EFFECTS OF HOST SEX AND PREGNANCY ON *TRICHINELLA ZIMBABWENSIS* INFECTION IN SPRAGUE-DAWLEY RATS AND BALB C MICE

by

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Preface

The experimental work described in this dissertation was carried out in the School of Life Sciences, University of Kwa-Zulu Natal, Durban, from January 2012 to December 2014, under the supervision of Professor S. Mukaratirwa and Dr B. Masola.

This study represents original work by the author and has not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

Declaration – Plagiarism

I, **Lerato Hlaka**…… . declare that,

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- Most importantly, the Almighty God for the opportunity to conduct and complete the study, obtaining results that show potential positive implications towards new control and treatment strategies for trichinellosis; the wisdom, the courage, the strength, protection and provision with resources.

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List of abbreviations

NBL = New-born larvae

Spp. = Species

BW = Body weight

BRU = Biomedical research unit

UKZN = University of KwaZulu-Natal

PI = Post-infection

ELISA = Enzyme-linked immunosorbent assay

SEM = Standard error of mean

ANOVA = Analysis of variance

LPG = Larvae per gram

P/A = per animal

RCI = Reproductive capacity index

Th = T-helper cell

IL = Interlukin

IFN-γ = Interferon gamma

TGF-β = Transforming growh factor beta

NK = Natural killer cell

PIBF = Progesterone induced blocking factor

FSH = Follicle-stimulating hormone

EGF = Epidermal growth factor

CBG = Corticosteroid binding globulin

IGF = Insulin-like growth factor

IgE = Immunoglobulin E

HGF = Hemopoietic growth factor

HPA = Hypothalamic pituitary adrenal axis

ES = Excretory-secretory

DHT = Dihydrotestosterone

P_4 = Progesterone

PRL = Prolactin

PR = Progesterone receptor

cAMP = cyclic nucleotide 3', 5'-adenosine monophosphate

LH = Luteinizing hormone

GR = Glucocorticoid receptor

GnRH = Gonadotropin releasing hormone

POMC = Proopimelanocortin

GABA = γ-aminobutric acid

MHC 1 = Major histocompatibility class 1

HLA = Human leukocyte antigen

NFκB = Nuclear factor kappa B

MSCs = Mesenchymal cells

CRH = Corticotrophin-releasing hormone

ACTH = Adrenocorticotropic hormone

IEN = Immuno-endocrine network

SOCS 1 = Suppressor of cytokine signalling 1

PRE = Progesterone response elements

SPRMs = Selective progesterone receptor modulators

DNA = Deoxyribonucleic acid

RNA pol 2 = Ribonucleic acid polymerase2

PGF2α = Prostaglandin F2α

P450scc = Cytochrome P450 cholesterol side-chain cleavage enzyme

Abstract

Trichinellosis is a zoonotic parasitic disease caused by nematode parasites of the genus *Trichinella*. *Trichinella* species infect a wide range of hosts including humans, domestic and wild animals. The main mode of human infection is through ingestion of raw or undercooked pork. The successful establishment and development of *Trichinella* parasite in a host is affected by various factors which include sex of host, environmental, immunological and hormonal.

The objective of this study was to determine the effect of host sex and pregnancy on the establishment and development of *Trichinella zimbabwensis* in Sprague-Dawley rats and Balb C mice respectively.

Rodents are the common reservoirs of *Trichinella spp.* in the domestic and sylvatic cycles and it was logical to determine the establishment and development of *Trichinella zimbabwensis* in Sprague-Dawley rats. The study on the effects of pregnancy and levels of progesterone and cortisol was done in Balb C mice and this was influenced by availability of mice in large quantities to conduct the experiments. Rats and mice are not widely different in their physiology and have been used interchangeably as host in previous *Trichinella* studies. Therefore it was important to use these animals as models for the study.

In order to determine the effect of host sex on the establishment and development of *T. zimbabwensis*, 50 Sprague-Dawley rats were divided into two groups (25 males and 25 females) and orally infected with 7 *Trichinella zimbabwensis* muscle larvae per gram (LPG) of animal live weight. On days 5, 10, 15, 20 and 25 post-infection (PI), five animals from each group were sacrificed and the numbers of adult parasites in the intestine as well as larvae in muscles were determined. To determine the effect of host pregnancy, 90 female Balb C mice were divided into 3 groups of 30 mice each. Group 1 animals were orally infected with 50 LPG on day 0 of trial; group 2 animals were mated on day 0, but were not infected; group 3 animals were mated on day 0 and infected with 50 LPG on day 7 post-mating. On days 0, 7, 14, 21 and 28 PI for groups 1; days 0, 7, 14, 21 and 28 post-mating for group 2; and days 7, 14, 21, 28 and 35 post-mating for group 3, six animals from each group were sacrificed and the numbers of adult parasites in the intestines as well as larvae in the muscles were determined in infected groups. In addition, levels of the hormones progesterone and cortisol were measured in all groups at the same intervals.

Results from the study showed a significantly higher number of *Trichinella* adults and larvae ($P < 0.05$) in male than in female Sprague-Dawley rats (four times higher adult worms and two times higher in muscle larvae in males than in females). On the other hand, pregnancy reduced the number of larvae establishing in muscles with progesterone levels significantly higher in pregnant than in non-pregnant Balb C mice ($P < 0.05$). This was attributed to the parasiticidal effect of progesterone against new-born larvae (NBL). This finding can be exploited when designing strategies to control and to treat the infection in rodents and humans. There were no significant differences in cortisol levels between pregnant and non-pregnant mice.

Keywords: Balb C mice, Sprague-Dawley rats, *Trichinella zimbabwensis*, parasite establishment, host sex, pregnancy, progesterone, cortisol.

1. Introduction

Trichinellosis is an important zoonotic parasitic disease caused by nematode species of the genus *Trichinella* which has a world-wide distribution, infecting both domestic and wild animals (Kapel, 2000; Pozio, 2000; Pozio, 2007). The parasite circulates in either a sylvatic or domestic transmission cycle, with a flow existing between the two cycles (Campbell, 1983). The main source of human infection is through ingestion of raw or undercooked infected meat from pigs, horses and wild animals (Gottstein *et al.,* 2009). Clinical signs of infection in humans and non-human primates include facial oedema, fever, diarrhoea, myalgia, inflammation, arthralgia and prostration (Clausen *et al*., 1996; Mukaratirwa *et al*., 2008).

1. 1 General background on Trichinella

1. 1. 1 Classification and geographical distribution of Trichinella species

Trichinella consists of 12 species and genotypes that are morphologically delineated into two main clades, encapsulated and non-encapsulated, according to the presence or absence of a collagen capsule that forms around the larvae when they encyst in skeletal muscles (Pozio *et al.*, 1992; Zarlenga *et al.*, 2006; Krivokapich *et al.*, 2012) (Table 1). The encapsulated clade consists of the *T. spiralis, T. nativa, T. britovi* and *T. murrelli*, T8, T6, T9 and T12 whilst the non-encapsulated clade consists of *T. pseudospiralis*, *T. papue* and *T. zimbabwensis* (Pozio *et al.*, 1992; La Rosa *et al.*, 2003). All *Trichinella* species infect mammals with *T. pseudospiralis* also infecting birds, whilst *T. papuae and T. zimbabwensis* also infecting reptiles (Pozio *et al.*, 2002; Taybouavone *et al*., 2008). All *Trichinella* species are deemed zoonotic except *T. zimbabwensis* with no record of human infection, though it has been shown to infect non-human primates (Mukaratirwa *et al*., 2008).

Table 1: Classification, distribution and host range of *Trichinella* species (La Rosa *et al*., 2003)

Species/ Genotypes	Capsule	Distribution	Hosts
T. spiralis	Yes	Cosmopolitan	Mammals
T. britovi	Yes	Palaearctic & Temperate regions	Mammals
T 9	Yes	Palaearctic & Temperate regions	Mammals
T 8	Yes	Palaearctic & Temperate region	Mammals
T 6	Yes	Arctic region	Mammals
T. nelsoni	Yes	Ethiopic region	Mammals
T. nativa	Yes	Arctic regions	Mammals
T. murelli	Yes	Nearctic &Temperate regions	Mammals
T. pseudospiralis	No	Palaearctic, Nearctic regions & Australia	Mammals & birds
T. papue	No	Papua New Guinea	Mammals & reptiles
T. zimbabwensis	No	Southern & Eastern sub-Saharan Africa	Mammals, reptiles & non-human primates
T 12	Yes	South America	Mammals

1. 1. 2 Life cycle

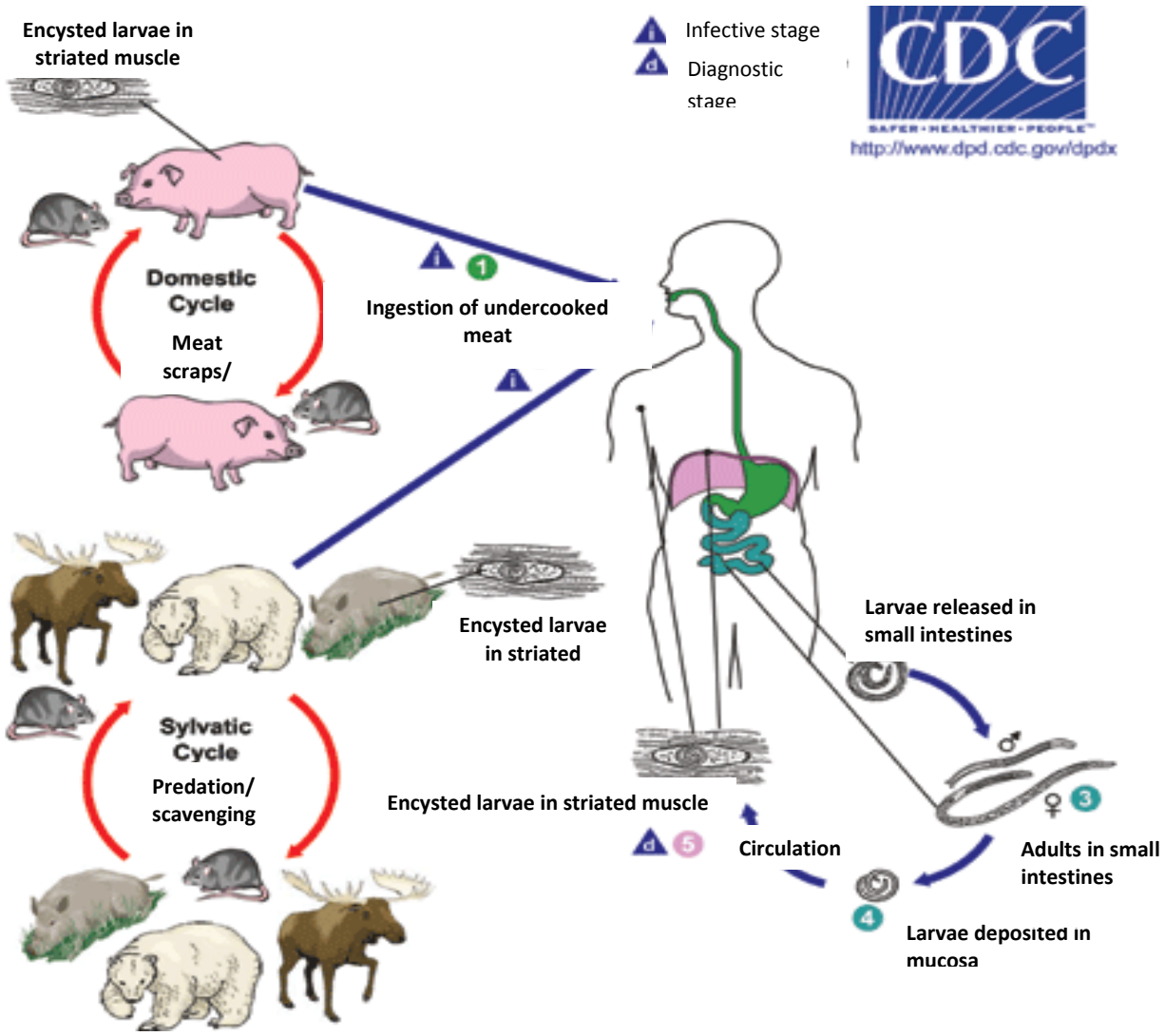


Fig. 1: Life cycle of *Trichinella* species (Smith, 2007)

All *Trichinella* species follow a similar life cycle where the parasite completes a full generation within the body of a single host (Capó & Despommier, 1996; Gottstein *et al.*, 2009) and within a host; the parasite undergoes an enteral and a parenteral phase (Fig. 1). In the enteral phase, the larvae are released from muscle tissue, through digestion of muscle tissue by the combination of pepsin and HCl and they colonize the columnar epithelial cells of small intestines in which parasite moulting to adult stage occurs (Fig. 1) (Despommier, 1975; Kozek, 1971).

The parenteral phase is characterized by migration of new-born larvae (NBL) from intestines to striated muscle tissue, via the lymphatic and blood vessels, and subsequent colonization of muscle tissues (Fig. 1) (Despommier, 1975). At this stage the muscle fibre modulates to form the nurse cell which serves as a protection and a source of nutrients for the larvae (Fig. 1) (Despommier, 1975).

Transmission of *Trichinella* spp. follows either a domestic or sylvatic transmission cycle (Fig. 1) (Campbell, 1983). Transmission of *Trichinella* species through the domestic cycle usually involves pigs, horses, rats and mice; the anthropophilic rodents acting as reservoirs for the parasite (Fig. 1) (Gottstein *et al.*, 2009). When infective larvae are ingested by wild animals, transmission follows a sylvatic route either through predation or scavenging (Fig. 1). The domestic route is usually the main mode of transmission for human infection (Gottstein *et al*., 2009). However, some sylvatic animals such as wild boars and bears were reported to be other sources of human infections (Gottstein *et al*., 2009).

Sylvatic cycles differ according to the *Trichinella* species and geographic distribution (Pozio, 2007). In the South and Eastern Africa, hypothetical sylvatic cycles for *T. nelsoni* and *T. zimbabwensis* have been reported (Mukaratirwa *et al*., 2013). The hypothesized life cycle of *T. nelsoni* include the predation and scavenging behaviour of spotted hyenas, lions, bush pigs and warthogs and the latter marks a possible route of human transmission through ingestion of raw or undercooked meat (Mukaratirwa *et al*., 2013). The hypothetical sylvatic cycle for *T. zimbabwensis* includes reptiles from Crocodylidae and Varanidae families which have scavenging and cannibalistic behaviour, as the main reservoirs (Mukaratirwa *et al.*, 2013).

The flow among cycles and the success of the parasite is influenced by various factors including geographical region, environmental factors, type of host or the changes in physiology of the host agent (Pozio, 2000).

1. 2 Factors affecting Trichinella establishment

Various factors have been reported to affect the successful establishment of *Trichinella* infection and transmission, depending on the type of species and the respective host involved (Pozio, 2000). These factors could be environmental, immunological, and physiological which includes the gender of the respective host and hormones present within the host (Wakelin *et al.*, 1994; Pozio, 2000; Escobedo *et al*., 2005).

1. 2. 1 Environmental factors

Environmental stress has been reported to affect the survival and transmission of the parasite according to the biological diversity of *Trichinella* genotypes and geographic distribution (Pozio, 2000). High humidity and low temperatures support *Trichinella* survival in decaying meat thus playing a significant role in the transmission pattern of the parasite (Pozio, 2013). Survival of these larvae under such conditions is influenced by their freeze tolerance capacity and anaerobic metabolism occurring in the nurse cell (Pozio, 2000). The collagen capsule allows the larvae to survive and remain infective when all other muscle cells have decomposed, with larvae reportedly remaining infective for up to four months in decaying muscle tissue of encapsulated species (Madsen, 1974). This is supported by the observation that infectivity can take place from a scavenged decomposing animal (Pozio, 2000; Pozio, 2013).

Larvae from encapsulated species of *Trichinella* also have high resistance to freezing temperatures in muscle tissue and it has been suggested that this is due to the protective effect of the high carbohydrate matrix of the nurse cell capsule (Lacour *et al.*, 2013). The thicker the capsule with chronic infection, the better the protection against freezing in encapsulated species (Lacour *et al*., 2013). *Trichinella nativa* which is found in arctic regions has been reported to survive for four years under freezing temperature of -18 ˚C (Kapel, 2000). However, in comparison to other genotypes, *T. spiralis* is less tolerant to extreme temperatures and degrading muscle tissue; factors which have been implicated to have an effect on its host range (Kapel, 2000).

Heat tolerance, freezing resistance and survival have been reported to be low in *T. pseudospiralis* compared to encapsulated species (Kapel, 2000). The ability of *T. papaue* and *T. zimbabwensis* to survive in both ectotherm and endotherms with varying natural

temperature ranges reveals a significant amount of tolerance to thermal stress (Hurnikova *et al*., 2004). *T. zimbabwensis* larvae isolated from experimentally infected fox meat kept under -5 °C was found to be still viable and infective to mice after four weeks of freezing (Hurnikova *et al*., 2004). Although external factors may have an influence on the infectivity, survival and persistence of the parasite, changes in the internal environment (host system) play a significant role in the establishment and survival of the parasite.

1. 2. 2 Host internal environment

1. 2. 2. 1 Immunological factors

Trichinella can ensure its survival by modulating host immunological responses by inducing Th2 (IL-4, IL-5 and IL-13) responses and the production of immune regulatory cytokines, IL-10, IFN-γ and TGF-β (Wakelin *et al.*, 1994; Morales *et al*., 2002; Beiting *et al*., 2007). Antibodies and cytokines have a significant role on successful establishment and development of *Trichinella* by implementing expulsion, reduction in numbers of worms, fecundity and killing of NBL (Wakelin *et al*., 1994). This depends on specific dose-dependent antigen expression, the developmental stage of parasite and the type of host (Wakelin *et al*., 1994, Wu *et al.*, 2013a). During muscular phase of *Trichinella* infection, an increase in $CD8^{+}$T-cell expression has been reported and the suppression of Th1 responses by the pro-inflammatory response-inhibiting cytokine, IL-10 during larval development within muscle cells, may reflect an adaptation to reduce intracellular killing of larvae during this stage of development (Morales *et al*., 2002; Beiting *et al*., 2007; Yang *et al*., 2013). IL-10 is a regulatory cytokine which plays a significant biological role in the protection against the intestinal stages of *T. spiralis* through suppression of IFN-γ activity against NBL thus ensuring their survival and development to parenteral stage (Helmby & Grencis, 2003).

Stimulation of immune responses may differ according to species and different species of *Trichinella* may use different molecules for larval formation and the activation of host immune-suppression at different developmental stages of the parasite (Wakelin *et al*, 1994; Wu *et al*., 2013a, b). These will in-turn influence how the parasite invades and survives in the host system (Wakelin *et al*., 1994; Wu *et al.*, 2013a, b). For example, *T. pseudospiralis* showed high IgE response at adult stage in the intestines, higher IL-4 in NBL and muscle larvae when re-stimulated with excretory-secretory (ES) protein antigens (Wu *et al.*, 2013a). The expression of pyroglutamyl-peptidase 1, 6 phosphogluconolactase and a serine protease

from adult worms and NBL contributes to the stronger immune suppression marked by *T. pseudospiralis* (Wu *et al.*, 2013a, b). A greater establishment of parasites are found in immune-compromised hosts as a result of impaired cytokine release (Culbertson, 1942).

1. 2. 3 Physiological and hormonal factors

1. 2. 3. 1 Host sex and sex hormones

Parasites have been shown to exploit host's hormonal micro-environment to ensure their survival, with the response being influenced by host species and sex (Escobedo *et al*., 2005). Host sex has been reported to play a significant role in determining the susceptibility/resistance of a host to a wide variety of protozoan, helminthic and arthropod infections and this has been attributed to sex hormones (Mankau & Hamilton, 1972; Reddington *et al*., 1981; Escobedo *et al.*, 2005).

It has been reported that males are usually more prone to parasite infections due to behavioural and ecological patterns as well as genetic and physiological differences between males and females (Zuk & McKean, 1996; Klein, 2004). Males usually get involved in behaviours such as aggression, dispersal and grouping which then increase the chances of contact with both ecto- and endoparasites (Klein, 2004). Males are also usually larger in size than females which may make them obvious targets for parasitism (Klein, 2004). The main physiological factor implicated in this male-biased infection is the immune-suppressive effect of testosterone which increases susceptibility and exposure to various parasitic infections (Folstad & Karter, 1992; Grear *et al*., 2009). *Plasmodium falciparum* merozoites produced an increased number of gametocytes after *in vitro* treatment with testosterone (Escobedo *et al*., 2005). In a study on the effects of sex hormones on *T. spiralis,* male rats presented a greater susceptibility to *T. spiralis* infection than female rats (Mankau & Hamilton, 1972). Male mice were reported to be also more susceptible to other helminths such as *Trichuris muris* and *Schistosoma mansoni* (Hepworth *et al*., 2010).

Although males are reported to be more susceptible than females to many parasites, there are parasite species for which males are more resistant to than females (Klein, 2004). According to Hernandez-Bello and colleagues (2011), male mice are less resistant to protozoa like *Plasmodium berghei, Tryponosoma cruzi,* and nematodes like *Strongyloides sp.* However, *in vitro* exposure of *Entamoeba histolytica* trophozoites to various concentrations of 17β-

estradiol, progesterone, testosterone and dihydrotestesterone (DHT), had little effect on the parasite viability and proliferation (Escobedo *et al.*, 2005).

In contrast, females have been reported to be less prone to parasitic infections when compared to male hosts (Grear *et al*., 2009). Female hormones are reported to interact with the immune system to elicit a stronger immune response characterized by higher antibody levels and a better adaptive immunity (Grear *et al*., 2009; Hepworth *et al.*, 2010). In a study conducted on *T. spiralis,* male rats injected with stilbestrol, a synthetic estrogenic compound used in treatment of female animals for infertility; displayed a marked decline in larval count as compared to normal male rats. On the contrary treatment of *T. crassiceps* cysticerci with 17β-estradiol increased its reproduction capacity; whereas testosterone and DHT reduced this function (Escobedo *et al*., 2005). Physiological changes within the female host will also determine the level of resistance to most parasitic infections. Pregnancy results in elevated levels of the hormone progesterone (Johnson, 2003).

1. 2. 3. 2 Pregnancy hormones

I. Physiology of progesterone

i. Synthesis and secretion of progesterone

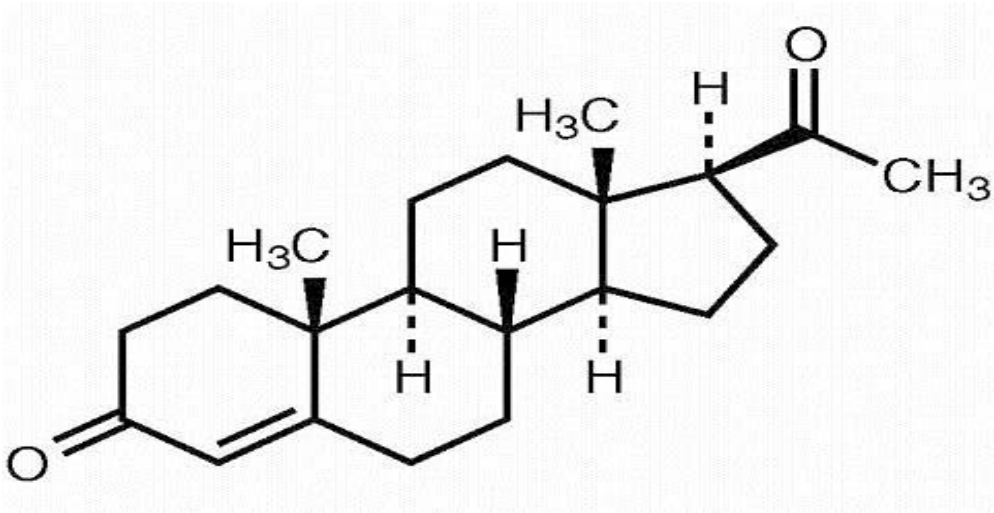


Fig. 2: Chemical structure of progesterone (M.P Biomedicals, 2014)

Progesterone also known as P_4, is a C21 steroid hormone containing a keto-group at C3 and a double bond at C4 and C-5 (Johnson, 2003) (Fig. 2). Like othe steroids, it is derived from cholesterol through a series of chain reactions in this case through the cleavage cholesterol by cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc) at carbons 21 and 22,

yielding 21-carbon progestins (Johnson, 2003) (Fig. 2). P_4 is mainly responsible for reproduction in females (Graham & Clarke, 1997). The ovary is the major organ responsible for the synthesis and secretion of female sex hormones mainly estrogen and P_4 and gives the cyclic changes that occur during reproduction cycle (Graham & Clarke, 1997).

Prior to ovulation, the granulosa cells in the primary follicle secrete estrogen and subsequent to follicle rupture and ovum release, granulosa cells mature to form corpus luteum which is responsible for secreting progesterone and estrogen at the later stage of the cycle (Graham & Clarke, 1997). P_4 prepares the uterus for the nurture and implantation of the newly fertilized ovum (Loeb & Quimby, 1989). If fertilization occurs, the corpus luteum continues to grow and function for the first trimester of pregnancy after which the placenta assumes the role hormone biosynthesis in humans (Johnson, 2003).

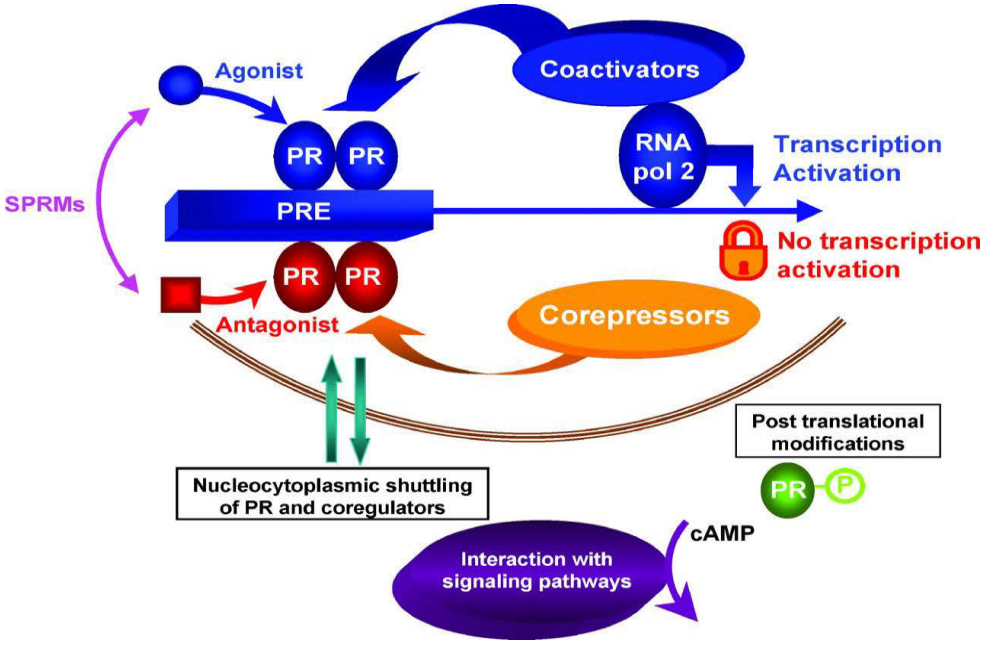
In females of different animal species, the secretion of P_4 is enabled by different mechanisms. In humans it is influenced by a number of hormones but mainly the gonadotropin hormone, lutropin hormone (LH) which is released from the pituitary gland together with the follicle-stimulating hormone (FSH) through stimulation by gonadotropin-releasing hormone (GnRH) (Johnson, 2003). While FSH is responsible for follicular development in the normal reproductive cycle at follicular phase, LH induces formation of the corpus luteum from remaining glomerulosa cells in the ruptured follicle at the luteal phase of the cycle and also induces the production of ovarian steroid hormones, estrogen and progesterone (Von Euler & Heller, 1963; Johnson, 2003). The latter is known to be mediated via the intracellular effects of LH on second messenger cAMP in the theca and granulosa cells (Johnson, 2003). As the follicle approaches ovulation, it accumulates follicular fluid that causes the follicular wall to expand due to proteolytic enzymes which are responsible for the digestion the collagen framework and other proteins of the intercellular matrix of the follicular wall (Johnson, 2003). One of the enzymes is plasmin, which appears in the form of its inactive precursor, plasminogen (Johnson, 2003). Plasminogen activator is secreted by granulosa cells in response to hormonal stimulation. Since they have acquired new receptors, granulosa cells respond to LH by secreting progesterone (Johnson, 2003).

Once released, P_4 is carried in the blood stream and the blood concentrations range from 0 mg/dL during the early pre-ovulation to as much as 2 mg/dL after the corpus luteum has formed (Von Euler & Heller, 1963). In cyclic rats and mice the levels, the maintenance of P_4 in circulation is influenced by binding plasmatic proteins such as globulin or albumin (Loeb

& Quimby, 1989; Johnson, 2003; Cabrera-Muñoz *et al*., 2010). Once released, progesterone is carried in the blood by transcortin (corticosteroid-binding globulin) in many species including humans (Graham & Clarke, 1997). In the uterine fluid of the rabbit, between days 3 and 12 of pregnancy, an additional P_4 carrier, uteroglobin, is present and has been postulated to protect the embryo during pregnancy (Loeb & Quimby, 1989; Graham & Clarke, 1997). In pregnant guinea pigs, a specific binding plasma protein has been described, which has greater affinity for P_4 than corticosteroid-binding globulin (CBG) forming a major progesterone-binding protein which is synthesized by the placenta. The protein is induced at day 15 and 20 of pregnancy and remains elevated until parturition at about day 65 (Loeb & Quimby 1989; Graham & Clarke, 1997).

Follicle-stimulating hormone (FSH); prolactin (PRL) [the milk production stimulating hormone]; prostaglandins (lipid compounds derived enzymatically from fatty acids and responsible for uterine contractility during pregnancy); and β-adrenergic agents (mediators of cAMP responsible for contraction and relaxation of smooth muscles), also play a role in the regulation of P_4 secretion (Graham & Clarke, 1997). Intermediates such as activin, which is stimulated by FSH for the inhibition of P_4 secretion by granulosa cells and follistatin produced by granulosa cells that binds to activin, contribute to a complex regulatory pattern of P_4 secretion (Graham & Clarke, 1997; Johnson, 2003). In the rat uterus, increased P_4 synthesis is accompanied by induction of ovarian follistatin gene expression at the start of blastocyst implantation, which appears to help in maintaining P_4 secretion (Graham & Clarke, 1997; Johnson, 2003; Pocock & Richards, 2004). However, it is not clear whether the expression of follistatin is stimulated by progesterone to prevent inhibition of progesterone secretion by activin or whether to prevent down-regulation of P_4 secretion from the corpus luteum (Graham & Clark, 1997). The onset of P_4 release occurs at the beginning of estrus in unfertilized rats and at the 3rd day of pregnancy in the case of fertilization (Finn & Martin, 1971).

ii. Mechanism of action



Key:

PR = Progesterone receptor

cAMP = cyclic nucleotide 3', 5'-adenosine monophosphate

PRE = Progesterone response elements

SPRMs = Selective progesterone receptor modulators

Fig. 3: Progesterone mechanism of action (Human Reproduction Update, 2014)

The functions of P_4 are mediated via its nuclear receptors, the progesterone receptor (PR) which modifies gene expression in the cell (Graham & Clarke, 1997; Cabrera-Muñoz *et al*., 2010; Mani & Oyola, 2012) (Fig. 3). PR is a member of a large family of ligand-activated nuclear transcription regulators, amongst which are receptors for steroids, retinoids, thyroid hormones, and vitamin D (Graham & Clarke, 1997).

P_4 functions in two ways, the classical and non-classical mechanisms. In the non-classical mechanism, P_4 binds to PR that is located in the plasma membrane (mPR) and in cytoplasm which mediates signalling via G-protein coupled pathways, inducing the production of second messengers such cAMP and the activation of kinases and modifying ion conductance in the cell (Fig. 3). In the classical mechanism P_4 interacts with PR which results in the conformational change of the receptor that induces dissociation of heat shock proteins leading to phosphorylation and dimerization of the receptor, DNA binding and recruitment of co-activators to facilitate the interaction with basal transcription apparatus RNA polymerase 2 (RNA POL 2) (Graham & Clarke, 1997; Johnson, 2003; Cabrera-Muñoz, 2010; Mani & Oyola, 2012) (Fig. 3).

The newly formed progestin-complexed PR dimer has high binding affinity for specific sequences in the DNA, known as progesterone response elements (PRE) in target genes resulting in transcription of those genes (Graham& Clarke, 1997; Cabrera-Muñoz *et al*., 2010; Mani & Oyola, 2012) (Fig. 3). Progesterone receptor antagonist (PA) on the other hand, competes with the agonist for PR binding to promote and activate dimerization and binding to specific PRE genes. However, PA recruits co-repressors that induce an altered conformation in PR that is transcriptionally inactive, resulting in a non-productive interaction of the receptor with DNA (Pocock & Richards, 2004; Mani & Oyola, 2012) (Fig. 3).

iii. Expression of PR isoforms

PR exists in two main forms a PR-A and PR-B characterised by molecular masses of about 81 kDa and 115 kDa respectively (Graham & Clarke, 1997; Cabrera-Muñoz *et al*., 2010; Mani & Oyola, 2012). Although both isoforms are encoded by a single gene, they are transcribed by distinct promoters, each of which gives rise to a distinct subgroup of PR mRNA subspecies (Graham & Clarke, 1997; Cabrera-Muñoz *et al*., 2010). Although both PR-A and PR-B are activators of target genes, PR-B tends to be a stronger activator of target genes than PR-A, while PR-A can act as a dominant repressor to B, suggesting that activation of target genes may be modulated via alterations in the ratio of PR-A and PR-B expression. Therefore high PR-A expression may result in reduced cellular responsiveness to progestins. However, there is an interspecies difference in the expression of PR isoforms in normal tissues (Graham & Clarke, 1997).

An equimolar expression of PR A and B is observed in chick oviduct and human uterus, whereas a predominant PR A over PR B in a 3: 1 ratio is observed in the rodent. Changes in the ratio of PR forms in the chick oviduct during late winter, or in aged non-laying animals, results in a measurable decrease in PR functional activity (Graham & Clarke, 1997). In human breast tumours, the ratio of expression of PR A and B proteins differs among patients (Graham & Clarke, 1997). This difference in relative expression of PR-A and PR-B marks the distinction in the functional capacities to activate transcription of target genes and in this way regulate different physiological processes in different tissues of different species (Johnson, 2003; Graham & Clarke, 1997; Cabrera-Muñoz *et al*., 2010; Mani & Oyola, 2012).

iv. Functions of P_4.

Maturation of oocytes and ovulation

Although the main role of progesterone is marked in reproduction, it plays various other significant roles in mammals. For example in the uterus and ovary, P_4 stimulates the release of mature oocytes through the activation of 3β-hydroxysteroid dehydrogenase, serine proteases, kallikrein, and plasminogen activator to stimulate ovulation (Graham& Clarke, 1997). The formation of the corpus luteum represents a distinct intraovarian process and appears to be progesterone-dependent marked by the expression of PR induced by LH in granulosa cells of mature pre-ovulatory follicles and the corpus luteum (Johnson, 2003).

Implantation

Progesterone is also responsible for facilitation of implantation and maintenance of pregnancy through the activation of enzymes that are responsible for the lysis of zona pellucida; activation of PRL, which is secreted by both the endometrial stroma and myometrium and is involved in decidualization of stromal cells in the uterus; and the control of growth factors that are responsible for uterine cell proliferation in early stages of pregnancy (Johnson, 2003).

Cellular sensitivity to autocrine/paracrine effects of growth factors is controlled by cell type-specific expression of growth factor receptors (Johnson, 2003). Stimulation of growth factor secretion by progesterone from the luminal and glandular epithelium in mouse endometrium promotes proliferation of the epidermal growth factor (EGF) receptor-positive blastocyst trophectoderm to facilitate implantation (Graham & Clarke, 1997, Pocock & Richards, 2004). It has been suggested that a paracrine influence on the growth and differentiation of the placental trophoblast, is exerted by the hemopoietic growth factor (HGF) and colony stimulating factor-I which is secreted from the luminal glandular epithelium in the mouse in a process regulated by estrogen and P_4 (Johnson, 2003). In the first two days of pregnancy in the mouse, insulin-like growth factor-I (IGF-I), secreted from the luminal and glandular epithelium of the uterus under estrogen stimulation may contribute to effects on the blastocyst (Johnson, 2003; Pocock & Richards, 2004). After this time, synthesis and secretion of IGF-I occur in the stroma and are induced by P_4, resulting in increased proliferation and enlargement of the uterus (Johnson, 2003; Pocock & Richards, 2004).

Maintenance of pregnancy

After implantation, P_4 maintains pregnancy through the suppression of myometrium contractility through mechanisms that include progesterone effects on intracellular calcium levels, prostaglandins, oxytocin (neurohypophysical hormone secreted by the pituitary gland that increases strength and frequency of uterus contractions) and relaxin (protein hormone, that is produced by ovaries for pelvic and cervical relaxation) (Johnson, 2003; Pocock & Richards, 2004). An increase in free intracellular calcium leads to myometrium contractility (Johnson, 2003). Progesterone then induces the secretion of calcitonin, a hormone which is involved in calcium homeostasis that lowers free calcium levels thereby preventing contraction in the uterus (Johnson, 2003). Increasing endogenous P_4 also decreases the expression of calbindin D9k mRNA, a calcium transporter and this action is mediated by PR, thus preventing increases in free intracellular calcium, contributing to myometrium contractility (Graham & Clarke, 1997). Calbindin D9k expression stimulated by estrogen in the rat can be blocked by treatment with progesterone agonist R5020 (Graham & Clarke, 1997).

P_4 also inhibits the synthesis and activity of prostaglandins (responsible for uterine contractility during gestation) through the stimulation of prostaglandin 15-dehydrogenase in sheep. Prostaglandin oxidation catalysed by this enzyme results in its inactivation, thus decreasing myometrium contractility (Schmidt-Nielsen, 1990). Progesterone also inhibits the effects of prostaglandins in the luteal phase of the human cycle and during pregnancy by decreasing the levels of F2α and E in the endometrium and by inhibiting the induction of F2α by estrogen (Johnson, 2003). Effects of prostaglandins are exerted by oxytocin receptors which are inhibited by progesterone through blocking the production of PGF2α. At the end of pregnancy, an increase in prostaglandin synthesis and production leads to decreased levels of P_4 leading to birth (Graham & Clarke, 1997; Johnson, 2003; Podock & Richards, 2004).

P_4 also interacts with the adrenergic system which also plays a role in the relaxation of the myometrium by increasing the transcription of β-adrenergic receptors in the myometrium of the rat during late pregnancy. This leads to an increase in sensitivity to adrenergic agents. Relaxin on the other hand, inhibits prostaglandin-induced myometrium contractility and thus contributes to the maintenance of pregnancy (Graham & Clarke, 1997; Johnson, 2003).

P_4 action in the breast and during lactation

In the mammary gland P_4 plays a role in the development of lobular-alveolar in preparation for milk secretion and suppression of milk protein synthesis before parturition (Johnson, 2003). *In vivo studies* suggested that progesterone plays a role in the rapid growth of the developing lobular-alveolar of the breast (Masters *et al*., 1977; Going *et al*., 1988; Clarke & Sutherland, 1990). This was evident when an increase in DNA synthesis in the late luteal phase of the normal cycle, which was consistent with an increase in the number of epithelial mitosis, also peaking at the end of the luteal phase (Masters *et al*., 1977).

Progesterone also acts synergistically with PRL, the milk secretion stimulating hormone, to prepare for lactation. During mid to late pregnancy P_4 acts as an anti-PRL to prevent the synthesis of milk proteins in humans (Graham & Clarke, 1997; Johnson, 2003). At the end of pregnancy, progesterone levels decrease resulting in parturition, then follows a sudden increase in PRL secretion, thus stimulating lactation (Johnson, 2003).

The protein, α-Lactalbumin, which also forms part of the lactose synthetase complex, is involved in lactogenesis after parturition and is induced by PRL, insulin, and glucocorticoids (Graham & Clarke, 1997; Johnson, 2003). During mid-pregnancy PR competes with glucocorticoid receptor (GR) in the α-lactalbumin gene to block the induction of α-lactalbumin expression by glucocorticoids. The increased levels of secondary messenger cAMP caused by progesterone during pregnancy are also capable of blocking the stimulation of α-lactalbumin synthesis (Graham & Clarke, 1997; Johnson, 2003). However, in the last days of pregnancy and the beginning of lactation, the synthesis of α-lactalbumin increases significantly exclusive of the induction by glucocorticoids or suppression by progestins, thus increasing the production of lactose (Graham & Clarke, 1997; Johnson, 2003).

The expression of β-casein (milk protein) mRNA is also blocked by P_4 during pregnancy through binding of pregnancy specific factors to the casein gene promoter. Synthesis of this protein is resumed at parturition when the repressor effect of P_4 is lost (Graham & Clarke, 1997; Johnson, 2003).

Progesterone action in the brain

Progesterone together with estrogen is reported to mediate specific functions required for sexually responsive behaviour through the stimulation of γ-aminobutyric acid (GABA) signaling pathways in specific areas of the brain (Graham& Clarke, 1997; Johnson, 2003; Mani & Oyola, 2012). Progesterone-mediated increases in $GABA_A$ receptor binding sites in the brain, areas where PR expression is low or absent, are suggested to contribute to the stimulation of lordosis behaviour in rats and hamsters, suppression of aggressive behaviour, and induction of the release of GnRH (Schmidt-Nielsen, 1990; Graham & Clarke, 1997; Johnson, 2003; Mani & Oyola, 2012).

Hormone-dependent sexual behaviour is stimulated when estrogen and progesterone treatment potentiates the release of norepinephrine release via stimulation by oxytocin from the ventromedial hypothalamus mediated by noradrenergenic projections; and also through affecting the synthesis of the β-endorphin precursor, proopiomelanocortin (POMC) (Graham & Clarke, 1997; Johnson, 2003). Estrogen down-regulates the synthesis while progesterone prevents this down-regulation (Graham & Clarke, 1997). β-endorphin decreases the pituitary secretion of LH and FSH, thus contributing to an increase in sexual receptivity (Graham & Clarke, 1997; Johnson, 2003).

During bone formation, P_4 plays in the modulation of bone mass through its effects on metalloproteinase via the expression of PR which regulates proteinase activity in osteoblasts and through binding to glucocorticoid receptors to prevent bone loss (Graham& Clarke, 1997).

Progesterone role in immune response

Progesterone is also involved in various immune-regulatory functions through interaction with immune cells to allow successful pregnancy maintaining immune balance between mother and foetus. Cabrera-Muñoz and colleagues (2010) reviewed that P_4 exerts an up-regulation to the expression of the molecules from the major histocompatibility class 1 (MHC1) and other molecules such as Human Leukocyte Antigen (HLA)-G and HLA-E in JEG-3 which play a significant role in the immune balance between mother and foetus in the trophoblast and mesenchymal stem cells (MSCs) during the first trimester of pregnancy (Cabrera-Muñoz *et al*., 2010). During pregnancy P_4 exerts activation of the nuclear factor

kappa B (NFκB) and an increase in the expression of the suppressor of cytokine signalling (SOCS1) protein in macrophages during innate immunity (Cabrera-Muñoz *et al*., 2010).

Other reported physiological roles of progesterone include maintenance of normal blood sugar levels, prevention of tumour diseases as well play anti-hormonal effects in parasitic diseases (Johnson, 2003; Cabrera-Muñoz *et al*., 2010).

II. Effects of progesterone on parasite infections

Sex steroids have been reported to play a role on parasitic infections through the modulation of host immune-endocrine network (IEN) and/or direct regulation of parasite reproduction and differentiation (Cabrera-Muñoz *et al*., 2010). P_4 has been reported to play a role on parasitic and viral diseases via both mechanisms (Cabrera-Muñoz *et al*., 2010). The elevated levels of P_4 during pregnancy has an impact on the immune system and incidentally on the resistance or susceptibility to parasite invasions (Lutton & Callard, 2006). The influence exhibited by P_4 on parasitic infections depends in part on P_4 and the parasite species involved. The increase in susceptibility to *Toxoplasma gondii* infection during pregnancy was associated with P_4's influence on immune cells. The immune phenotype was suggested to be as a result of the up-regulation of progesterone-induced blocking factor (PIBF), which inhibits CD8 and NK cell activity, and by the down-regulation of IL-12 caused by P_4 in infected macrophages (Prigione *et al*., 2006; Jones *et al*., 2008). Fleming & Conrad (1989) observed a higher number of worms in ewes infected with the nematode *Haemonchus contortus* subsequent to treatment with P_4.

In contrast, a protective effect of P_4 in other parasitic infections in different host species has also been reported (Cabrera-Muñoz *et al*., 2010). Administration of the P_4 analogue molecule, medroxiprogesterone acetate to female golden hamsters (*Mesocricetus aureatus)* infected with *Schistosoma haematobium* resulted in a decreased number of recovered worms and egg load when the contraceptive was administered with antischistosomial atorvastin (Soliman & Ibrahim, 2005). P_4 administration to ovarectomized female wild rodent *Calomys callosus* resulted in reduced parasite loads of *Trypanosoma cruzi,* suggesting that P_4 restricts *T. cruzi* establishment and development (do Prado *et al*., 1998). A similar helminthotoxicity effect of P_4 has been reported for *T. spiralis* (Nuñez *et al*., 2005; Hernàndez-Bello *et al.*, 2011). *In vitro* exposure of NBL to doses of P_4 resulted in the induction of parasite mortality suggesting

that P_4 can have direct effects on helminthic parasites (Nuñez *et al*., 2005). The toxicity and permissive effects of P_4 therefore depend on the parasite and the host infected (Cabrera-Muñoz *et al*., 2010).

III. Effects of progesterone on Trichinella

The release of progesterone in pregnant animals has also been reported to have an effect on the migration success of *Trichinella spiralis* larvae (Nuñez *et al*., 2008; Hernàndez-Bello *et al.*, 2011). The restrictive effects of P_4 via its direct regulatory effects on the reproduction and differentiation of parasites, has also been reported for *T. spiralis* (Cabrera-Muñoz *et al*. 2010; Hernàndez-Bello *et al.*, 2011). *In vitro* studies showed 35-50 % inhibition of the development of the *T. spiralis* which was attributed to repression of the Ts-Cav-1 gene that is implicated in the maturing and development of NBL (Nuñez *et al*., 2005; Hernàndez-Bello *et al.*, 2011). This is achieved through activation of effector cells that are responsible for the death of NBL in an antibody-independent manner (Nuñez *et al*., 2005).

The interaction between the hormonal micro-environment and immune system determines resistance to *Trichinella* infection; and changes in the levels of steroid hormones during menstrual/oestral cycles influence the composition of cells in the immune system regarding the cytokines they release (Nuñez *et al*., 2002). Th2 responses due to pregnancy, working synergistically with Th2 responses from *T. spiralis* infection, have an effect on parasite reproduction and differentiation, resulting in higher mortality rates in NBL (Nuñez *et al*., 2002; Cabrera-Muñoz *et al*., 2010; Hernàndez-Bello *et al.*, 2011). Therefore, pregnancy resulted in increased resistance against these pathogens compared to virgin mice (Hernandez-Bello *et al.*, 2011). Despite the immune-protective effects stimulated during pregnancy against *T. spiralis* NBL, congenital transmission of the parasite is still possible (Nuñez *et al*., 2002).

IV. Physiology of cortisol

i. Synthesis and secretion

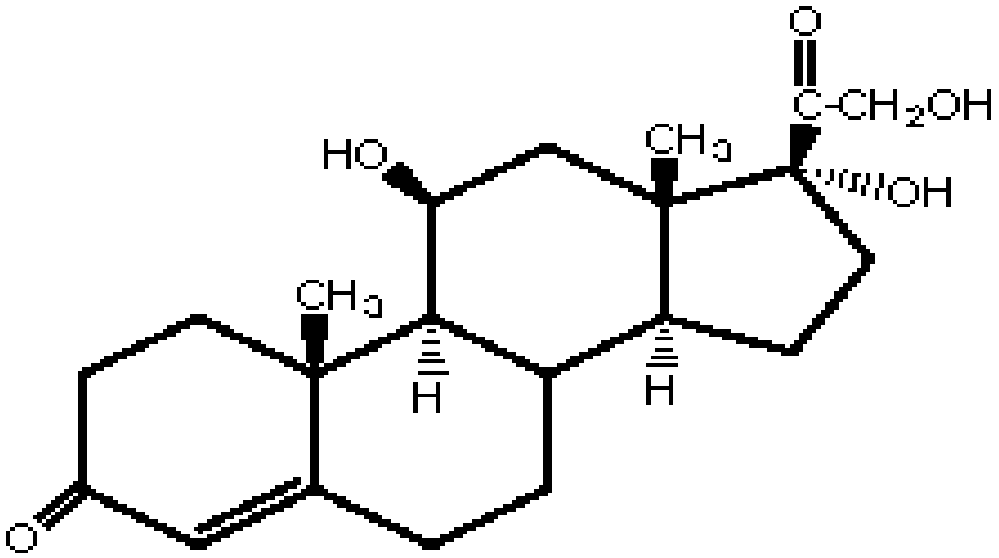


Fig. 4: Chemical structure of cortisol (www.gngfy.org/index.php?title=cortisol)

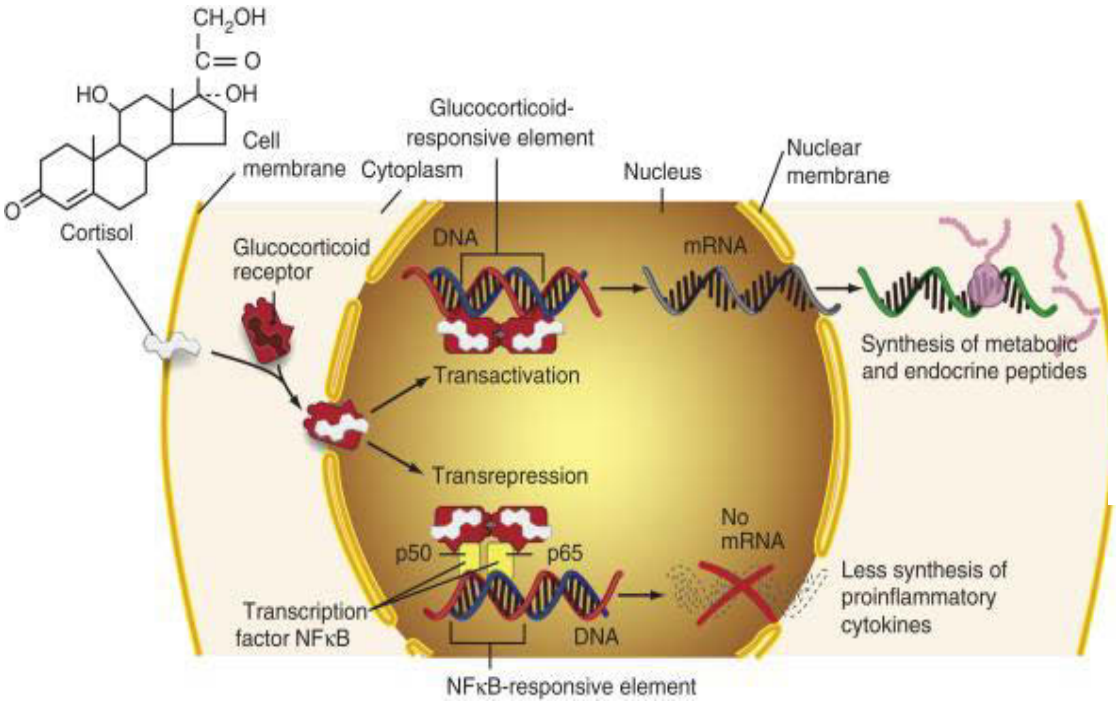
In response to physiological stress and physiological stressors, the adrenal glands, stimulated from the hypothalamic pituitary adrenal axis (HPA) secrete the glucocorticoid steroid, cortisol to restore homeostasis following stress exposure (Pocock & Richards, 2004). The secretion of corticotropin-releasing hormone (CRH) by the hypothalamus triggers anterior pituitary secretion of adrenocorticotropin hormone (ACTH) which is carried by the blood to the adrenal cortex where it is stimulated for the secretion of glucoocorticoids. Cortisol and, to a lesser extent, corticosterone are the main physiological glucocorticoids and are so named due to their ability to maintain carbohydrate reserves. Corticosterone is the major glucocorticoid that is secreted by the adrenal cortex of rats and mice. The secretion of glucocorticoids is influenced by diurnal pattern, stress levels and light and dark cycles. Once released they are carried in the blood mostly bound to proteins, including corticosteroid binding globulin (CBG) and albumin; or carried unbound which makes it easier to pass through plasma membranes where it binds intracellular glucocorticoid receptors.

Synthesis of cortisol takes place in the zona fasculata of the adrenal cortex, while synthesis of corticosterone takes place in the zona glomerulosa (Johnson, 2003). Like all other steroid hormones, glucocorticoids are derived from cholesterol. During cortisol synthesis, ACTH increases the concentration of cholesterol in the inner mitochondrial membrane by stimulating the synthesis of steroid acute regulatory (STAR) protein (Johnson, 2003). The

STAR protein plays significant role in the cholesterol conversion step to pregnenolone by presenting cholesterol to the oxidizing enzyme cytochrome P450. The STAR protein has a very short half-life and the stimulation of its synthesis appears to the essential regulated step in cortisol synthesis (Johnson, 2003).

Cortisol is metabolized by the enzyme 11-β hydrosteroid dehydrogenase (11-βHSD) and P450c11β expressed by cells of the zona fasciculata and reticularis and can only oxidize only carbon 11. Corticosterone, deoxycorticosterone and aldesterone are metabolized by P450c11AS which catalyses oxidation of both carbon 18 and 11 and P450c17 which catalyses the oxidation of carbon 17 in the cells of the zona glomerulosa (Johnson, 2003). The enzyme 11-βHSD is also responsible for the conversion of cortisol to its inactive metabolite, cortisone; and the reverse reaction from cortisone to cortisol (Johnson, 2003).

ii. Mechanism of action



Key:

DNA = Deoxyribonucleic acid

mRNA = messenger Ribonucleic acid

NFκB = Necrosis factor κappa B

Fig. 5: Cortisol mechanism of action (Huisman *et al*., 2006)

Most of serum cortisol is bound to proteins, including corticosteroid binding globulin and serum albumin (Johnson, 2003). Free cortisol that is unbound to protein passes easily through the cellular membranes, where it binds to intracellular cortisol receptors (Fig. 5). Cortisol either works through a classic genomic mechanism when it passes through the cell membrane and binds to GR and heat shock protein in the cytoplasm (Fig. 5) (Huisman *et al*., 2006). This

complex migrates into the nucleus where it binds to glucocorticoid-responsive elements which are regions of DNA that are bound and activated by the glucocorticoid receptor-glucocorticoid complex (Fig. 5). Binding to glucocorticoid-responsive elements leads to either activation or suppression of transcription of target genes; processes which are termed transactivation or transrepression respectively (Fig. 5) (Huisman *et al*., 2006). Transactivation leads to transcription of DNA and is the key mechanism of the adverse metabolic and endocrine effects of glucocorticoids; whereas transrepression leads to the suppression of DNA transcription when the GR binds to NFκB-responsive elements which are regions of DNA that are bound and activated by the transcription factor nuclear factor κB, which consists of protein 50 (p50) and protein 65 (p65) and is the key mechanism of the anti-inflammatory mechanism of glucocorticoids (Fig. 5) (Johnson, 2003; Huisman *et al*., 2006). The non-genomic mechanisms of glucocorticoids occur either via non-specific physicochemical association with the plasma membrane, a receptor-mediated activity or plasma membrane bound form of GR (Fig. 5) (Huisman *et al*., 2006).

iii. GR isoforms

There are two isoforms derived from the gene that encodes for GR, GR-α and GR-β and the alpha isoform binds glucocorticoids, sheds its associated proteins and migrates to the nucleus where it can form homo-dimers that bind to responsive elements in target genes (Johnson, 2003). The beta isoform on the other hand, cannot bind hormone and it is located in the nucleus, but cannot bind DNA, however it can dimerize with the alpha isoform and limit or inhibit the ability of the alpha isoform to activate transcription (Johnson, 2003).

iv. Functions of cortisol

The main functions of cortisol include anti-inflammation through inhibition of the release of proteolytic enzymes from damaged cells, inhibition of pro-inflammatory eicosanoids like prostaglandins and leukotrienes production, and inhibiting the ability of white blood cells to proliferate and respond to IL-1 and 2 (Johnson, 2003). It also increases blood glucose levels through the down-regulation of insulin during glucose synthesis and aids in fat, carbohydrate and protein metabolism (Brunton & Russell, 2008; Castro *et al*., 1980). It also plays a role in water balance as well as foetal lung development during pregnancy (Johnson, 2003).

Energy and metabolism

Cortisol promotes gluconeogenesis by counteracting insulin, contributing to hyperglycaemia-causing gluconeogenesis and inhibiting the peripheral utilization of glucose resulting in insulin resistance by decreasing translocation of glucose transporters mainly GLUT-4 to the plasma membrane (Johnson, 2003; Pocock & Richards, 2004). However, cortisol increases glycogen synthesis in the liver through hepatic actions that enhance the flow of glucose precursors through existing enzymatic machinery; and further induces the synthesis of additional gluconeogenesis and glycogen-forming enzymes along with enzymes required to convert amino acids to usable precursors of carbohydrate (Johnson, 2003). Cortisol also defends against hypoglycaemia by decreasing the utilization of glucose by muscle and adipose tissue and lowers the responsiveness of these tissues to insulin. However excess exposure to cortisol which may be as a result of chronic stress or excess secretion may lead to diabetes mellitus as seen in patients with Cushing's syndrome (Pocock & Richards, 2004).

During protein and fat metabolism, cortisol promotes proteolysis and inhibits protein synthesis in the muscles and lymphoid tissue, causing amino acids to be released in the blood (Johnson, 2003). Furthermore it increases blood glycerol concentration through interaction with other hormones to increase lipolysis in the adipose tissue and thus inhibit lipid synthesis (Johnson, 2003).

Water balance

Cortisol also plays a role in the water balance activity by the kidneys acting as a diuretic hormone to maintain rates of glomerular filtration via the direct effects on glomeruli or glomerular blood flow; or by the inverse stimulation and production of the atrial natriuretic hormone (Johnson, 2003). On the other hand, when the effect of cortisol is shuttered or in the absence of cortisol, the anti-diuretic hormone, vasopressin secretion is increased (Johnson, 2003).

Lung development

One of the main roles of cortisol during late pregnancy is the formation of the foetus lungs. It plays a role in the maturation of the alveoli and the production of surfactant (Johnson, 2003). Although the foetal adrenal glands are capable of secreting cortisol at the 24^{th} week of pregnancy, the major secretory products of the foetal adrenal gland at this stage are androgens that serve as precursors for placental estrogen synthesis (Johnson, 2003). The main problem pertaining to preterm delivery when there are still insufficient concentrations of

cortisol in the placenta is the condition known as respiratory distress syndrome which is caused by impaired pulmonary mechanics leading to incomplete alveolar development and production of surfactant. Supplementation of large doses of cortisol to mothers at risk of premature labour reduces the chance of respiratory distress (Pocock & Richards, 2004).

Anti- inflammatory effects of cortisol

The anti-inflammatory effect of cortisol has been reported as one of its major functions in the body. This is mediated via the modulation of inflammation at local sites of injuries (Johnson, 2003). Inflammation is initiated, sustained and amplified by large number of chemical mediators which include cytokines which are mainly produced by cells of the hematopoietic and immune systems, but can be secreted by any cell; prostaglandins and leukotrienes, mainly released from vascular endothelial cells and macrophages (Johnson, 2003). These can have pro and anti-inflammatory effects depending on the particular compound formed and the cell type acted on. Free cortisol concentrations then increase at the site of tissue injury compared to those in general circulation. The partial degradation of CBG by proteolytic enzyme elastase secreted by mononuclear leukocyte decreases its affinity for binding cortisol and thus increases the concentration of free cortisol at the local site of injury (Johnson, 2003). The inflammatory mediators up-regulate the expression of 11β-HSD1 to convert inactive cortisone to active cortisol (Johnson, 2003)

Suppression of immune system

One of the established effects of cortisol is the suppression of immune responses during infections via its interaction with inflammatory cytokines IL-1 and TNF-α; and by decreasing levels of circulating lymphocytes and white blood cells, particularly, eosinophils (Johnson, 2003; Pocock & Richards, 2004). Cortisol plays a role as negative modulators of IL-1 and TNF-α by inhibiting their production; interfering with signalling pathways, and through the inhibition of the functionality of their products (Johnson, 2003). Cortisol also interferes with the synthesis and releases of other pro-inflammatory cytokines such IFN-γ, IL-2, IL-6 and IL-8 (Johnson, 2003). Production of IL-1 and TNF-α and their effects on target cells are mediated by the activation of genes by NFκB. In the inactive state NFκB resides in the cytoplasm where is bound to NFκB inhibitor IκB (Johnson, 2003). Cortisol then interferes with the effects of IL-1 and TNF-α by promoting the synthesis of IκB which traps NFκB in the cytosol, thus interfering with the ability of NFκB to enter nucleus and activate target

genes (Johnson, 2003). The effect of cortisol and its inactive metabolite, cortisone on the suppression were seen in various diseases including intestinal nematode infections. These effects were also reported during infections with *Trichinella*.

V. Effects of cortisol on Trichinella

In *Trichinella spiralis* infected rats, high exogenous doses of cortisone have been shown to be immune-suppressant (Brunton & Russell, 2008). Cortisone and hydrocortisone have been shown to suppress cellular responses to *T. spiralis* within the intestinal wall thus allowing for an increased parasite establishment and persistence resulting in more larvae migrating to the musculature (Coker, 1955; 1956; Stewart *et al*., 1982; Duran *et al*., 1986).

Most documented studies are on the effect of exogenous and endogenous cortisol in pregnant and non-pregnant mice and rats infected with encapsulated *T. spiralis* (Stewart *et al*., 1982; Duran *et al*., 1986). No studies have been conducted to show the effects of endogenous cortisol on the establishment and larvae migration of *Trichinella spp.* in a natural host. Furthermore, studies conducted on the effects of progesterone on *Trichinella* have mostly focused on comparing levels of infection between pregnant and non-pregnant animals. *In vitro* studies, on the direct effects of progesterone on the parasite stages of development have been done, however, no studies have been done on the effect of different endogenous progesterone concentrations in a natural host system (Hernàndez-Bello *et al.*, 2011; Nuñez *et al*., 2005).

2. Objectives

The main objective of the study was to determine the effects of host sex and pregnancy on *T. zimbabwensis*. In order to achieve this objective, the study was divided into two specific objectives;

1) To determine and compare the establishment and development (adult worms and muscle larvae) of *T. zimbabwensis* in male and female Sprague-Dawley rats.
2) To determine the establishment and development (adult worms and muscle larvae) of *T. zimbabwensis* pregnant and non-pregnant Balb C mice and the levels of progesterone and cortisol in the two groups.

3. Materials and Methods

3. 1 Study animal

A total of 50 Sprague-Dawley rats (25 males & 25 females) aged six weeks old [130-150g body weight (BW)] and 90 female Balb C mice (three groups of 30) aged six weeks [25-30g (BW)] were used for the study. The use of two different animal species was based on the objective of the study and significance. Rodents are the common reservoirs of *Trichinella spp.* in the domestic and sylvatic cycles (Gottstein *et al*., 2009) and it was logical to determine the establishment and development of *Trichinella zimbabwensis* in Sprague-Dawley rats. The study on the effects of pregnancy and levels of progesterone and cortisol was done in Balb C mice and this was influenced by the availability in large quantities to perform the experiments. Furthermore, rats and mice are similar in physiology and have been used interchangeably as hosts in previous *Trichinella* studies (Mankau & Hamilton, 1972; Mukaratiwa *et al*., 2003). Therefore, it was important to use these animals as models for the study.

All the animals were weighed at start of experiment with the weight rounded off to the nearest gram. The animals were housed at the Biomedical Research Unit (BRU) of the University of KwaZulu-Natal (UKZN), in a room subjected to a 12h light/12h dark cycle at a temperature range of 22-24 °C with feed and water provided *ad libitum*. Animals were randomly selected into groups of five animals per cage according to gender for the first study, while animals for the second study were randomly selected into three groups of 30 and were further separated into six animals per cage according to their respective groups. Cage bedding was changed every second day to ensure a clean and stable environment. Ethical clearance for the study was obtained from the UKZN Ethics Committee (069/12/Animal).

3. 2 Parasite strain

The *T. zimbabwensis* strain used in our study was originally isolated from a naturally infected crocodile (*Crocodylus niloticus*) and has been maintained at BRU in rats. For preparation of infective material for the study animals, carcasses of infected rats were digested using a modified protocol of the HCl-pepsin digestion method described by Pozio *et al*., (2002). Muscle tissue from each rat was weighed and for every 100 g of tissue sample, 16 ml of 25% HCl, 20 g of 0.7 U/mg Pepsin(from porcine gastric mucosa), 2L H_2O was used to prepare the digestive fluid. Muscle tissue was digested for 35 min at 37° C in a 2L beaker on a magnetic

stirrer. After sedimentation for 40 min in a separating funnel, the larvae were collected by flushing the bottom 40 ml from the funnel into a 50 ml cylinder. After cleaning the digestion fluid with an additional 10 min sedimentation step, the top 30 ml supernatant in the cylinder was removed and the resulting suspension containing larvae was transferred to a marked petri-dish. The number of larvae was determined using a dissecting microscope 20 x objective. The number of larvae per gram (LPG) of muscle tissue per rat was calculated. The experimental animals were infected by a once-off gastric gavage of 7 LPG of animal for Sprague-Dawley rats and 50 LPG for Balb C mice.

3. 3 Study Design 1

Experiment 1: ***Effect of host sex on parasite establishment and development in Sprague-Dawley rats***

Fifty Sprague-Dawley rats were randomly selected into 25 males and 25 females and infected with *T. zimbabwensis* larvae by gastric gavage at a dose of 7 LPG per animal using an 18 G curved oral dosing needle. On days 5, 10, 15, 20 and 25 post-infection, five animals were humanely sacrificed from each of the two groups using a terminal dose of halothane, to determine establishment of infection in the intestines and in the muscle tissue. Infection was defined as the establishment of larvae in the muscle cells as a result of successful mating of adult parasites in the intestine and was detected by the digestion of muscle tissue (Takumi *et al.*, 2010).

Recovery of adult worms from the intestines was achieved using a modification of the protocol by Mukaratirwa *et al*., (2003). The small intestines were immersed in 0.85 % saline solution, split open longitudinally with scissors and washed with H_2O under 212µm sieve. After washing, the intestinal tissues were further incubated for 1hr at 37 ºC and then re-washed under a 212 µm. The washings were viewed under dissecting microscope at 20 x objective for adult worm counts; whereas larvae in muscles were recovered using the modified artificial digestion protocol as described by Pozio *et al*., (2002).

3. 4 Study Design 2

Experiment 2: Effects of pregnancy on parasite establishment and development in Balb C mice

Ninety female mice were randomly divided into three groups. In the first group (group 1); animals were infected, but not mated. The second group (group 2); animals were mated but were not infected. The third group (group 3); animals were both mated and infected. Infected animals were given a parasite dose of 50 LPG of animal by gastric gavage. Group 4 of neither mated nor infected animals was added to the study as a control group. Animals in group 1 were infected on day 0 of trial. Mating in group 2 and 3 females by pairing the female with male Balb C mice on day 0 of trial in a 2:1 ratio and enclosed in the respective cages for 24 hours to allow for mating. Confirmation of conception was done by checking for a vaginal plug twice a day following mating (morning and afternoon) (Cui *et al*., 2006) and presence of a vaginal plug was taken as 1st day of pregnancy. On day 7 of pregnancy, animals from group 3 were infected with the same dose of larvae as group 1.

On days 0, 7, 14, 21, 28 post-infection for group 1; days 0, 7, 14, 21 and 28 post-mating for group 2 and on days 7, 14, 21, 28 and 35 post-mating for group 3, six animals were sacrificed from each group using a terminal dose of halothane. Recovery of adult worms from the intestines was done using a modification of the protocol described by Mukaratirwa *et al.*, (2003) and muscle larvae were recovered as described by Pozio *et al*., (2002).

On days of sacrifice, blood was collected from mice on the days of sacrifice by cardiac puncture into 1 ml blood tubes containing clotting activator gel (dvac gel and clot activator tubes). The blood was subject to centrifugation using a Thermo scientific Heraeus Labofuge 200 microprocessor controlled table top centrifuge at 132 xg for 5 - 10 minutes. Following centrifugation, serum was collected into Eppendorf micro-centrifuge tubes and stored and -56 to -60 °C for later analysis. Serum cortisol and progesterone concentrations were measured using ELISA. Baseline levels of cortisol and progesterone at day 0 of trial were extrapolated from values obtained from group 2 prior to mating. Immediately after birth, pups were humanely sacrificed and screened for muscle larvae using the aforementioned method by Pozio *et al*., (2002).

Progesterone levels for group 4 were extrapolated from normal standard average progesterone levels of mice during estrous cycle, which were derived from Loeb & Quimby (1989), assuming that the experimental animals were at estrus on day 0 of trial [See appendix, Fig. 11 & Table 6 (pg. 63 & 64)]. Cortisol levels for group 4 were estimated from normal average cortisol levels of mice according to the normal serum cortisol level range values in mice that were derived from Dracott & Smith (1979), relative to the time of day blood samples were collected from the other groups when taking into account the diurnal concentrations of cortisol [See appendix, Table 7 (pg. 64)] Normal serum cortisol levels in mice ranges from 3 - 12 µg/100 ml (Dracott & Smith, 1979) which is equivalent to 3 000 - 12 000 pg/ml during the diurnal pattern of cortisol. The levels of blood cortisol peak in the early morning (approximately 8 am) and during the day reaching its lowest levels at between midnight and 4 am.

3. 5 Measuring serum cortisol and progesterone levels

Serum progesterone concentrations were measured using Demeditec Progesterone rat/mouse ELISA kit (No. DEV9988) from Biocombiotech. This is a solid-phase enzyme-linked immunosorbent assay based on competitive binding. An unknown amount of progesterone present in the sample and a defined amount of progesterone conjugated to horseradish peroxidase compete for the binding site of progesterone antiserum coated in the wells of the microplate. The assay procedure was conducted according to the manufacturer's instructions. A progesterone standard series (0, 0.4, 1.5, 6.5, 25 and 100) ng/mL was provided with the kit.

The 96 well microplate was divided by columns according to the days of sacrifice, however the first two columns were reserved for duplicates of the standards. 25 µL of each calibrator and sample were dispensed into appropriate wells. 50 µL of incubation buffer was added to each well, followed by the addition of 100 µL enzyme conjugate. The microplate was then incubated for 1hr at room temperature on a microplate mixer. The microplate was then washed four times after incubation. After adding 200 µL of tetramethylbenzidine (TMB) substrate solution to initiate the blue colour-forming reaction, the plate was further incubated for 30 minutes, followed by addition of 50 µL of 2 N hydrochloric acid stop solution to each well to yield a yellow colour. The microplate absorbance of each well was measured using a microplate reader at 450 nm. The concentration of progesterone was inversely proportional to the optical densities measured. Standards were used to plot standard curve of absorbance

versus concentration with the progesterone concentrations of samples determined through interpolation. The concentrations of progesterone in samples were determined using immunoassay software that measured optical densities and concentrations in samples. To convert to SI units (nmol/L), the following formula was used:

Progesterone conc. (ng/mL) × 3 = nmol/L.

The assay was chosen for it high sensitivity as it can detect as low as 0.04 ng/mL concentrations of progesterone from the zero calibrator. The cross-reactivity to related steroid compounds are given as percentages at 50 % displacement compared to progesterone: androstenedione (< 0.10 %); androsterone (< 0.10 %); corticosterone (0.30 %); 11-deoxycorticosterone (1.80 %); 5α-dihydrotestosterone (< 0.10 %); prednisone (< 0.10 %); estosterone (0.14 %); estradiol (< 0.10); estriol (< 0.10%); prednisolone (< 0.10 %); and pregnenolone (5.5 %).

Serum cortisol concentrations were measured using the Enzo Life Sciences Cortisol EIA kit (No. ADI-900-071) purchased from Biocombiotech, which measures the amount of cortisol in serum, plasma, saliva, urine and faeces samples from any species. For quantitative measure of cortisol, the kit uses a monoclonal antibody to cortisol that binds in a competitive manner to cortisol in a sample or an alkaline phosphatase molecule which has cortisol covalently attached to it. The assay procedure was conducted according to manufactures instructions. A 100 000 pg/mL standard solution of cortisol was provided with the kit and diluted with assay buffer to a standard series of (10 000, 5 000, 2 500, 1 250, 625, 313, 156) pg/mL to be used in the assay procedure.

The 96 well microplate was divided by columns; the first column was reserved for the Assay buffer, NSB, Bo, Total activity (TA) and blank wells according to the manufacturer's instructions. The second and third columns were reserved for the duplicates of the standards. The remainder of the wells were reserved for the samples and divided into columns according to the days of sacrifice. 100 µl of sample and standards were dispensed into appropriate wells. After a simultaneous incubation at room temperature, excess reagents were washed away and 200 µL of ρ-Nitrophenyl Phosphate (PNPP) substrate was added and a yellow colour generated. After a short incubation time the enzyme reaction was stopped with

trisodium phosphate solution. The microplate absorbances were read at 405 nm. The intensity of the bound yellow colour was inversely proportional to cortisol concentrations either of the standards or the samples. The measured optical densities were used to calculate concentrations of cortisol. Standards were used to plot standard curve of percentage of bound cortisol versus concentrations of standards with the cortisol concentrations of samples determined through interpolation. The following equation was used to calculate the concentrations of cortisol:

1. The average net Optical Density (OD) bound for each standard and sample was calculated by subtracting the average NSB OD from the average OD bound:

Average Net OD = Average Bound OD - Average NSB OD

2. The binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), was calculated using the following formula:

Percent Bound = Net OD x 100 Net Bo OD

3. Using Logit-Log paper, Percent Bound versus Concentration of Cortisol for the standards curve was plotted. Approximating a straight line through the points, the concentration of cortisol in the unknowns was determined by interpolation.

The assay has a sensitivity of 56.72 pg/mL, which allows for detection of low cortisol that range from 156 - 10 000 pg/mL from biological matrices. The cross-reactivity to related steroid compounds are given as percentages: Cortisol (100 %); Prednisolone (122.35 %); Corticosterone (27.68 %); 11-Deoxycortisol (4.0 %); Progesterone (3.64 %); Prednisone (0.85 %); Testosterone (0.12 %) and < 0.10 % with Androstenedione, Cortisone and Estradiol.

3. 6 Data analysis

The parameters measured were the number of adult *T. zimbabwensis* in the small intestines, reproductive status of adult female parasite recovered from the intestines and the reproductive capacity index (RCI). The RCI was calculated as the number of muscle larvae were recovered

divided by the number of larvae inoculated (Krivokapich *et al*., 2012). The reproductive status of each female parasite from the intestines was evaluated by observing in a female parasite the presence or non-presence of larvae in the uterus. A female parasite was considered as "gravid" when there was presence of larvae in the uterus and as "non-gravid" when there was no presence of larvae in the uterus.

All data were expressed as mean ± standard error of mean (SEM) using Microsoft Office Excel 2010. Two-way analysis of variance (ANOVA) with accompanying Tukey's post-hoc analysis for parametric data was used to compare mean values of adult worms and mean values of muscle larvae between male and female rats over time of exposure to infection. One-way ANOVA was used to compare mean number of female, gravid, non-gravid and male adult worms recovered within and between groups of animals (male and female rats). The statistical package from Graph-Pad Prism 5 was used and $P < 0.05$ was considered significant. Two-way ANOVA accompanied by Tukey's post-hoc analysis for parametric data was used to compare mean concentrations of cortisol and mean concentrations of progesterone in pregnant and non-pregnant mice over time. One-way ANOVA was used to compare mean number of female, gravid, non-gravid and male adult worms recovered between and within each group of animals (pregnant and non-pregnant). A non-parametric Spearman correlation analysis was used to examine the relationship between parasite establishment and the levels of progesterone and the relationship between parasite establishment and of cortisol levels post-infection. The statistical package SPSS 21 was used and $P < 0.05$ was considered significant.

4. Results

4. 1 Effects of host sex on T. zimbabwensis establishment in Sprague-Dawley rats

4. 1. 1 Establishment of in the gastrointestinal tract

The establishment of adult parasites of *T. zimbabwensis* in male and female Sprague-Dawley rats is shown in Table 2. The establishment of adult worms was significantly higher in male rats than in female rats at days 5 PI ($P > 0.05$). There were no significant differences in the number of adult worms between males and females at day 10 PI. No adults were observed in both groups after day 10 PI (Table 2 and Fig. 6).

The mean ± SEM numbers of adult female and male of *T. zimbabwensis* and the reproductive status of the female parasites in male and female Sprague-Dawley rats are shown in Table 3. The mean number of female adult worms was significantly higher ($P < 0.05$) than male adult worms in male rats at day 5 PI. The mean number of gravid females recovered in male rats at day 5 PI was significantly higher ($P < 0.05$) than mean number of non-gravid females (Table 3). A similar trend was observed in male rats at day 10 PI (Table 3). The mean number of female adult worms was significantly higher ($P < 0.05$) than male adult worms in female rats at day 5 with a significantly higher ($P < 0.05$) number of gravid females recovered than non-gravid females (Table 3). A similar trend in female rats was observed at day 10 PI; however no non-gravid females were recovered on that day. The female to male ratio of adult worms in male rats was higher, 35:1 at day 5 post-infection than 6:1at day 10 post-infection; whilst it was the reverse in female rats (Table3). In female rats, the female to male ratio was lower, 29:1 at day 5 post-infection than 94:1 at day 10 post-infection (Table 3).

4. 1. 2 Establishment of T. zimbabwensis larvae in the muscles

The establishment of *T. zimbabwensis* muscle larvae was significantly higher ($P < 0.05$) in male rats than in female rats at day 25 PI. No larvae were recovered on other days of sacrifice except at day 25 PI (Table 2). Reproductive capacity index (RCI) was significantly higher in male rats than in females (Table 2) as indicated by the number of larvae recovered.

Table 2: Mean (± SEM) of *Trichinella zimbabwensis* adult worms and muscle larvae recovered from male and female Sprague-Dawley rats at different days post-infection and the reproductive capacity index

Groups	No. of animals	Infection dose (LPG) p/a	Day sacrificed post-infection	Mean ± SEM of total adult worms recovered	Mean ± SEM number of larvae recovered	RCI
Males	5	7	5	$545.8^{a} \pm 88.85$	0	0
Females	5	7	5	$84.6^{b} \pm 24.03$	0	0
Males	5	7	10	$156^{a} \pm 24.95$	0	0
Females	5	7	10	$38.2^{a} \pm 23.20$	0	0
Males	5	7	15	0	0	0
Females	5	7	15	0	0	0
Males	5	7	20	0	0	0
Females			20	0	0	0
Males	5	7	25	0	$2366.40^{b} \pm 608.72$	2.37
Females	5	7	25	0	$981.80^{c} \pm 85.99$	0.98

SEM = standard error of means, p/a = per animal, RCI = reproductive capacity index, LPG = larvae per gram of animal

Different superscript letters indicate significant difference ($P < 0.05$)

Table 3: Distribution by sex of *Trichinella zimbabwensis* adult worms recovered from the intestines of male and female Sprague-Dawley rats and reproductive status of the female parasites at different days post-infection

Groups	No. of animals per group	Mean larval dose (LPG) p/a	Day sacrificed post-infection	Mean ± SEM adult worms recovered				Female/Male Ratio
				Gravid Females	Non-Gravid Females	Females	Males	
Males	5	7	5	$503.6^{a} \pm 88.09$	$27.2^{d} \pm 1.46$	$530.8^{a} \pm 89.55$	$15.0^{e} \pm 3.23$	35:1
Females	5	7	5	$74^{b} \pm 23.78$	$7.8^{e} \pm 3.60$	$81.8^{b} \pm 27.23$	$2.8^{e} \pm 0.37$	29:1
Males	5	7	10	$106.2^{c} \pm 11.43$	$28.6^{d} \pm 8.57$	$134.8^{c} \pm 20.00$	$21.6^{d} \pm 5.65$	6:1
Females	5	7	10	$37.8^{d} \pm 23.05$	0 ± 0.00	$37.8^{d} \pm 23.05$	$0.4^{f} \pm 0.25$	94:1

SEM = standard error of mean, p/a = per animal, LPG = larvae per gram of animal

Different superscript letters indicate significant difference ($P < 0.05$)

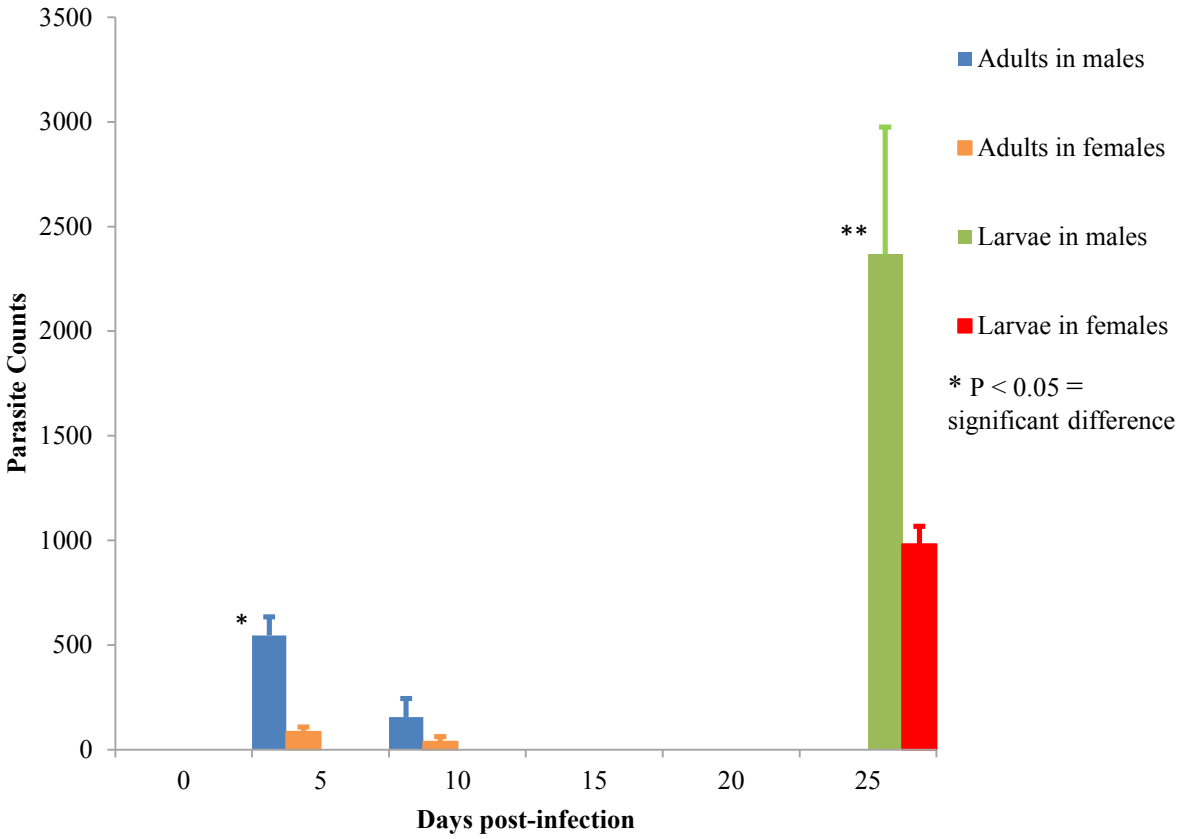


Fig. 6: Comparison of establishment of *Trichinella zimbabwensis* in male and female Sprague-Dawley rats at various days post-infection

4. 2 Effects of pregnancy on parasite establishment and development in Balb C mice

4. 2. 1 Establishment and development of T. zimbabwensis in pregnant and non-pregnant Balb C mice

The establishment and development of *T. zimbabwensis* in pregnant and non-pregnant Balb C mice is shown in Table 4. There were no significant differences ($P > 0.05$) in mean number of adult worms between non-pregnant and pregnant Balb C mice at days 7 and 14 PI (Table 4). There were no significant differences ($P > 0.05$) in number of males, females, gravid and non-gravid adult worms between non-pregnant and pregnant animals at days 7 and 14 PI (Table 4).

The number of female adult worms seemed higher than male adult worms in pregnant mice at day 7 PI, with gravid females higher than non-gravid females (Table 5); however the differences were not significant ($P > 0.05$). The number of female adult worms was equal to the number of male adult of worms at day 14 PI in pregnant mice (Table 5). Only gravid females were recovered and no observation of non-gravid females at day 14 PI (Table 5). The same trends were observed in non-pregnant mice at days 7 and 14 PI, except on day 14 where the number of adult females were higher than male adult worms (Table 5). The female to male ratio was higher, 8:1 at day 7 PI than 1:1 at day 14 PI in pregnant mice. A similar trend was observed in non-pregnant mice, female to male ratio was higher, 32:1 at day 7 PI than 9:1 at day 14 PI. There were no muscle larvae recovered in pregnant mice whereas there were larvae recovered from non-pregnant mice at days 21 and 28 post-infection (Table 4).

4. 3 Levels of progesterone and cortisol in pregnant and non-pregnant Balb C mice

Pregnant mice, as expected, had significantly higher progesterone than the non-pregnant mice ($P < 0.05$) at days 7 and 14 post-mating, peaking at day 7 after which levels decreased and were at lowest at day 21, slightly increased from day 21 to 28 and decreased again from day 28 to day 35 post-mating in pregnant, infected mice; whereas in pregnant, non-infected mice levels decreased until last day of sacrifice and were at lowest at day 28 post-mating (Fig. 7). Peak concentrations in pregnant, infected and pregnant, non-infected mice at day 7 post-mating were 219.5 ± 10.78 and 219.5 ± 10.49nmol/L, respectively (Fig. 7). Progesterone levels were thus at peak levels at day 0 of infection and lowest at day 14 PI in pregnant mice. Progesterone levels in non-pregnant, infected mice increased from day 0 to 7 PI after which

levels decreased and were at lowest at day 14 PI. Levels increased after day 14 until the last day of sacrifice, 28 PI (Fig. 7). There was no correlation observed between infection establishment and progesterone levels on days of sacrifice PI ($r = - 0.210$; $P > 0.05$) (Fig. 9).

There were no significant differences observed in cortisol levels between the three experimental groups. However, levels seemed to be higher in pregnant animals compared to non-pregnant animals except at day 28 post-infection in the non-pregnant, infected group (Fig. 8). Cortisol levels were at peak at 14 days post-mating in pregnant, non-infected mice. Levels seemed to be higher in pregnant, non-infected than pregnant infected mice throughout the study, except at days 0 and 7 post-mating where levels were equal in both groups. Cortisol reached peak at day 35 post-mating in pregnant, infected mice (Fig. 8). No correlation was observed between infection establishment and cortisol levels on days of sacrifice ($r = 0. 139$; $P > 0.05$).

Table 4: Mean (± SEM) of *Trichinella zimbabwensis* adult worms and muscle larvae recovered from pregnant and non-pregnant Balb C mice at different days post-infection and the reproductive capacity index (RCI)

Groups	No. of animals	Mean larval dose (LPG) p/a	Days post- infection	Mean ± SEM adult worms recovered	Mean ± SEM larvae recovered	RCI
Pregnant	6	50	7	11.5^{a} ± 2.72	0	0
Non- pregnant	6	50	7	26.5^{a} ± 4.43	0	0
Pregnant	6	50	14	1.8^{a} ± 1.28	0	0
Non- pregnant	6	50	14	2.0^{a} ± 0.93	0	0
Pregnant	6	50	21	0	0	0
Non- pregnant	6	50	21	0	859 ± 305.09	0.66
Pregnant	6	50	28	0	0	0
Non- pregnant	6	50	28	0	3063± 335.60	2.62

SEM = standard error of means, RCI = reproductive capacity index, LPG = larvae per gram of animal

Table 5: Distribution by sex of *Trichinella zimbabwensis* adult worms recovered from the intestines of pregnant and non-pregnant Balb C mice and the reproductive status of female parasites at different days post-infection

Groups	No. of animals per group	Days of sacrifice post-infection	Mean larval dose (LPG)	Mean ± SEM adult worms recovered				Female/ Male Ratio
				Gravid Females	Non-Gravid Females	Total females	Males	
Pregnant	6	7	50	$9.7^{a} \pm 2.85$	$0.5^{a} \pm 0.34$	$10.2^{a} \pm 2.98$	$1.3^{a} \pm 0.72$	8:1
Non- pregnant	6	7	50	$25.5^{a} \pm 4.15$	$0.3^{a} \pm 0.211$	$25.8^{a} \pm 4.23$	$0.8^{a} \pm 0.31$	32:1
Pregnant	6	14	50	$1.8^{a} \pm 1.28$	0 ± 0	$1.8^{a} \pm 1.28$	$1.8^{a} \pm 1.28$	1:1
Non- pregnant	6	14	50	$1.8^{a} \pm 0.87$	0 ± 0	$1.8^{a} \pm 0.87$	$0.2^{a} \pm 0.17$	9:1

SEM = standard error of means, LPG = larvae per gram

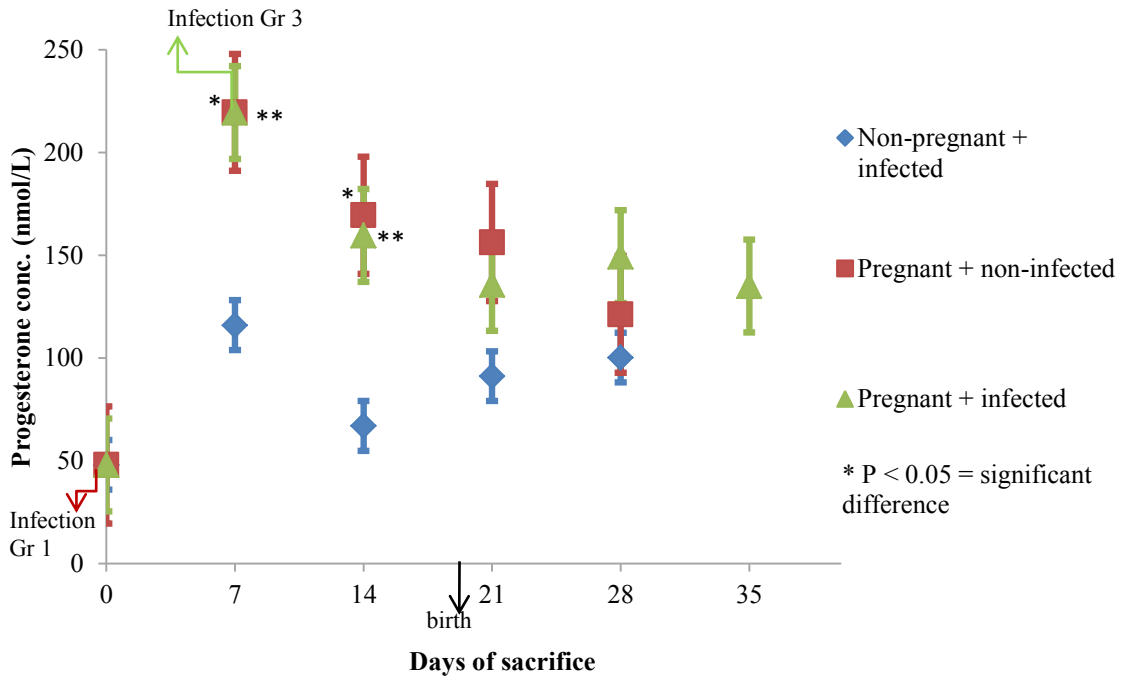


Fig. 7: Serum progesterone levels (nmol/L) in pregnant and non-pregnant Balb C mice infected with *Trichinella zimbabwensis*

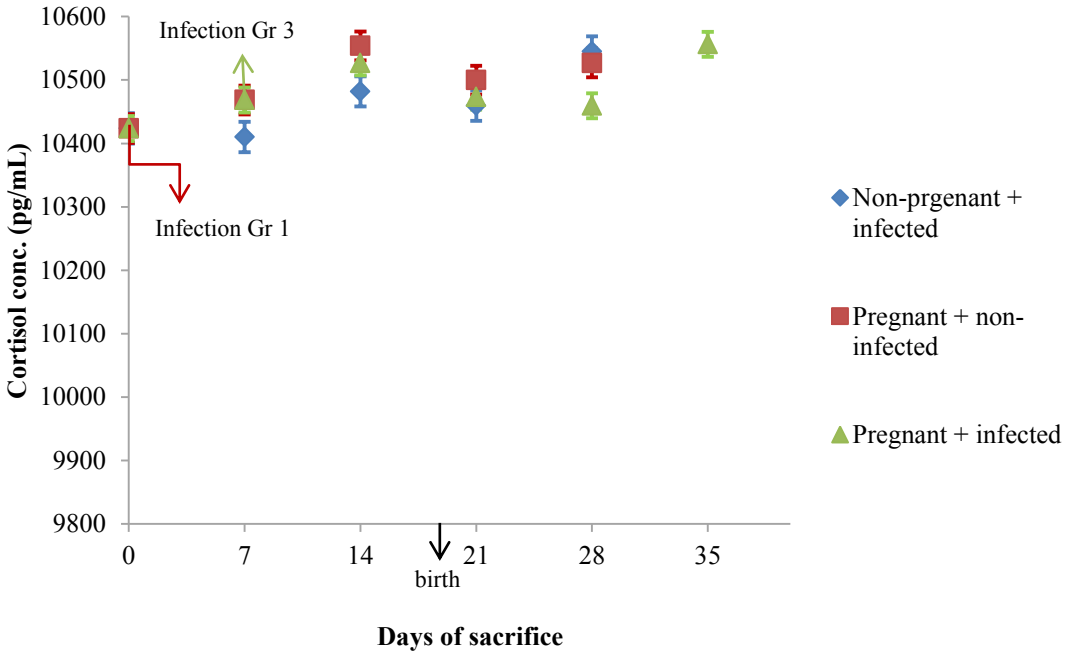


Fig. 8: Serum cortisol level (pg/mL) in pregnant and non-pregnant Balb C mice infected with *Trichinella zimbabwensis*

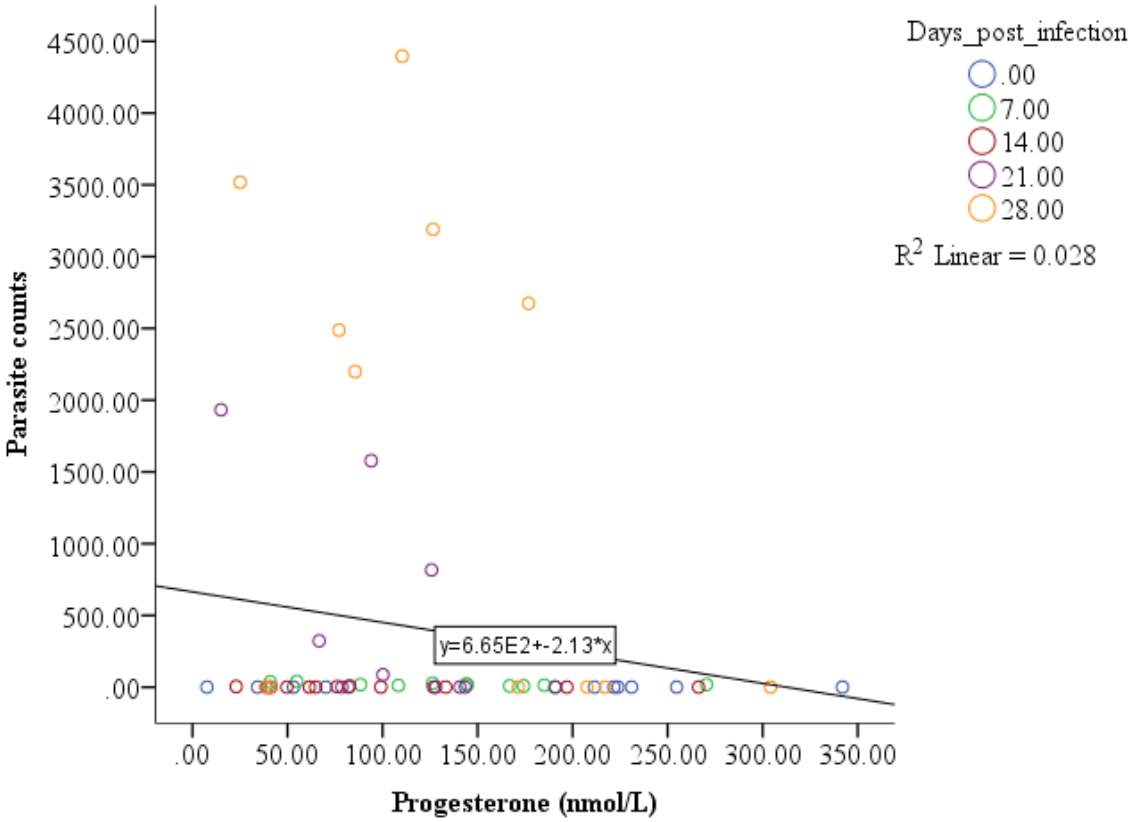


Fig. 9: Comparison of *Trichinella zimbabwensis* establishment (adults and muscle larvae) in relation to serum progesterone levels between pregnant and non-pregnant Balb C mice

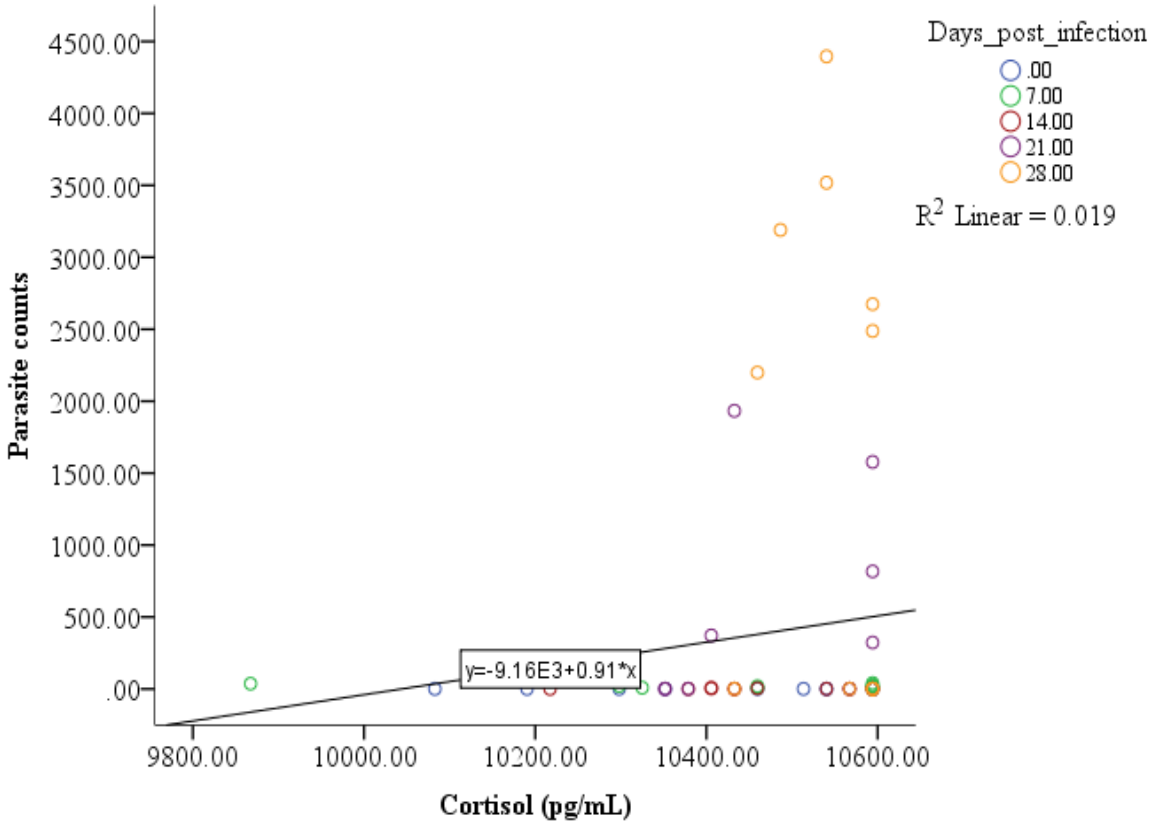


Fig. 10: Correlation between *Trichinella zimbabwensis* infection (adult worms and muscle larvae) and serum cortisol levels in Balb C mice

4. 4 Congenital transmission

The mean gestation period for groups 2 and 3 was 19 days and group 2 had an average number of 10 ± 1.51 pups while group 3 had an average of 8 ± 0.88 pups. All the pups born from dams of the different groups were negative for congenital infection.

5. Discussion

Results from this study showed that establishment of *T. zimbabwensis* in Sprague-Dawley rats is influenced by host sex, with a higher establishment of adults and larvae in males than in females (Table 2; Fig. 6). These results are in agreement with the results by Mankau & Hamilton (1972) on *T. spiralis* where establishment was three times higher in males than females. This suggests that there is a sex difference in the response to *Trichinella* infection with susceptibility and intensity more biased towards males than females and resistance more towards females. This sexual difference in exposure and susceptibility in parasitic infections has also been reported for several other parasite species in different host species with prevalence and intensity more biased towards males than females (Klein, 2000; Klein, 2004). However, these male-biased infections are parasite and/or host specific (Klein, 2000; Klein, 2004).

In a variety of host species including humans, rodents, birds and lizards, male biased infection rates are mostly detected in protozoan and nematode parasites (Klein, 2004). In humans, it is believed that these male-biased infections are the leading cause of increased death rates among men as compared to women (Owens, 2002). The prevalence and intensity of infection with *Leishmania*, *Plasmodium*, *Entamoeba*, *Necator*, and *Schistosoma* parasites, for example, is higher among males than females (Klein, 2004). *Plasmodium* species have been reported to cause sexual dimorphism in vertebrate host species (Landgraf *et al*., 1994). Although the incidences of infection are generally similar between sexes in humans, sex differences were reported in the intensity of infection in which men had higher parasitaemia than women (Wilding *et al*., 1995). Studies of rodent malarias where mice were infected with *P. berghei* or *P. chabaundi,* males showed higher mortality rates than females (Benten *et al*., 1997). Epidemiological studies on *Leshmania* infections revealed that men are more susceptible to infection than women (Weigle *et al*., 1993). Studies reported in pre-pubertal children, showed that boys are more prone to develop visceral leshmaniasis than girls (Shiddo

et al., 1995). Experimental studies on *Leshmania* in mice showed that males are more susceptible to *L. major* and *L. mexicana* than females (Satoskar *et al*., 1998). Studies on *Schistosoma* in humans revealed that among children and adults, the intensity and prevalence of infection was higher in males than females (Degu *et al*., 2002).

Although the prevalence, intensity and susceptibility to parasitic infections have been reported to be more biased towards males, there are parasites for which this sex difference is reversed. For example, female mice *(Mus musculus)* are more susceptible to infections of *Schistosoma mansoni*, *Babesia macroti, Taenia crassiceps* and *Toxoplasma gondii* (Pung & Luster, 1986; Larralde *et* al., 1995; Aguilar-Delfin *et al*., 2001). Female mice infected *T. gondii* developed brain inflammation and had more probable deaths following infection than males (Walker *et al*., 1997). Studies on *Taenia crassiceps* infected mice revealed that females developed more cysticerci than males (Larralde *et al*., 1995). Although in humans *S. mansoni* establishes better in males than in females, female mice are more susceptible to this parasite than males (Eloi-Santoz *et al*., 1992).

It is suggested that sex differences in exposure and susceptibility to infections could be attributed to genetic, behavioural and social patterns of hosts which may underlie the likelihood of males to be obvious targets to parasitism (Klein, 2000; Klein 2004). Experimental evidence suggested that females possess resistance genes than males (Satoskar *et al*., 1998). For example, studies of backcross and F2 recombinant inbred strains of mice (from parental C57BL/6 and DBA/2 mice) revealed that the effect of *Scl-2* (single locus on chromosome 4 that maps "no lesion growth") on resistance to *L. mexicana* is more pronounced among female than male mice (Satoskar *et al*., 1998).

In addition to genetic differences, behavioural variation between the sexes can result in differential exposure and contact with pathogens (Klein, 2000). Males tend to be more involved in aggressive behaviours and this increases chances of exposure to pathogens and may underlie the increased prevalence of infections among males as compared to females. Aggressive encounters are hypothesized to be a mode of transmission for several pathogens (Klein, 2000; Klein, 2004). For example, simian immunodeficiency virus is spread among colonies of male mandrills (*Mandrillus sphinx*) via aggressive encounters (Klein, 2004). Similarly, hantaviruses are hypothesized to be spread through aggression in rodents (Klein, 2000; Klein, 2004). In wild-caught rats (*Rattus norvegicus*), the severity and prevalence of

wounding is higher in adult males as they engage more in aggressive behaviours than juvenile males or females, and is correlated with the prevalence and intensity of hantavirus infection (Klein, 2000; Klein, 2004). Studies of mice (*Mus musculus*) suggest that high ranking, dominant males engage in more aggressive encounters and are more susceptible to infection with nematodes, such as *Heligmosomoides polygyrus*, protozoa, including *Babesia microti*, and viruses, such as herpes simplex, than less aggressive subordinate males (Klein, 2000; Klein, 2004). Therefore, greater involvement in aggression results in greater chances of exposure to infections (Klein, 2000; Klein, 2004).

Dispersal is another behavioural pattern that influences greater exposure to parasitism in males than females (Klein, 2000; Klein, 2004). For example, among Belding's ground squirrels (*Spermophilus beldingi*) and prairie voles (*Microtus ochrogaster*), males leave their natal burrow just before they become reproductively active and females remain at their natal range (Klein, 2000). This may increase the chances of exposure and susceptibility to foreign parasites (Klein, 2000). The act of dispersal is more likely to stimulate aggression among certain animal species, especially in the wild as the animals like to mark and protect their territories. Such behaviours may increase the chances of cannibalism and predation (Klein, 2000). These could be probable modes of transmission for *Trichinella spp.* especially in the sylvatic cycle and this may underlie the male-biased *Trichinella* infections as seen in the present study (Table 2; Fig. 6). However, results from the present study and previous studies on sex differences in *Trichinella* infected rodents represent male-biased infections that occur in controlled environments. It was suggested that these observations on sex differences in parasite infections including *Trichinella* were mediated by the differences in immune responses and the immune-endocrine interactions that exists between males and females among different host species during parasitic infections and these in-turn have been linked to circulating sex-steroids (Roberts *et al*., 1996; Klein, 2004).

Sex differences in immune function are well distinguished in vertebrates (Klein, 2004). Males generally express lower innate response, antibody-mediated responses and cellular responses than females during parasitic infections (Klein, 2004). Innate immunity is characterised as the first line of defence against parasites. These responses do not require prior exposure or sensitization; they can be initiated immediately subsequent to a novel parasite (Klein, 2004). Humoral immune responses are characterised by antibody production via B-cells and are usually greater in females than males. Cell-mediated immune responses also differ between

males and females. T-cells, in particular CD4 + helper T-cells (Th cells), are functionally and phenotypically heterogeneous and can be differentiated based on the cytokines they release (Klein, 2004). Reliance on subsets of Th cells (i.e. Th1 or Th2 cells) to overcome infection differs between males and females, with females reportedly exhibiting higher Th2 responses (i.e. higher IL-4, IL-5, IL-6, and IL-10 production) than males (Klein, 2004).

Studies of both humans and rodents illustrate that inflammatory immune responses are generally higher in females than males (Roberts *et al*., 1996). In the present study, the high intensity of both the adult stages recovered from the gastrointestinal tract at the early stages of infection and the developmental larval stages of *T. zimbabwensis* recovered from the muscles at the late stages of infection in male *Sprague-Dawley* rats as compared to females (Table 2), suggests that females have a stronger immunity than males. This may also be one of the primary factors to the sex difference observed by Mankau & Hamilton (1972) in rats infected with *T. spiralis*. Typically, during *Trichinella* infection, the parasite modulates host immunological responses by inducing Th2 (IL-4, IL-5 and IL-13) responses and the production of regulatory cytokines, IL-10, IFN-γ and TGF-β (Wakelin *et al.*, 1994; Morales *et al*., 2002; Beiting *et al*., 2007). Antibodies and cytokines contribute to successful establishment and development of *Trichinella* by implementing expulsion, reduction in numbers of worms, fecundity and killing of NBL (Wakelin *et al*., 1994). In the present study, the reduction in the number of adult worms and muscle larvae in females as compared to males, suggests that the immunity employed by females relies more on Th2 responses which further suggests that females have greater immunity against *Trichinella* infections than males in rodent species.

Studies on other parasites in different host species reported similar observations. Female mice infected with *Schistosoma mansoni* showed stronger delayed-type hypersensitivity reactions than males (Eloi-Santoz *et al*., 1992). The number and activity of cells associated with innate immunity also differs between the sexes (Klein, 2004). The activity of phagocytic cells, including macrophages and neutrophils which can kill parasites by generating reactive oxygen metabolites and nitric oxide, as well as by secreting enzymes is higher in females than males among humans and lizards (Klein, 2004). Elevated humoral immunity was observed in female mice infected with the parasite *Giardia muris*, where infection rates were lower and antibody production higher as compared to males (Klein, 2004).

Clinical and experimental evidence suggests that sex hormones regulate the immune system, based on observations that include the existence of sexual dimorphism in immune responses; the alteration of immune response due to gonadectomy and sex steroid replacement; alterations of immune responses during pregnancy when amounts of sex steroid hormone is increased and the presence of specific receptors for gonadal steroids in the organs that are responsible for immune responses (Grossman, 1985; Klein, 2004; Escobedo *et al*., 2011). It is therefore this interaction between immune system and sex steroid hormones that play a significant role in determining how different sexes respond to infections and this immune-endocrine interaction may be parasite-specific (Grossman, 1985; Lutton & Callard, 2006).

The male-biased parasitism observed for *T. zimbabwensis* in the present study (Table 2; Fig. 6), might be due to the immune-suppressive effect of the androgenic hormone, testosterone, as it has been reported to be immune-suppressive during helminth infections (Klein, 2000; Grear *et al*., 2009; Hepworth *et al*., 2010). Testosterone has been reported to stimulate the expression of IL-18 which is an IFN-γ inducing cytokine which has pleiotropic effects including the suppression of Th2 immunity and also inhibits the IL-13 mediated worm expulsion of *Trichuris muris* and *T. spiralis* infection, resulting in high larval burden in males than females (Hepworth *et al.*, 2010). In present study, this may have contributed to the success of worm establishment to both male and female adult worms, and the delay in worm expulsion; hence the higher numbers of adult worms observed in males than in females *Sprague-Dawley* rats (Table 2 & 3; Fig. 6). The high establishment of male and female adult worms at day 5 PI as shown by female to male ratios of adult worms (Table 3) suggested that there were greater chances of mating in the gastrointestinal tract of male rats than female rats, resulting in high larval burden in the muscles as shown by RCI (Table 2; Fig. 6).

The effect of testosterone on parasite-host interactions has also been reported for various other parasite species (ecto- and endoparasites) in diverse host species (Klein, 2004). It has been reported that testosterone increases the reproductive success of male lizards and stimulate increased energy expenditure during the mating season as males increase territorial behaviours such as endurance, movement and expression of morphological secondary sex characteristics (Klein, 2000). As activity increases and males patrol larger territories, they may become exposed to more parasites, leading to increased burdens. Furthermore, testosterone affects parasite loads via inhibition of immune function directly and indirectly by

increasing reproductive energy expenditure at the cost of immune maintenance (Klein, 2004). Experimentally elevated testosterone decreased leukocyte counts and suppresses the cell-mediated immune response and humoral immune responses leading to higher parasite loads. Testosterone-implanted male western-fence lizards, (*Sceloporus occidentalis*) infected with sub-adult western black-legged ticks (*Ixodes pacificus*), showed high parasite load as compared to control males (Pollock *et al*., 2012).

The immune-modulatory effect of testosterone through the synthesis of anti-inflammatory cytokines (IL-10); inhibitory effects on transcriptional factors (NFκB) that mediate the production of pro-inflammatory and anti-parasitic cytokines; and through increasing the expression and translation of stress proteins and apoptosis factors, has been reported for *Plasmodium sp.* and *Leshmania sp*. (Klein, 2004). The immune-suppression effect of testosterone is associated with the increased susceptibility to *Plasmodium* infections in male than female hosts (Klein, 2004). In mice infected with *P. chabaudi* or *P. berghei*, castration of males reduced, whereas exogenous administration of testosterone increased mortality (Benten *et al*., 1997). In addition to increased mortality rates, male mice recovered from *P. chabaudi*-induced weight loss, anaemia, and hypothermia slower and had lower antibody responses than females (Benten *et al*., 1997). In experimental mice infected with *L. major* or *L. maxicana*, castration of males reduced, whereas administration of testosterone to females increased susceptibility to *L. major* (Satoskar *et al*., 1998). Males were more susceptible than females to infection with *L. mexicana* and this sex difference appeared to be mediated by the effects of estrogens on the synthesis of IFNγ and production of Th1 responses (Mock & Nacy, 1988). Therefore, these immune-modulatory effects of sex steroids are influenced by the concentration of the relative sex steroids in a host (Klein, 2004).

Additionally, Mankau & Hamilton (1972) showed that injection of testosterone derived compounds in females infected with *T. spiralis* increased larval burden compared to non-treated females, whereas treatment of male with estrogen compound stilbesterol reduced larval burden compared to non-treated males. This finding contributes to the resistance effect of estrogen against parasitic infections. Estrogen has been reported to be associated with greater adaptive immunity resulting in reduced parasite burden in females (Grossman, 1985; Mankau & Hamilton, 1972). During parasitic helminth infections 17 β-estradiol (E_2) was reported to act directly on a variety of lymphocytes including $CD4^+$T-cells, $CD8^+$,

macrophages, dendritic cells (DC) and B-cells, acting towards a B-cell maturation mechanism by suppressing T-suppressor cell to enhance antibody production, thus enhancing Th2 protective immunity against *T. spiralis* resulting in reduced larval burden (Hepworth *et al*., 2010; Lutton & Callard, 2006). These findings could also explain the reduced larval burden observed in female Balb C mice infected with *T. zimbabwensis* in the present study (Table 2; Fig. 6).

During protozoan parasite infection in mice, female resistance to infections caused by *L. mexicana* was positively associated with estrogen concentrations (Mock & Nacy, 1988). Resistance against *L. mexicana* was related to the effects of estrogen on increased transcription of IFN-γ mRNA in mice. Depletion of IFN-γ using monoclonal antibodies or administration of recombinant IFN-γ in males, sex differences in disease progression can then be reversed (Mock & Nacy, 1988). In contrast, females are not resistant to all infections; female mice treated with pharmacological doses of 17β-estradiol are more susceptible to *Toxoplasma gondii* than non-treated females or males (Pung & Luster, 1986). Increased susceptibility to *T. gondii* in estrogen-treated mice may be due to reduced innate immunity (e.g. NK cell and macrophage activity) and lower cytokine responses (e.g. IL-12 and IFNγ production) (Pung & Luster, 1986).

Changes in the amounts of sex steroids present influence how the immune system responds and in-turn influence the success of infections (Lutton & Callard, 2006). In this study, pregnancy reduced the chances of successful establishment of *T. zimbabwensis* in the muscles of Balb C mice (Table 4). There were no significant differences in the number of adult worms in non-pregnant and pregnant mice (Table 4 & 5). Although gravid adult worms established in both pregnant and non-pregnant animals, parasite could not complete its development in pregnant animals as muscle larvae did not establish in this group of animals (Table 4). As expected, progesterone levels were higher in pregnant animals than in non-pregnant animals (Fig. 7) and these results were in line with the findings by Nuñez and colleagues (2005) on *T. spiralis* infection where parasite burden was reduced in pregnant mice compared to virgin mice.

The results were associated with the parasiticidal effect of progesterone through the modulation of the immune system or through direct effects on the parasite. The latter was

confirmed through *in vitro* studies when human sera of pregnant women was able to induced NBL death despite the lack of anti-NBL antibodies in their sera and when NBL mortality was induced by direct exposure to doses of P_4 (Nuñez *et al*., 2008). Progesterone was reported to act on synergistically with Th2 immunity in antibody-dependent cell cytotoxicity, targeting the induction of effector cells that are responsible for mortality of NBL in pregnant animals resulting in reduced or no larval establishment in the muscles (Nuñez *et al*. 2005).

These results are in line with the observations from the present study. In the present study, the levels of progesterone were significantly higher at day 0 (at peak levels) and at day 7 PI (days 7 and 14 post-mating) in pregnant mice than in non-pregnant mice (Fig. 7). It is possible that since levels were high during infection and during adult stage development, mating and shedding of larvae, that progesterone might have acted on the reproduction of the parasite when migration was starting in the intestines. However, correlation analysis showed no correlation between infection and progesterone levels (Fig.9). Perhaps the reason why there was no linear relationship between the two variables was that there may have been another variable with an influence that was not measured. Since estrogen has been shown to have an effect on *Trichinella* establishment and reduction of larval burden, these observations could also be associated with the elevated levels of estrogen during pregnancy when compared to virgin mice. Estrogen and may be working synergistically with progesterone against the establishment of *Trichinella zimbabwensis* larvae in the muscles.

Despite the helminthotoxity and cytotoxic effects of progesterone against migrating larvae, congenital transmission of the parasite is still possible (Nuñez *et al*., 2002). Although congenital transmission has been reported in *Rottus norvegicus* rats infected with *T. zimbabwensis* (Matenga *et al*., 2006); in the present study, no congenital transmission was observed in Balb C mice infected with 50 LPG at day 7 post-mating. This observation could have been influenced by the larval dose upon infection as observed by Matenga and colleagues (2006) where dams were infected with 2000 *T. zimbabwensis* larvae/rat at days 4, 6 and 10 post-mating and only observed 1-3 larvae per positive offspring whilst in the present study a lower dose of 50 LPG of animal was used; or it could be influenced by the stage of pregnancy at which infection occurred; and the levels of progesterone in the host system (Nuñez *et al*., 2002).

The parasiticidal effect of progesterone has also been reported on *Schistosoma haematobium* infected female golden hamsters (*Mesocricetus aureatus*) treated with 0.1 ml/kg

medraxyprogesterone acetate at days 7 and 35 PI (Soliman & Ibrahim, 2005). Treatment caused tegument damage and reduced the number of animals in treated groups; thus suggesting that progesterone causes impairment in the development of these parasites (Sliman & Ibrahim, 2005). However, the reverse was reported for *Taenia solium* cysticerci exposed to P_4 *in vitro* (Escobedo *et al*., 2010). Treatment induced scolex invagination which is the initial step to adult worm development, suggesting that progesterone promotes *Taenia solium* worm development (Escobedo *et al*., 2010).

On the contrary, treatment of female golden hamsters with progesterone reduced adult worm recovery and tapeworm length and increased proliferation rate of leukocytes from spleen and mesenteric lymph nodes (Escobedo *et al*., 2011). These cells showed high expression of IL-4, I-6 and TNF-α at the duodenal mucosa; suggesting that progesterone protects hamsters from *T. solium* tapeworm establishment by improving intestinal mucosal immunity (Escobedo *et al*., 2011). These findings suggest that direct effect of progesterone on parasite and the effect when interacting with immune system could be different for certain parasites and this could be parasite and host specific. The contradicting results observed in *T. gondii* infected pregnant women when circulating progesterone levels were elevated, showing down-regulation of T-cell mediated responses, suggested that progesterone like estrogen is permissive to *T. gondii* infections (Prigione *et al*., 2006). These findings suggest that the interaction of progesterone with the immune system is also parasite and host specific.

Although, host sex hormones affect responses to infection, parasites can have an effect on hormone signalling within the host. In the present study, infection increased progesterone levels following infection and remained higher in non-pregnant, infected mice (Fig. 7) when compared to levels in normal animals (control) (Table 6, see appendix) except at day 0 of trial, suggesting that infection might have caused and disrupted oestrus cycle. This further suggests that the immune-regulatory effects of progesterone for different parasites species are influenced by specific expression of antigens of a particular parasite (Klein, 2004). Seemingly, infection also increased cortisol levels in non-pregnant, infected mice (Fig. 8) as cortisol levels were significantly higher in non-pregnant, infected than control group as shown by values on Table 7 (see appendix). On the other hand, no significant differences were observed between cortisol levels in pregnant mice when compared to non-pregnant, infected mice (Fig. 8). These findings suggest that stimulation of cortisol release was equivalent in pregnant animals and non-pregnant, infected animals and this might be due to

the stress caused by infection on host. Cortisol release might have been stimulated as a result of the antigens expressed by the parasite sending signals of the presence of a stressor to the HPA.

Cortisone has been reported to decrease the expulsion rate of intestinal helminthic infections, including *Trichinella* due to immune-suppressive effects during the intestinal stages of the parasite (Coker 1955; Stewart *et al*., 1982; Duran *et al*., 1986). In wild adult male chimpanzees (*Pan troglodytes schweinfurthii*) of Uganda, high intestinal parasites was directly associated with elevated cortisol levels (Muehlenbein & Watts, 2010). In the present study, there were no significant differences in cortisol levels or establishment of adult worms in pregnant and non-pregnant, infected animals and adult parasites did not persist longer in the intestines (Table 4 & 5; Fig. 8). Furthermore, correlation analysis showed no correlation between cortisol levels and infection (Fig. 10). Previous studies on the other hand showed that exogenous supplementation of hydrocortisone resulted in greater establishment of *T. spiralis* adult worms in the enteral phase of the parasite, persistence of the adult worms and increased fecundity resulting in high larval burdens in the muscles (Coker, 1955; Steward *et al*., 1982).

Results from the present study suggest that cortisol might not have had an impact on the infection of *T. zimbabwensis* despite evidence from previous studies that exogenous hydrocortisone has influences establishment of *T. zimbawensis* in pregnant Sprague- Dawley rats. In a study conducted on the effect of exogenous hydrocortisone *T. zimbabwensis* in pregnant Sprague-Dawley infected with 300 *T. zimbawensis* larvae/animal at day 9 post-mating and treated with a standard dose of 2mg/ kg BW at day 0 of trial and every second day following the first dose; a greater establishment of adult worms was observed in pregnant, treated animals compared to pregnant, non-treated animals (Mukaratirwa *et al.*, 2011, pers comm.). In that study, adults persisted until day 19 post-infection in the pregnant, treated group compared to the pregnant, non-treated group (Mukaratirwa *et al*., 2011, pers comm.). In the present study, pregnant Balb C mice were infected with 50 LPG of *T. zimbabwensis* at day 7 post-mating and no supplementation of exogenous hydrocortisone as the objective of the study was to detect the effects of endogenous cortisol in the establishment of *T. zimabwensis*. Results from the present study suggest that endogenous levels in Balb C mice were too low to have an effect on the parasite, thus suggesting that the effects of cortisol on intestinal parasites is also concentration depended.

6. Conclusion

The role of host sex and sex/steroid hormones in the establishment and development of *T. zimbabwensis* in the host was revealed in the study. These hormones can be strategically used to control and prevent *Trichinella zimbabwensis* targeting the different developmental stages of the parasites. The differences that were observed in the intensity of infection between males and females suggest that susceptibility and responses to parasitic infections are gender specific. The differences in immune responses that exists among the sexes, suggest that males and females may have different responses to treatment which may also be host and parasite specific. This means that a treatment approach may be influenced by the antigens expressed by the parasite; immune responses stimulated; and sex/steroids hormones present and manipulated by the parasite within the host system.

Seemingly, female hormones stimulate a more resistance effect on *Trichinella sp.* The present study and several other studies on *Trichinella* and the effects of pregnancy in mammals including humans associated progesterone with the low larval burdens observed at the larval stages of the parasite. It can thus be concluded that progesterone has protective effects against trichinellosis and can be used as a treatment, targeting the developmental stages of the parasite, the NBL. However, according to results from the present study, it was suggested that progesterone in a natural host may not be acting individually, but synergistically with other female sex hormones such as estrogen, against *Trichinella* development and establishment. Future studies could measure the amounts of both estrogen and progesterone over time of infection during pregnancy to determine the effects of these hormones on *Trichinella* development and at exactly what stage they will have an effect. Perhaps, the effects of these hormones could be investigated by both measuring and supplementing different doses of these hormones to see what levels would cause an effect in a natural host. This could be achieved by having different groups of females, both pregnant and non-pregnant that would be supplemented a standard dose per group over time of infection. In this way the amount of hormones that have an effect at the different stages of infection would be controlled.

The use of progesterone as possible treatment might only be positive at the early stages of infection during the migration stages. The use of progesterone as a treatment strategy would require intensive research on the effects of this hormone on males of different host species as

this hormone is only endogenous to females and might cause adverse effects on the physiology of the opposite sex. Furthermore, the concentrations of progesterone that causes an effect on the parasite might differ among host species. Perhaps the concentrations obtained from this study could be used as baseline levels for treatment strategies during the early stages of infection.

On the other hand, cortisol or corticosterone in the case of the rodent models used in this study did not have an effect on *T. zimbabwensis* development, although reported to have immune-suppressive effects in the intestinal stages of the parasite. Thus, the effects of pregnancy on *T. zimbabwensis* observed in the present study can be attributed to the effects of progesterone and possibly elevated estrogen levels that are occurring during pregnancy. However, it cannot be concluded whether or not cortisol and corticosterone can be used as treatment to *T. zimbabwensis* infection during pregnancy, since exogenous administration of hydrocortisone has been reported to cause persistence of adult stage infection in the intestines of a host; whilst successful treatments of *T. spiralis* with cortisone and ACTH at the muscle stages of the parasite in human patients with clinical trichinellosis due to the anti-inflammatory properties of cortisone have been documented (Sadusk, 1954). Perhaps, corticosterone in rodents and cortisol in primates could also be used as treatment only at the muscle stages of the parasite. However, further studies would be required to substantiate the independent effects of cortisol or corticosterone on the enteral and on the parenteral phases of the parasite independently, so as to extrapolate the concentrations that would cause an effect and use them as baseline levels when forming treatment drugs.

It is also important to know the stage of infection and the stage of pregnancy before attempting or initiating treatment, since administration of such hormones may have detrimental effects on the host physiology during certain stages of pregnancy. Cortisol, for example, is important for the lung development of the foetus at late pregnancy; supplementation of this hormone, resulting in high concentrations may lead to miscarriages if at early pregnancy. Supplementation of progesterone at muscle stages of infection may not cause an effect, but increase progesterone in the host system which may delay parturition if at late stages of pregnancy. Therefore, future studies aim to intensively research on how the various sex and steroid hormones affect both sexes is required and how these hormones could be used as new treatment or in combination with current commercially available drugs.

7. References

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8. Appendix

8. 1 Additional figures and tables

8. 1. 1 Estimated P_4 levels for group 4 derive from average progesterone levels during estrous cycle in normal mice

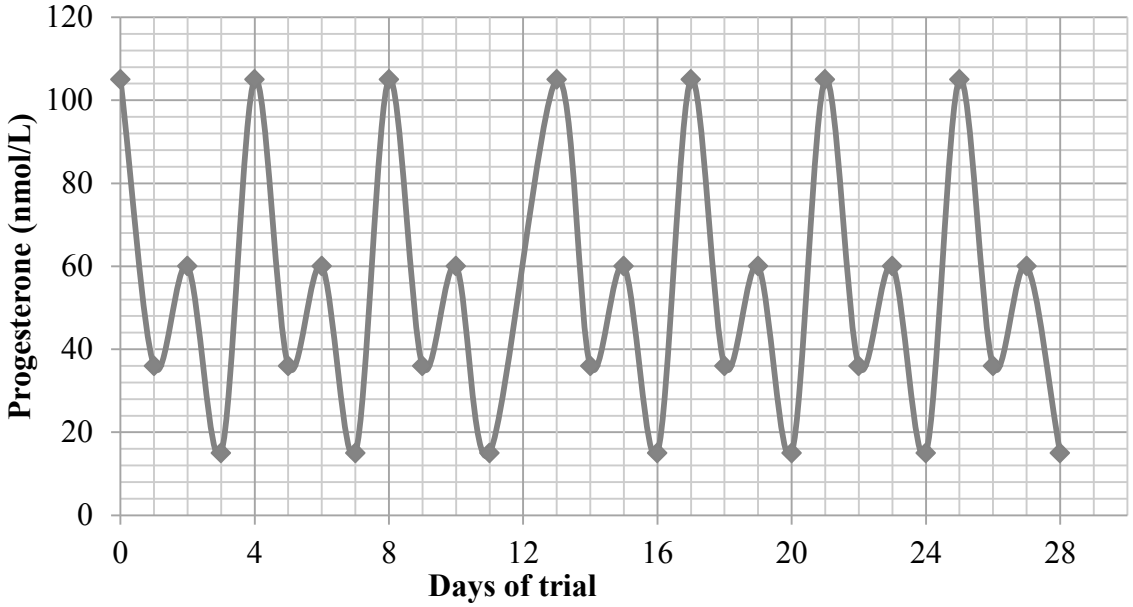

Figure 11: Standard average progesterone concentrations in mice during estrous cycle (Loeb & Quimby, 1989)

8.1.2 Estimated progesterone levels for group 4

Table 6: Estimated average normal progesterone concentrations in mice

Days of Sacrifice	Estimated progesterone concentration (nmoL/L)
0	105
7	7*
14	36*
21	105
28	15*

* Significant difference ($P < 0.05$) when compared to experimental groups

8. 1. 3 Estimated cortisol levels for group 4

Table 7: Estimated average normal concentrations in mice in relation to diurnal variation of blood cortisol

Days of blood collection	**Time of blood collection**	**Estimated cortisol concentration (pg/mL)**
0	9 : 30 AM	10 000*
7	9 : 10 AM	10 100*
14	9 : 45 AM	9 800*
21	9 : 30 AM	10 000*
28	9 : 10 A	10 100*

* Significant difference ($P < 0.05$) when compared to experimental groups