucks in groups A and B. Each buck in group A was inoculated via the jugular vein with 1×10^6 and the bucks in group B with 2×10^6 *T. b. brucei* while the bucks in the control group C, were each administered 1 ml of normal saline via the same route. The number of *T. b. brucei* for inoculation was estimated according to the method of Paris *et al.* (1982) as modified by Mutayoba *et al.* (1994).

3.3.3 Clinical Examination

The experimental animals were examined daily for clinical signs of trypanosomosis (e.g. pyrexia, lacrimation, anaemia, emaciation etc).

3.3.4 Determination of the Rectal Temperature (^oC)

The rectal temperature for each experimental animal was assessed daily in the morning between 8.00 and 9.00 am using a clinical digital thermometer. The thermometer was inserted into the rectum and tilted to touch the rectal mucosa. After a beep, the thermometer was removed and the body temperature changes were read and recorded in degree centigrade (^oC).

3.3.5 Sample Collection and Processing

3.3.5.1 *Blood sample*

A total of 5 ml of blood was collected from each of the experimental animals via the jugular vein using sterile 10 ml syringe and 21G X 1½ⱡ needle. The blood was carefully dispensed into clean, labeled Bijou bottles for analyses as follows:

1. Two millilitres of the blood sample was dispensed into Bijou bottles containing EDTA, for the determination of PCV and the detection of parasitaemia.

2. Three millilitres of the blood sample was dispensed into Bijou bottles without anticoagulant and was allowed to clot in the refrigerator. The blood sample was then centrifuged at 2000 *g* for five minutes to obtain serum which was aspirated into two serum vials, and stored at -20 ^{O}C for glucose and insulin assay.

The blood samples were collected once daily, from day 2 pre-infection to day 7 post-infection (p.i.) thereafter, every 3 days to the end of the 40-day experimental period that started 2 days pre-infection and ended 37 days p.i.

3.3.5.2 *Tissue Sample*

At the end of the experiment, all the bucks in groups A, B and C, were euthanized. Postmortem examination was carried out according to standard procedure. The gross lesions were observed and recorded. Tissue samples were collected from the pancreas of the bucks and preserved in 10% buffered neutral formalin for histopathological studies.

3.3.6 Laboratory Analysis

3.3.6.1 *Determination of the level of Parasitaemia*

The level of parasitaemia was estimated using haematocrit centrifugation technique as described by Woo (1970). The scores used are as presented below:

1. + = up to 10 trypanosomes in buffy coat or plasma layer seen per field.
2. ++ = 10 - 20 trypanosomes in buffy coat or plasma layer seen per field.
3. +++ = numerous trypanosomes in buffy coat or plasma layer seen per field.
4. ++++ = massive trypanosomes in buffy coat or plasma layer seen per field.

3.3.6.2 *Thin Blood Smear for Trypanosome Parasite Identification*

A drop of blood was placed on a clean and grease-free microscope slide. A thin smear of blood was made by sliding another slide on the first slide. The smear was air dried and fixed in methanol for about 3 minutes. The slide was stained in 10 % Giemsa stain for 25 - 30 minutes, rinsed in distilled water, dried and examined microscopically under oil immersion at x 100 objective. The identification of the *T. b. brucei* was done morphologically as described by Hoare (1972).

3.3.6.3 *The Determination of Packed Cell Volume (PCV)*

The PCV was determined using standard microhaematocrit centrifugation technique and the values were read by Hawksley microhaematocrit reader (Gellman, Hawksley Ltd, England) as described by Coles (1986). The packed cell volume was determined daily for the first 10 days thereafter, every 3 days to the end of the experiment**.**

3.3.6.4 *The Assay of Serum Glucose*

The quantitative assay of total serum glucose was carried out as described by Trinder (1959) using glucose oxidase reagent kit (Teco diagnostics® USA).

3.3.6.5 *The Assay of Serum Insulin*

The quantitative assay of serum insulin was carried out as per the instructions of the manufacturer of the Mercodia Bovine Insulin ELISA kit (Mercodia AB, Sweden).

3.3.7 Histopathological Analysis

Tissue samples of 0.5 cm in size were collected from the pancreas of each buck. The tissue samples were immediately preserved in 10 % buffered neutral formalin (BNF). After 48 hours of fixation, the tissue samples were processed, embedded in paraffin wax and sectioned at 5 microns. The sections were mounted on clean grease-free glass slides and stained with haematoxylin and eosin (H & E) stains as described by Luna (1968). The stained slides were examined microscopically at x 40 objective. Histopathological lesions were observed and recorded. Photomicrographs of the lesions were taken under Olympus® microscope at x 40 objective with a digital camera.

3.3.8 Statistical Analysis

One way analysis of variance (ANOVA) and Student's t-test using GraphPad Prism version 4.0 for windows from Graph Pad software, San Diego California, U.S.A. (www.graphpad.com) was used to test levels of significance between the mean values obtained from the infected groups compared to the control. Tukey post hoc test analysis was done using the Tukey multiple comparison tests. The values of $p<0.05$ were considered as statistically significant (Steel *et al;* 1997).

CHAPTER 4

RESULTS

4.1 CLINICAL SIGNS

The observed clinical signs among the bucks in the infected groups A and B were similar and included intermittent pyrexia, pale ocular mucous membrane, mucoid ocular discharges, diarrhea, reduced and or selective feed intake, reduced body weight gain, rough hair coat, lymphadenopathy of the prescapular lymph nodes, lethargy, drowsiness and death. One death was recorded in each group A and B, on day 22 p.i. and on day 25 p.i. respectively, before the end of the experimental period.

4.2 MEAN PARASITAEMIA LEVELS (+)

The parasitaemia was observed by day 1 p.i. of the experimental bucks in both groups A and B. However, the initial wave of parasitaemia in group A was higher than that in group B. The Parasite disappeared from the peripheral blood by day 2 p.i. and reappeared by day 3 p.i. with a parasitaemia level (1+) in both groups A and B. Thereafter, there were fluctuations in parasitaemia producing intermittent waves. There was statistical significant difference ($p < 0.05$) in the mean parasitaemia values between the bucks in groups A and B by day 7 p.i. and day 19 p.i. The parasitaemia in group A reached its peak value (2+) by day 28 p.i., while the peak value (2+) in group B was attained by day 19 p.i. The parasitaemia levels in both groups A and B returned to zero by day 31 p.i. and day 34 p.i., respectively. There was no statistically significant difference ($p > 0.05$) in the mean parasitaemia values between the bucks in groups A and B at the end of the experiment (Figure 4.1; Appendices I & II).

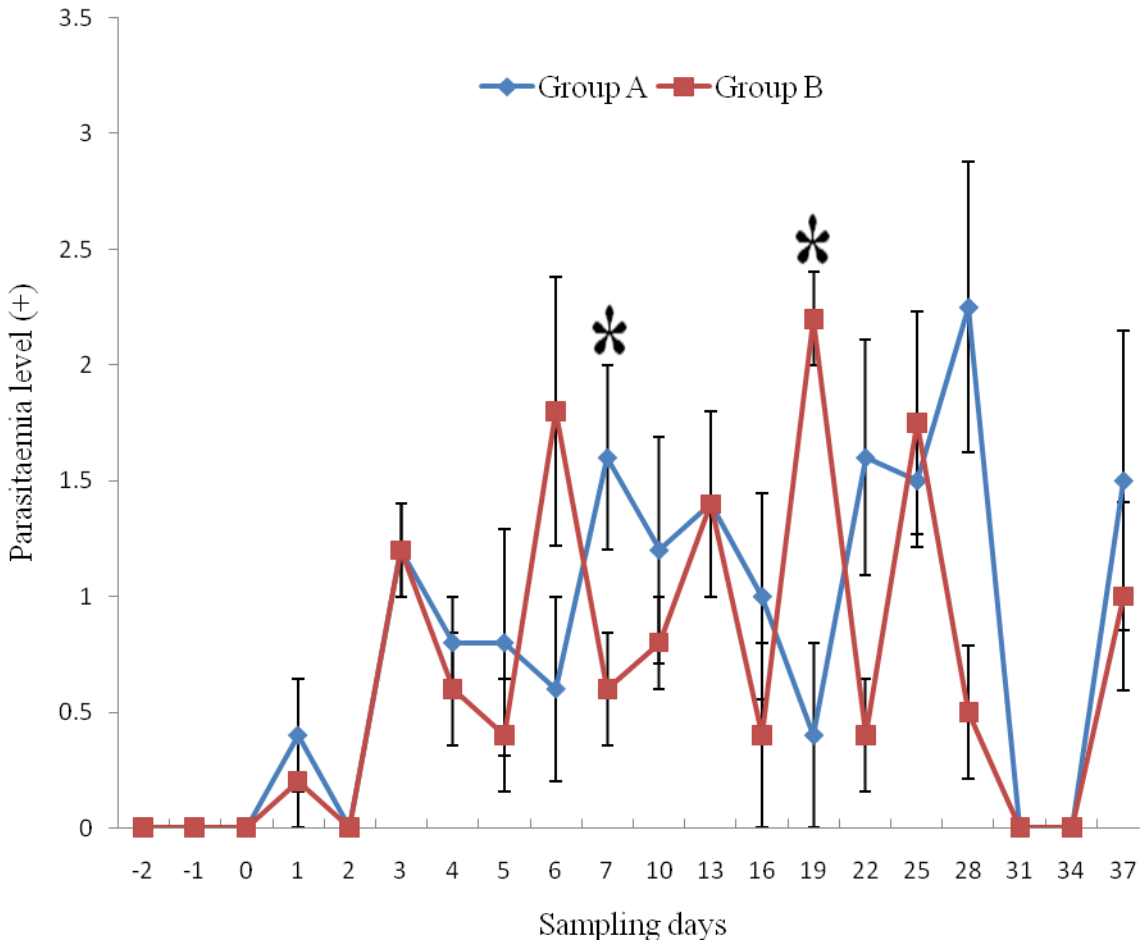


Figure 4.1: Mean parasitaemia (+) of the *T. b. brucei* infected groups A and B bucks. Note * = $p < 0.05$.

4.3 MEAN RECTAL TEMPERATURE (^{O}C)

At the beginning of the experiment, the pre-infection mean rectal temperature values of the bucks in groups A, B and C were 39.6 ± 0.0 ^{O}C, 39.6 ± 0.2 ^{O}C and 39.5 ± 0.1 ^{O}C, respectively. By day 1 p.i., the mean rectal temperature values of the infected groups began to rise steadily and by day 3 p.i, the mean rectal temperature values in both groups A and B reached 41.0 ± 0.1 ^{O}C and 40.4 ± 0.4 ^{O}C, respectively. Thereafter, there were fluctuations in the mean temperature values of the infected groups A and B that continued to the end of the experiment. The mean rectal temperature values for the control group remained relatively constant. The maximum mean temperature rise in groups A and B were observed by day 3 p.i. (41.0 ± 0.1 ^{O}C) and day 31 p.i. (40.5 ± 0.1 ^{O}C), respectively. At the end of the experiment, the mean rectal temperature values of the bucks in groups A, B and C were: 40.3 ± 0.2 ^{O}C, 40.1 ± 0.3 ^{O}C and 38.7 ± 0.1 ^{O}C, respectively. There was statistical significant difference ($p < 0.001$) in the mean temperature values of the infected bucks in groups A and B when compared to the control group C at the end of the experiment (Figure 4.2; Appendices III, IV & V).

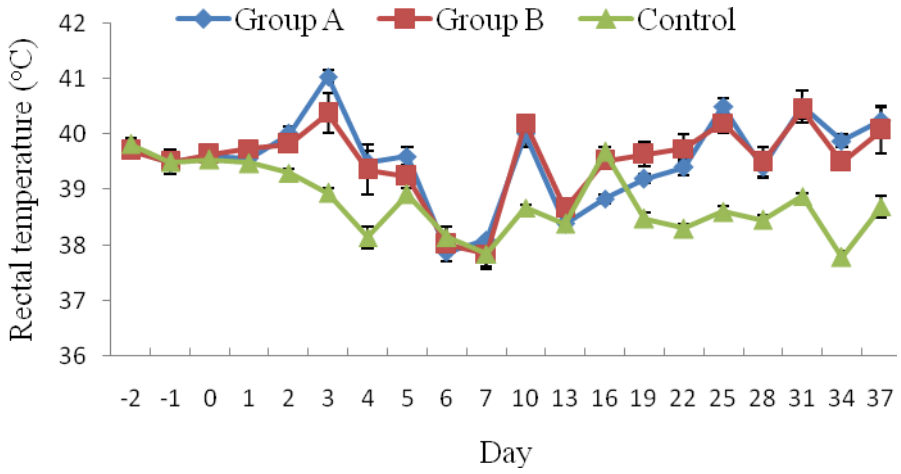


Figure 4.2: Mean rectal temperature values (°C) of the control and the *T. b. brucei* - infected groups A and B bucks.

4.4 MEAN PACKED CELL VOLUME (PCV %)

The pre-infection mean PCV of the bucks in the experimental groups A, B and C were 27.4 ± 1.4%, 26.6 ± 1.6% and 26.6 ± 1.5%, respectively. By day 3 p.i., the mean PCV of the infected groups A and B began to decline at different levels of significance to the end of the experiment when compared with the control group C. At the end of the experiment, the mean PCV in the infected groups A and B declined to 19.5 ± 1.9% and 19.8 ± 3.0%, respectively, while that of the bucks in the control group C was 30.4 ± 2.3%. There was statistical significant decline ($p < 0.05$) in the mean PCV of the infected groups A and B when compared with the control group C (Figure 4.3; Appendices VI, VII & VIII).

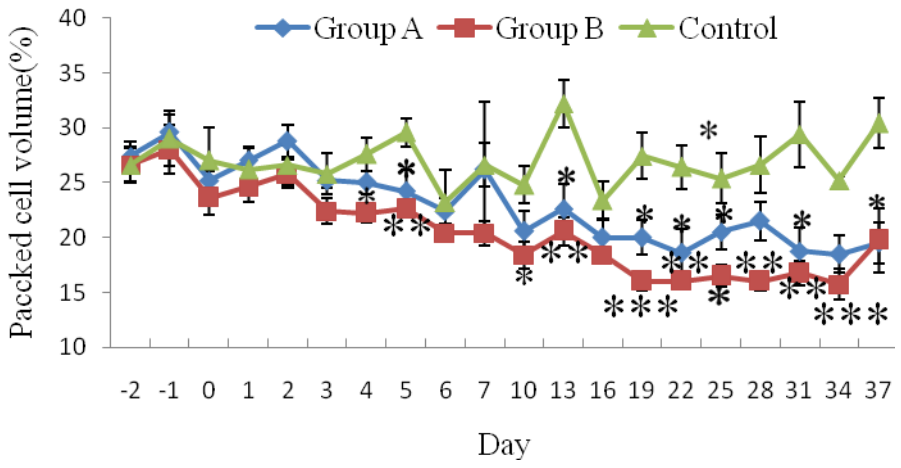


Figure 4.3: Mean Packed cell volume (%) of the control and the *T. b. brucei* - infected groups A and B bucks. Note * = $p < 0.05$, * * = $p < 0.01$ and *** = $p < 0.001$.

4.5 MEAN SERUM GLUCOSE CONCENTRATIONS (mmol/L)

The pre-infection mean serum glucose concentrations of the bucks in groups A, B and C were: 2.83 ± 0.01 mmol/L, 3.10 ± 0.04 mmol/L and 2.85 ± 0.13 mmol/L, respectively. By day 13 p.i., the mean serum glucose concentrations in groups A and B began to increase relative to the control group C. The increase in the mean serum glucose concentrations of the bucks in groups A and B continued to the end of the experiment while the mean serum glucose concentrations of the bucks in the control group C maintained a relatively constant profile. There was statistically significant increase ($p < 0.05$) in the mean serum glucose concentration of the bucks in groups A and B by day 22 p.i. and day 34 p.i., respectively when compared with the control group C. At the end of the experiment, the mean serum glucose concentrations of the bucks in groups A, B and C were: 3.68 ± 0.28 mmol/L, 3.10 ± 0.30 mmol/L, and 3.14 ± 0.43 mmol/L, respectively. There was no statistically significant difference ($p > 0.05$) in the mean serum glucose concentrations of the *T. b. brucei* - infected bucks in groups A and B, and the bucks in the control group C. The cumulative mean serum glucose concentrations of the bucks in groups A, B and C at the end of the experiment were: 2.90 ± 0.07 mmol/L, 2.88 ± 0.08 mmol/L and 2.62 ± 0.06 mmol/L. There was statistical significant increase ($p < 0.05$) in the cumulative mean serum glucose concentrations of the infected groups A and B when compared to the control group C (Figure 4.5). There was a positive correlation ($r = 0.242$) between the mean serum glucose concentrations and parasitaemia level of infected group A, whereas the mean serum glucose and the parasitaemia concentrations level of infected group B correlated negatively ($r = -0.019$) with each other. However, the level of correlation was non-significant ($p < 0.8162$, $p < 0.8460$) in both groups A and B (Figures 4.4; Appendices IX, X & XI).

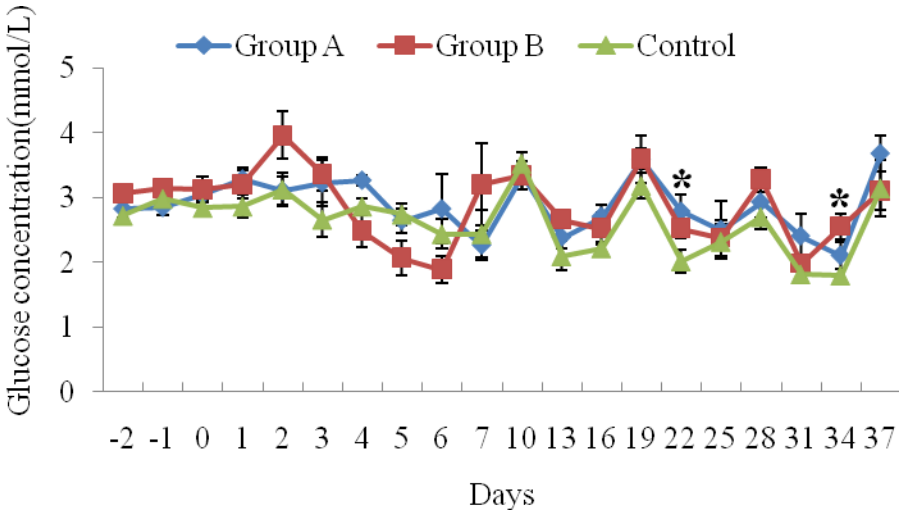


Figure 4.4: Mean serum glucose concentrations (mmol/L) of the control and the *T. b. brucei* - infected groups A and B bucks. Note * = $p < 0.05$.

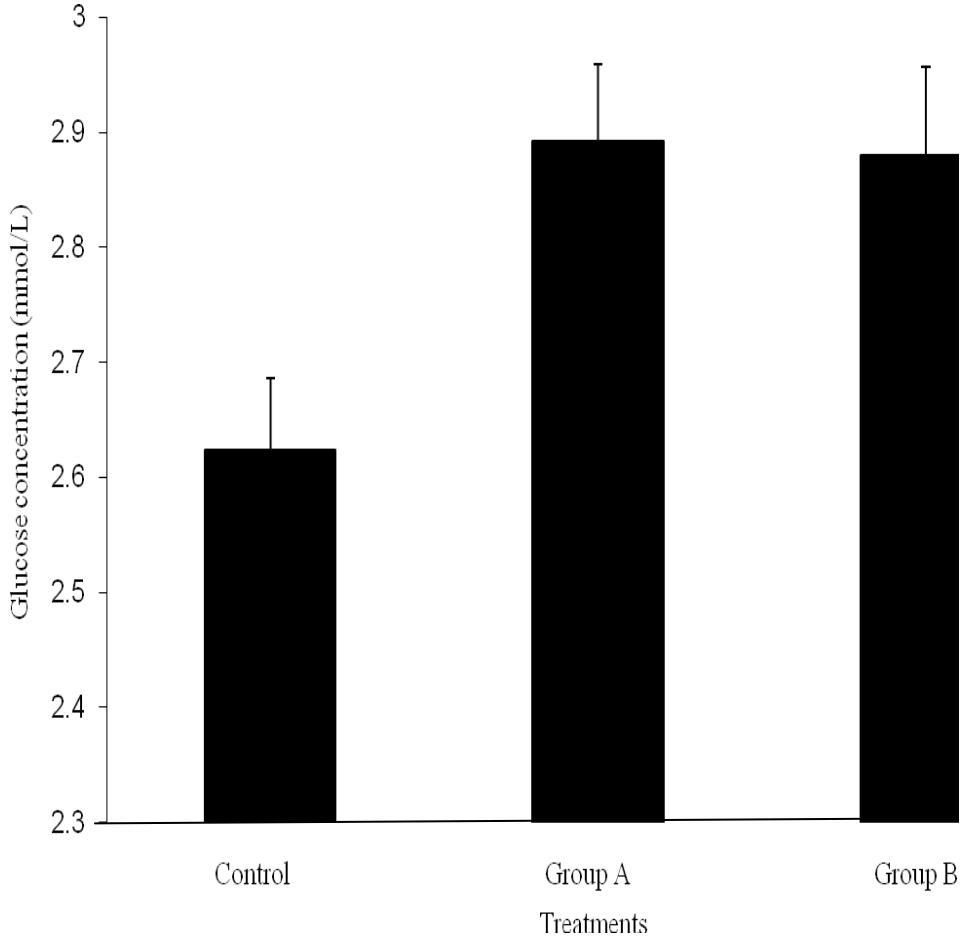


Figure 4.5: Cumulative mean serum glucose concentrations (mmol/L) of the control and the infected *T. b. brucei* groups A and B bucks. Note groups A and B = $P < 0.05$.

4.6 MEAN SERUM INSULIN CONCENTRATIONS (µg/L)

The pre-infection mean serum insulin concentrations of the bucks in groups A, B and C were: 0.1326 ± 0.0216 µg/L, 0.1772 ± 0.0104 µg/L and 0.2158 ± 0.0054 µg/L, respectively. By day 13 p.i., the mean serum insulin concentrations in groups A and B began to increase relative to that of the control group C which maintained a constant profile to the end of the experiment. By day 16 p.i., there was a statistical significant increase ($p < 0.05$) in the mean serum insulin concentration of the *T. b. brucei* - infected bucks in the group A when compared with the control group C. At the end of the experiment, the mean serum insulin concentrations of the bucks in the groups A, B and C were: 0.8974 ± 0.1126 µg/L, 0.6480 ± 0.4032 µg/L and 0.3467 ± 0.0941 µg/L, respectively. There was no statistical significant difference ($P > 0.05$) in mean serum insulin concentrations among the groups. The cumulative mean serum insulin concentrations in groups A, B and C, at the end of the experiment were: 0.3642 ± 0.0395 µg/L, 0.3131 ± 0.0306 µg/L, and 0.2192 ± 0.0128 µg/L, respectively. There was a statistically significant increase ($p < 0.01$) in the cumulative mean serum insulin concentrations of the bucks in group A when compared with that of the bucks in the control group C (Figure 4.7). The mean serum insulin concentrations of the infected bucks in groups A and B negatively correlated ($r = -0.1139$, $p < 0.2717$ and $r = -1143$, $p < 0.2700$) with the mean serum glucose concentrations of the infected groups A and B, respectively. However, a direct correlation ($r = 0.1350$, $p < 0.1923$; $r = 0.1809$, $p < 0.0793$) was observed between serum insulin concentrations of the *T. b. brucei* - infected bucks in groups A and B, when compared with their respective parasitaemia values in the groups. The level of correlation was insignificant in both cases (Figures 4.6; Appendices XII, XIII & XIV).

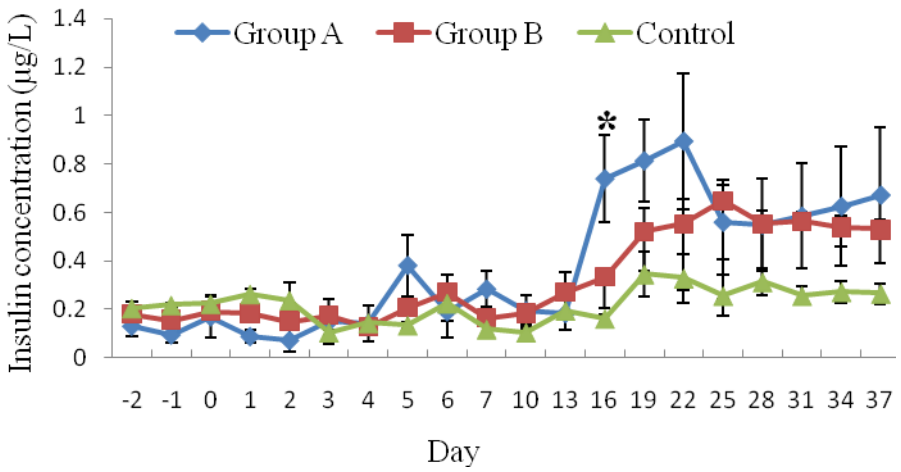


Figure 4.5: Mean insulin concentrations (µg/L) of the control and the *T. b. brucei* - infected groups A and B bucks. Note * = $p < 0.05$.

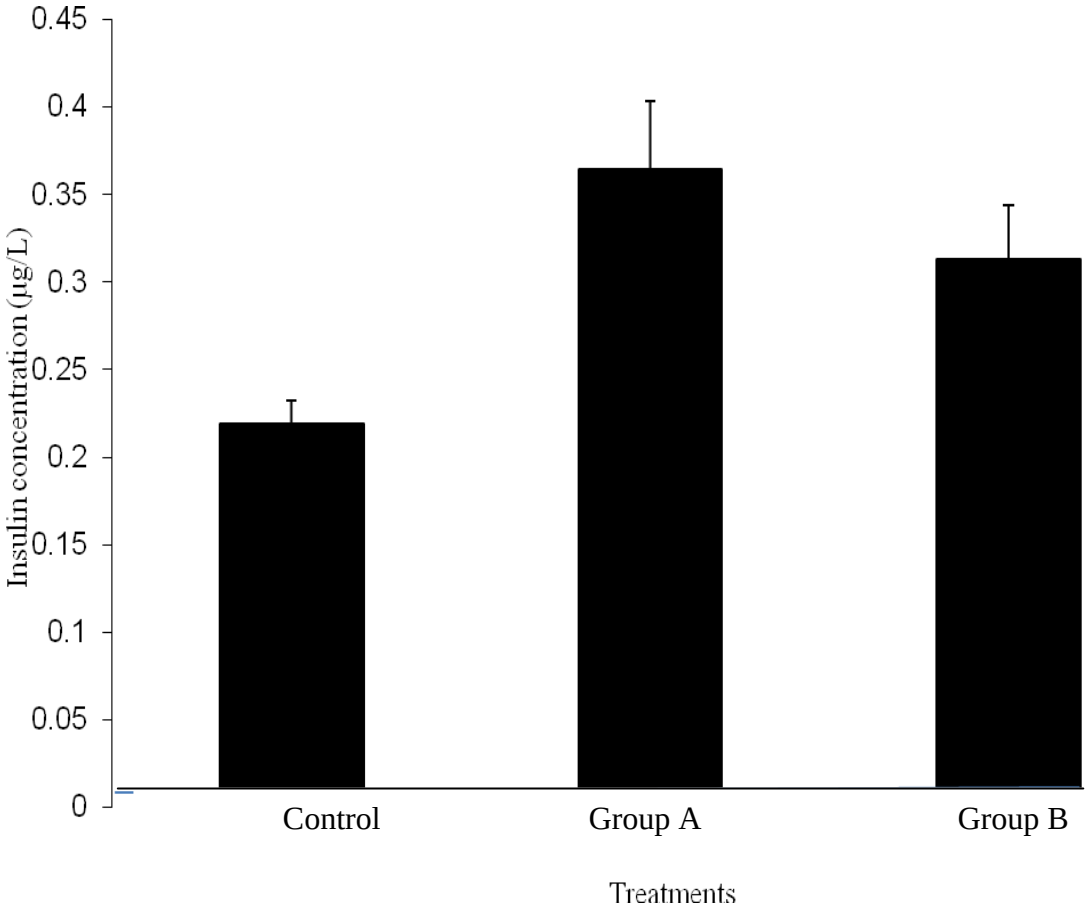


Figure 4.7: Cumulative mean serum insulin concentrations (µg/L) of the control and infected *T. b. brucei* groups A and B bucks. Note group A = $p < 0.01$.

4.7 GROSS PATHOLOGICAL CHANGES

The observed gross lesions in the *T. b. brucei* - infected bucks in groups A and B that were euthanatized at the end of the experiment and the 2 that died before the end of the experiment were similar. The lesions included pale mucous membrane and pale carcasses (anaemia), pale pancreas, lymphadenopathy, splenomegaly, hepatomegaly, necrotic areas on the liver, hydropericardium (4.5 -6 ml straw coloured), red hepatization of the apical lobe of the right side of the lung, adhesion of the diaphragmatic lobe of the right side of the lung to the epicardium and diaphragm. Serous atrophy of the mesenteric fats, fats around the coronary groove of the heart and perirenal fat, haemorrhagic areas in the meninges, orchitis, ingesta in the trachea and bronchi, and watery faeces in the intestine.

4.8 HISTOLOGICAL CHANGES

The observed pancreatic microscopic lesions of the *T. b. brucei* - infected bucks in group A were focal degenerative changes in the acinar cells and the islets of Langerhans, while diffused degenerative changes in the acinar cells and the islets of Langerhans were the lesions observed in the group B bucks (Plate I & II). The pancreases of the control bucks were normal (Plate III).

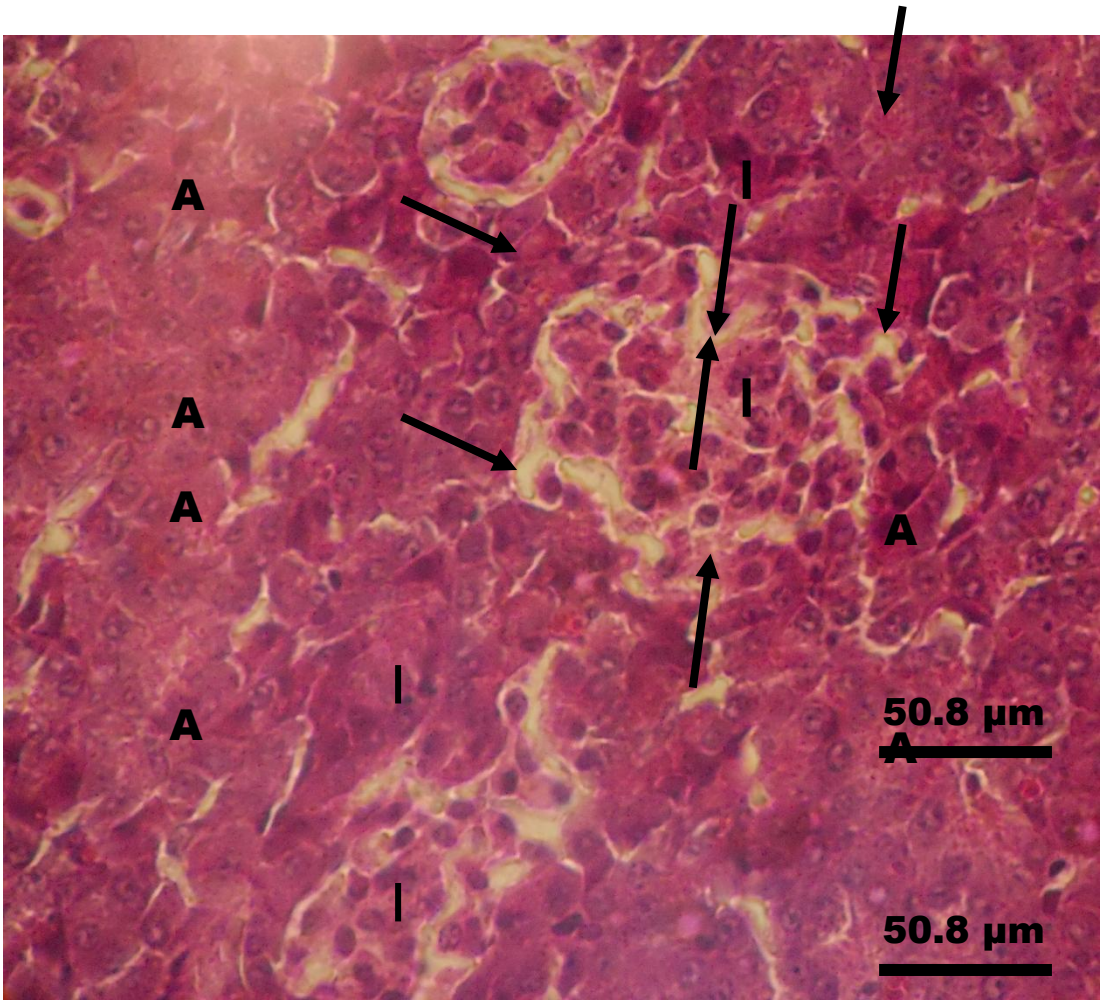


Plate I: Photomicrograph of a section of a pancreas from the group A buck experimentally infected with 1 x 10^6 *T. b. brucei*. Note the degenerated areas of islets of Langerhans (**arrows**) and acinar cells (**A**). H & E stain.

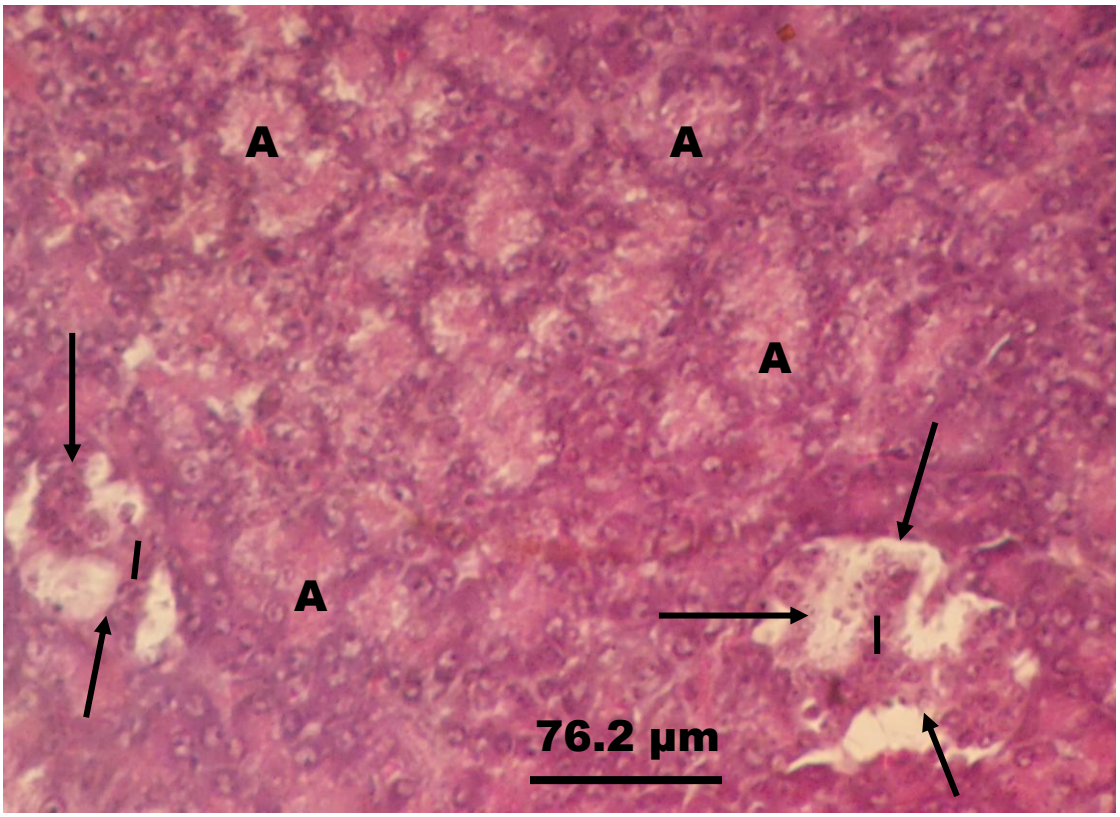


Plate II: Photomicrograph of a section of a pancreas from a group B buck experimentally infected with 2 x 10^6 *T. b. brucei.* Note the degenerated areas of islets of Langerhans (**arrows**) and acinar cells (**A**). H & E stain.

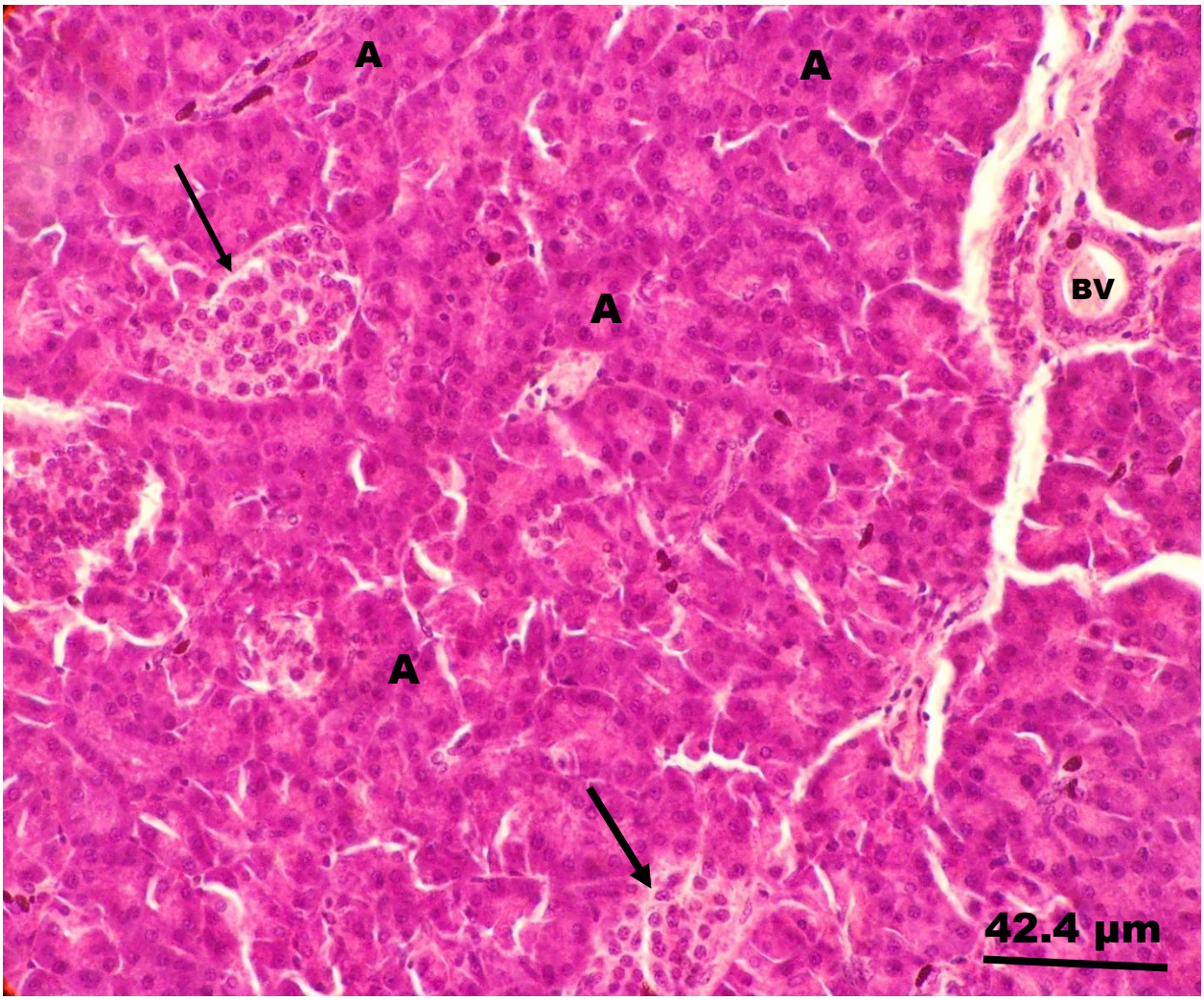


Plate III: Photomicrograph of a section of a pancreas from the control buck. Note the organized cellular morphology of the islets of Langerhans (**arrows**), the acinar cells (**A**) and the blood vessel (**BV**). H & E stain.

CHAPTER 5

DISCUSSION

In this study, parasitaemia was observed one day after the infection of the experimental bucks. This observation is similar to an earlier finding by Ikede and Losos (1972), in which *Trypanosoma brucei* were detected in the blood smear from infected cattle one day post-infection (p.i.). *Trypanosoma brucei brucei* has relatively short incubation period and generally, varying prepatent periods of 2 – 6 days p.i in wildlife and domesticated animals, have been reported (Omeke and Onuora, 1992; Allam *et al*., 2006; Adeiza *et al*., 2008; Mbayal *et al*., 2009). Anosa (1988) and Maudlin *et al.* (2004), reported that the varying prepatent period was largely dependent on the immune status of the host, the pathogenicity of the trypanosome and route of inoculation used (Murray and Dexter, 1988). The relatively very short prepatent period observed in this study could probably, be indicative of the high virulence of the *T. b. brucei*, the dose of the trypanosome and route of inoculation used in the experimental bucks.

The levels of parasitaemia observed coincided with the rise in rectal temperature and the decline in packed cell volume of the *T. b. brucei* - infected bucks of groups A and B. The parasitaemia and the clinical signs observed in the study are consistent with the observations of Anosa (1988), Biryomumaisho and Katunguka-Rwakishaya (2007) and Adeiza *et al.* (2008). Similar clinical signs have been reported in *T. brucei* - infected Red fronted gazelles (Mbayal *et al*., 2009) and rats (Sulaiman and Adeyemi, 2010). The onset of anaemia was related to the first wave of parasitaemia and the severity was associated with the levels and initial peaks of parasitaemia as also earlier reported (Esievo *et al*., 1982; Seifert, 1996; Mbayal, *et al*.,

2009). The trypanosome parasites present in the blood produced numerous changes in the cellular and biochemical constituents of blood (Igbokwe and Mohammed, 1992; Taiwo *et al*., 2003). The *T. brucei* infection like other trypanosome infections precipitated increased red blood cell destruction which resulted in anaemia and tissue damage (Igbokwe and Nwosu, 1997; Takeet and Fagbemi, 2009). These changes together with the attempt by the host to destroy the parasite are presumably responsible for the clinical manifestations of African trypanosomosis (Igbokwe and Nwosu, 1997; Omotainse and Anosa, 2009; Takeet and Fagbemi, 2009). The pyrexia and the clinical features observed in this study could be attributed in part, to the toxic effect of the metabolites produced by the trypanosome parasites and the resultant trypanolytic crisis (Takeet and Fagbemi, 2009).

The significant, gradual and steady fall in the mean PCV levels observed in the two groups of the *T. b. brucei* - infected bucks, below the pre-infection levels in this study agrees with the observations made in mixed *T.brucei* and *Cowdria ruminantium* infected West African Dwarf goats (Adejinmi and Akinboade, 1999, 2000), *T.brucei* - infected Savannah Brown goats (Adeiza *et al*., 2008), pigs (Adamu *et al*., 2009) and rats (Sulaiman and Adeyemi, 2010). The decline in the PCV levels observed in the infected groups may be due to acute haemolysis induced by the proliferating *T. b. brucei,* and could also explain the anaemia observed in the infected bucks of groups A and B. The onset of anaemia and the extent, to which the packed cell volume declined, correlated closely with the appearance, height and duration of parasitaemia (Luckins and Gray, 1978). Anaemia is the most prominent feature of trypanosomosis in both animals and man, and may be observed as pallor of the mucous membranes (Saror, 1980; Ogunsanmi and Taiwo, 2001; Cadiolli *et al*., 2006). It was responsible for the development of most tissue and cellular degenerative changes and disorders that were

found in trypanosome - infected animals (Logan-Henfrey *et al*., 1992; Igbokwe *et al*., 1994).

Serum biochemical changes were observed to be associated with trypanosome infections in animals, and several factors were found to influence the nature and severity of these changes (Anosa, 1988 a, b). In this study, the significant increase ($p<0.05$) in the mean serum glucose concentrations of the *T .b. brucei* - infected groups of bucks when compared with the control group that was observed, contradicts the reports of hypoglycaemia in *T. vivax* - infected cattle (Kadima *et al*., 2000), *T. brucei* - infected sheep (Taiwo *et al*., 2003), Vervet monkeys (Ngure *et al*., 2008) and in *T. evansi* - infected camels (Sazmand *et al.*, 2011). Anosa (1988) suggested excessive utilization of the blood glucose by trypanosomes for their metabolism to account for the hypoglycaemia observed during trypanosomosis. Cadioli *et al*. (2006) attributed hypoglycaemia to increased metabolic rate induced by pyrexia and hepatocyte degeneration during trypanosome infections. However, Hilali *et al*. (2006) observed no significant changes in the mean serum glucose levels in *T. evansi* - infected buffalo calves. Similarly, Ukwueze and Anene (2011) observed no effect on the glycaemic status of either normal or diabetic rats infected with *T. brucei* and *T. congolense*. The significant increase in the mean serum glucose concentrations observed in this study may be multifactorial in origin. It could probably be partly, due to the degenerative changes in the pancreas observed in this study. The degenerative changes in the pancreas could denature and possibly render the biosynthesized insulin in the β- cells inefficient. The *T. b. brucei* infection could also cause some cellular damage and possible metabolic and biochemical changes in the insulin target organs leading to some enzymatic alterations and inhibition at the receptor sites. The net effect will be an improper absorption of glucose by tissue cells and inappropriate glucose storage in the

liver, adipose tissues and the skeletal muscles with the resultant increase in the serum glucose observed. Insulin deficiency and decrease in insulin ability to stimulate uptake of glucose in tissues due to dysfunction of pancreatic β – cells have been reported (Zlatoš, 1996; Sasaki 2002). The *T. b. brucei* induced stressor effect on the infected bucks could be another factor. Glucose metabolism is markedly altered during sepsis. Hyperglycaemia is well documented in situations of stress such as in systemic infection and shock (Igarashi *et al.,* 1992). This is believed to be due, in part to the increase in certain hormones, such as cortisol, which is released during stress and could impair glucose tolerance with resultant hyperglycaemia (Zilva and Pannall, 1984; Igarashi *et al.,* 1992). However, in this study, blood cortisol level was not evaluated.

In this study, a significant increase ($p < 0.01$) in the mean serum insulin concentrations of the *T. b. brucei* – infected bucks in groups A and B when compared with the control group C was observed. This finding could be attributed to the possible metabolic and biochemical changes in the insulin receptors and insulin target organs due to the *T. b. brucei* infection as earlier stated. Dysfunction of pancreatic β – cells may cause alterations and or inhibitions in the insulin receptor and glucose transport genes with resultant insulin resistance in target tissues and a concomitant hyperglycaemia and hyperinsulinaemia. Insulin resistance as a result of either reduced number of normal insulin receptors, or dysfunction of insulin receptors due to abnormal insulin binding to the α - subunit or defective tyrosine kinase activity in the β – subunit of insulin receptors has been reported (Zlatoš, 1996). Accumulation of amylin, an amyloidogenic peptide in circulation due to pancreatic β – cells dysfunction was also reported to cause insulin resistance (Zlatoš, 1996). However, the evaluation of either tyrosine kinase activity or hyperamylinaemia and amylin deposition in the pancreatic β – cells was not carried out in this study.

The gross lesions observed in the two groups of *T. b. brucei* – infected bucks in this study were similar. The observations were consistent with the clinical observations in trypanosome - infected cattle, sheep and goats (Losos and Ikede, 1972; Taylor and Authie, 2004; Omotainse and Anosa, 2009). Similar observations were also reported in *T. brucei* - infected goats (Biryomumaisho and Katunguka-Rwakishaya, 2007) and in *T. vivax* - infected cattle (Fatihu *et al*., 2009). The gross lesions in animal trypanosomosis are non-pathognomonic (Taylor and Authie, 2004). African trypanosome infection produced multiple alterations in the immunological and biochemical homeostasis of infected human and animal hosts, eventually leading to death (Mansfield, 1990; Taylor and Authie, 2004). The specific mechanisms leading to these multiple pathological aberrations are still been investigated (Saror, 1980). However, it is generally agreed that anaemia in both man and animal trypanosomosis is predominantly the result of haemolytic crisis, in which the erythrocytes are being destroyed by an expanded mononuclear phagocytic system (Igbokwe *et al*., 1994; Adamu *et al*., 2009). The pathological changes observed in this study might be at least in part, due to the effect of the toxic metabolites produced by the *T.b.brucei* and their resultant trypanolytic crisis as earlier reported (Takeet and Fagbemi, 2009).

Focal and diffused degenerative changes were observed in both the pancreatic acinar cells and the islets of Langerhans in the *T. b. brucei* - infected groups A and B bucks. However, the observed microscopic lesions were more marked in the infected group B than group A bucks. Acute pancreatitis in hamsters experimentally infected with *T. cruzi* was reported by Santos *et al*. (1999). The pathology of the pancreas due to African trypanosomosis in domestic animals and particularly in goats has not been well documented. There is therefore dearth of reports on the pathological changes in the pancreas due to trypanosome infections. However, it has been reported that *T.*

brucei like most other trypanosome infections induce immune reactions that precipitated increased erythrophagocytosis, anaemia and tissue destruction (Murray *et al.*, 1974 a, b; Facer *et al.*, 1978; Igbokwe and Nwosu, 1997; Vincendeau and Bouteille, 2006). Marked proliferative changes reflecting immunologic responses have also been reported as the usual observations in most organs in *T. brucei* infections (Anosa and Kaneko, 1983). Maré (2004) in a review stated that, anaemia and other dramatic changes such as inflammation, degeneration and tissue necrosis were observed in *T. brucei* infections. These changes were as a result of tissue invasion by trypanosomes. The pancreas is a parenchymal tissue with high metabolic rate and oxygen requirement. The anaemia observed in this study therefore, could have induced tissue hypoxia and particularly in the pancreas with the resultant degenerative changes.

CHAPTER 6

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 SUMMARY

This study has provided more evidence that *T. b. brucei* is pathogenic to Savannah Brown bucks and can cause clinical disease with clinical manifestations such as pyrexia, pale ocular mucous membranes, mucoid ocular discharges, diarrhea, reduced and or selective feed intake, emaciation, rough hair coat, lymphadenopathy, lethargy, conjunctivitis, drowsiness and death.

A very short prepatent period of one day p.i. was observed in the two infected groups with the initial parasitaemia wave higher in groups A than B. The mean PCV values in the infected groups A and B declined significantly ($p<0.001$) from 27.4 ± 1.4% and 26.6 ± 1.6% to 19.5 ± 1.9% and 19.8 ± 3.0%, respectively when compared to the control group C, which remained relatively constant throughout the experimental period. The decline in the PCV levels observed in the infected groups might be due to acute haemolysis induced by proliferating trypanosome parasites and could also explain the anaemia observed in the infected groups of the bucks.

The study revealed a significant increase in the mean serum concentrations of glucose in the infected bucks compared to the control. Also, there was a significant increase in mean serum insulin concentrations in the *T. b. brucei* infected bucks unlike in the control.

Diffused and focal degenerative changes in the acinar cells and islets of Langerhans, and congested blood vessels were the microscopic lesions observed from

the H and E stained sections of the pancreas of the infected bucks. No observable lesions were found in the H and E stained sections of the pancreas of the bucks from the control group.

6.2 CONCLUSIONS

In conclusion, the study on experimental *T. b. brucei* infection in bucks produced:

1. An infection with a very short prepatent period of one day.
2. Clinical disease associated with pyrexia, anaemia and death.
3. Gross and microscopic lesions in the pancreas.
4. Concurrent increase in serum glucose and insulin concentrations.

6.3 RECOMMENDATIONS

Based on the findings of this research and the need to throw more light on it, further study is recommended to:

1. Evaluate the pathology of the pancreas in other animal species (e.g. dogs and pigs) experimentally infected with *T. b. brucei.*
2. Evaluate the serum concentrations of other hormones of the endocrine pancreas of bucks experimentally infected with *T. b. Brucei* e.g. glucagon.
3. Evaluate the concentrations of the enzymes of the exocrine pancreas (e.g. amylase, lipase, elastase, trypsinogen etc) of bucks experimentally infected with *T. b. brucei.*

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APPENDICES

Appendix I: Mean parasitaemia levels (+) of the infected *T. b. brucei* group A bucks

Days PI	A1	A2	A3	A4	A5	Mean ± SEM
-2	0	0	0	0	0	**0 ± 0**
-1	0	0	0	0	0	**0 ± 0**
0	0	0	0	0	0	**0 ± 0**
1	1	0	0	0	1	**0.4 ± 0.3**
2	0	0	0	0	0	**0 ± 0**
3	1	1	1	1	2	**1.2 ± 0.2**
4	1	1	1	0	1	**0.8 ± 0.2**
5	2	0	2	0	0	**0.8 ± 0.5**
6	1	0	2	0	0	**0.6 ± 0.4**
7	2	1	1	3	1	**1.6 ± 0.4**
10	0	1	1	3	1	**1.2 ± 0.5**
13	1	0	2	2	2	**1.4 ± 0.4**
16	0	2	2	1	0	**1 ± 0.5**
19	2	0	0	0	0	**0.4 ± 0.4**
22	1	2	0	3	2	**1.6 ± 0.5**
25	2	1	_	1	2	**1.5 ± 0.3**
28	1	2	_	4	2	**2.3 ± 0.6**
31	0	0	_	0	0	**0 ± 0**
34	0	0	_	0	0	**0 ± 0**
37	2	0	_	1	3	**1.5 ± 0.**

Appendix II: Mean parasitaemia levels (+) of the infected *T. b. brucei* group B bucks

days PI	B1	B2	B3	B4	B5	Mean ± SEM
-2	0	0	0	0	0	**0 ± 0**
-1	0	0	0	0	0	**0 ± 0**
0	0	0	0	0	0	**0 ± 0**
1	0	0	0	1	0	**0.2 ± 0.2**
2	0	0	0	0	0	**0 ± 0**
3	1	2	1	1	1	**1.2 ± 0.2**
4	0	1	1	0	1	**0.6 ± 0.3**
5	0	0	0	1	1	**0.4 ± 0.3**
6	0	3	2	1	3	**1.8 ± 0.6**
7	1	1	0	1	0	**0.6 ± 0.3**
10	1	1	1	0	1	**0.8 ± 0.2**
13	2	0	1	1	1	**1 ± 0.3**
16	0	2	0	0	0	**0.4 ± 0.4**
19	3	2	2	2	2	**2.2 ± 0.2**
22	0	1	1	0	0	**0.4 ± 0.3**
25	2	1	1	_	3	**1.8 ± 0.5**
28	0	1	1	_	0	**0.5 ± 0.3**
31	0	0	0	_	0	**0 ± 0**
34	0	0	0	_	0	**0 ± 0**
37	1	1	2	_	2	**1.5 ± 0.3**

Appendix III: Mean rectal temperature values (°c) of the infected *T. b. brucei* group A bucks

Days	A1	A2	A3	A4	A5	Mean ± SEM
-2	39.6	39.8	39.5	39.9	39.8	**39.7 ± 0.1**
-1	39.5	39.5	39.7	39.3	39.4	**39.5 ± 0.1**
0	39.5	39.6	39.6	39.7	39.6	**39.6 ± 0.0**
1	39.8	38.6	39.7	39.9	39.8	**39.6 ± 0.3**
2	40.3	39.5	39.9	40.1	40.2	**40 .0 ± 0.2**
3	41.1	40.9	41.2	41.3	40.7	**41.1 ± 0.1**
4	39.3	40.1	39.1	39.0	39.9	**39.5 ± 0.2**
5	39.9	39.4	40.0	39.6	39.1	**39.6 ± 0.2**
6	38.4	37.8	38.1	37.3	37.8	**37.9 ± 0.2**
7	38.0	38.0	38.3	38.1	38.1	**38.1 ± 0.1**
10	39.7	39.9	40.4	40.3	39.7	**40 .0 ± 0.2**
13	38.4	38.9	38.9	38.6	39.2	**38.8 ± 0.1**
16	39.3	39.7	39.7	39.9	39.8	**39.7 ± 0.1**
19	38.9	39.6	39.0	39.0	39.5	**39.2 ± 0.2**
22	39.1	39.7	39.9	39.4	39.8	**39. 6 ± 0.2**
25	40.0	40.5	_	40.8	40.7	**40.5 ± 0.2**
28	39.4	39.9	_	38.6	39.7	**39.4 ± 0.3**
31	40.4	40.7	_	40.7	40.2	**40.5 ± 0.1**
34	39.2	40.3	_	39.9	40.1	**39.9 ± 0.2**
37	39.9	40.5	_	40.1	40.5	**40.3 ± 0.2**

Appendix IV: Mean rectal temperature values (°c) of the control group C bucks

Days PI	B1	B2	B3	B4	B5	Mean ± SEM
-2	39.5	39.5	39.5	39.9	40.1	**39.7±0.1**
-1	39.3	38.9	39.5	39.5	40.3	**39.5±0.2**
0	39.3	39.6	39.6	39.5	40.2	**39.6±0.2**
1	39.5	39.8	39.7	39.6	40.1	**39.7± 0.1**
2	39.4	40.0	40.1	39.8	39.8	**39.8 ± 0.2**
3	39.2	41.2	40.8	39.9	40.8	**40.4 ± 0.4**
4	40.9	38.4	39.9	38.8	38.8	**39.4 ± 0.5**
5	39.6	39.1	39.1	39.2	39.2	**39.2 ± 0.1**
6	37.6	38.0	38.8	37.1	38.6	**38.0 ± 0.3**
7	37.5	37.4	38.0	37.7	38.6	**37.8 ± 0.2**
10	39.8	40.3	40.0	40.8	40.1	**40.2 ± 0.2**
13	38.6	38.7	38.3	38.9	38.8	**38.7 ± 0.1**
16	39.6	39.6	38.9	39.3	40.2	**39.5 ± 0.2**
19	40.1	39.2	38.9	39.8	40.2	**39.6 ± 0.3**
22	40.2	39.1	39.6	39.8	40.0	**39.7 ± 0.2**
25	40.9	40.1	39.6	_	40.2	**40.2 ± 0.3**
28	39.8	39.5	39	_	39.7	**39.5 ± 0.2**
31	40.7	40.4	40.1	_	40.6	**40.5 ± 0.1**
34	39.8	39.6	38.3	_	40.3	**39.5 ± 0.4**
37	40.4	40.0	39.7	_	40.2	**40.1 ± 0.3**

Appendix V: Mean temperature values (^{o}c) of the control group C bucks

Days PI	C1	C2	C3	C4	C5	Mean ± SEM
-2	40.0	39.5	39.7	40.1	39.8	**39.8 ± 0.1**
-1	39.8	39.2	39.4	39.4	39.7	**39.5 ± 0.1**
0	39.8	39.2	39.5	39.5	39.7	**39.5 ± 0.1**
1	39.6	39.5	39.4	39.4	39.5	**39.5 ± 0.0**
2	39.2	39.5	39.3	39.4	39.1	**39.3 ± 0.1**
3	38.9	39.2	39.1	38.7	38.8	**38.9 ± 0.1**
4	38.3	38.3	38.6	37.4	38.1	**38.1 ± 0.2**
5	38.8	39.2	39.1	38.7	38.8	**38.9 ± 0.1**
6	37.6	38.4	38.6	37.7	38.4	**38.1 ± 0.2**
7	38.1	37.3	38.8	37.5	37.5	**37.8± 0.3**
10	38.8	38.5	39.0	38.4	38.7	**38.7 ± 0.1**
13	38.3	38.5	38.5	38.1	38.5	**38.4 ± 0.1**
16	39.0	38.8	39.0	38.8	38.6	**38.8 ± 0.1**
19	38.6	38.4	38.7	38.3	38.4	**38.5 ± 0.1**
22	38.2	38.5	38.5	38.3	38	**38.3 ± 0.1**
25	38.5	38.9	38.7	38.5	38.4	**38.6 ± 0.1**
28	38.4	38.5	38.6	38.3	38.5	**38.5 ± 0.1**
31	38.6	39.1	39.2	38.8	38.7	**38.9 ± 0.1**
34	38.0	38.1	38.1	37.1	37.6	**37.8± 0.2**
37	38.7	38.9	38.7	38.6	38.6	**38.7 ± 0.1**

Appendix VI: Mean packed cell volume (%) of the infected *T. b. brucei* group A bucks

Days PI	A1	A2	A3	A4	A5	Mean ± SEM
-2	32	29	25	25	26	**27.4 ± 1.4**
-1	33	29	25	33	28	**29.6 ± 1.5**
0	28	24	24	26	24	**25.2 ± 0.8**
1	30	25	24	30	26	**27.0 ± 1.3**
2	33	28	25	31	27	**28.8 ± 1.4**
3	28	24	25	25	24	**25.2 ± 0.7**
4	29	24	23	25	24	**25.0 ± 1.1**
5	30	24	18	25	24	**24.2 ± 1.9**
6	25	22	18	24	23	**22.4 ± 1.2**
7	50	0	14	23	22	**21.8 ± 8.2**
10	25	20	14	22	22	**20.6 ± 1.8**
13	28	24	14	23	24	**22.6 ± 2.3**
16	23	22	14	21	20	**20.0 ± 1.6**
19	22	20	11	21	17	**18.2 ± 2.0**
22	24	21	11	20	17	**18.6 ± 2.2**
25	24	22	_	19	17	**20.5 ± 1.6**
28	25	24	_	19	18	**21.5 ± 1.8**
31	24	20	_	14	17	**18.75 ± 2.1**
34	22	19	_	14	19	**18.5 ± 1.7**
37	25	18	_	17	18	**19.5 ± 1.9**

Appendix VII: Mean packed cell volume (%) of the infected *T. b. brucei* group B bucks

Days PI	B1	B2	B3	B4	B5	Mean ± SEM
-2	31	29	27	24	22	**26.6 ± 1.6**
-1	29	33	32	25	21	**28.0 ± 2.2**
0	23	26	28	22	19	**23.6 ± 1.6**
1	28	24	25	26	20	**24.6 ± 1.3**
2	29	24	27	27	22	**25.8 ± 1.2**
3	26	24	22	21	19	**22.4 ± 1.2**
4	25	23	22	21	20	**22.2 ± 0.9**
5	24	24	22	22	21	**22.6 ± 0.6**
6	21	22	18	21	20	**20.4 ± 0.7**
7	20	21	24	20	17	**20.4 ± 1.1**
10	20	21	19	18	14	**18.4 ± 1.2**
13	20	25	20	21	17	**20.6 ± 1.3**
16	20	19	17	18	18	**18.4 ± 0.5**
19	18	16	16	17	13	**16.0 ± 0.8**
22	18	16	16	16	14	**16.0 ± 0.6**
25	18	18	16	_	14	**16.5 ± 1.0**
28	16	18	16	_	14	**16.0 ± 0.8**
31	16	20	15	_	16	**16.8 ± 1.1**
34	14	20	15	_	14	**15.8 ± 1.4**
37	20	16	15	_	28	**19.8 ± 3.0**

Appendix VIII: Mean packed cell volume (%) of the control group C bucks

Days PI	C1	C2	C3	C4	C5	Mean ± SEM
-2	30	29	28	23	23	**26.6 ±1.5**
-1	32	33	34	22	24	**29.0 ± 2.5**
0	30	37	25	20	23	**27.0 ± 3.0**
1	30	31	26	21	23	**26.2 ± 2.0**
2	30	31	26	21	25	**26.6 ± 1.8**
3	29	31	25	21	23	**25.8 ± 1.9**
4	32	30	27	25	24	**27.6 ± 1.5**
5	34	29	30	29	26	**29.6 ± 1.3**
6	32	26	24	20	14	**23.2 ± 3.0**
7	32	28	28	0	25	**22.6 ± 5.8**
10	29	28	24	20	23	**24.8 ± 1.7**
13	37	33	33	24	34	**32.2 ± 2.2**
16	17	27	25	25	23	**23.4 ± 1.7**
19	24	31	29	21	32	**27.4 ± 2.1**
22	26	30	31	25	20	**26.4 ± 2.0**
25	30	24	30	25	18	**25.4 ± 2.2**
28	33	25	30	27	18	**26.6 ± 2.5**
31	25	25	32	25	40	**29.4 ± 3.0**
34	25	24	26	26	25	**25.2 ± 0.3**
37	30	25	26	34	37	**30.4 ± 2.3**

Appendix IX: Mean serum glucose concentrations (mmol/L) of the infected *T. b. brucei* group A bucks

Days P I	A1	A2	A3	A4	A5	Mean ± SEM
-2	3.2	2.6	3.1	2.6	2.6	**2.82 ± 0.12**
-1	2.5	3.1	2.7	2.8	3.1	**2.84 ± 0.12**
0	2.9	3	3	3.3	3	**3.04 ± 0.07**
1	3.5	3	3	3.9	3	**3.28 ± 0.18**
2	2.6	3	3	3	3.9	**3.10 ± 0.22**
3	2.1	3.5	3.5	4.2	2.8	**3.22 ±0.36**
4	3.1	3.4	3.1	3.5	3.2	**3.26 ± 0.08**
5	2.9	2.8	2.7	1.9	2.9	**2.64 ± 0.19**
6	3.4	1.7	2.8	1.7	4.5	**2.82 ± 0.53**
7	1.9	1.9	2.8	2.8	1.9	**2.26 ± 0.22**
10	2.8	3.7	2.8	3.7	3.7	**3.34 ± 0.22**
13	2.1	2.8	2.8	2.1	2.1	**2.38 ± 0.17**
16	3.3	2.3	2.3	2.8	2.8	**2.70 ± 0.19**
19	2.5	3.6	3.1	4.1	4.6	**3.58 ± 0.37**
22	3.5	3	2.2	2.2	3	**2.78 ± 0.25**
25	2.6	1.3	-	2.6	3.5	**2.50 ± 0.45**
28	2.3	2.8	-	3.3	3.3	**2.925 ± 0.24**
31	2	2	-	2.2	3.4	**2.40 ± 0.34**
34	1.8	1.8	-	2.1	2.7	**2.10 ±0.21**
37	3.4	3.4	-	3.4	4.5	**3.68 ± 0.28**

Appendix X: Mean serum glucose concentrations (mmol/L) of the infected *T. b. brucei* group B bucks

Days P I	B1	B2	B3	B4	B5	Mean ± SEM
-2	3.3	3.1	3	3	2.9	**3.06 ± 0.07**
-1	3.1	3.2	3.3	2.9	3.2	**3.14 ± 0.07**
0	2.9	3	2.9	2.8	3	**2.92 ± 0.04**
1	3.9	2.6	3.5	3	3	**3.20 V 0.23**
2	4.3	4.7	2.6	4.3	3.9	**3.96 ± 0.36**
3	4.2	3.5	2.8	3.5	2.8	**3.36 ±0.26**
4	1.9	2.6	1.9	3	3	**2.48 ± 0.25**
5	1.3	2.4	1.8	1.9	2.9	**2.06 ± 0.27**
6	2.5	1.9	1.2	1.9	1.9	**1.88 ± 0.21**
7	4.7	4.7	1.9	1.9	2.8	**3.20 ± 0.63**
10	3.7	3.7	2.8	2.8	3.7	**3.34 ± 0.22**
13	2.8	2.8	2.1	2.8	2.8	**2.66 ± 0.14**
16	2.3	2.3	3.3	1.9	2.8	**2.52 ± 0.24**
19	3.6	3.6	4.1	3.6	3.1	**3.60 ± 0.16**
22	3	2.6	2.2	2.6	2.2	**2.52 ± 0.15**
25	2.6	2.2	1.7	-	3	**2.38 ± 0.28**
28	3.3	2.8	3.3	-	3.7	**3.28 ± 0.18**
31	2.2	2	1.7	-	2	**1.98 ± 0.10**
34	2.1	3	2.4	-	2.7	**2.55 ± 0.19**
37	3.4	2.2	3.4	-	3.4	**3.10 ± 0.30**

Appendix XI: Mean serum glucose concentrations (mmol/L) of the control group C bucks

Days P I	C1	C2	C3	C4	C5	Mean ± SEM
-2	2.8	2.8	2.7	2.4	2.9	**2.72 ± 0.09**
-1	3	3.1	2.8	3	3	**2.98 ± 0.05**
0	2.8	2.6	3	2.7	3.1	**2.84 ±0.09**
1	2.6	2.6	2.6	3	3.5	**2.86 ± 0.18**
2	2.6	3.9	2.6	3	3.5	**3.12 ± 0.26**
3	2.1	2.8	2.1	2.8	3.5	**2.66 ± 0.26**
4	2.6	2.7	2.7	3	3.3	**2.86 ± 0.13**
5	2.9	3	2.8	2.1	2.9	**2.74 ± 0.16**
6	2.8	2.8	2.8	1.9	1.9	**2.44 ± 0.22**
7	1.9	2.8	1.9	3.7	1.9	**2.44 ± 0.36**
10	2.8	3.7	3.7	3.7	3.7	**3.52 ± 0.18**
13	2.1	2.1	2.1	2.8	1.4	**2.10 ± 0.22**
16	2.3	2.3	2.3	1.9	2.3	**2.22 ± 0.08**
19	2.5	3.6	3.6	3.1	3.1	**3.18 ± 0.20**
22	2.2	2.2	2.2	2.2	1.3	**2.02 ± 0.18**
25	2.6	3	2.6	1.7	1.7	**2.32 ± 0.26**
28	2.8	3.3	2.3	2.3	2.8	**2.70 ± 0.19**
31	2	1.7	2	1.7	1.7	**1.82 ± 0.07**
34	1.8	1.8	1.8	2.1	1.5	**1.80 ± 0.09**
37	4.5	3.4	3.4	2.2	2.2	**3.14 ± 0.43**

Appendix XII: Mean serum insulin concentrations (µg/l) of the infected *T. b. brucei* group A bucks

Days P I	A1	A2	A3	A4	A5	Mean ± SEM
-2	0.01133787	0.09750567	0.25170068	0.12358277	0.17460317	**0.1318 ± 0.0399**
-1	0.1462585	0.12244898	0.16893424	0.0600907	-0.0192744	**0.09567 ± 0.0340**
0	0.069161	0.04421769	0.14058957	0.51247166	0.0861678	**0.1705 ± 0.0869**
1	0.00226757	0.05895692	0.0952381	0.15306122	0.13718821	**0.0893 ± 0.0273**
2	0.00113379	0.02154195	0.0521542	0.24943311	0.04195011	**0.0732 ± 0.0449**
3	0.04875283	0.07596372	0.10657596	0.51247166	0.00566893	**0.14989 ± 0.0923**
4	-0.0147392	0.41269841	0.15646259	0.10090703	0.05328798	**0.1417 ± 0.0734**
5	0.32539683	0.8015873	0.49092971	0.22902494	0.06122449	**0.3816 ± 0.1260**
6	0.23582766	0.56802721	-0.0226757	0.17573696	-0.0022676	**0.1909 ± 0.1066**
7	0.22562358	0.41043084	0.41496599	0.3537415	0.01473923	**0.2839 ± 0.0755**
10	0.17006803	0.20748299	0.24376417	0.36848073	-0.0147392	**0.1950 ± 0.0621**
13	0.17120181	0.26190476	0.25170068	0.52721088	0.11111111	**0.2646 ±0.0712**
16	1.07029478	0.12131519	0.9138322	1.03628118	0.54988662	**0.7383 ± 0.1797**
19	1.13492063	0.27777778	1.13265306	0.94671202	0.56802721	**0.8120 ±0.1688**
22	0.87528345	0.17913832	1.73015873	1.25283447	0.42517007	**0.8925 ± 0.2792**
25	0.68480726	0.1893424	_	0.90702948	0.45578231	**0.5592 ± 0.1539**
28	0.57256236	0.35147392	_	1.07369615	0.19501134	**0.5482 ±0.1915**
31	0.88095238	0.58276644	_	1.29705215	0.40362812	**0.7911 ± 0.1953**
34	0.67460317	0.90589569	_	1.2845805	0.35714286	**0.8056 ± 0.1953**
37	1.02380952	0.91156463	_	1.07823129	0.57596372	**0.8974 ± 0.1126**

Appendix XIII: Mean serum insulin concentrations (µg/L) of the infected *T. b. brucei* group B bucks

Days P I	B1	B2	B3	B4	B5	Mean ± SEM
-2	0.17346939	0.19954649	0.08163265	0.11451247	0.33446712	**0.1807 ± 0.0437**
-1	0.13038549	0.14965986	0.16439909	0.19727891	0.14739229	**0.1578 ± 0.0112**
0	0.23015873	0.26417234	0.17687075	0.17346939	0.12131519	**0.1932 ± 0.0247**
1	0.24943311	0.24376417	0.13605442	0.14739229	0.14399093	**0.1841 ± 0.0256**
2	0.12585034	0.56349206	0.12698413	0.23469388	0.1292517	**0.2361 ± 0.0845**
3	0.12698413	0.21315193	0.16666667	0.26303855	0.10997732	**0.1759 ±0.0281**
4	0.11678005	0.07936508	0.17913832	0.1462585	0.12358277	**0.1290 ± 0.0165**
5	0.0952381	0.10884354	0.43310658	0.29251701	0.10544218	**0.2070 ± 0.0674**
6	0.10997732	0.14058957	0.57823129	0.39909297	0.12358277	**0.2702 ± 0.0937**
7	0.17120181	0.14399093	0.20634921	0.1893424	0.1122449	**0.1646 ± 0.0167**
10	0.16099773	0.22902494	0.12698413	0.33333333	0.07029478	**0.1841 ± 0.0453**
13	0.15986395	0.3276644	0.63151927	0.12698413	0.11111111	**0.2714 ± 0.0979**
16	0.0952381	0.78004535	0.18480726	0.15306122	0.46598639	**0.3358 ±0.1281**
19	0.38548753	1.15192744	0.44217687	0.41156463	0.2154195	**0.5213 ± 0.1625**
22	0.16780045	1.64058957	0.31746032	0.37755102	0.26303855	**0.5533 ±0.2740**
25	1.85714286	0.25396825	0.26303855	_	0.21768707	**0.6480 ±0.4032**
28	1.10997732	0.1814059	0.42403628	_	0.50340136	**0.5547 ± 0.19736**
31	0.61564626	0.57936508	0.60090703	_	0.46031746	**0.5641 ± 0.0354**
34	0.46031746	0.77664399	0.42857143	_	0.49319728	**0.5400 ± 0.0800**
37	0.5600907	0.52154195	0.52380952	_	0.52947846	**0.5337 ± 0.0089**

Appendix XIV: Mean serum insulin concentrations (µg/L) of the control group C bucks

Days P I	C1	C2	C3	C4	C5	Mean ± SEM
-2	0.12585034	0.1462585	0.27097506	0.24943311	0.234694	**0.2054 ± 0.0291**
-1	0.23129252	0.18707483	0.22335601	0.24036281	0.20861678	**0.2181 ± 0.0094**
0	0.14399093	0.25623583	0.17460317	0.2585034	0.28571429	**0.2238 ± 0.0273**
1	0.32993197	0.27664399	0.2324263	0.28798186	0.19274376	**0.2640 ± 0.0236**
2	0.18367347	0.15759637	0.20408163	0.53514739	0.10997732	**0.2381 ± 0.0759**
3	0.07823129	0.10090703	0.09750567	0.14172336	0.10884354	**0.1054 ± 0.0104**
4	0.12018141	0.12018141	0.14399093	0.13945578	0.19954649	**0.1447 ± 0.0146**
5	0.1462585	0.10090703	0.11337868	0.12471655	0.18707483	**0.1345 ± 0.0151**
6	0.51020408	0.14512472	0.13945578	0.13492063	0.19954649	**0.2259 ± 0.0720**
7	0.17913832	0.17006803	0.07482993	0.07596372	0.09183673	**0.1183 ± 0.0232**
10	0.10544218	0.06575964	0.13265306	0.08049887	0.14172336	**0.1052 ±0.0146**
13	0.11111111	0.10997732	0.09977324	0.13718821	0.51927438	**0.1955 ±0.0812**
16	0.21768707	0.12244898	0.15079365	0.15192744	0.16893424	**0.1624 ± 0.0157**
19	0.48412698	0.17687075	0.25170068	0.17346939	0.64739229	**0.3467 ± 0.0941**
22	0.69614512	0.15986395	0.21201814	0.18820862	0.39115646	**0.3295 ± 0.1002**
25	0.58049887	0.10997732	0.21995465	0.13265306	0.2414966	**0.2569 ± 0.0847**
28	0.46031746	0.1984127	0.28911565	0.20408163	0.41609977	**0.3136 ± 0.0538**
31	0.3968254	0.18480726	0.2244898	0.20521542	0.27324263	**0.2569 ± 0.0379**
34	0.37414966	0.26417234	0.27324263	0.10997732	0.34240363	**0.2728 ± 0.0457**
37	0.25623583	0.33333333	0.13605442	0.37755102	0.226757	**0.2660 ± 0.0421**

Appendix XV: The assay of serum glucose

The principle of the assay

The principle is based on an enzymatic reaction sequence and is as follows:

$$\beta\text{-D-Glucose} + H_2O + O_2 \xrightarrow{\text{Glucose Oxidase}} H_2O_2 + \text{D-Gluconic Acid}$$

$$H_2O_2 + \text{4-Aminoantipyrine} + \text{p-HBS} \xrightarrow{\text{Peroxidase}} \text{Iminoquinone} + H_2O$$

β-D-Glucose is oxidized by glucose Oxidase to produce D-Gluconic Acid and hydrogen peroxide. The hydrogen peroxide is then oxidatively coupled with 4-Aminoantipyrine and phenol substitute, p-HBS, in the presence of peroxidase to yield a red quinoneimine dye. The amount of coloured complex formed is proportional to glucose concentration and can be measured photometrically.

Test components

Glucose reagent:

1. Glucose oxidase (15µl/ml)
2. Peroxidase (horseradish) (1.2µl/ml)
3. mutarotase (4.0µl/ml)
4. 4-Aminoantipyrine (0.38mM)
5. p-Hydroxybenzene sulfonate (10mM)
6. Non-reactive ingredients.

Glucose standard: (100mg/dl β-D-glucose).

Test procedure

1. Preparation of the reagent was according to instructions.
2. The test tubes were labeled accordingly: blank, standard and test samples.
3. 1.0 ml of glucose reagent was pipetted into all the tubes and incubated at $37^{O}C$ in a water bath for 5 minutes.
4. 0.005ml (5µl) of the samples was pipetted into the respective tubes, mixed and incubated at $37^{O}C$ for ten minutes.
5. After incubation, the spectrophotometer was zeroed with the reagent blank and the absorbance of all tubes were read at 500nm and recorded.

Calculations

The concentration of glucose in the test sample was calculated as follows:

Absorbance (test sample)/Absorbance (standard) x concentration of standard (mg/dl) = concentration of test sample (mg/dl).

To obtain results in SI units (mmol/L), the concentration of the test sample in mg/dl was multiplied by 10 to convert deciliter to litre, and the value gotten was divided by 180, the molecular weight of glucose i.e.

X mg/dl x 10/180 = X mg/dl x 0.0556 mmol/L.

Appendix XVI: The assay of serum insulin

Principle of the serum insulin ELISA

Mercodia Bovine Insulin ELISA is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation, insulin in the sample reacts with peroxidase-conjugated anti-insulin antibodies and the anti-insulin antibodies bound to the micro titration well. After a simple washing step that removes unbound enzyme labeled antibody, the bound conjugate is detected by reaction with 3,3-5,5-tetramethylbenzidine (TMB). The reaction is stopped by the addition of acid, giving a colorimetric endpoint that can be read spectrphotometrically.

Test components

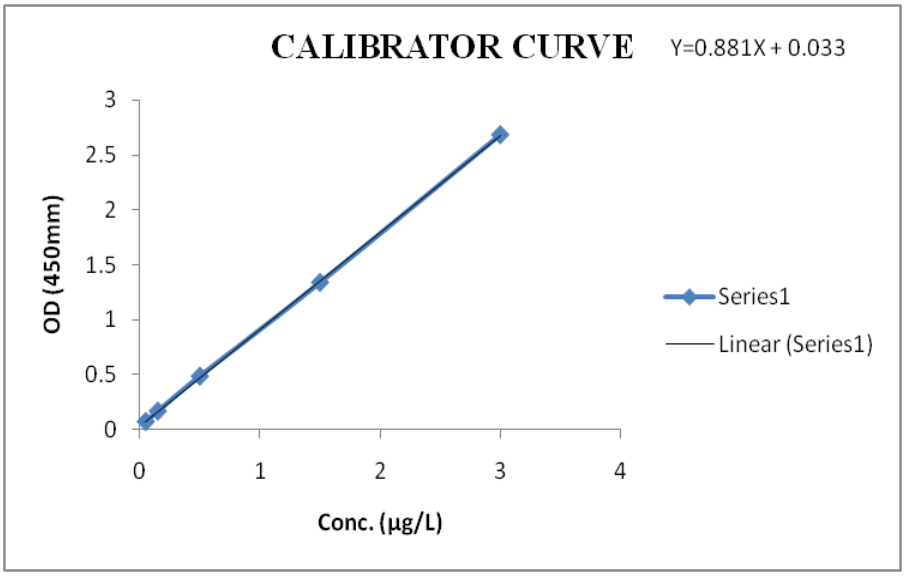
1. Coated plate (mouse monoclonal anti-insulin) with 96 wells.
2. Calibrators/standards: six vials(0,1,2,3,4,5) for bovine insulin at concentrations 5ml, 0.05 µg/L, 0.15 µg/L, 0.5 µg/L, 1.5 µg/L, 3.0 µg/L respectively.
3. Enzyme conjugate 11x (peroxidase conjugated mouse monoclonal anti-insulin) (1.3ml vial).
4. Enzyme conjugate buffer (13ml vial).
5. Wash buffer 21x (50 ml bottle).
6. Substrate TMB (22 ml bottle).
7. Stop solution (0.5 M H_2SO_4) 7ml vial

Test procedure

1. Before starting the assay, all reagents were brought to room temperature before use.
2. Enzyme conjugates 1x and wash buffer solutions were prepared.
3. Sufficient micro plate wells were also prepared to accommodate calibrators and test samples
4. 25 µl each of calibrators and test samples were pipetted into appropriate wells
5. 100 µl of enzyme conjugate 1x solution was added into each well that already contain calibrators and test samples
6. The reaction volume was incubated on a plate shaker (700-900rpm) for 2 hours at room temperature (18-25^{o}C)
7. After incubation, the reaction volume was discarded by inverting the micro plate over a sink
8. 350 µl of wash buffer 1x solution was then added to each well
9. The wash buffer 1x solution was discarded and excess liquid was removed by tapping the microplate firmly several times against absorbent paper
10. The washing process was repeated 5 times, however, avoiding prolonged soaking during the washing
11. 200 µl of substrate TMB was added into each well
12. This reaction volume was incubated for 15 minutes at room temperature (18-25 ^{o}C)
13. 50 µl of stop solution H_2SO_4 was added into each well and the plate was placed on the shaker for about 5 seconds to ensure thorough mixing
14. The optical density of the reaction was read at 450 nm using colorimeter within 30 minutes and the results were calculated.

Appendix XVII: Standard curve

Mean conc. µg/L	A 450
0.05	0.073
0.15	0.168
0.5	0.486
1.5	1.34



X= y - a /b, where, y=OD, a=-intercept and b=slope.

slope = 0.881 and Intercept = 0.033.

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Wells	Identity	A450	Mean conc. µg/L
B	Blank	0.291	0
S1	calibrator 0	0.064	5
S2	calibrator 1	0.073	0.05
S3	calibrator 2	0.168	0.15
S4	calibrator 3	0.486	0.5
S5	calibrator 4	1.34	1.5
S6	calibrator 5	2.685	3