HAEMATOLOGICAL, BLOOD GLUCOSE AND INSULIN PROFILE IN SPRAGUE-DAWLEY RATS EXPERIMENTALLY INFECTED WITH *TRICHINELLA ZIMBABWENSIS* AND *PLASMODIUM BERGHEI* ANKA

by

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Submitted in fulfilment of the academic requirements for the degree of Master of Science

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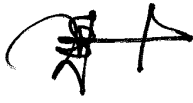College of Agriculture, Engineering and Sciences

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September 2017

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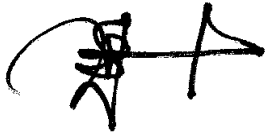**Main Supervisor**

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PREFACE

The research contained in this thesis was completed by the candidate, from February 2016 to September 2017, while based in the Discipline of Parasitology, School of Life Sciences, University of KwaZulu-Natal, Westville Campus, under the supervision of Professor S. Mukaratirwa and Dr P. Murambiwa.

The contents of this study represent original work by the author, and have not been submitted in any form to another tertiary institution, except where the work of others is acknowledged in the text.

Signed: Professor Samson Mukaratirwa
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DECLARATION

I Achasih Quinta Nkemzi declare that:

The research reported in this thesis, except where otherwise indicated, is my original research. This was part of research project on "Tissue-dwelling helminths and *Plasmodium berghei* co-infection in laboratory rats. The *P. berghei* parasitaemia, haematology, adult worm and muscle larvae burden data graphs were shared and are common with studies by my colleagues (Yanga Mdleleni and Ekuyikeno Silas Umo) who were also looking at other aspects except the once I did). All of us participated equally in generating these parameters.

This thesis has not been submitted for any degree or examination at any other university. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then their words have been re-written but the general information attributed to them has been referenced. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

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Abstract

Malaria and helminth co-infection have been widely reported by other researchers. Despite the increasing knowledge on co-infection, the impact of helminths and malaria on humans and laboratory animal models remains uncertain. Furthermore, studies have associated co-infection to the induction of hypoglycemia. Accordingly, the effect of *Plasmodium berghei* ANKA and *Trichinella zimbabwensis* co-infection on haematology, blood glucose and insulin profiles was investigated in male Sprague-Dawley rats. The study was investigated using 168 Sprague-Dawley rats that were assigned into four groups of 42 rats each; control (C), *P. berghei* (Pb), *T. zimbabwensis* (Tz) and *P. berghei* (Pb) + *T. zimbabwensis* (Tz) co-infection groups. The Tz group was orally infected with 3 muscle larvae/g animal body weight of Tz strain (Code ISS1209) at day 0. The Pb group received (1×10^5 PRBCs) of *P. berghei* ANKA using intraperitoneal injection and Pb + Tz co-infection group received 3 muscle larvae/g animal body weight at day 0 followed by intraperitoneal injection of 1×10^5 PRBCs at day 28 post-infection with Tz while the Control group was uninfected. *Plasmodium berghei* parasitaemia (%), Tz adult worms (AWs) and muscle larvae (ML), blood glucose, liver and muscle glycogen, insulin concentration as well as the cumulative body weight were determined. Results showed a significant increase in parasitaemia for Pb + Tz co-infection at day 3 and 4 PI compared to Pb group. Adult Tz worms and ML recovered showed no significant difference ($P > 0.05$) between the co-infection and the Pb group ($P > 0.05$). Blood glucose levels for co-infected group were also not significantly different compared to the control although the Pb group showed a decrease in blood glucose at day 7 and 14 PI. The co-infection group showed a higher mean glycogen accumulation compared to Tz group although there was no significant difference between the two groups. Tz group and Pb + Tz co-infection groups showed a decrease in liver glycogen at day 7 Pl and at day 14 PI. The Pb + Tz co-infection group showed a significant decrease in glycogen compared to the control. After Pb infection, there was a decline in insulin for both Tz and Pb + Tz co-infection whereas at day 7 only the Pb + Tz co-infection group presented a decrease in insulin compared to the control. Therefore, the current study revealed that co-infection increased Pb parasitaemia and glycogen levels without decreasing the host blood glucose concentration. Early increase in Pb parasitaemia demonstrated that co-infection could alleviate parasitaemia severity in the host.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

ANOVA = Analysis of variance

AW = Adult worm

BRU = Biomedical research unit

C = Control

DNA = Deoxyribonucleic acid

EDTA = Ethylenediaminetetraacetic acid

ELISA = Enzyme-linked immunosorbent assay

g = grams

HCl = Hydrochloric acid

HCT= Haematocrit

iRBC = Infected red blood cell

LPG = Larvae per gram

 Mg = milligram

ML = Muscle larvae

NBL = New born larvae

Pb = *Plamodium berghei*

PCR = Polymerase chain reaction

PI = Post-infection

Pmol/l = Picomole per liter

PRBC = Parasitized red blood cell

RBC= Red blood cell

SEM = Standard error of mean

Spp. = Species

SSA= Sub-Saharan Africa

STH = Soil transmitted helminths

Tz = *Trichinella zimbabwensis*

UKZN = University of KwaZulu-Natal

UV – Ultraviolet

WBC = White blood cell

CHAPTER 1: GENERAL INTRODUCTION

1.1 Background of the study

Malaria and trichinellosis are two common parasitic infections that pose an enormous health and economic burden to developing countries, especially Sub-Saharan African countries (Boraschi et al. 2008; Florey et al., 2012). The prevalence and burden of these diseases is correlated to little or no scientific attention, unavailability of vaccines and poor socio-economic conditions in affected countries that hinder the effective control and management of the diseases (Boraschi et al., 2008). According the World Health Organization world malaria report 2017, the current global death toll of malaria is 445,000 deaths and 216 million cases worldwide. Thus, highlighting the fact that malaria still presents a major global mortality, morbidity and burden in affected regions which still needs attention (Utzinger et al., 2012).

Equally, trichinellosis which has been reported to be an emerging and re-emerging zoonotic disease, is estimated to be a threat to 10 million people in some parts of the world (Utzinger et al., 2012; Mukaratirwa et al., 2016). This zoonotic disease is caused by species of the genus *Trichinella*, a tissue-dwelling nematode (Pozio et al., 2006; Mukaratirwa et al., 2016; Onkoba et al., 2016a), through eating raw or undercooked meat (Mukaratirwa et al., 2013). The detection of *Trichinella* parasite in wild and/or domestic animals and humans (Mukaratirwa et al., 2016) is indicative of the economic importance of this disease to humans, pig production and food safety, since it is associated with animals and their products (Gottstein et al., 2009). Therefore, trichinellosis has an effect on meat and meat products trading industries across the world (Gottstein et al., 2009). Despite the devastating effect of malaria and trichinellosis on the human host, their occurrence in the same area exposes the host to the transmission of both infections and co-infection is inevitable (Onkoba et al., 2015). As one of the highly endemic regions for both malaria and helminths, there is an overlap between both diseases in Sub-Saharan Africa (SSA), and individuals living in such an environment are prone to both malaria-helminthiasis infections such as trichinellosis (Graham et al., 2005; Su et al., 2005; Melo et al., 2010; Salazar-Castañon et al., 2014). Although, no human cases of trichinellosis have yet been reported in Southern Africa, the parasite has a likelihood of causing future outbreak of human trichinellosis in SSA region because

of its ability to affect non-human primates thus increasing the risk through, movement of animal products, poor animal rearing system and interaction between humans and wildlife/livestock (Mukaratirwa et al., 2008; Mukaratirwa et al., 2013; Onkoba et al., 2016a).

However, current studies have revealed that malaria and helminthiasis co-infection can decrease the severity of malaria, especially with *P. falciparium* (Melo et al., 2010). But, the interaction of parasites and their host might be synergistic or antagonist (Telfer et al., 2008) which could impact each other directly or indirectly (Cox, 2001) by either aggravating or amending the disease within human host (Graham et al., 2005). This could result in implications such as morbidity or mortality for both hosts (Salazar-Castañon et al., 2014). More so, recent studies on vertebrates have demonstrated that parasitic co-infection could have a valuable influence on disease severity, development and transmission dynamics (Telfer et al., 2008; Knowles, 2011). However, for a better knowledge of epidemiology, it is important to understand the cellular, molecular, immunological and physiological mechanisms in which the parasites infect the same host (Knowles, 2011), and how the host immune system can counteract this mixed interaction (Druilhe et al., 2005).

1.2 Motivation

Although several studies have attempted to explain the patterns and mechanisms of co-infection between malaria and helminths, these surveys have recorded series of pathological disorder associated to malaria and trichinellosis. Malaria is known to cause a range of pathological complications, which are associated with metabolic impediments, such as impaired kidney functions, cardiovascular and glucose homeostasis. Glucose homeostatic derangement caused by malaria is hypoglycemia, initiated by the high parasite demand for glucose from its host (Smith et al., 2005). However, suggested mechanisms responsible for the cause of hypoglycemia have been the depletion of gluconeogenic substrate such as thiamine and liver damage (Murambiwa et al., 2013). Similarly, *Trichinella* infection has been reported to have an influence on blood glucose level and this has been demonstrated in mice (Wu et al., 2009), dogs (Reina et al., 1989) and humans (Buşilă et al., 1967) infected with *Trichinella* species. The cause of hypoglycemia, has been associated to the rapid growth of the parasite larvae during migration and when located in

their final niche (muscles), thus the parasite necessitates huge amount of glucose for their sustainability there by affecting the host glucose store (Wu et al., 2009; Gumede et al., 2003). Co-infection studies on helminths and malaria have demonstration differential pathological feedback (Onkoba et al., 2015). These feedbacks are always contradictory and non-heterogonous, and there is also paucity of data from local and global burden estimates (Utzinger et al., 2012). It is against this background that this study seeks to understand the effects of *Plasmodium berghei* ANKA and *T. zimbabwensis* co-infection on blood glucose concentration in Sprague-Dawley rats.

1.3 Aim

- To determine the effect of *Plasmodium berghei* ANKA and *Trichinella zimbabwensis* co-infection on blood glucose and insulin profile in male Sprague-Dawley rats.

1.4 Specific objectives

- To determine the effect of *P. berghei* ANKA and *T. zimbabwensis* co-infection on blood glucose and insulin levels in male Sprague-Dawley rats.

- To determine the effect of *P. berghei* ANKA and *T. zimbabwensis* co-infection on liver and gastrocnemius muscle glycogen levels in male Sprague-Dawley rats.

1.5 Thesis structure

The thesis is structured in the form of chapters. Chapter 1 introduces the study, study motivation, aims and objectives of the study.

Chapter 2 gives the literature review that reviews all current literature associated to the study and highlights the research gaps in the area.

Chapter 3 gives the materials and methods of the study and provides details of the experimental procedure and data and analysis.

Chapter 4 reports the results and provides graphical representation and synthesis of all data that was analyzed.

Chapter 5 Reports on the discussion, in this chapter the results are discussed in view of other studies.

Chapter 6 reports on general conclusion and recommendation.

CHAPTER 2: LITERATURE REVIEW

2.1. Introduction

The prevalence of parasitic infection such as helminths is widespread in humid and tropical region, where they are easily transmissible. Likewise, parasitic protozoa such as malaria (*Plasmodium* spp.) are common in this region, with both infections having a huge impact in the world, particularly when they co-exist in the same host (Supali et al., 2010). Infections caused by these parasites within the host result in a variety of biochemical reactions such as modulation of energy metabolism, anemia, inflammations and nutrient deficiencies (Saric et al., 2010). Parasitic infection in animals and humans has been postulated to induce hypoglycemia, which has been reported in cases of *Trichinella* infection (Nishina et al., 2004), and malaria (Smith et al., 2005). Severe clinical conditions in patients are known to be exacerbated by hypoglycemia, such conditions include malaria, malnutrition, and intoxication (Barennes et al., 2005). The above conditions can further be aggravated and result in high morbidity and mortality rate as reported in sub-Saharan Africa (Barennes et al., 2005). Hypoglycemia associated with malaria has a significant contribution to approximately 22500 deaths recorded in African children under the age of five years (Barennes et al., 2005). Cases of comatose children have as well been linked to hypoglycemia, and the mechanism driving this is still obscure. In order to understand the driving mechanism, several suggestions have been reported to tie to the cause of hypoglycemia; these include stress, hypercatabolism, anaerobic glycolysis, gluconeogenesis (Barennes et al., 2005), and drug treatment such as quinine have also been described as a possible mechanisms leading to hypoglycemia in African children (Murambiwa et al., 2013).

In this regard, this section presents a review on the effects of malaria, helminthiasis and co-infections on glucose and insulin levels and how they are associated with hypoglycemia in the host, in order to establish research gaps.

2.2 Malaria infection and epidemiology

Malaria is one of the most severe and deadly diseases of humans and animals in the world (Benelli, 2016), ranking third among infectious diseases, next to Acquired Immune Deficiency Syndrome (AIDS) and tuberculosis (TB) (Murambiwa et al., 2013). Malaria infection is caused by the genus *Plasmodium*, a parasitic protozoan distributed by infected female *Anopheles* mosquitoes (Salazar-Castañon et al., 2014; Benelli, 2016). Species of *Plasmodium* that cause malaria infection in humans include; *P. falciparum, P. vivax, P. malariae, P. ovale,* and *P. knowlesi* (Brooker et al., 2007). However, most deadly species are *P. vivax* and *P. falciparum*, with *P. falciparum* being the most deadly species in Sub-Saharan Africa (Melo et al., 2010). The wide distribution of these pathogens across the globe is responsible for morbidity and mortality, especially in tropical countries (Salazar-Castañon et al., 2014). According to recent studies on malaria incidence, it is estimated to be a threat to close to 3.3 billion people and 1.2 deaths recorded (Melo et al., 2010). Most of the mortality is noticeable in the African continent, particularly in Sub-Saharan Africa, among children below the age of 5 years and pregnant women (Onkoba et al., 2015). The Sub-Saharan region therefore serves as the core of parasite transmission. Even though recent studies on malaria have documented a marked decline in mortality up to about 54% in the African continent (Benelli, 2016), the disease still remains a serious public health threat that needs attention.

2.3 Life cycle of Plasmodium spp.

The transmission of malaria to the host (vertebrates such as humans), is through the *Anopheles* female mosquitoes (Crawley and Nahlen, 2004). Most life cycles of *Plasmodium* spp. occur through infected female *Anopheles* mosquito bite during blood meal, which releases infective sporozoites through the dermis into the peripheral blood circulation of the host. Sporozoites then migrate to the hepatocytes, where they duplicate through asexual schizogony to produce schizonts containing merozoites. The schizont ruptures and releases several merozoites which enter the blood stream. But, for the other species such as *P. vivax* and *P. ovale* the hypnozoites are formed which remain dormant in the liver until future stage; however, this does not happen with *P.*

falciparum (Trampuz et al., 2003). The process occurring in the liver is referred to as exo-erythrocytic cycle which is usually asymptomatic as shown in Figure 1.

The intra-erythrocytic or erythrocytic stage commences when the merozoites leave the liver and enter red blood cells (RBCs), developing into a ring form which later matures into trophozoites. During the ring stage, the parasite lies dormant for a while and becomes metabolically active after about 15 hours post invasion. There is therefore an increasing rate of metabolison and biosynthesis in the infected RBCs to finally mature into trophozoites (Kirk, 2004; Kirk, 2001). By so doing, it converts hemoglobin of the RBCs to aminoacid, and is also dependent on other nutrient sources for survival (Ramasamy, 1998; Milner et al., 2012). The malaria parasite relies on the host energy store because it has only a single mitochondrion but lacks a functional citric acid cycle; so it is dependent uniquely on glycolysis for its energy requirements (Kirk, 2004; Kirk, 2001). Trophozoites then undergo schizogony to form merozoites. The parasitized red blood cells (PRBCs) are lysed to release abundant merozoites, which enter blood circulation to recommence a new cycle of schizogony within the RBCs. Some of the merozoites in the blood stream develop into the male and female gametocytes (See Figure 1).

These gametes are ingested by the mosquito during subsequent blood meal and undergo gametogenesis in the mosquito midgut. During this process, the gametocytes undergo fertilization to produce motile ookinete. The ookinete crosses the epithelial cell of the midgut into the haemocoele and develops into oocyst. The oocyst then produces sporozoites. The sporozoites formed in the mosquito migrate to the salivary glands ready for re-infection of the vertebrae host in another blood meal and the cycle resumes. The life cycle of malaria is similar in all species of *Plasmodium*. During the life cycle, the erythrocytes are modified in several ways. One of the ways is by deriving energy through anaerobic glycolysis of glucose to lactic acid contributing to manifestation of hypoglycemia and lactic acidosis (Milner et al., 2012). It is during this asexual cycle that most clinical manifestations are encountered.
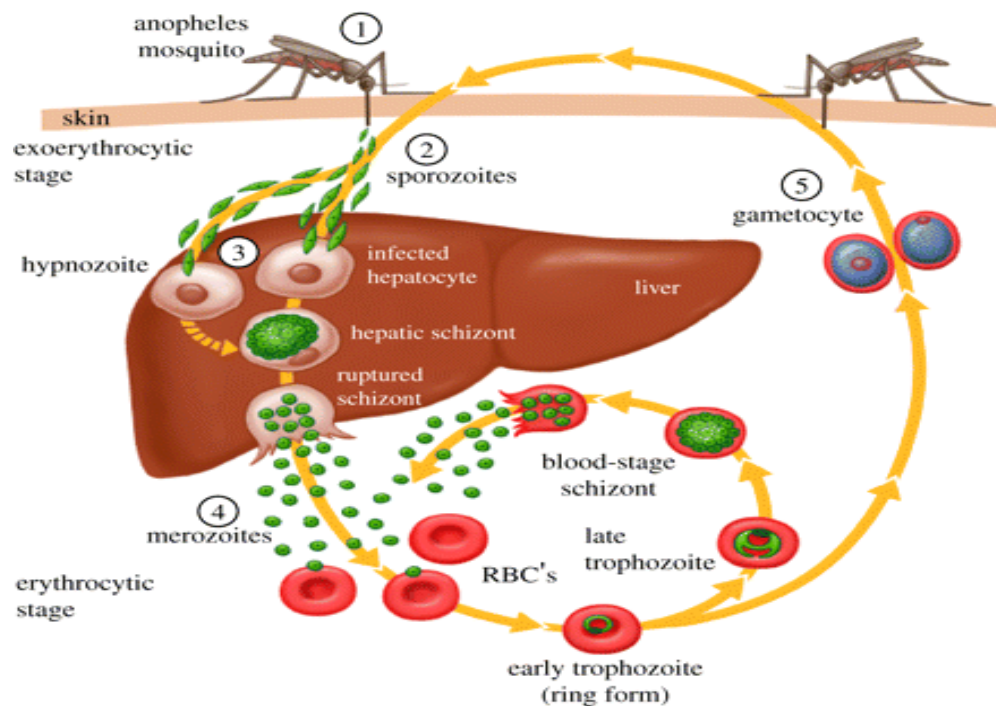
Figure 1: Life cycle of *Plasmodium* spp. Hill (2011)

2.3.1 Description of clinical features of malaria

Clinical symptoms of malaria are felt during the erythrocytic stage of the lifecycle (Rosenthal, 2008). Most of these symptoms ascend from the rupture of the schizont and destruction of the erythrocytes. However, the cycle of malaria might occur in some stages without specific symptoms and is a major concern in clinical and pathogenesis studies (Idro et al., 2010). Therefore, malarial manifestations may show resemblance with other viral infections, thus, making it difficult to diagnose (Trampuz et al., 2003). The most common clinical symptoms observed in infected individuals are; fever, headache, body weakness, prostration (Das, 2008). Additionally, vomiting, nausea, abdominal pain, myalgia, mild diarrhea, jaundice may occur (Trampuz et al., 2003). In cases of severe malaria there are no specific symptoms as well, but most are characterized by severe anemia, metabolic acidosis, jaundice, hyperlactemia, hypoglycemia, renal failure, pulmonary oedema, haemoglobinuria, abnormal bleeding, cerebral malaria and electrolyte

imbalance (Idro et al., 2010). Hypoglycemia and acidosis result from metabolic impediments (Trampuz et al., 2003), which are often associated with cerebral malaria during severe illness (Rosenthal, 2008).

The above-mentioned symptoms might evolve speedily over a duration of an hour or days. In situations where all these complications are combined in a particular host, death comes as fast as possible probably within a few hours (Trampuz et al., 2003). Therefore, it is necessary for proper evaluation of a patient infected with malaria for any of these signs to avoid the disease severity which might lead to death through instant treatment (Trampuz et al., 2003). Although, studies have revealed that proper evaluation of clinical outcome in a patient could be influenced by other factors such as pregnancy, age, virulence of the parasite strain, inoculation host genetic factor and immunity, all these need to be taken into consideration before treatment (Legesse et al., 2004).

2.3.2 Host-parasite interaction and pathophysiology of malaria

The pathogenesis and clinical outcomes induced by *P. falciparum* is a multi-complex interaction (Buffet et al., 2011). Complications usually arise from an interplay between parasite and host, and might lead to multiple responses within the host. These responses could either be mechanical, humoral or immunological set to eliminate parasite but end up affecting the host (Elsheikha and Sheashaa, 2007; Mishra and Das, 2008). Once the host is infected, parasite-induced red blood cell alteration and microcirculatory abnormalities which lead to local and systemic reaction occur (Buffet et al., 2011). Malaria infection is known to be associated with severe anemia, cerebral malaria and metabolic acidosis, hypoglycemia, hypovolemia (Mockenhaupt et al., 2004).

Hypovolemia, which has been identified as significant feature, might be exacerbated by the severe anemia caused by red blood cells destruction, metabolic acidosis, and microvascular impediment by sequestrated parasites resulting in the decrease delivery of oxygen to tissue (Mockenhaupt et al., 2004). Microcirculation perturbations might lead to decrease in metabolites exchange and hypoxia, and may trigger the release of pro-inflammatory and anti-inflammatory cytokines mediators (Clark and Cowden, 2003; Mockenhaupt et al., 2004; Murambiwa et al., 2013). Hypoxia has also been attributed to the disproportionate release of pro-inflammatory and anti-inflammatory

cytokine, hence, it has been associated to be the main factor in the pathogenesis of malaria (Clark and Cowden, 2003). Thus, understanding the host immune response is a vital contribution that will help unveil protective immunological strategies. Another main feature of the life cycle of *P. falciparum* is cytoadherence, whereby the mature parasitized red blood cells (trophozoites and schizonts), are capable of displaying significant change by and/or adhering to the endothelial cells (Rosenthal, 2008; Buffet et al., 2011; Cobbold et al., 2016). This could favor the parasite because the process can possibly obscure the passage of abnormal erythrocyte to the spleen (Rosenthal, 2008). On the other hand, the adherence of mature PRBCs on small blood vessel obstructs the peripheral blood circulation, contributing to the pathogenesis of malaria (Buffet et al., 2011; Cobbold et al., 2016). However, the early stages (rings) of the infection present slight modification of adhesion or deformability, which can be seen within the blood circulation; hence, the level of peripheral parasitaemia could be quite low, since not all the intra-erythrocytic stages of the parasite are present in the blood circulation (Sherman et al., 2003; Rosenthal, 2008).

2.3.3 Role of cytoadherence and sequestration of *Plasmodium falciparum* in pathogenesis of malaria

Sequestration is a phenomenon in which the mature parasites adhere to the endothelial cells, blood cells and uninfected red blood cells. This is mediated by multiple host receptor recognized by parasite adhesine (Buffet et al., 2011). The accumulation of these parasites within the organs leads to variable clinical outcomes such as cerebral malaria, non-cardiogenic pulmonary edema and renal failure (Rosenthal, 2008). The consequence of sequestration may lead to mechanical obstruction of blood flow, which might lead to hypoxia, the release of parasites toxins and inflammatory mediators and cerebral oedema and neurological deficits (Beeson and Brown, 2002). In addition, parasite sequestration may concentrate metabolic disturbances and trigger pro-inflammatory response to a particular organ such as the brain contributing to the severe clinical outcomes (Beeson and Brown, 2002). Studies have shown that individuals who progress to coma, might regain consciousness easily, therefore, this state could not be linked to neurological sequelae but rather signifying that the pathology of cerebral malaria is associated to metabolic components (Beeson and Brown, 2002).

2.4 Host–pathogen interaction and nutrient competition

The host supports the parasite by providing a suitable environment for development, and this is based on an ecological and evolutionary perspective (Smith et al., 2005). Disease infection caused by pathogens and its manifestation comprises a series of ecological processes that occur within the host, which include, invasion, prey-predation dynamics, resources competition and consumption (Smith et al., 2005). Hence, host infection with pathogen, no matter the intensity of infection, decreases the host nutrients. Host nutrient supply is therefore strongly correlated to pathogen population growth and dynamics and pathological outcome (Smith et al., 2005). Increase in population of parasite results in increased demand of the host internal nutrient store (Goater et al., 2013), while the host will suffer especially when valuable nutrients such as glucose are depleted (Smith et al., 2005). Competition of the large parasite population size and a single host for glucose uptake, results in hypoglycemia (Binh et al., 1997).

2.4.1 Glucose metabolism by parasitized red blood cells during malaria

The fact that the parasite within the infected erythrocytes need to survive and multiply, requires large amount of energy. Since the parasite has the ability to reproduce within 48 hours it therefore relies on the host energy store (Ginsburg and Stein, 2004). Thus, the parasite need to develop mechanisms to obtain nutrients from the host cytosol, consequently there is competition between metabolic and biosynthetic machinery of the host (Kirk, 2001). Most of the energy is critically required during the invasion of RBCs (ring stage) and the demand increases during the maturity to trophozoite (Kirk, 2001) which results in infected RBCs undergoing alteration of membrane transport (Sherman et al., 2003). Increasing membrane permeability of the infected red blood cell (iRBC) is of vital important to the parasites survival within the host, because the parasite is known to have a single mitochondrion and no functional citric acid cycle and hence dependent on glycolysis (Kirk, 2001) to meet its metabolic demand that cannot be provided by other endogenous sources from uninfected RBCs (Ginsburg and Stein, 2004). In such cases the developing parasites within the RBCs restructures the host cell, which becomes suitable for the transportation of large

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number of proteins, there by facilitating nutrient transport, trafficking and evasion of host immune system (Cobbold et al., 2016). According to Kirk (2001) the amount of glucose used and lactic acid production in iRBCs increases 100 times compared to uninfected RBCs.

2.4.2 Role of insulin in glucose metabolism

Insulin is a hormone produced by pancreatic β- cells of the islet of Langerhans in response to elevated blood glucose and aminoacid. It is also well known to be the most potent anabolic hormone involved in tissue development and maintenance of the body glucose homeostasis (Saltiel and Kahn, 2001). The glucose regulatory hormones insulin and glucagon help to maintain the plasma glucose within narrow physiological range (Leto and Saltiel, 2012), by the interplay of the glucose-lowering action of insulin and the glucose-raising action of four counter regulatory hormones (catecholamines, cortisol, glucagon and growth hormone) (Thien et al., 2006). Insulin plays a vital role in most insulin-responsive sites by enhancing glucose uptake and inhibiting gluconeogenesis and glycogenlysis and reducing hepatic glucose uptake while increasing the rate of glucose uptake into striated muscles and tissues (Pessin and Saltiel, 2000; Saltiel and Kahn, 2001). Glucose uptake from the circulatory system and into targeted tissue and fat is mediated by insulin- stimulated translocation transporter GLUT4 isoform to the cell surface (Leto and Saltiel, 2012), thereby impacting lipid metabolism by increasing lipid synthesis in the liver and fat cells via the reduction of fatty acid from triglycerides in fat and muscles (Pessin and Saltiel, 2000).

Despite the anabolic potentials of insulin in glucose metabolism, inadequate release of insulin in normal circulatory system in order to control glucose uptake leads to a defect in signals transduction, referred to as insulin resistance. Several mechanisms have been reported to be associated with insulin resistance, the molecular targets and intracellular signaling system that are modified during insulin resistance have received attention, yet, no evidence for the basic mutation in any signaling pathways (Pessin and Saltiel, 2000). However, insulin signaling mechanism has been reported to be involved in several glucose and lipid metabolism (Pessin and Saltiel, 2000) by participating in different processes such as cell growth and differentiation, apoptosis, protein lipid and glucose anabolism and catabolism. Insulin signaling pathway has been reported to play a central role in transporting glucose from blood to responsive tissues and stimulating the conversion

of glucose to glycogen to storage organs (Youngren, 2007). According to Wu et al. (2009), this pathway is activated by insulin, which binds with the receptor on cell surfaces of the insulin responsive tissues such as muscle. The insulin signaling pathway stimulates the binding process through the tyrosine phosphorylation of IR, insulin receptor substrate-1 (IRS-1) and IRS-2, which further activates phosphatidylinositol 3-kinase (PI3-K) (Youngren, 2007). Later the PI3-K also activates V-akt murine thymoma viral oncogene homologue (Akt) (Wu et al., 2009). Activated Akt then stimulates glucose uptake into cells by the help of glucose transporter 4(Glut4) from the intracellular storage sites to the plasma membrane (Wu et al., 2009).

Glucose transporter 4 (Glut4) facilitates glucose uptake in muscle, and adipose tissue, and is expressed by activating the insulin receptor (MacIver et al., 2008). Insulin signaling has so far been reported in incident of malaria (Luckhart and Riehle, 2007) and Trichinella using experimental mice (Wu et al., 2009), which was underscored to induce hypoglycemia. Hypoglycemia in mice has been associated with hyperinsulinemia and its severe pathological outcome (Luckhart and Riehle, 2007).

2.4.3 Effect of malaria on host glucose stores

One of the most common complications during severe *P. falciparum* malaria is the host nutrient consumption by the parasite, and glucose is one of such nutrient (Smith et al., 2005). Consumption from the host glucose store by pathogenic protozoa is known to induce hypoglycemia (Smith et al., 2005; Thien et al., 2006), which implies that hypoglycemia is associated with the high rate of morbidity and mortality. In addition, most of the fatal incidences of hypoglycemia have been reported to have a higher frequency in children than adults, which has been observed in cases of comatose children with cerebral malaria (Barennes et al., 2005; Zijlmans et al., 2009). However, the pathological mechanism responsible for an imbalance between glucose production and utilization is still not clear (Dekker et al., 1997).

During the life cycle of malaria in the vertebrate host, the erythrocytes are modified so as to derive energy through anaerobic glycolysis from glucose to lactic acid contributing to manifestation of hypoglycemia and lactic acidosis (Milner et al., 2012; Mehta et al., 2005). The iRBCs are solely

dependent on the host energy to meet the energy demand of the parasite, there by depleting the host energy store (Mehta et al., 2005) by competing with the metabolic and biosynthetic machinery of the host (Kirk, 2001). It is unquestionable that hypoglycemia is bound to occur since there is increased depletion of glucose by the parasite without sufficient replenishment from the host. According to Thien et al. (2006), the parasite intake of host glucose is known to be a contributing factor rather than a causative factor of hypoglycemia. Current reports have shown that iRBCs could use greater amount of glucose compared to non-infected RBCs (Mehta et al., 2005; Zijlmans et al., 2009). In addition, a severely ill person requires higher glucose demand compared to the parasite (Zijlmans et al., 2009). This has been demonstrated by Kochar et al. (1998) in a study of severe malaria cases in India, which reported that the mortality rate was higher in patients with low blood glucose (less than 2.2mmol/l) and a lower mortality rate in patients with blood levels greater than 3.3mmol/l.

Hypoglycemia has well been reported to be exacerbated by hyperinsulinemia caused by quinidine in malaria treatment, as quinine stimulates insulin release (Trampuz et al., 2003).

According to Thien et al. (2006),

> Plasma glucose concentration is maintained in the relatively narrow physiological range by the interplay of the glucose-lowering action of insulin and the glucose-raising action of four counter regulatory hormones (catecholamines, cortisol, glucagon and growth hormone).

These hormones are involved in glucose homeostasis but the key counteracting regulators are insulin and glucagon. Insulin is involved in decreasing blood glucose while there are numerous glucose-increasing hormones (König et al., 2012). Although glucagon plays a primary counter-regulatory role in hypoglycemia, epinephrine plays the secondary role and others such as thyroxine and cortisol play a minor role for the liver in conditions where there is decrease in effectiveness and response by glucagon (König et al., 2012). The concentration of insulin and glucagon in the plasma is released depending on the blood glucose concentration. Insulin functions by increasing glucose uptake pathways such as glycolysis and glycogenesis and decreases the production pathways such as gluconeogenesis and glycogenolysis, whereas a contrary process occurs for glucagon and other hormones (König et al., 2012). Other gluconeogenic substrates of importance

are thiamine and depletion of them has been reported by Krishna et al. (1999), and it is assumed to be caused by an increase in serum transketolase activities in patients with malaria in response to increased parasite glycolytic pathways. This results in decrease of host aerobic glycolysis and induces lactic acid accumulation (Murambiwa et al., 2013).

Another possible cause of gluconeogenic substrate depletion resulting in hypoglycemia during malaria could be attributed to liver damage. Since the liver plays a vital role in glucose homeostasis by breaking down insulin receptor complex, any damage of the hepatocyte might delay the action of insulin receptor recycling, which might induce hypoglycemia (Murambiwa et al., 2013). According to Dekker et al. (1997), the hepatocellular destruction could occur during the rapid multiplication and change of phase within the liver by the malaria parasite. Therefore, the presence of hepatocyte cells within the extracellular fluid might be possible due to permeability of intracellular content of damaged hepatocytes. This is supported by studies carried out by Kausar et al. (2010) in which they observed an increase in liver enzymes such as alkaline transaminases, aspartate, alkaline phosphatase, suggesting the probability of liver damage.

2.4.4 Diagnosis, treatment and prevention of malaria

Most fatal records of malaria in many countries are attributed to late diagnosis and treatment. Therefore, for proper management of the disease, accurate diagnosis is required. Normally, malaria diagnosis is done by identifying the parasite or antigen/product in the blood, but is influenced by several factors. Some of these factors could be the different species causing malaria and their endemicity, level of transmission, the different stages of erythrocyte cycle, immunity, drug resistance, parasite sequestration and many more (Tangpukdee et al., 2009).

However, the most widely used method of malaria diagnosis is based on clinical symptoms, which is known to be the traditional method. The majority of clinical diagnosis is done based on the patient clinical signs and symptoms and also on the physical examination (Tangpukdee et al., 2009). However, the first symptoms of malaria are non-specific and show similarities with other viral and bacterial infections. This implies that the close similarities of malaria sign and symptoms

with other diseases make malaria diagnosis challenging. Furthermore, the non-specificity of clinical diagnosis might lead to misdiagnosis and eventually might result in wrong treatment.

Another possible method of malaria diagnosis other than clinical pathology method is by using different laboratory techniques. The most common used laboratory techniques are microscopic diagnosis of thick and thin blood smears. Other concentration techniques include quantitative buffer coating (QBC), rapid diagnostic tests (example OptiMAL, ICT, Para-HIT-f, ParaScreen, SD Bioline, Paracheck) (Tangpukdee et al., 2009) and molecular diagnostic methods such as polymerase chain reaction (PCR). Some advantages and shortcomings of these methods have been described, related to sensitivity, specificity, accuracy, precision, time consumed, cost-effectiveness, labor intensiveness, the need for skilled microscopists and technicians (Tangpukdee et al., 2009). Proper malaria control necessitates immense efforts and some of the currently used intervention strategies to control malaria include, use of insecticide-impregnated bed nets, house spraying and antimalaria drug administration to infected individuals (Brooker et al., 2007).

2.5 Trichinellosis

Trichinellosis is a meat-borne zoonosis, caused by parasitic tissue-dwelling nematode worm of the genus *Trichinella* (Mukaratirwa et al., 2016). The species are widely spread across the globe, without any documented incidence of the parasite infection in the Antarctica (Pozio et al., 2006; Pozio et al., 2007; Mukaratirwa et al., 2013). The detection of *Trichinella* parasite in wild and/or domestic animals and humans across the world reveals that it is a public health threat (Dupouy-Camet and Murrell, 2007; Mukaratirwa et al., 2013). Also considering the fact that humans possess different ethnic habits regarding consumption of meat which could either be eaten raw or undercooked, hence promoting the transmission of the parasite (Pozio et al., 2007; Mukaratirwa et al., 2013). Although, other factors such as humoral, environmental and physiological might as well favor the transmission of *Trichinella* infection, the disease is regarded to be a huge burden to the public health sectors and the economy by the impact it causes to humans, pig production and food safety and meat trade (Gottstein et al., 2009). The disease burden levied to the society has prompted massive control and eradication efforts of the zoonotic pathogen from the food chain in developed countries (Gottstein et al., 2009) with little effort being made in developing countries.

2.5.1 Classification of Trichinella species

Current taxonomical studies on the genus *Trichinella* have been based on the epidemiology, genetic diversity and zoogeographical distribution, resulting in the identification of eight species and four genotype (Gottstein et al., 2009; Mukaratirwa et al., 2013). The 12 recognized taxa are biologically and genetically categorized into two separate groups; the encapsulated and non-encapsulated species based on the collagen capsule formed around the larvae within the muscular tissues of the host (Gottstein et al., 2009; Mukaratirwa et al., 2013). The encapsulated species include (*Trichinella spiralis*, *Trichinella nativa*, *Trichinella britovi*, *Trichinella murrelli* and *Trichinella nelsoni*) affecting mammals uniquely and two non-encapsulated species *Trichinella pseudospiralis* which infects birds and mammals only and *Trichinella papuae* and *T. zimbabwensis* that affect both mammals and reptiles (Mukaratirwa et al., 2008; Gottstein et al., 2009; Ludovisi et al., 2013). Out of the eight classified species, six and one genotype have been detected in humans (Arango et al., 2015), although there are no current documented cases of the prevalence of *Trichinella* infection in humans and animal in Sub-Saharan Africa, most infections were associated with *Trichnella spiralis* (Pozio et al., 2007). According to Mukaratirwa et al. (2013) the cosmopolitan, *T. spiralis* and *T. pseudospiralis* still remain undocumented in the Sub-Saharan region. However, recent reports have documented species such as *T. britovi*, *T. nelsoni*, *T. zimbabwensis* and *Trichinella* T8 to be present in Sub-Saharan Africa (Mukaratirwa et al., 2013). *Trichinella zimbabwensis* was first observed in Nile crocodiles (*Crocodylus niloticus*) and a carnivorous mammal (*Panthera leo*) (Mukaratirwa et al., 2013; Mukaratirwa et al., 2016). However, experimental studies have shown that *T. zimbabwensis* is infective to caimans, pythons, varans, and turtles (Pozio et al., 2007), rodents and domestic pigs (Mukaratirwa and Foggin, 1999) and non-human primates (Mukaratirwa et al., 2008).

2.5.2 Geographical distributions and epidemiology of trichinellosis

The distribution of the zoonotic pathogen *Trichinella* in animals and humans in the world, have been meat-borne (Mukaratirwa et al., 2013), and it is noticeable among ethnic communities around

the world due to their difference culinary habits with regards to meat consumption, which could either be raw or undercooked (Murrell and Pozio, 2011). The global impact of the disease have shown that 65 818 human trichinellosis incidence and 42 deaths have been reported world-wide and sub-Saharan Africa only contributing 0.04% of human trichinellosis and 3.6% mortality (Murrell and Pozio, 2011). According to recent reviews, eight species and four genotypes are documented across the world (Gottstein et al., 2009; Pozio et al., 2009) yet, there is minimal newly reported cases of *Trichinella* spp. infection in animals and humans in sub-Saharan African region (Mukaratirwa et al., 2013), except in a single review where ten sub-Saharan African countries were reported to have *Trichinella* spp. in animals and humans (Pozio et al., 2007). The species that have been described in sub-Saharan Africa (SSA) include *T. britovi*, *T. nelsoni*, *T. zimbabwensis* and *Trichinella* T8, however, the cosmopolitan *T. spiralis* and *T. pseudospiralis* have not yet been reported in SSA (Mukaratirwa et al., 2013).

In West Africa, *T. britovi* has been reported in Guinea in true civet and palm civet and isolation of the Guinea strain through multiplex-PCR has been shown to be alike to the European reference strain (Pozio et al., 2005; Pozio et al., 2007). Also, the species that was responsible for the *Trichinella* infection in animal and humans in Senegal could possibly have been *T. britovi* (Pozio et al., 2005). Beside the documented incidence of *T. britovi* in West Africa, studies on *Trichinella* spp. in this region is scanty (Pozio et al., 2005; Pozio et al., 2007). *Trichinella nelsoni* has been reported to be restricted SSA countries (Pozio et al., 2005; Pozio et al., 2007) such as in Kenya, South Africa and Tanzania (Pozio et al., 1997; Marucci et al., 2009). In southern Africa *Trichinella* T8 has been reported in South Africa and Zambia and its presence in these countries could perhaps be linked to the passive introduction from Europe (La Rosa and Pozio, 2000). The occurrence of encapsulated larvae of *T. britovi* has been detected in wild carnivore, no animal or human case has been recorded, and in Algeria *T. britovi* was found in and domestic pig (Pozio et al., 2007). Two species have been detected in Egypt i.e. *T. spiralis* in stray dogs and domestic pigs and *T. britovi* in wolves (Pozio et al., 2007).

Trichinella zimbabwensis has wide distribution in SSA, particularly in Southern Africa, and has been observed in countries such as Zimbabwe, Ethiopia, Mozambique and South Africa (Mukaratirwa and Foggin, 1999; Pozio et al., 2002; La Grange et al., 2013). However, knowledge of the geographical range of *T. zimbawensis* is increasing based on the increase in number of

identified isolates (Pozio et al., 2009). The *T. zimbabwensis* larvae was first detected in the muscle of farmed crocodiles (*Crocodylus niloticus*) in Zimbabwe (Pozio et al., 2002; Pozio et al., 2009) which represented the first observation of *Trichinella* in naturally infected reptiles and in poikilothermic animals (Pozio et al., 2009), so far, no human infection has been reported. In South Africa, the prevalence of *T. zimbabwensis* has been recorded in wild crocodiles in the Kruger National Park (KNP). In addition, two *Trichinella* taxa, *Trichinella nelsoni* and *Trichinella T8* have been found in Lions (*Panthera leo*) and spotted hyenas (*Crocuta crocuta*) in the KNP (Marucci et al., 2009; La Grange et al., 2013). Experimental studies have revealed that *T. zimbabwensis* can infect mammalian host such as rodents, pigs, carnivores and non-human primates (Mukaratirwa et al., 2008). Globalization of trade could intensify the danger of spread of the parasite through increased mobility of individuals, wildlife and livestock in and out of *T. zimbabwensis* endemic zones of southern Africa (Mukaratirwa et al., 2013). Therefore, it is of great epidemiological and public health interest. However, the control, surveillance and treatment of this disease is impeded by the range of domestic, synanthropic and sylvatic reservoir animals exacerbated by lack of a vaccine, licensed and efficient drugs against the muscle larvae and appropriate diagnostic tools which could be used to determine early infection (Onkoba et al., 2016b).

2.5.3 Host range and mechanism of spread of trichinellosis

The parasite *Trichinella* has a vast range of hosts (Kapel and Gamble, 2000; Murrell and Pozio, 2000). An understanding of the host range and geographical distribution of a parasite is vital and the fact that *T. zimbabwensis* reside in muscle tissue of several mammalian host and its wide distribution, suggests that there could be possible mechanism propelling its wide distribution. One such mechanisms could be related to ethnic meat eating habits (Mukaratirwa et al., 2013) or alternatively the spread of trichinellosis could possibly be through the scavenging activities of a variety of mammals on infected dead animals or cannibalism. According to Mukaratirwa et al. (2013) reports from field observation have demonstrated that *Trichinella* infection in wild carnivore sub-Saharan Africa, have maintained the sylvatic cycle through predation, scavenging and cannibalism.

2.6 Life cycle of Trichinella spp.

The life cycle of all *Trichinella* spp. have an identical sequence to that of *T. spiralis*. The cycle occurs in host vertebrates such as (mammals, reptiles and birds) including humans (Mitreva et al., 2004). Human trichinellosis is transmitted by eating raw or undercooked infected meat from pigs, wild boars, dogs, horses, walruses, bears and foxes (Gottstein et al., 2009) as shown in (Figure 2 A). The cycle comprises of two phases within the host which includes the intestinal and enteral phase or enteric and muscle phase (Capo and Despommier, 1996; Mitreva et al., 2004) occuring within the host body (Figure 2 B). Transmission from one host to the other occurs by consumption of infective muscle larva L_1 (enteric phase). The enteric phase begins after ingestion of infective muscle larvae (ML_1) (Mitreva et al., 2004; Wu et al., 2009), while in the stomach the ingested larvae are released by the action of pepsin hydrochloric acid and move to the small intestine where it invades the mucosal columnar epithelial cells creating an infection (Gottstein et al., 2009). The larva then undergoes development into an adult parasite and this occurs within a short period of time 2 days (Gottstein et al., 2009). In the course of development, complex interaction with the host is initiated before it finally matures to an adult parasite (Mitreva et al., 2004). Copulation between the male and female is known to occur between 30-34 hours post-ingestion of larvae (Capo and Despommier, 1996). The gravid female worm produces numerous immature first stage larvae or newborn larvae (NBL) in the intestinal mucosal. The NBL then enters the host portal venous and are dislodged via blood circulation into the host tissues (Gottstein et al., 2009). The muscle phase (parenteral) comprises significant transformations of the striated muscles by the first stage larvae causing intracellular infection thus inducing series of changes in the host (Mitreva et al., 2004). Muscle cells infected by parasite cause pathological problems, thus transforming the muscle cells into another type of cell called nurse cell (see Figure 2) (Wu et al., 2009). Subsequently the process of calcification occurs over a period of time (weeks, months, or years) (Gottstein et al., 2009).
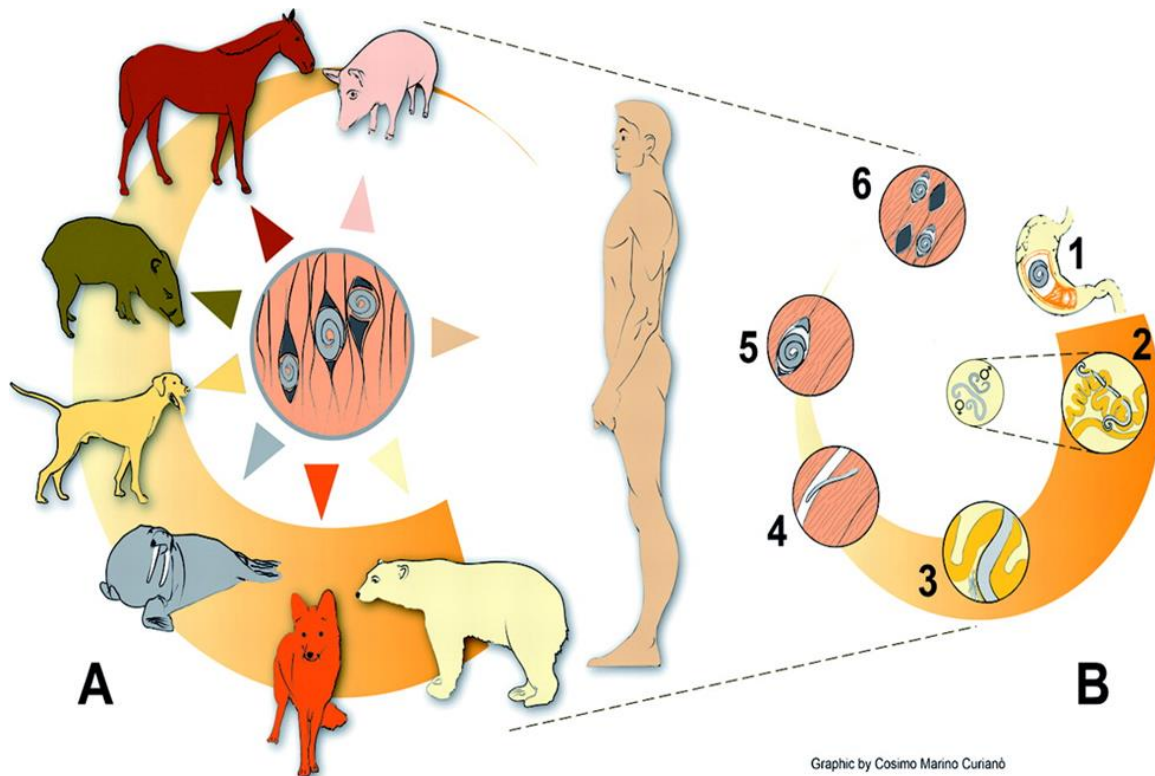
Figure 2: The life cycle of *Trichinella* spp. Gottstein et al. (2009).

2.6.1 Clinical and pathological characterization of trichinellosis

Manifestation of trichinellosis infection in humans is often subclinical, however the clinical manifestation presented often varies based on the phase in which the parasite occurs and the number of larvae consumed (Arango et al., 2015). This could result into a series of clinical outcome, which ranges from asymptomatic to mortality (Gottstein et al., 2009). The asymptomatic signs are normally indistinct due to the low intensity of the parasite consumed but when higher number of larvae are consumed, instantly there is gastroenteritis which is associated with diarrhea and abdominal pains which commence the acute phase of the disease (Gottstein et al., 2009).

During the enteric phase, the basic signs which are noticeable after consuming infected larvae include diarrhea, vomiting, and abdominal discomfort. Other signs that follow happens within 1-6 week in the systemic phase after larvae ingestion. This is followed by fever, myalgia, facial or

periorbital oedema, headache, fatigue, and weakness (Arango et al., 2015). Additional signs that might be observed in the course of infection include conjunctivitis, sub-lingual and maculopapular hemorrhages. The differential diagnosis of trichinellosis includes influenza, dermatomyositis, and viral gastroenteritis (Arango et al., 2015) and these clinical manifestations are commonly observed during the acute phase of infection. Observable complications in the chronic phase include encephalitis, neurological symptoms which are often subacute and secondary infections such as bronchopneumonia and sepsis, which usually occur later during infection (Gottstein et al., 2009).

Initiation of clinical pathology is noticeable when the larvae is released into the intestinal mucosa, and migrates into vessels and the distribution to the final site of predilection, which are the skeletal muscles (Gottstein et al., 2009). Migratory larvae and their metabolites cause instant reaction, consequently resulting to immunological, pathological and metabolic disturbances and associated clinical manifestation observed (Gottstein et al., 2009). In addition, pathological complications arise in parenteral phase due to series of changes induced by the developing larval in muscle cells, some of these changes are dissolution and disappearance of myofibers, proliferation and differentiation of satellite cell, de-differentiation of muscle cells, re-entry and arrest of cell cycle and transforming it into nurse cell (Wu et al., 2009). Formation of nurse cells in the encapsulated species involves complex process such as growth, regeneration, differentiation, proliferation of cell cycle, regulation and transformation (Wu et al., 2008).

2.6.2 Effect of trichinellosis on host glucose metabolism

Parasitic infection depend on host environment for development (Smith et al., 2005), thereby inflicting variable disease signs and symptoms. Disease manifestations may arise as a result of invasion, predator-prey relationship, resource competition and consumption (Bohannan, 2000). The invasion of *Trichinella* larvae in muscle tissues, causes direct and indirect negative effects on host. Direct effects occur during larva migration while indirect effects arise due to inflammatory responses of the host (La Grange and Mukaratirwa, 2014). One of such effect of *Trichinella* parasite on the host is the depletion of glucose which leads to hypoglycemia in the host (Wu et al., 2009).

Hypoglycemia caused by *Trichinella* has been reported in humans (Buşilă et al., 1967) and experimental animals such as dogs (Reina et al., 1989) and mice (Wu et al., 2009) although, the mechanism is still not clear. A hypothetical postulation from previous reviews states that "high glucose consumption by rapid growing parasites causes "hypoglycemia" (Wu et al., 2009). Some of the recent studies on trichinellosis have highlighted hypoglycemia in human host to be attributed to the rapid proliferation of larvae within the muscle cell, total increase of glycogen in infected tissues, huge amount of glycogen found in larvae, glucose absorbance by larvae during incubation in-vitro and metabolic activities associated with glucose metabolism in nurse cell (Montgomery et al., 2003; Wu et al., 2009) which could possibly be the causes of hypoglycemia in host. This is further supported by a study carried out by Wu et al. (2009) on *T. spiralis* and *T. pseudospiralis* infected mice and there was an observable decrease in blood glucose in the infected host, moreover, the kinetics of the blood glucose levels during infection also ties with the growth of muscle larvae and encystment of muscle cell.

An alternative mechanism might be associated with tissues destruction as a result of parasite invasion, which could further lead to increase in membrane permeability, and hence leakage of fluid to adjacent cells or tissues (Gottstein et al., 2009; La Grange and Mukaratirwa, 2014). This process has been linked to an increase of creatine phosphokinase (CPK), lactate dehydrogenase (LDH), aspartate transaminase (AST) and alanine transaminase (ALT) in the blood (Kociecka, 2000), suggesting the possibility of leakage of vital nutrient component such as glucose, resulting in hypoglycemia.

Furthermore, nutrients are required for larvae invasion and growth, therefore, the larvae could proliferate by consuming one of such nutrient as glucose and according to Wu et al. (2008), the increase expression of insulin receptor in infected muscle tissue as shown by cDNA microarray is indicative of the high glucose demand within the cells. Still, the insulin signaling pathways increase demand for glucose, thus play a role in the conversion of glucose from blood to responsive tissues by increasing the number Glut4 in the membrane increasing glucose uptake for storage as glycogen (Youngren, 2007). Insulin responsive tissues such as adipose tissues and skeletal muscles binds with insulin at the receptor surface.

2.6.3 Diagnosis of trichinellosis

Human trichinellosis disease often present a complicated diagnosis, since there are no specific clinical signs and symptoms, especially during the early stages, but the parasite detection at the later or chronic phase is also not easy (Gottstein et al., 2009). More so, the disease becomes more challenging to humans to resolve, since diagnosis in live animals is nonspecific as well, regardless of the amount of *Trichinella* spp. larvae ingested by host. Therefore, preventing human trichinellosis is by meat inspection to detect the larvae (Mukaratirwa et al., 2013). The non-specificity of the disease has resulted in late diagnosis (Janse and Waters, 1995). However, suggested possibilities to avoid conflicting diagnosis of trichinellosis is to delineate the signs and symptoms from that of other diseases that might show some similarities, thus making diagnosis complicated.

Diagnosis of *Trichinella* infection in humans is centered on clinical, laboratory and epidemiological findings (Kociecka, 2000). According to Gottstein et al. (2009) one of the three findings (clinical, laboratory and epidemiological) should point to the presumptive diagnosis of trichinellosis. The key clinical signs reported include, fever, muscle pain, facial oedema, gastrointestinal symptoms, retinal and subconjunctival hemorrhages, eosinophils and sublingual oedema. Experimental findings of trichinellosis in the laboratory could be based on muscle biopsies of *Trichinella* larvae and *Trichinella*-specific antibody response by indirect immunofluorescence, ELISA or Western blot (Mukaratirwa et al., 2013). Epidemiological investigations should tie with laboratory confirmation accompanied with evidence of consumption of under-cooked or raw meat or animal product by patients (Gottstein et al., 2009; Mukaratirwa et al., 2013). In SSA there is insufficient epidemiological reports of human trichinellosis due lack of expertise by physicians which is often obscured by the early diagnosis of the disease. Since most of the documented reports in SSA are based on clinical findings, muscle biopsies and an epidemiological link to ingestion of meat from bush pigs and/or warthogs this still leaves a gap in early diagnosis of trichinellosis (Mukaratirwa et al., 2013).

2.6.4 Treatment and control of trichinellosis

Human trichinellosis treatment can be tackled based on the following criteria; the clinical severity of infection and species of the parasite involved (Mukaratirwa et al., 2008). Most recommended anthelmintic drugs for patients with trichinellosis are mebendazole, albendazole, glucocorticosteroids and preparations that compensate for proteins and electrolyte loss (Kociecka, 2000; Gottstein et al., 2009). The first two drugs are not recommended during pregnancy and children below 2 years, so the best drug for this group is pyrantel, but it is active only against adult worms in the gut and has no effect against muscle larvae (Dupouy-Camet and Murrell, 2007; Bruschi and Dupouy-Camet, 2014). The most commonly use steroid is prednisolone, which may alleviate the general symptoms of the disease (Bruschi et al., 2002).

For an appropriate control of trichinellosis, quality meat inspection which involve trichinoscopy for the detection of larvae in muscle, community awareness campaign and public health education (Dupouy-Camet and Murrell, 2007; Enemark et al., 2015) is recommended. The targeted group for awareness should involve consumers and hunters of bush meat from protected areas, who need to be informed on the health implications and danger when ingesting raw or undercooked meat without inspection. The other safe and acceptable method to prepare these meats is by irradiation to inactivate *Trichinella* larvae and according to Gottstein et al. (2009) the methods such as use of microwave, curing, drying and smoking are considered unsafe for meat preparation as they do not kill the larvae. More so the freezing is an inappropriate measure to control trichinellosis although it can inactivate certain *Trichinella* spp. muscle larvae, but there are freeze resistant species like *T. britovi* (Gottstein et al., 2009).

2.7 Malaria-trichinellosis co-infection

Malaria and helminthiasis, which includes trichinellosis, are common parasitic infections, which are rampant in tropical and sub-tropical countries (Figure 3). The convergence of these parasite in the same epidemiological zone often result in mixed infection or polyparasitism (Supali et al., 2010). And as such, malaria is known to have a higher prevalence amongst the poor rural communities (Su et al., 2005; Brooker et al., 2007; Onkoba et al., 2015), where there is high

predominance of soil transmitted-helminths (STHs) as shown in Figure 3 (Mwangi et al., 2006; Melo et al., 2010; Knowles, 2011). Individuals living in such epidemiological zones of co-endemicity are predisposed to greater possibility of multiparasitism or polyparasitim, or co-infection with both malaria and helminth infections (Figure 3) (Graham et al., 2005; Su et al., 2005; Melo et al., 2010; Salazar-Castañon et al., 2014; Onkoba et al., 2015).
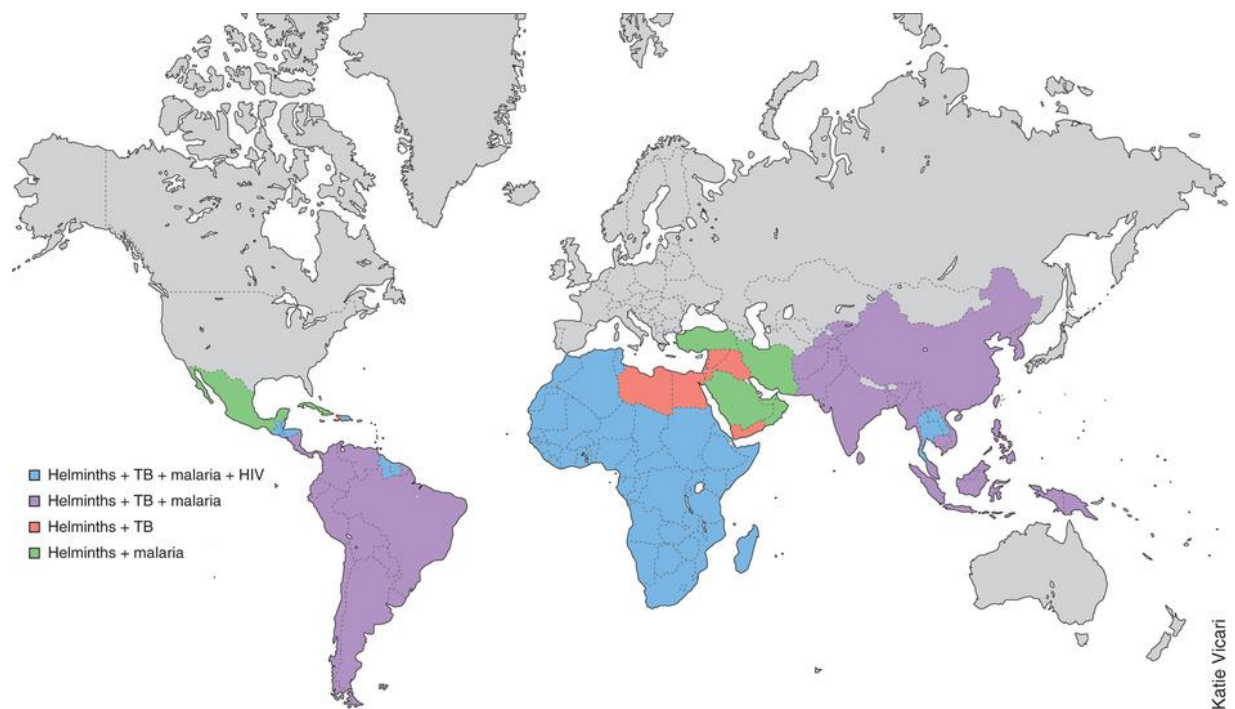
Figure 3: Map showing the epidemiological zones of malaria, helminths, tuberculosis and HIV Salgame et al. (2013).

The transmission of mixed infections with more than one parasite is common in endemic areas, whereby there is a high probability of both parasites to co-exist within the same host (Cox, 2001). According to Cox (2001), mixed infections often lead to the association of two or more parasites belonging to different species and possessing different genotypes suggesting that their interaction within the host might be of variable degrees, either synergistic or antagonist (Telfer et al., 2008) and could impact each other directly or indirectly, either by aggravating or ameliorating the disease within the human host (Cox, 2001; Graham et al., 2005). The combination of different parasites

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within a host can have an impact depending on the group of parasites and the site of predilection in the host.

Despite the host's health implications of co-infections, several epidemiological and laboratory studies on parasitic co-infection with malaria and helminths have never been harmonised, making it difficult to have a harmonized solution in targeting co-infection issues. The extreme deviation in conclusions from these studies can be attributed to the fact that the majority of the studies laid more interest on the immunological aspects, and not considering the non-immunological aspect (Onkoba et al., 2015). Human and animal studies have documented contrasting results. Some presented that malaria-helminth can exacerbate malaria severity (Le Hesran et al., 2004; Supali et al., 2010). An example has been demonstrated in co-infection studies with *P. falciparum* and *Schistosoma mansoni* resulting in an increase in hepatomegaly and splenomegaly of the host (Booth et al., 2004), and increased malaria frequency after infection with these parasites (Faye et al., 2008). While others have presented that it could enhance protection against malaria disease severity (Nacher, 2008; Onkoba et al., 2015), and a few reports have revealed no association between the parasites (Lyke et al., 2006; Mulei, 2011).

The potential for a parasite to successfully commence an infection on the host depends on its initial immune response (Telfer et al., 2008). Although, most literature has shown that helminths are capable of modulating the immune system of its host in order to survive (Ateba-Ngoa et al., 2016), invading parasite often encounter a new immune environment which could determine the possibility of prior and present infection state (Salazar-Castañon et al., 2014). This can be supported by reports from other co-infection studies, which state that a single parasite cannot have a direct influence on disease outcome and establishment of the second parasite, thus, implying that a single parasite only plays a role in changing the immune response in a way that will probably be to the advantage of the second parasite establishing itself within the host (Onkoba et al., 2015).

However, based on past review, the presence of Th1 and Th2 effector response is reversibly suppressed, in which theoretically, Th2 response are evoked towards helminth infection and is capable of suppressing pro-inflamatory Th1 response that causes immunopathology in *Plasmodium* infection (Salazar-Castañon et al., 2014). Therefore, helminth infections have been identified in numerous studies to have a strong potential in diminishing the immune response to

unassociated antigens of other parasites such as *Plasmodium* parasite (Metenou et al., 2011; Ateba-Ngoa et al., 2016), which could imply that individuals with chronic helminth infection are suspected to have a decrease in malaria specific immune response to the host (Ateba-Ngoa et al., 2016). Different malaria outcomes with different species of helminth have been reported in previous studies. Parasitic infection and its implication with regard to glucose homeostasis has been shown in the case of malaria (Smith et al., 2005) and *Trichinella* (Wu et al., 2009). The combined effect of malaria-*Trichinella* coinfection on host glucose metabolism is still obscure. In addition, there is paucity in knowledge on host glucose metabolism and its adaptive immune responses induced by *P. berghei* ANKA and *T. zimbabwensis* co-infection, acting as a barrier against the advancement and improvement of diagnostic tools, surveillances, and control and management strategies of the coinfected parasites in SSA. Based on this criterion, the present study seeks to further investigate the impact of *P. berghei* and *T. zimbabwensis* co-infection on host glucose and insulin store.

CHAPTER 3: MATERIALS AND METHODS

3.1 Chemicals and accessories

All Chemicals used in this study were sourced as shown:

Isoform inhalation (Safeline Pharmaceuticals (PTY) LTD, Weltevreden Park, Rooderport, South Africa), The Ultrasensitive Rat Insulin ELISA kit (Mercodia AB, Sylveniusgatan 8A SE-754-50 Uppsala, Sweden); potassium hydroxide (KOH), sodium sulphate (Na_2SO_4), anthrone, glycogen powder from rabbit liver (Sigma-Aldrich chemical company Germany), Giemsa stain (Merk (Pty) Ltd 1Friesland drive, Modderfontein, Gauteng, 1645 South Africa) sulphuric acid (H_2SO_4) (BDH Chemicals LTD, Poole, England); Pepsin (from porcine gastric mucosa), hydrochloric acid (HCl). All chemical reagents were of analytical grade purchased from standard commercial pharmaceutical suppliers.

3.2 Study animals

Male Sprague-Dawley rats (90-150g) used in the experiment were bred and housed in the Biomedical Resource unit in the University of KwaZulu-Natal, Westville campus and maintained under standard laboratory conditions ($22 \pm 2°C$, CO_2 content of < 5000 p.p.m., relative humidity of 55 ± 5 % and illumination cycles of 12 hours light and 12 hours darkness and noise level of < 65 decibels). The animals were allowed to have free access to heat-sterilized rodent pellet chow (Meadows, Pietermaritzburg, South Africa) and clean water was supplied *ad libitum*.

3.3 Ethical considerations

All experimental procedures in this study were conducted with the approval of Animal Ethics Research Committee of the University of KwaZulu-Natal under the ethical clearance number AREC/018/016 PD. Where applicable, we adhered to ARRIVE guidelines for reporting in vivo animal experiments (Kilkenny et al., 2012).

3.4 Experimental design

The effect of *P. berghei* ANKA and *T. zimbabwensis* on blood glucose concentration was evaluated in 168 male Sprague-Dawley rats of body weight (90-150g). The animals were randomly divided into four different groups of 42 rats per group: Control group (n = 42), *Trichinella zimbabwensis* group (n = 42), *Plasmodium berghei* group (n = 42) and co-infection group (n = 42). Each group was further subdivided into n = 6 in a cage (Figure 3). The animal in each of the cages for all the groups had equal amount of food and water that was given daily. The beddings of the cages was cleaned after every 2 days. The experimental animals were grouped as follows:

1) The control group (C) which was non-infected was given deionized water.
2) The *Plasmodium berghei* group (Pb) was infected with 1×10^5 *P. berghei* ANKA parasitized RBCs through intraperitoneal injection (IP).
3) The *Trichinella zimbabwensis* group (Tz) was infected with 3 muscle larvae/g animal body weight of crocodile-derived *T. zimbabwensis* strain (Code ISS1209) by oral gavage on day 0 of the experimental study using an 18 G curved oral dosing needle.
4) The co-infection group (Pb + Tz) was infected with 3 muscle larvae/g animal body weight of *T. zimbawensis* strain (Code ISS1209) through oral gavage at day 0 of the experiment and at day 28 the rats where co-infected with (1×10^5 PRBCs) of *Plasmodium berghei* ANKA using intraperitoneal injection. The experimental design is outlined in the following schematic diagram below (Figure 4).
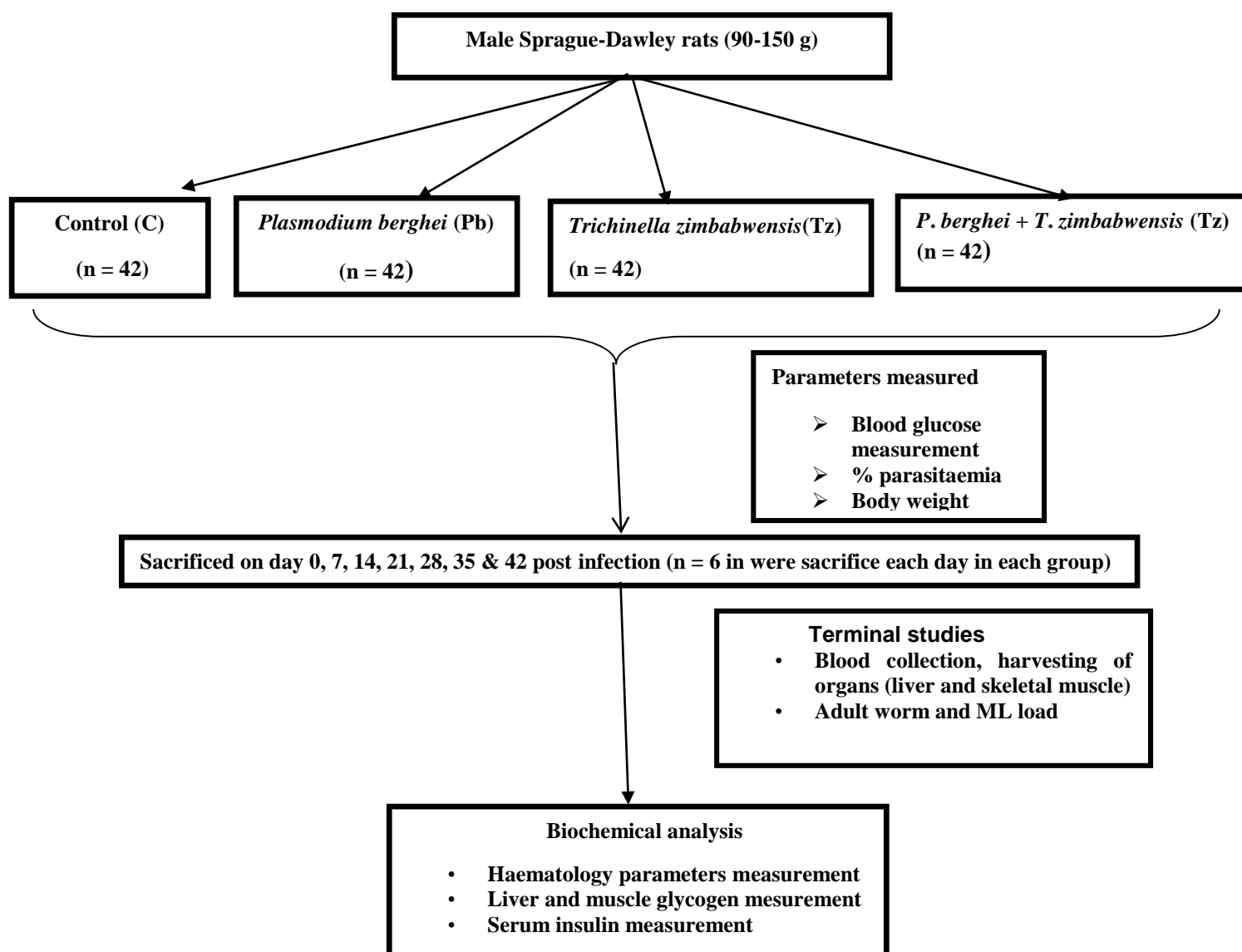
Figure 4: A schematic diagram showing the 42-day experimental protocol design. *Trichinella zimbabwensis* induction was done on day 0 of the 42-day experimental protocol while *P. berghei* induction was done on day 28 of the experimental study, coinciding with presence of *Trichinella* muscle larvae in the rats skeletal muscle.

3.4.1 *Plasmodium berghei* induction

Plasmodium berghei ANKA strain used to induce malaria infection in rats was kindly donated by the University of Cape Town, South Africa. Infection was induced in the Sprague-Dawley rats (90-150g) through single intra–peritoneal injection of *P. berghei* (1×10^5 parasitized RBCs) (Gumede et al., 2003). The control animals were injected with phosphate buffer saline vehicle. Confirmation of infection was done through microscopic examination of Giemsa-stained thin smears of rat-tail blood.

3.4.2 *Trichinella zimbabwensis* induction

Trichinella zimbabwensis parasite strain (Code ISS1209) were isolated from crocodile (*Crocodylus niloticus*) and maintained in male Sprague-Dawley rats at the Biomedical Research Unit (BRU) of the University of KwaZulu-Natal. *Trichinella zimbabwensis* larvae from infected rat carcasses were isolated using the artificial modified method of HCl-pepsin digestion according to (Mukaratirwa et al., 2003). The larvae recovered were used to infect experimental animal, and infective dose of each experimental rat was 3 muscle larvae/g animal body weight administered orally as described by (Mukaratirwa et al., 2001).

3.5 Haematological profile

Blood samples were collected from all the animals group through cardiac puncture and transferred into EDTA tubes for measurement of haematology parameters, using AC.T 5 diff Beckman-coulter counter machine (Maimi Florida USA) which automatically determine the red blood cell (RBC) count, white blood cell (WBC) count and percentage (%) haematocrit.

3.6 Parasitaemia determination

Thin blood smears were prepared each day on microscope glass slides from Pb mono-infection and Pb + Tz co-infection group rats, using blood from rats tail prick post-infection with *P. berghei*.

The smears were allowed to the air to dry freely for about 10 seconds and were fixed with 80 % methanol for approximately 30 seconds. After fixation the slides were stained using freshly prepared Giemsa stain (20 % Giemsa with 80 % 6.2 buffer) for 35-45 minutes and rinsed with water. Slides were allowed to air dry and parasite identification was done using standard compound microscope at 100X objective with immersion oil. Average percentage parasitaemia was obtained by counting the total number of RBCs and total of iRBCs in 5 different microscopic field of view and calculated as follows;

$$\% \text{ Parasitaemia} = \frac{\text{Total number of iRBC counted}}{\text{Total number of RBCs counted}} \times 100$$

A percentage parasitaemia of 15-20 % was considered a stable state for malaria.

3.7 Trichinella muscle larvae burden and adult worm counts

Trichinella parasite was considered to have successfully reached host muscle on day 28 post infection. Larvae determination was confirmed by digestion of the muscle tissue following the procedures of modified artificial digestion as described by (Mukaratirwa et al., 2003). Briefly, digestion procedure involves; Per each 100g of muscle tissue collected from the rat carcasses a digestive fluid mixture made of 16 ml of 25 % HCl, 20 g of pepsin and 2 liters of distilled water was prepared. The temperature of the digestive fluid was maintained at 37° in a beaker containing a magnetic stirrer for 35 minutes followed by sedimentation for 40 minute in a separating funnel, 40ml of the digestive mixture was collected and allowed to settle for 10 minutes. Subsequent separation of the digestive fluid was carried out for another 10 minutes, and supernatant removed allowing the suspension containing the larvae which is transferred into a Petri-dish for quantification of larvae under a dissecting microscope at 20x magnification. The adult worms were also recovered from the experimental rats intestine using a modified protocol as described by Pozio et al. (2002). To achieve this, the intestines were split open longitudinally, using a sharp dissecting scissors and submerged in 0.85% saline solution. The intestines were then incubated overnight at 27° and later washed with distilled water under a 212 μm sieve. The process of washing was

repeated 3 times. After washing the parasites were viewed and counted using a Zeiss Stemi DV4 Stereo microscope at 20X.

3.8 Blood glucose concentration measurement

Blood was collected by tail prick using a sharp sterile needle daily at 0900 am for glucose measurement. These measurements were taken every three days from day 0 to day 42 as i.e; 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, and 42 and on the day of sacrifice (0, 7, 14, 21, 28, 35, and 42). Blood glucose measurement was done using a calibrated Accu-Check® Active (Roche, Randburg, South Africa) glucometer (Smith and Marais, 2004).

3.8.1. Liver and muscle glycogen measurement

The concentration of glycogen in the liver and muscle tissue was determined following a modified protocol as described by Ngubane et al. (2011). In this study 0.25-0.5g of tissue sample were homogenized in 2ml of 30% KOH (30g/100ml) and boiled at 100°C for 30 minute. Then, 0.15ml of 10% Na_2SO_4 (10g/100ml) was added into the tissue homogenates and vortexed. From each sample, 2μl was added to 2μl of 95% of ethanol, 1ml of de-ionized water and 4ml anthrone reagent (0.5g in 250ml of concentrated H_2SO_4) to extract the glycogen from the liver and muscle sample. Following similar procedure of tissues sample preparation, blanks were prepared, where the tissue samples in the blanks were replaced with 2μl of de-ionised water. Glycogen powder from rabbit liver was used to prepare standards solutions (10-2000 mg/1) which were also treated in the same way as tissue samples described above. Subsequently, all prepared solutions were vortexed for 10 minutes before measuring the absorbance at 540nm using a Spectrostar Nano microplate reader (BMG Labtech Germany). A glycogen standard curve was prepared and used for the extrapolation of all tissue glycogen concentrations.

3.8.2 Serum insulin measurement

To determine insulin concentration, blood samples were collected from the heart through cardiac puncture on each day of sacrifice (0, 7, 14, 21, 28, 35 and 42 PI) into the heparin tubes for serum. The blood samples were later centrifuged to separate the plasma and the serum. The measurement of insulin concentration was further done using Mercodia Ultrasensitive Rat Insulin ELISA Kit on the separated blood sera. All the reagents used were supplied by the manufacturer and the procedures for insulin assay was conducted as follows: The Insulin kit consists of a 96 well plate coated with mouse monoclonal anti-insulin, enzyme conjugate 11X, enzyme conjugate buffer, wash buffer 21X, calibrators 1,2,3,4,5, substrate 3,3',5,5'-tetramethylbenzidine (TMB) and a stop solution. The kit has a two sided solid phase immunoassay which function based on a technique of direct sandwich, in which two monoclonal antibodies are directed against separate insulin determinants on the insulin molecule. In the course of incubation, insulin in the sample reacts with peroxidase-conjugated anti-insulin antibodies bounds to a microtitration well. A simple washing steps was done to removes unbound enzyme labeled antibody. The bound conjugates were detected by reacting with 3, 3'-5, 5' tetramethylbenzidine. The reaction was stopped by adding sulphuric acid (0.5M) to give a calorimetric end point that was read in spectrophotometer microplate reader (BMG Labtech Germany) at 450nm. The determination of each sample was performed in duplications of all standards. Subsequently an insulin standard curve was done and use to extrapolate the insulin concentration.

3.9 Body weight determination

The body weight of experimental animals were measured using a daily calibrated digital balance (Germany) for all groups (C, Pb, Tz, and Pb + Tz) after every three days (0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, and 42) and on the day of sacrifice (0, 7, 14, 21, 28, 35, and 42).

3.9.1 Terminal Studies

All the four experimental groups (C, Pb, Tz, Pb + Tz) were terminated by sacrificing (n = 6) from each of the group after every 7 days (0, 7, 14, 21, 28, 35, and 42). The animals were sacrificed by anaesthetizing them in an anesthetic chamber with 100mg/kg of isofor inhalation anesthetic for 3-5 minutes. Blood samples were collected by cardiac puncture and put in heparin tubes which was later centrifuged to separate the serum. Separated blood sera was placed into Eppendorf micro-centrifuge tubes and stored in -80°C Ultra-Bio Freezer in the University of KwaZulu-Natal and was later used to measure insulin concentration. The following organs were harvested (kidneys, liver, pancreas, heart, spleen, muscles) and weighed using a calibrated gravimetrical balance.

3.10 Data Analysis

The data obtained from adult worms recovered in the intestines and the number of muscle larvae were counted in five microscopic field of view and their mean values were used for analysis. The level of malaria parasitaemia was assessed from five different fields of view to obtain the mean in percentage which was used for the analysis. The muscle and liver glycogen OD values were extrapolated from the standard curve OD values and the mean of the extrapolated data was analyzed.

The differences in the measured parameters; blood glucose, insulin, liver glycogen, muscle glycogen, % parasitaemia, % hematocrit and *Trichinella* muscle larvae burden in all the groups were expressed as mean ± standard error (SEM). Statistical significance between the control, malaria, *Trichinella* and co-infection experimental groups were determined by Two Way Analysis of Variance (ANOVA) followed by multiple comparison Bonferroni post-test analysis. All the statistical analysis and graphs were done using a GraphPad InState software (version 5.00, Graphpad software, San Diego, California, USA). $P < 0.05$ was considered statically significant.

CHAPTER 4: RESULTS

4 General

This chapter describes the following:

Percentage parasitaemia in *P. berghei* infected experimental animals, *Trichinella* muscle larvae burden, haematology parameters, cumulative body weight, blood glucose concentration in all the experimental groups, liver and muscle glycogen concentration of the experimental groups and serum insulin concentration of the experimental groups as presented in subsequent sections.

4.1 Percentage parasitaemia after P. berghei infection

The mean parasitaemia and standard error of mean after *Plasmodium berghei* induction of the experimental groups are shown in Figure 5. The result shows that the percentage (%) *P. berghei* parasitaemia increases for both *P.berghei* mono-infection and *P. berghei + T. zimbabwensis* co-infection groups at day 1 PI and 2 PI respectively ($P > 0.05$). Subsequently, at day 3 PI and 4 PI % *P. berghei* parasitaemia was elevated for *Plasmodium berghei* (Pb) + *Trichinella zimbabwensis* (Tz) co-infection compared to *P. berghei* mono-infection ($P < 0.01$). However, we observed that the peak % parasitaemia for *P. berghei* mono-infection group was reached earlier at day 7 PI (67.4 %) whilst the co-infection group peaked % parasitaemia occurred later at day 8 PI (69.2 %) with no significant difference ($P > 0.05$). After the peak % *P. berghei* parasitaemia was attained both Pb mono-infection and Pb + Tz co-infection groups decreased progressively till the end of the experiment (day 14 PI) but the co-infection group still maintained a higher % *P. berghei* parasitaemia compared to the *P. berghei* mono-infection group ($P > 0.05$).
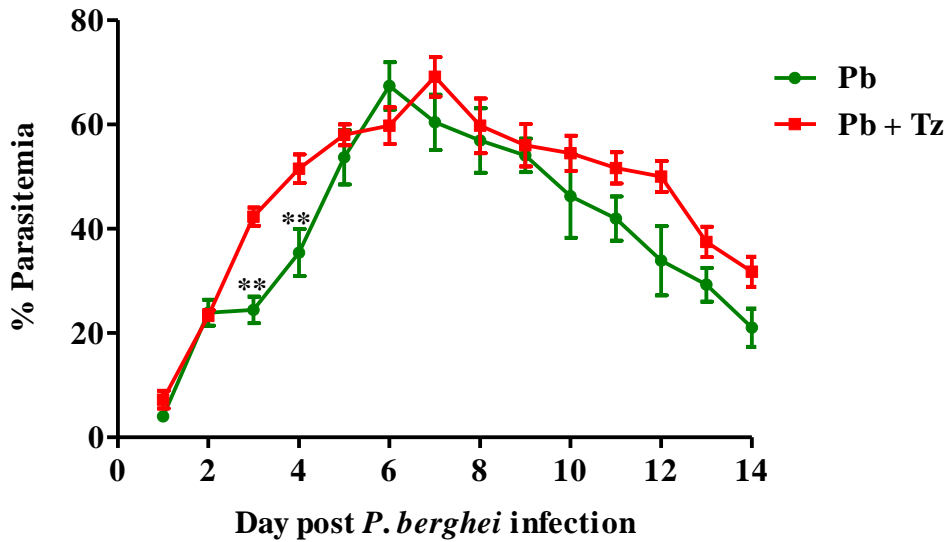
Figure 5: Mean percentage parasitaemia (± SEM) in male Sprague-Dawley rats infected with *Plasmodium berghei* mono-infection (Pb) and *P. berghei* (Pb) + *Trichinella zimbabwensis* (Tz) co-infection. Day 0 of *P. berghei* induction represent day 28 post *Trichinella zimbabwensis* infection in rats when the muscle larvae are in the rat muscles. ** indicates $P < 0.01$ when comparing *P. berghei* mono-infection and *P. berghei* + *Trichinella zimbabwensis* co-infection**.** Pb = *Plasmodium berghei* and Tz = *Trichinella zimbabwensis*.

4.2 Trichinella zimbabwensis adult worms and muscle larvae burden

The number of *Trichinella zimbabwensis* adult worms recovered from the intestines of the experimental rats infected with *Trichinella zimbabwensis* (Tz) mono-infection and *Plasmodium berghei* (Pb) + *T. zimbabwensis* (Tz) co-infection at day 7 PI, 14 PI and 21 PI are shown in Figure 6. The results show that there was no significant differences ($P > 0.05$) between number of adults recovered from the Tz mono-infection and Pb + Tz co-infection. However, the Tz mono-infection had slightly higher mean number of adult worms recovered (18.5 AW) compared to Pb + Tz co-infection (15 AW) at day 7 PI whereas at day 14 PI few AW were recovered showing no statistical significance ($P > 0.05$). At day 21 PI no AW or ML was recovered from both Tz mono-infection and Pb + Tz co-infection groups. The number of muscle larvae burden increases progressively from day 28 PI, 35 PI, and peaking at day 42 for both Tz mono-infection and the Pb + Tz co-

infection with no significant difference ($P > 0.05$) when compared to each other. At day 42 ML (lpg) recovered from the Tz mono-infection was higher (97.4 lpg) compared to the Pb + Tz co-infection (71.6 lpg) although not statistically significant ($P > 0.05$).
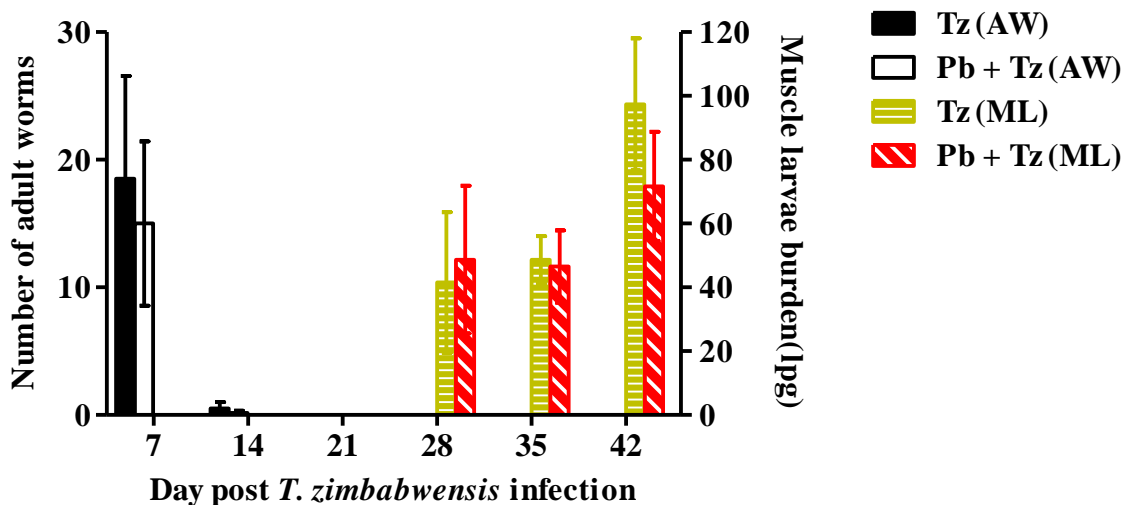
Figure 6: Mean number of adult worms (± SEM) of *Trichinella zimbabwensis* recovered at day 7 PI, 14 PI and 21 PI and Mean (± SEM) muscle larvae burden recovered at day 28 PI, 35 PI and 42 PI. After Tz mono-infection and Pb + Tz co-infection in male Sprague-Dawley rats. All values are expressed as mean ± SEM (n = 6 in each group). AW = Adult worms; ML = Muscle larvae; lpg = larvae per gram of muscle; Tz = *Trichnella zimbabwensis*; Pb = *Plasmodium berghei*.

4.3 Cumulative body weight gain

The cumulative body weight changes of experimental animal after post infection are shown in Figure 7. We observed that *P. berghei* (Pb) + *Trichinellla zimbabwensis* (Tz) co-infection group gained weight steadily from day 0 PI up to day 42 PI. Significantly, the cumulative weight gained was ($P < 0.001$) at day 14 PI, 21 PI, 28 PI respectively, ($P < 0.01$) at day 35 PI while day 42 PI was ($P < 0.05$) compared to the control group. *Trichinellla zimbabwensis* (Tz) mono-infection, also present a steady weight gained from day 0 PI to 42 PI, with a significant weight gain at day

14 PI ($P < 0.001$), 28 PI, ($P < 0.01$) and 42 PI ($P < 0.05$) compared to control. The *P. berghei* mono-infection correspondingly shows a consistent weight gained from day 0 PI up to day 35 PI showing no significant difference ($P > 0.05$) compared to the control. Subsequently, there was a significant ($P < 0.001$) weight loss at day 42 PI for Pb mono-infection compared to the control.
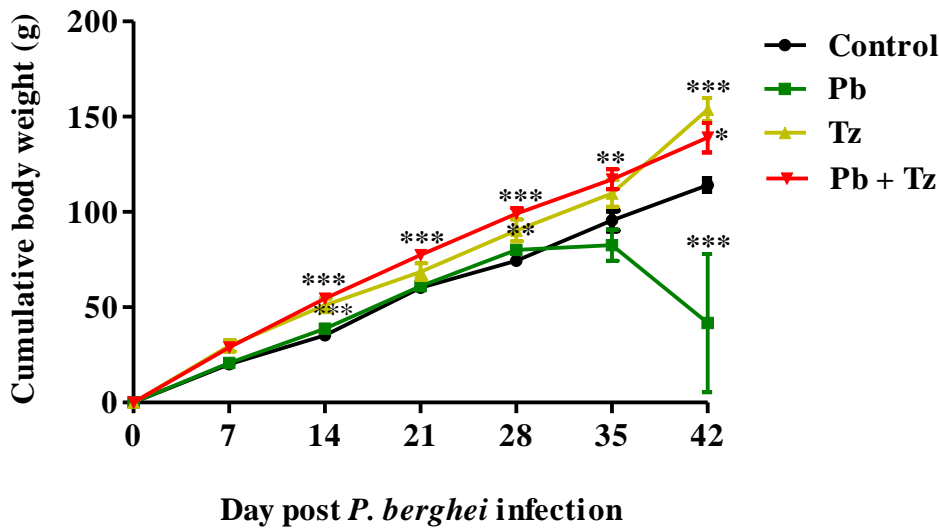
Figure 7: Cumulative body weight of male Sprague-Dawley rats infected with *Plasmodium berghei* (Pb) mono-infection, *Trichinella zimbabwensis* (Tz) mono-infection, *P. berghei* (Pb) + *Trichinellla zimbabwensis* (Tz) co-infection and non-infected control (C). All values were expressed as mean ± SEM (n = 6 in each group). Level of significance * $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$ was obtained by comparing with the control. Tz = *Trichnella zimbabwensis*; Pb = *Plasmodium berghei*.

4.4 Blood glucose concentration

The mean blood glucose after *P. berghei* infection were compared between the control and the infected groups (*Trichinella zimbabwensis* mono-infection (Tz), *Plasmodium berghei* mono-infection (Pb), *Trichinella zimbabwensis* (Tz) + *Plasmodium berghei* (Pb) co-infection) are shown

in Figure 8. At day 0 PI the mean blood glucose concentration for all infected groups showed no significant difference ($P > 0.05$) compared to non-infected control group. The blood glucose later decreases significantly ($P < 0.05$) at day 7 PI for Pb mono-infection whereas the Tz group and Pb + Tz groups showed no significant difference ($P > 0.05$) compared to the control. Later at day 14 PI the blood glucose dropped significantly ($P < 0.001$) for Pb mono-infection while Tz mono-infection and Pb + Tz co-infection still presented no significant difference ($P > 0.05$) compared to the control.

.

Figure 8: Mean blood glucose concentration (± SEM) in male Sprague-Dawley rats infected with *Trichinella zimbabwensis* mono-infection (Tz), *Plasmodium berghei* mono-infection (Pb), *Plasmodium berghei* (Pb) + *Trichinella zimbabwensis* (Tz) co-infection and non-infected control groups. Day 0 of *P. berghei* induction represent day 28 post *Trichinella zimbabwensis* infection in rats when the muscle larvae are in the rat muscles. Values are presented as means and vertical bars indicate mean ± SEM (n = 6 in each group). Level of significance * $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$. Tz = *Trichnella zimbabwensis*; Pb = *Plasmodium berghei*.

4.4.1 Liver glycogen concentration

The liver glycogen concentration after *P. berghei* infection are shown in Figure 9. At day 0 PI all infected groups (*Trichinella zimbabwensis* mono-infection (Tz), *Plasmodium berghei* mono-infection (Pb), *Plasmodium berghei* (Pb) + *Trichinella zimbabwensis* (Tz) co-infection) showed no significant difference ($P > 0.05$) compared to non-infected control. Significantly, the liver glycogen concentration decreases at day 7 PI for Pb + Tz co-infection and Tz mono-infection ($P < 0.01$ and $P < 0.001$) respectively while the Pb mono-infection present no significant difference ($P > 0.05$) compared to the control group. We also observed a significant ($P < 0.05$) decrease in liver glycogen concentration at day 14 PI for Pb + Tz co-infection group compared to the control group, while the Tz mono-infection group liver glycogen concentration was restored back ($P < 0.05$) compared to the control. The *P. berghei* mono-infection infection had no significant change ($P > 0.05$) compared to the control at day 14 PI.
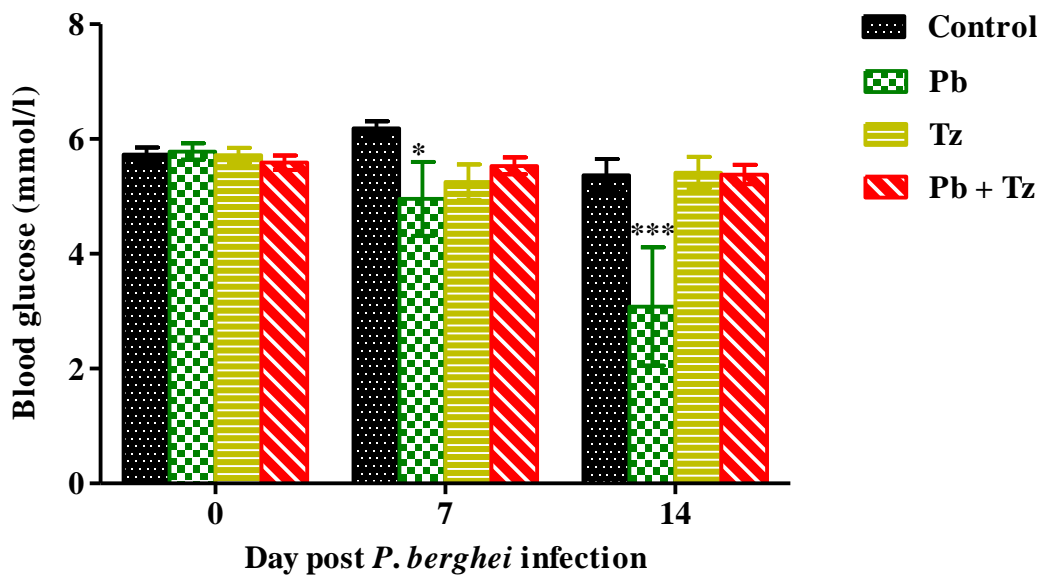

Figure 9: Mean liver glycogen concentration (± SEM) in male Sprague-Dawley rats infected with *Trichinella zimbabwensis* mono-infection (Tz), *Plasmodium berghei* mono-infection (Pb),

Plasmodium berghei (Pb) + *Trichinella zimbabwensis* (Tz) co-infection and non-infected control groups. Day 0 of *P. berghei* induction represent day 28 post *Trichinella zimbabwensis* infection in rats when the muscle larvae are in the rat muscles. Values are presented as means and vertical bars indicate SEM (n = 6 in each group). Level of significance indicated as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Tz = *Trichnella zimbabwensis*; Pb = *Plasmodium berghei*.

Muscle glycogen concentration

The muscle glycogen concentration (± SEM) presented no significant difference ($P > 0.05$) between the *T. zimbabwensis* mono-infection and *Plasmodium berghei* (Pb) + *Trichinella zimbabwensis* (Tz) co-infection (Figure 10). Muscle glycogen accumulation increased progressively from day 0 PI, 7 PI and 14 PI for both infected groups (Tz mono-infection and Pb + Tz co-infection). However, the co-infection group presented a higher mean muscle glycogen value at day 7 PI and 14 PI (0.244 and 0.279 mg/tissue) respectively compared *T. zimbabwensis* single infection (0.185 and 0.19 mg/ tissue) respectively.

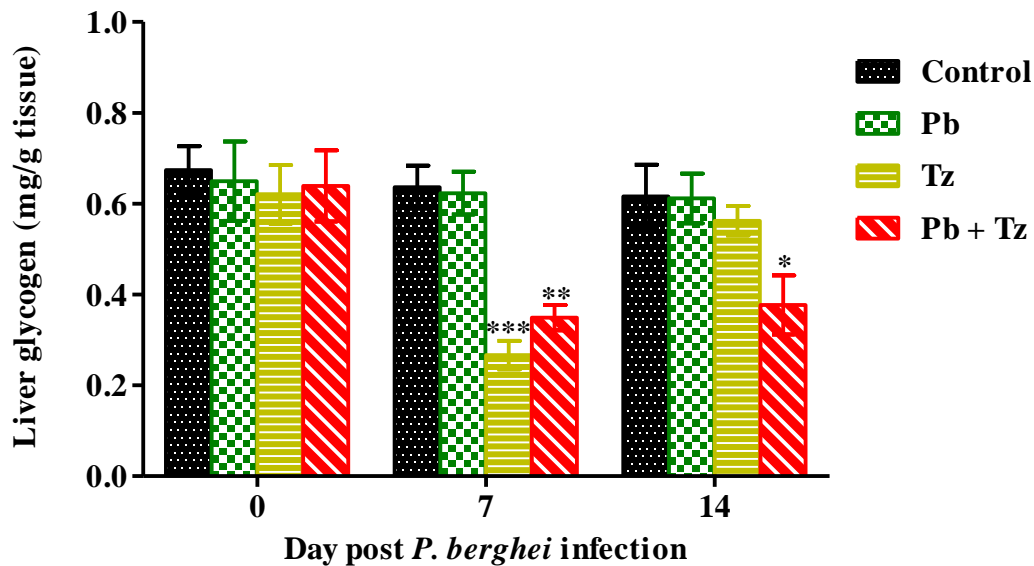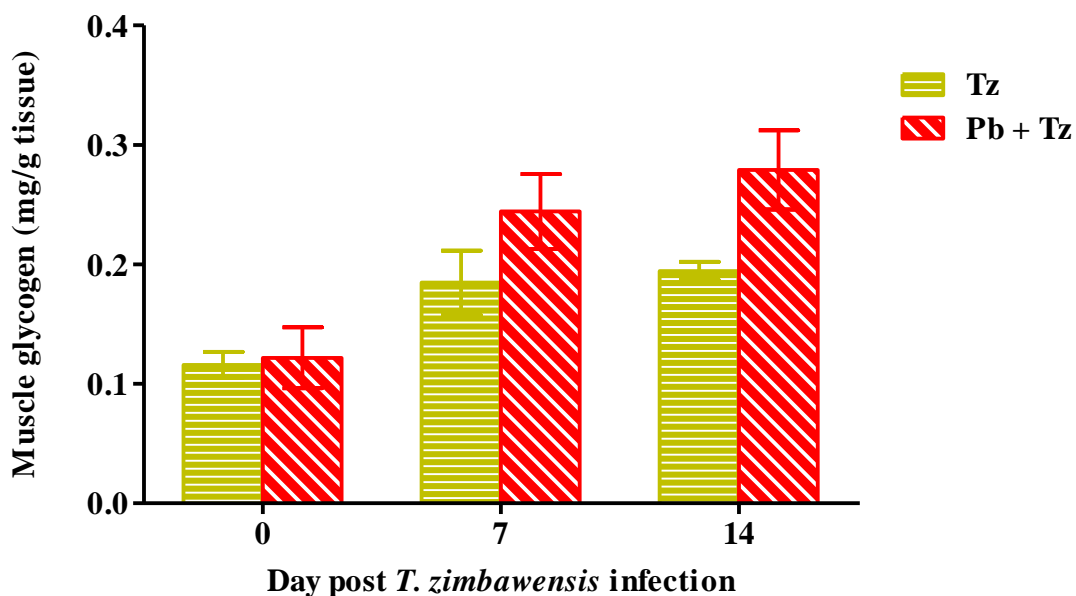**Figure 10:** Muscle glycogen concentration (± SEM) in male Sprague-Dawley rats infected with *T. zimbabwensis* mono-infection and *Trichinella zimbabwensis* (Tz) + *Plasmodium berghei* (Pb)

co-infection. Day 0 of *P. berghei* induction represent day 28 post *Trichinella zimbabwensis* infection in rats when the muscle larvae are in the rat muscles. Values are presented as means and vertical bars indicate SEM (n = 6 in each group); Level of significance ($P > 0.05$) obtained by comparing Tz mono- infection and Tz + Pb co-infection. Tz = *Trichnella zimbabwensis*; Pb = *Plasmodium berghei*.

4.4.2: Haematology measurements.

Mean red blood cells count (RBC), white blood cell count (WBC) and % haematocrit are shown in Figure 11 below. In Figure 11 A; we observed that at day 0 PI there was no significant ($P > 0.05$) change in RBC count in all infected group (*P. berghei* mono-infection (Pb), *Trichinella* mono-infection (Tz) and *Plamodium berghei* (Pb) + *Trichinella* infection (Tz) co-infection) compared to the control group. This was followed by a decrease in RBC count at day 7 PI for *Trichinella* mono-infection (Tz) and *Trichinella* infection (Tz) + *Plamodium berghei* (Pb) co-infection group ($P < 0.001$) respectively compared to control while Pb mono-infection also decrease at day 7 PI although not significant when compared to the control. The co-infection group (Pb + Tz) and Pb mono-infection start to increase again at day 14 PI and was statistically non-significant ($P > 0.05$) when compared to control group while the Tz group also increases but, still there was a significant difference ($P < 0.05$) compared to the control.

We observed no significant difference ($P > 0.05$) of WBC count in all infected groups at day 0, PI, 7 PI and 14 PI (Figure 11 B) compared to control. However, at day 7 PI and 14 PI there was increase in WBC count for Tz mono-infection, Pb mono-infection and Pb + Tz co-infection at day 7 PI and 14 PI but, still not significant ($P > 0.05$).

The percentage (%) haematocrit shown in (Figure 11 C) indicated that at day 0 PI all infected groups were not statistically significant ($P > 0.05$) compared to the control group. Significantly at day 7 PI *P. berghe*i mono-infection (Pb), *T. zimbabwensis* mono-infection (Tz) and *P. berghei T. zimbabwensis* co-infection (Pb + Tz) decreases significantly ($P < 0.01$ and $P < 0.001$) respectively. At day 14 PI the Pb, Tz, and Pb + Tz group increases and were not significant compared to the

control although the Tz and Pb+ Tz was higher than the control the was no statistical difference in all of these groups.
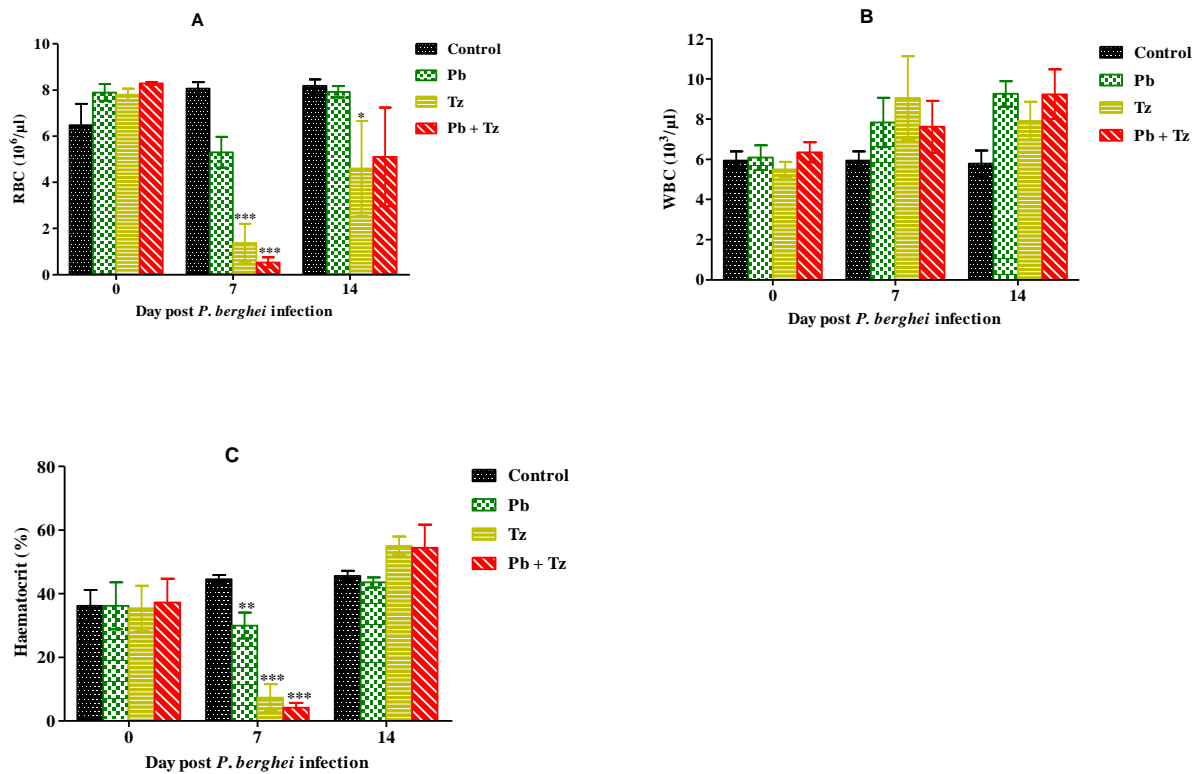
Figure 11: Mean (± SEM) red blood cell (RBC); % haematocrit and white blood cell (WBC) concentrations in experimental rats infected with *Trichinella zimbabwensis* mono-infection (Tz), *Plasmodium berghei* mono-infection (Pb), *Plasmodium berghei* (Pb) + *Trichinella zimbabwensis* (Tz) co-infection and non-infected control groups. Day 0 of *P. berghei* induction represent day 28 post *Trichinella zimbabwensis* infection in rats when the muscle larvae are in the rat muscles. Values are presented as means and vertical bars indicate SEM (n = 6 in each group). Level of significance indicated are * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ was obtained by comparing with the control. Tz = *Trichnella zimbabwensis*; Pb = *Plasmodium berghei*.

4.5 Serum insulin concentration

Insulin concentration decreases significantly ($P < 0.01$) for both *Trichnella* mono-infection (Tz) and *Plasmodium berghei* (Pb) + *Trichinella zimbabwensis* (Tz) co-infection at day 0 PI while *P. berghei* mono-infection show no significant change ($P > 0.05$) compared to the non-infected control (Figure 12). Later at day 7 PI there was a significant ($P < 0.05$) decrease in insulin for co-infection group only, however, the Tz mono-infection and Pb mono-infection decreases but was not statistically significant ($P > 0.05$) compared to the control. At day 14 PI there was no significant ($P > 0.05$) change in insulin concentration for all infected group Pb mono-infection, Tz mono-infection and Pb + Tz co-infection comparison to the control.
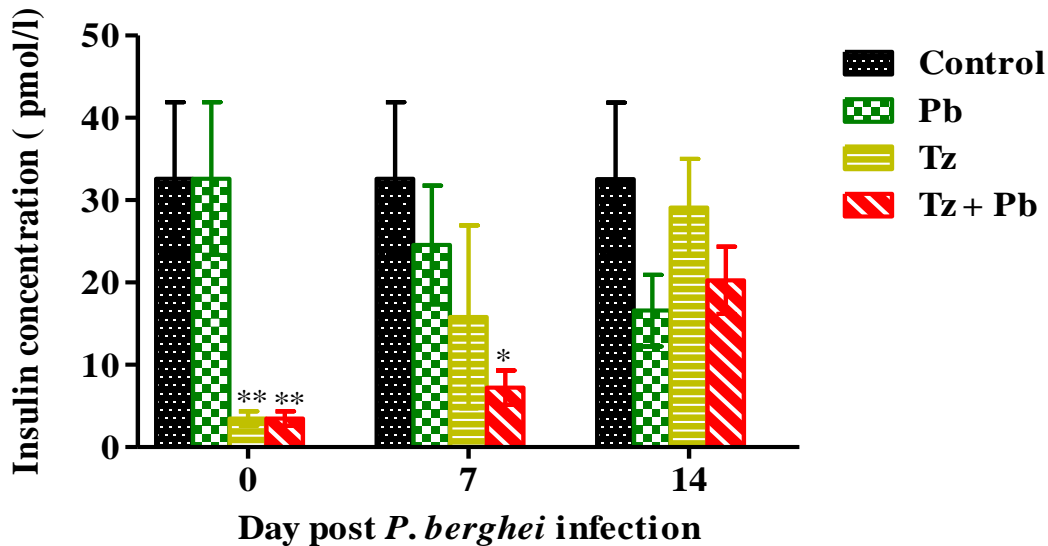
Figure 12: Insulin concentrations in experimental male Sprague-Dawley rats infected with *Trichinella zimbabwensis* mono-infection (Tz), *Plasmodium berghei* mono-infection (Pb), *Plasmodium berghei* (Pb) + *Trichinella zimbabwensis* (Tz) co-infection and non-infected control groups. Day 0 of *P. berghei* induction represent day 28 post *Trichinella zimbabwensis* infection in rats when the muscle larvae are in the rat muscles. Values are presented as means and vertical bars indicate SEM (n = 6 in each group). Level of significance * P < 0.05, ** P < 0.01, *** P < 0.001 by comparing with the control. Tz = *Trichnella zimbabwensis*; Pb = *Plasmodium berghei*.

CHAPTER 5: DISCUSSION

Findings from the current study show that the development of Pb in male Sprague-Dawley rats co-infected with Tz is enhanced. Our study agrees with similar studies although co-infection was with a protozoan parasite. For example, mice co-infected with *Trypanosoma brucei* and *P. berghei* showed a rapid development of *P. berghei* with an increased *P. berghei* parasitaemia for the co-infected mice than only *P. berghei* infected (Ademola and Odeniran, 2016). In another study by Bucher et al. (2011), they also reported increased early parasitaemia for co-infected *S. mansoni* and *P. berghei* mice than single infected *S. mansoni* mice. The elevated parasitaemia for the co-infection group suggests that *T. zimbabwensis* chronic infection might potentially predispose the host to more severe infection.

The occurrence of adult worms(AW) in the intestine of Tz mono-infection and co-infection persisted up to day 14 PI and no worms were recovered at day 21 PI onwards. Previous study by Onkoba et al. (2016a) have also demonstrated the persistence of AW of *T. zimbabwensis* in the intestine of mice at day 7 PI and 14 PI and a fewer number of ML at day 21. The non-appearance of AW at day 21 PI in the current study suggests that the majority of AW might have been eliminated by the host immune system. More so, day 21 PI might also indicate the appropriate time that most adult worms will have been killed by the host immune system. According to Bruschi and Chiumiento (2012), the successful establishment of *Trichinella* parasite in a host, primarily depends on their ability of the larvae to escape the host immune response. In addition, the persistence of AW in the intestine of hosts with recurrent exposure to infection has been thought to be associated with the down regulation of the immune response of the intestine or premonition state induced by continuous antigen stimulation (Bruschi and Chiumiento, 2012), thus suggesting that immune mechanism play a role in the process of deworming.

Steady weight gain was observed in rats infected with Tz mono-infection and Pb + Tz co-infection group. However, the Pb mono-infected group had weight loss at day 42 PI compared to the control. Previous studies have shown that malaria and helminthiasis co-infection can decrease the severity of malaria, especially with *P. falciparium* (Melo et al., 2010). The fact that chronic Pb + Tz co-infection was associated with weight gain confirms the synergistic interaction of parasites and their host during pathogenesis of malaria (Telfer et al., 2008). Therefore, we speculate that Tz + Pb co-

infection may possibly ameliorate the disease severity inflicted by *P.berghei* within the host (Graham et al., 2005).

There was a reduction in RBCs count and percentage haematocrit in rats infected with Pb mono-infection, Tz mono-infection and Pb + Tz co-infection a week after infection, with an increase in the subsequent week. Previous studies have reported malaria infection to be associated with anemia (Mishra et al., 2002; Maina et al., 2010). Certainly, the manifestation of anemia during malaria infection might have a direct effect by reducing the percentage haematocrit and RBCs. However, it is thought that most malaria induced anemia ascend from the rupture of schizonts and destruction of erythrocytes (Idro et al., 2010). Contrary to another study which reported that the pathogenesis of anemia caused by malaria infection does not depend on peripheral blood destruction (Ademola and Odeniran, 2016). Surely, increase in parasitaemia, may result in excessive haemolysis of parasitized RBCs thereby inducing anaemia. In our study, it is shown that increased parasitaemia in infected groups is consistent with reduction of RBCs and haematocrits. Following previous studies, anemia increases with increase parasite density (Ademola and Odeniran, 2016). Thus, the pathophysiology of anemia involves multiple mechanisms which are inconclusive such as, destruction of RBCs (Clark and Cowden, 2003) and decreased production of RBCs (Ademola and Odeniran, 2016). However, the current study could not provide appropriate mechanisms involved in RBCs and haematocrit reduction. Additionally, we observed increased WBCs for single infection groups (Pb and Tz) and co-infection. Akin-Osanaiye et al. (2015) also reported increases in white blood cell count and parasitaemia during infection of albino mice and no significant increase after treatment. This is suggestive that the white blood cell plays an integral part during parasitic infections by regulating the host immune system to combat foreign pathogens directly via the process of phagocytosis. Therefore, it is certain that an increase in WBC due to co-infection helps in the expulsion of the *P. berghei* parasite there by suppressing the effect on the host. Our study affirm that *T. zimbabwensis* promotes the production of WBCs, which helps in suppressing the *P.berghei* parasite there by counteracting the pathological effect due to the protozoan parasite.

Co-infection with *P.berghei* and *T. brucei* has demonstrated a blood glucose lowering effect in mice (Ademola and Odeniran, 2016). Contrary to our study, co-infection with *P. berghei* (Pb) + *T. zimbabwensis* (Tz) did not alter the blood glucose level, likewise *Trichinella* (Tz) mono-infection, however, the *P.berghei* (Pb) mono-infection showed a reduction in blood glucose. *Plasmodium berghei* parasite has been reported to cause hypoglycemia in mice (Wu et al., 2009). Our study asserts that, *P. berghei* parasite decreases blood glucose which manifests after peak parasitaemia by which we assume that the parasite is dependent on the host energy store and thereby creating an imbalance between the host's energy supply and demand (Kirk, 2001; Mehta et al., 2005). According to Olszewski et al. (2009) metabolic disturbance in glucose is a complex balance between supply, utilization, transportation and storage.

Across the parasite membrane there is increasingly uptake of glucose through the process of glycolysis facilitated by hexose transporters Glut4 through the process of facilitated diffusion (MaIver et al., 2008, Ademola and Odeniran, 2016). The invasion of *Plasmodium* parasite into the RBCs (ring stage) requires energy consumption which increases during maturity (trophozoites) (Kirk, 2001). A slight decrease in glucose concentration observed at day 7 PI may suggest the invasion of RBCs by *P. berghei* while the persistent decline on day 14 PI coincides with the maturity of the trophozoites which needs a large amount of energy. This study showed that Pb mono-infection had a blood glucose lowering effect which was not associated to a decrease in liver glycogen compared to the other three groups, suggesting that there could have been other mechanisms involved such as hepatocyte damage caused by the parasite (Murambirwa et al., 2013). Several mechanisms have been proposed in previous studies as cause of hypoglycemia, such as the depletion of the glyconeogenic substrate, thiamine, by malaria parasite (Krishna et al., 1999), others include hepatocellular damage caused by the malaria parasite (Murambiwa et al., 2013). Liver damage is caused by the entry of the parasite into the hepatic cells by in the course of replication (Murambiwa et al., 2013). Since the liver plays a major role in the glucose utilization, it is therefore certain that damage of the hepatic cells might turn might induce hypoglycemia (Onyesom and Agho, 2011). According to Onyesom and Agho (2011) hypoglycemia could also be attributed to organ congestion, sinusoidal blockage and inflammation of pancreatic cells. Contrary to other studies that has illustrated the occurrence of hypoglycemia during co-infection of helminths and malaria (Segura et al., 2009; Ademola and Odeniran, 2016), our study illustrated

that co-infection with *T. zimbabwensis* may regulate blood glucose lowering effect of *P. berghei*, although the mechanism involved could not be elucidated.

The current study shows elevated glycogen accumulation in Pb + Tz co-infected muscle cell of experimental animals compared to Tz mono-infected rats. This indicates that *T. zimbabwensis* co-infection might cause higher glycogen accumulation, however, glycogen accumulation between Tz mono-infection and Pb + Tz co-infection was not significant suggesting that *T. zimbabwensis* ML play a vital role on glycogen accumulation in the skeletal muscles (Gottstein et al., 2009) since the larvae require more energy for proliferation. A previous study has reported an increase in glycogen accumulation at day 8 PI and 18 PI in mice infected with *T. spiralis* and *T. pseudospiralis* infections and depleted at day 28 PI and 48 PI, although the glycogen accumulation for *T. spiralis* lasted longer than that of *T. pseudospiralis* infections (Wu et al., 2009). Similarly, our study observed steady increases in glycogen accumulation from day 0 PI peaking at day 14 PI, suggesting that *T. zimbabwensis* ML development was demanding higher energy levels. The mechanism involved in high glycogen accumulation is still not clear and among the hypothesized mechanisms include increase glucose uptake through insulin signaling pathways (Wu et al., 2009). Insulin plays a vital role in most insulin-responsive sites by enhancing glucose uptake glucose uptake in striated muscles and tissues (Pessin and Saltiel, 2000; Saltiel and Kahn, 2001).

The co-infection group and Tz mono-infection reduced liver glycogen concentration at day 7 PI whereas only the co-infection group still presented a significant decrease at day 14 PI compared to the control. Since, the liver plays a major role in glucose homeostasis by breaking down insulin receptor complex found at the cell surface where it is internalized for degradation and recycling of glucogeogenic substrate back to the membrane, this suggest that depletion of gluconeogenic substrate during Pb infection could be attributed to liver damage (Murambiwa et al., 2013). According to Dekker et al. (1997), the hepatocellular destruction could occur during the rapid multiplication and change of phase within the liver by the malaria parasite. This is supported by studies carried out by Kausar et al. (2010) in which they observed an increase in liver enzymes such as alkaline transaminases, aspartate, alkaline phosphatase, suggesting the probability of liver damage. However, the current study could not investigate the histopathological effect of *P. berghei* parasite on the liver and hence conclusive mechanisms could not be drawn.

Results from this study show that the level of serum insulin was reduced in Tz mono-infection and Pb + Tz co-infection group compared to control. At day 14 PI the serum insulin level in infected groups increases back to normal suggesting that in the later days of the infection the insulin level might have been restored by regulating enteritis and pancreatobiliary secretory motor functions (Onkoba et al., 2016). Similar observation have been reported in mice infected with *T. spiralis* and *T. pseudospiralis* (Wu et al., 2009). This is in contrast to another study that shows an increase in insulin in mice infected with *T. zimbabwensis* compared to the control (Onkoba et al., 2016). Normally, insulin functions in regulating blood glucose level and when there is an increase in blood glucose, insulin is released from the pancreatic β- cells of the islet of Langerhans targeting responsive tissues to stimulate glucose uptake (Leto and Saltiel, 2012). However, Wu et al. (2009) suggested that the ML in muscle cell exploits the host metabolic processes to increase glucose uptake and maintain a high glycogen content in infected muscle cells by increase expression of insulin signaling factors in infected muscle cells rather than increasing insulin concentration. Therefore, in cases of inadequate release of insulin, alternative mechanism has been reported such as intracellular signaling systems that have been modified and are involved in glucose and lipid metabolism (Pessin and Saltiel, 2000). Insulin signaling pathways have been suggested to play a main role in glucose transportation to responsive tissues and also facilitate the conversion of glucose to glycogen in storage organs (Youngren, 2007). Therefore, it can be speculated that the observed increased glycogen concentration in muscle tissues in the current study, was not insulin dependent, suggesting that there could be other functional mechanism in play. Further research in this aspect is recommended to draw an appropriate conclusion.

CHAPTER 6: CONCLUSION

The current study demonstrated that Tz mono-infection and Tz + Pb co-infection did not have blood glucose lowering effect in the host. We also reported hypoinsulinemia for the aforementioned groups, which was later restored to normal. Co-infected rats with *P. berghei* and *T. zimbabwensis* resulted in rapid development of Pb parasitaemia than the *P. berghei* mono-infection accompanied by decrease in RBC and haematocrit. Additionally, co-infection group showed an increase in glycogen content and body weight gain. The fact that hypoglycemia was not observed in co-infected male Sprague-Dawley rats suggest that there are other possible mechanisms through which tissue dwelling parasite upregulates the glucose store without decreasing the blood glucose concentration. This process necessitates further research in order to elucidate the specific mechanisms and pathways involved.

6.1 Recommendation

The results from the current study will contribute to knowledge on how tissue dwelling helminth can modulate *P. berghei* parasite during co-infection while adapting to the hosts metabolism and immune system without causing hypoglycemia. Therefore, further study on the histological parameters and insulin signaling pathways should be addressed in future research.

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8. APPENDIX

8.1 Graph showing the insulin standard curve
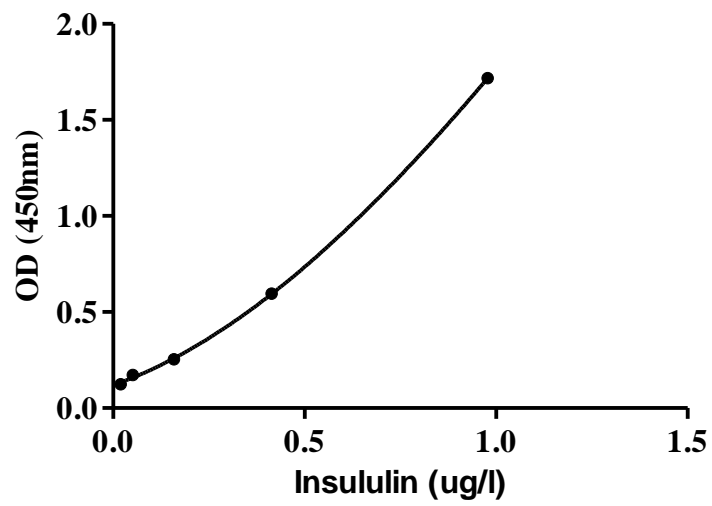
8.2 Ethical clearance approval form 2016

UNIVERSITY OF ™
KWAZULU-NATAL

INYUVESI
YAKWAZULU-NATALI

16 May 2016

Dr Pretty Murambiwa
School of Life Sciences
Westville Campus

Dear Dr Murambiwa,

Protocol reference number: AREC/018/016PD
Project title: Immune responses in experimental animal paradigms co-infected with *Trichinella. zimbabwensis* and *Plasmodium berghei*
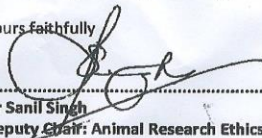
Full Approval – Research Application

With regards to your revised application received on 05 May 2016. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 16 May 2017.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

....
Dr Sanil Singh
Deputy Chair: Animal Research Ethics Committee

/ms

Cc Supervisor: Professor S Mukaratirwa
Cc Academic Leader Teaching & Learning: Professor A Olaniran
Cc Registrar: Mr Simon Mokoena
Cc NSPCA: Ms Jessica Light
Cc BRU – Dr Sanil Singh

Animal Research Ethics Committee (AREC)
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Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

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8.3 Ethical clearance form 2017

UNIVERSITY OF ™
KWAZULU-NATAL

INYUVESI
YAKWAZULU-NATALI

12 June 2017

Dr Pretty Murambiwa (16834)
School of Life Sciences
Westville Campus

Dear Dr Murambiwa,

Protocol reference number: AREC/018/016PD
Project title: Immune responses in experimental animal paradigms co-infected with *Trichinella. zimbabwensis* and *Plasmodium berghei*

Full Approval – Renewal Application

With regards to your renewal application received on **31** May 2017. The documents submitted have been accepted by the Animal Research Ethics Committee and **APPROVAL** for the renewal has been granted.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In **case** you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 12 June 2018.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

Dr S Islam, PhD
Chair: Animal Research Ethics Committee

/ms

Cc Supervisor: Professor S Mukaratirwa
Cc Dean & HoS: Professor A Olaniran
Cc Registrar: Mr Simon Mokoena
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