STUDIES ON THE EFFECTS OF *TRYPANOSOMA CONGOLENSE* INFECTION ON THE REPRODUCTIVE FUNCTION OF THE RAM

BY

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SUMMARY

This thesis describes a series of studies carried out in Scottish blackface rams experimentally infected with *Trypanosoma congolense* stabilates 57/10 and 57/11 (originally imported from ILRAD, Kenya as ILRAD 1180) with the primary aim of determining the effects of infection on the function of the hypothalamo-pituitary-gonadal axis. The studies also investigated the possibility that pyrexia is responsible for inducing gonadal endocrine and exocrine dysfunctions in infected animals. In addition the effect of infection on the function of the hypothalamo-pituitary-adrenal (HPA) axis was assessed in order to determine whether reproductive dysfunction generally seen during trypanosomiasis is related to stress caused by the infection.

Chapter I comprises an introduction and a literature review on trypanosome-induced reproductive dysfunctions with emphasis on pyrexia and changes in the HPA axis. Chapter II describes the two experiments carried out in rams infected with *T. congolense* and the general materials and methods used in these studies.

Chapter III describes the effects of infection on semen characteristics and pathology of various reproductive organs such as the testis, cauda epididymis, prostate and pituitary gland. It was found that *T. congolense* induced a progressive deterioration of semen quality in terms of an increased percentage of abnormal spermatozoa in the ejaculate. Progressive non-inflammatory degenerative changes were observed in the testis and prostate gland. The cauda epididymis showed varying degrees of decreased sperm reserve. Trypanosome-induced pyrexia led to an elevation of scrotal temperature in infected rams, suggesting that the changes in the gonads could have been due to increased testicular temperature. Indeed, similar changes were observed in the semen and gonads of uninfected rams following artificial elevation of testicular temperature by scrotal insulation. The pituitary gland showed changes associated with increased basophilic degranulation in infected rams.

Changes in plasma concentrations of reproductive hormones in the same rams are described in Chapter IV. It was observed that soon after the onset of parasitaemia, which occurred within 1 - 2 weeks of infection, plasma testosterone concentration declined and levels remained low throughout the infection period. This reduction in plasma testosterone concentration was associated with a progressive and marked decline in testosterone pulse amplitude and testosterone secretion after injection of gonadotrophin-releasing hormone (GnRH) was also depressed throughout the infection period. By four weeks after infection, declining plasma testosterone concentration was

accompanied by a significant increase in plasma luteinizing hormone (LH) pulse amplitude and increased pituitary responsiveness (LH secretion) to exogenous GnRH. As the infection progressed up to 8 weeks, the plasma LH concentration declined. This could not be associated with some aspects of gonadal steroid feedback as similar LH changes were observed in infected rams which had been castrated. Neither was the decline in plasma LH concentration caused by the inability of the pituitary gland to secrete and release LH as secretion of LH in response to exogenous GnRH was not impaired throughout the infection period. It was therefore concluded that the decline in plasma LH concentration after 8 weeks of infection was possibly induced by a progressive impairment of the ability of the hypothalamus to synthesize and/or release GnRH.

Gonadal steroidogenesis in infected rams was investigated in the *in vitro* experiments described in Chapter V. This work showed that the alteration in plasma testosterone concentration following infection was associated with a decline in Leydig cell steroidogenesis, possibly mediated by increased testicular temperature affecting testosterone biosynthetic enzymes. However, by 4 weeks after infection, reduced plasma testosterone in infected animals was exacerbated by the impaired ability of the testes to release testosterone into the circulation resulting in a significant increase in intratesticular testosterone content. A similar increase was also observed in scrotal-insulated rams and it was therefore suggested that changes in intratesticular testosterone in infected rams at 4 weeks of infection was associated with a trypanosome-induced increase in testicular temperature perhaps through an effect on testicular blood flow.

The effects of *T. congolense* infection on the function of the HPA axis in rams and the relationship between this and the changes in the hypothalamo-pituitary-gonadal axis are described in Chapter VI. The onset of parasitaemia stimulated a significant increase in plasma cortisol concentration which was followed within 3 - 6 week of infection by a decline in plasma cortisol levels and a reduced ability of the pituitary to secrete adrenocorticotrophin hormone (ACTH) after injection of corticotrophin-releasing hormone (CRH). Thereafter, the activity of the HPA axis was increased in step with the fluctuating parasitaemia. CRH stimulation of the HPA axis had no effect on LH secretion but reduced the plasma concentration of testosterone indicating the possible aggravation of *T. congolense*-induced reproductive disorders by stress-induced cortisol.

The general discussion and conclusions drawn from all the experiments are presented in Chapter VII.

It can be concluded that *T. congolense* causes a very profound dysfunction of the hypothalamo-pituitary-gonadal axis in rams through actions at various sites. These effects may be partly associated with trypanosome-induced pyrexia and are exacerbated by increased plasma cortisol concentrations resulting from the activation of the HPA axis.

DECLARATION

The work presented in this thesis is original and has been carried out solely by the author, except where collaboration with others has been acknowledged

Benezeth Mugyabùso Mutayoba

December, 1993

DEDICATION

TO

MY PARENTS,

MY WIFE,

SALOME

MY CHILDREN,

AMON AND MUGISHA

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CHAPTER VI

EFFECTS OF *TRYPANOSOMA CONGOLENSE* INFECTION ON THE PITUITARY-ADRENAL FUNCTION OF THE RAM

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ABBREVIATIONS

22ROHC	22R-hydroxycholesterol
α	alpha
ACTH	adrenocotrophic stimulating hormone
BSA	bovine serum albumin
cAMP	cyclic adenine monophosphate
cm	centimetre(s)
CNS	central nervous system
CO_2	carbon dioxide
cpm	counts per minute
CRH	corticotrophin-releasing hormone
CV	coefficient of variation
Δ^4	double bond between carbons 4 and 5
Δ^5	double bond between carbons 5 and 6
DAR	donkey anti-rabbit
dbCAMP	dibutyryl cyclic adenine monophosphate
DNA	Deoxyribonucleic acid
FAO	Food and Agricultural Organization
FSH	follicle stimulating hormone
γ	gamma
G	gauge
g	gravitational force
gm	gramme(s)
GnRH	gonadotrophin-releasing hormone
h	hour(s)
H&E	haematoxylin and eosin
hCG	human chorionic gonadotrophin
HPA	hypothalamo-pituitary-adrenal
IAA	indole-3-acetic Acid
IFN	interferon
IL	interleukin

ILA	indole-3-lactic acid
ILRAD	International Laboratory for Research on Animal Diseases
IU	International units
kD	kilodalton
kg	kilogram(s)
km	kilometre(s)
l	litre (s)
LH	luteinizing hormone
LW	liveweight
M199	medium 199
min	minute
ml	millilitre(s)
µmol	micromole
MSB	Martius Scarlet Blue
NC	not calculated
ND	not detectable
ng	nanogramme(s)
nmol	nanomole(s)
NRS	normal rabbit serum
^{o}C	degrees celsius
OFG	Orange Fuchsin Green
PBS	phosphate buffered saline
PCV	packed cell volume
pmol	picomole(s)
r^2	correlation coefficient
s.e.m.	standard error of the mean
s.w.g	standard wire gauge
SAPU	Scottish Antibody Production Unit
SD	standard deviation
ß	beta
TC	testicular circumference

TNF	tumour necrosis factor
TOL	tryptophol
µg	microgramme (s)
µl	microlitre(s)
w/v	weight/volume
WHO	World Health Organisation

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CHAPTER I

INTRODUCTION AND GENERAL LITERATURE REVIEW

1.1. INTRODUCTION

Trypanosomiasis is a disease of humans and animals caused by protozoan organisms of the family Trypanosomatidae. Although trypanosomiasis is a serious constraint to development throughout the tropical region and some temperate areas, it exerts the greatest socio-economic effects in sub-Saharan Africa. This region provides suitable geographical factors which favour the survival of the vector flies of the genus *Glossina* (tsetse flies) which are responsible for cyclical transmission of the trypanosomes. Tsetse-transmitted trypanosomiasis is classified as severe in the majority of the 37 sub-Saharan countries affected, where it figures among the first three priority veterinary diseases (Jordan, 1992). With increasing human populations and the growing demand for land and food in these areas, human beings and their livestock have been forced to encroach into tsetse habitats increasing the human and veterinary trypanosomiasis risks. It has been estimated that about 10 million km^2 or 40% of tropical Africa is infested with tsetse flies and these areas are among the most fertile areas in the continent (Wilson *et al.*, 1963; Luckins, 1992). Losses to livestock production in Africa due to trypanosomiasis in meat alone amount to US$5 billion annually (FAO-WHO-OIE, 1982). However, despite the concerted and specific control measures directed against the parasite and the tsetse flies since the early 1960s, the pattern of disease incidence has remained much the same in many African countries. The situation may deteriorate further due to decline in national efforts because of shrinking resources and changes in climatic conditions affecting both crop and animal productivity.

1.1.1. Classification of Trypanosomes

The principal causative organisms of trypanosomiasis are flagellate protozoa of the Order Kinetoplastida, Class Mastigophora, Family Trypanosomidae and the genus *Trypanosoma* (Stephen, 1986). The pathogenic trypanosomes in the genus *Trypanosoma* are subdivided into two sections, Stercoraria and Salivaria, according to their development in the vector and transmission by either the saliva (Salivaria) or faecal contamination of the wound caused by the bite of the vector (Stercoraria). Each section

is further subdivided into subgenera and species. The classification of trypanosomes of veterinary and medical importance according to Losos (1986) is as follows:

Salivaria			
Subgenus	*Duttonella*	Species	*Trypanosoma vivax*
Subgenus	*Nannomonas*	Species	*Trypanosoma congolense*
			T. simiae
Subgenus	*Trypanozoon*	Species	*Trypanosoma brucei brucei*
			T. b. rhodesiense
			T. b. gambiense
			T. b. evansi
			T. b. equiperdum
Subgenus	*Pycnomonas*	Species	*Trypanosoma suis*
Stercoraria			
Subgenus	*Schizotrypanum*	Species	*Trypanosoma cruzi*

The infectivity of various trypanosomes has been well documented (Hoare, 1972; Losos, 1986; Stephen, 1986). Briefly, all salivarian trypanosomes have a wide range of domestic hosts. *T. congolense, T. b. brucei* and *T. b. evansi* infect cattle, sheep, goats, horses, donkeys, camels, pigs, dogs and cats and in addition *T. b. evansi* infects water buffalo. *T. vivax* infects all the animals infected by other tsetse-fly transmitted species with the exception of the dog, cat and pig. *T. b. equiperdum* is restricted to horses and donkeys. *T. simiae* infect pigs, camels, sheep and goats and *T. suis* infect only pigs. *T. cruzi* infects man causing Chagas disease in South and Central America. In domestic animals, natural infections caused by *T. cruzi* do not occur with the exception of dogs, cats and rarely in pigs.

Among the salivarian group, *T. vivax, T. congolense* and *T. b. brucei* are the main cause of African animal trypanosomiases (nagana) in cattle and other domestic animals. *T. b. gambiense* and *T. b. rhodesiense* infect man and cause human trypanosomiasis (sleeping sickness) in Africa.

1.1.2. Distribution of Trypanosomes within the Body

Depending on the degree of parasitaemia and the distribution of trypanosomes in the host tissues, tsetse-transmitted pathogenic trypanosomes can be divided into two main groups. One group of trypanosomes leave the blood during parasitaemia and infect the intercellular tissue fluids of various tissues and organs. This group include the *brucei*-like organisms, namely *T. b. brucei, T. b. rhodesiense, T. b. gambiense* (Goodwin, 1970; Losos and Ikede, 1972). The other group includes those species regarded as being confined to the vasculature of infected hosts (haematic trypanosomes) and include *T. congolense* and *T. vivax* (Losos and Ikede, 1972; Losos *et al.*, 1973; Banks, 1978).

Several reports are now available which have conclusively shown the capacity of *T. vivax* to invade and cause inflammation in tissues and especially the heart of several animals (Bungener and Mehlitz, 1977; van den Ingh and de Neijs-Bakker, 1979; Masake, 1980; Kimeto *et al.*, 1990). There are reports of *T. vivax* being found also in lymph nodes (Fiennes, 1950; Emery, *et al.*, 1980), the pituitary gland (Fiennes, 1950) and in cerebro-spinal fluids and aqueous humour (Whitelaw *et al.*, 1988). *T. vivax* infections of the tissue appear to differ from those caused by *T. b. brucei* in that they do not localize throughout the body of the host (Losos, 1986). In earlier studies by Hornby (1952), *T. congolense* was reported to localise in small blood vessels. It is now known that these organisms attach themselves by their flagellae to the endothelial cells of small blood vessels of the host (Bungener and Muller, 1976; Banks, 1978). Due to this attachment the population in the microvasculature may be a thousandfold higher than that found in the general circulation (Banks, 1978; Tizard *et al.*, 1978b). Apart from an extravascular phase in the skin (Gray and Luckins, 1980; Emery and Moloo, 1981) and occasional dissemination in cerebro-spinal fluid (Masake *et al.*, 1984), *T. congolense* appears not to leave the circulatory system (Ssynyonga and Adam, 1975; Tizard *et al.*, 1978a, 1978b; Murray and Dexter, 1988; Murray, 1989; Abebe *et al.*, 1993a).

Although parasitaemia occurs throughout the course of infection, blood parasite concentration varies with different species and strains of trypanosomes and with various species of the hosts. Parasitaemia is usually lower with those trypanosomes that localise

in tissues than with those species which remain in the circulation (Losos and Ikede, 1972).

1.1.3. African Animal Trypanosomiases.

Comprehensive reviews on the epizootiology, ecology, virulence, control and economic importance of African animal trypanosomiasis (nagana) in various regions of Africa are available (McLennan, 1970; Ford, 1971; Herbert and Parratt, 1979; Losos, 1986; FAO, 1992). The precise nature of each disease depends not only on the species and strain of trypanosome involved but also on the susceptibility of various hosts (Stephen, 1970; Losos, 1986; Stephen, 1986).

From the assessment of the pathogenicity of nagana trypanosomes, it is generally accepted that *T. vivax* is associated with acute infections in ruminants in West Africa while most infections in East Africa are more chronic (Fiennes, 1950; Fairbairn, 1953; Stephen, 1970). *T. congolense* is regarded to be more pathogenic in East Africa than in West Africa (Losos and Ikede, 1972; Tizard *et al.*, 1978b). Nevertheless, highly virulent and haemorrhagic forms of *T. vivax* have been reported in Eastern Africa including Tanzania (Cornell, 1936), Kenya (Mwongella, *et al.*, 1981; Wellde *et al.*, 1983) and Somalia (Dirie *et al.*, 1988). *T. b brucei* is less pathogenic in domestic animals than the other two species and is widely distributed in both West and East Africa (Tizard *et al.*, 1978b).

1.1.4. African Trypanosomiases in Small Ruminants

Sheep and goats play an important role in many agricultural systems in Sub-Saharan Africa. Due to their small size, lower feed requirements and diverse feeding habits, they can occupy a wide range of ecological habitats ranging from semi-arid to humid regions. Over 90% of sheep and goats in the Sub-Saharan Africa are found in East and West Africa where they provide 30% of the meat and 15% of milk consumed (ILCA, 1990, 1991). Many of the areas occupied by small ruminants are infested with tsetse flies which transmit the pathogenic trypanosomes to all species of domesticated livestock. Although tsetse-transmitted trypanosomiasis is recognised as serious constraint to cattle productivity, the situation with regard to sheep and goats is less clear

(Murray *et al.*, 1984). It has been suggested that small ruminants are rarely affected under natural conditions since tsetse flies seldom feed on them, when compared to large animals (Mathewman, 1980; Stephen, 1986). Kramer (1966) and MacLennan (1970) also indicated that trypanosomiasis is not a serious problem in small ruminants. However, field surveys have recently indicated that both disease incidence and mortality in small ruminants might be higher than was previously believed (Makumyaviri *et al.*, 1989; Kanyari *et al.*, 1986; Mawuena, 1988; Kalu, 1991). In addition, experimental studies have shown that small ruminants are equally susceptible to infection with pathogenic trypanosomes as large animals (McGuire *et al.*, 1985; Whitelaw *et al.*, 1985; Mutayoba *et al.*, 1989a).

It has also been shown from field studies in Kenya that the economic effects of trypanosomiasis in sheep and goats, which accrue from retarded growth rates, poor reproductive performance and death might be substantial (Griffin and Allonby, 1979b; Kanyari *et al.*, 1983). Mahmoud and Elmalik (1977) emphasized that sheep and goat may act as a reservoir for the disease which can be readily transmitted to other domestic animals in the field. On several occasions small ruminants have also been found to be a reservoir of human trypanosomes (Berl *et al.*, 1982; Okuna and Mayende, 1983; Scott *et al.*, 1983; Noireau *et al.*, 1986; Kageruka *et al.*, 1991).

The course and outcome of trypanosome infections in small ruminants depends on whether the infection is natural or experimentally-induced (Griffin, 1978). This is largely because most of the tsetse-transmitted natural infections are not caused by one species of trypanosome (Griffin and Allonby, 1979a) and mixed infections are common (MacKenzie and Cruickshank, 1973; Stephen, 1986).

The pathology and pathogenesis of experimental trypanosomiasis in sheep and goat has been well documented (Losos and Ikede, 1972; Ikede and Losos, 1975; Ingh *et al.*, 1976; Kaaya *et al.*, 1977; Masake, 1980). Sheep and goats have often been used as a convenient ruminant host in studies of experimental trypanosomiasis, serving as a model of diseases in cattle (Kilgour *et al.*, 1975; Bouitelle *et al.*, 1988). Indeed, in recent years an increasing number of reports on experimental small ruminant trypanosomiases has become available, most of these reports are on *T. congolense* and

T. vivax as these are thought to be more pathogenic to small ruminants than the *T. brucei* sub-group (Losos and Ikede, 1972; Verma and Gautam, 1977).

The disease syndromes caused by *T. congolense* have been described in naturally infected sheep and goats (Griffin and Allonby, 1979a; MacKenzie and Cruickshank, 1973) and in experimentally infected goats (Mutayoba *et al.*, 1989) and sheep (MacKenzie and Cruickshank, 1973; Katunguka-Rwakishaya, 1992). According to the duration of infection, the disease had been divided into acute, subacute and chronic forms. The acute infection lasts for about 4-6 weeks and is characterised by rapid decline in packed cell volume (PCV), widely fluctuating body temperature and parasitaemia and culminates either in death or in some cases self cure or progress to a chronic infection. The subacute infection lasts for about 6 - 12 weeks and is characterised by a marked but steady decline in PCV and liveweight and fluctuating body temperature. Infected animals may die during this period or may develop a chronic infection. The chronic infection lasts over 12 weeks and is characterised by a gradual limited recovery in haemogram and liveweight parameters but infected animals become emaciated and are generally in poor condition. Death may occur or animals may recover spontaneously without treatment. It is, however apparent that sheep and goats can commonly support a *T. congolense* infection for several months. In most cases death occurs following secondary complications resulting from mixed trypanosome infections (Stephen 1986; Losos, 1986), bacterial infections (MacKenzie and Cruickshank, 1973) or due to stress associated with inadequate nutrition (Katunguka-Rwakishaya, 1992).

1.2. PATHOPHYSIOLOGY OF AFRICAN TRYPANOSOMIASIS: EFFECTS ON REPRODUCTIVE FUNCTION

A considerable amount of research on trypanosomiasis has been carried out for several years and the pathogenic effects associated with the disease have been well elucidated. However, whereas the literature abounds with reports on the pathology and pathogenesis of the lesions in large organs such as the spleen, liver, heart and brain, less attention has been paid to the pathophysiology and pathogenicity of trypanosome-induced reproductive dysfunction although it is known that reproductive disorders are consistently present among the clinical features of human (Apted, 1970) and animal (Hornby, 1921;

Stephen, 1966) trypanosomiases. Although many animals suffering from acute trypanosome infections invariably die if left untreated, chronically infected animals may survive for many months sometimes showing few overt clinical signs of the disease. In recent years an increasing number of reports have confirmed that such animals develop varying degrees of reproductive impairment and severely affected animals may become sterile. Since reproductive pathophysiological changes associated with *T. congolense* and *T. vivax* infections are closely similar and are different from those caused by *T. brucei*-group, reproductive anomalies caused by *T. congolense* and *T. vivax* have been reviewed together in the following subsection.

1.2.1. Reproductive Changes in the Male

1.2.1.1. Changes in Libido and Ejaculation Time

Although the effects of trypanosomiasis on semen and testicular pathology in animals and man have been well documented (reviewed by Ikede *et al.*, 1988) changes in libido and ejaculation time of infected animals have been rarely reported. Infection with trypanosomes at an early age causes a delay in reaching sexual maturity in cattle (Stephen, 1966; Roberts and Gray, 1973). Male animals dying from the infection or killed at one year of age had juvenile testes and had shown no libido. Detailed studies on the effects of *T. vivax* and *T. congolense* on reaction (ejaculation) time in zebu bulls have recently been reported by Sekoni *et al.* (1988) and Sekoni *et al.* (1990a). The reaction time, defined as the period taken for the bull to ejaculate following massage of the scrotal contents and accessory organs per rectum, was significantly increased in infected bulls from pre-infection range values of 24.3 - 60.13 sec to 14.25 - 267.25 sec within 9 - 12 weeks of infection. The increased reaction time in *T. congolense*-infected bulls had not returned to pre-infection levels by 10 weeks after Novidium$^{(R)}$ (homidium chloride) treatment given 12 weeks post-infection. Loss of libido has also been reported in boars experimentally infected with either *T. congolense* or *T. b. brucei* (Omeke and Onuora, 1992) and in humans suffering from sleeping sickness (Apted, 1970).

1.2.1.2. Testicular Changes

The testicular pathology induced by *T. congolense* and *T. vivax* infections in animals has been well documented, but the pathogenesis of the associated gonadal lesions still remains unclear. Depending on the virulence of invading serotypes and degree of susceptibility of the host, the onset of testicular lesions may be slow or dramatic (Ikede *et al.*, 1988). Lesions are however, progressive leading to marked testicular degeneration, atrophy and aspermia. Losos and Ikede, (1970) and Isoun and Anosa (1974a, 1974b) observed testicular necrosis, calcification and plugging of seminiferous tubules by spermatozoa in sheep and goats infected with *T. vivax*. Shrinkage and desquamation of the seminiferous germinal epithelium and dislodgement of spermatocytes into the lumen were later reported in goats infected with *T. congolense* (Kaaya and Oduor-Okelo, 1980; Waindi *et al.*, 1986; Gombe, 1989) and in sheep and goats infected with *T. vivax* (Anosa and Isoun, 1980). Similar lesions were reported in bulls infected with *T. vivax* (Isoun *et al.*, 1975; Masake, 1980) and *T. congolense* (Grundler *et al.*, 1988; Sekoni, 1990; Sekoni *et al.*, 1990b) and boars infected with either *T. congolense* or *T. b. brucei* (Omeke, 1991; Omeke and Onuora, 1992). Recent studies in cattle (Sekoni *et al.*, 1990b) have shown that *T. congolense* produced more severe testicular degenerative changes than *T. vivax*. However, in most uncomplicated cases, testicular degenerative lesions induced by *T. congolense* and *T. vivax* are usually simple, and are accompanied by minimal inflammatory changes (Anosa and Isoun, 1980; Sekoni *et al.*, 1990b).

The effects of *T. congolense* and *T. vivax* on Leydig cell pathology have not been adequately described. Waindi *et al.* (1986) noted an apparent diminution of Leydig cell numbers in Toggernburg goats acutely infected with *T. congolense* whereas no apparent changes in Leydig cells were reported in sheep and goats infected with *T. vivax* (Anosa and Isoun, 1980) and goats and boars infected with *T. congolense* (Kaaya and Oduor-Okelo, 1980; Omeke and Onuora, 1992, respectively). Further studies are still needed to ascertain the integrity of the Leydig cells in haematic trypanosome infections.

As mentioned earlier, there is conclusive evidence showing that *T. vivax* may localise in extravascular spaces of several organs including the heart, pituitary, lymph nodes and also in cerebrospinal fluid but there are no reports of *T. vivax* being located extravasculary in the testicular interstitium. Recently, Omeke and Onuora (1992)

reported the localization of *T. congolense* in the testis of the boar at necropsy, but there was no mention whether these parasites were found extravascular in histological sections.

Clinical and pathological testicular anomalies caused by *T. b. gambiense and T. b. rhodesiense* in man were described by Apted (1970). These lesions were characterised by a combination of scrotal dermatitis, orchitis and periorchiditis and localization of the trypanosomes in the testicular tissue and surrounding tissue soon after infection. Reduced gonadal weights and reproductive potential in male *Microtus montanus* (Voles) infected with *T. b. gambiense* were also reported by Ashman and Seed (1974).

T. b. brucei has also been found to localize in the scrotal skin, hydrocoel fluid, tunica vaginalis, testes, epididymis and spermatic cord provoking a nonsuppurative granulomatous inflammation in sheep (Ikede and Losos, 1972a; Ikede, 1979), horses (Ikede *et al.*, 1973), rabbits (Van den Ingh and Van Dijk, 1975; Ikede and Akpavie, 1982), mice (Anosa and Kaneko, 1984) and monkeys (Peruzzi, 1928). Due to extravascular localization *T. b. brucei* infections in animals are associated with more marked testicular pathology than those caused by *T. congolense* or *T. vivax*. Testicular changes induced by *T. b. brucei* are characterised by progressive diffuse atrophy of the seminiferous tubules associated with intertubular oedema, desquamation of germinal epithelial cells, reduction in spermatogenic activity, thickened tubular basement membrane and fibrosis (Ikede, 1979). In severely affected animals there is denudation of spermatogonia and seminiferous tubules are lined only by vacuolated Sertoli cells (Losos and Ikede 1970; Ikede, 1979; Ikede and Akpavie, 1982). Multiple foci of tubular calcification and hyalinization have also been reported (Ikede, 1979). Intratesticular interstitial and perivascular infiltration of lymphocytes (Moulton and Sollod, 1976), macrophages (Anosa and Kaneko, 1984) and other mononuclear cells (Ikede and Losos, 1972a; Ikede, 1979; Anosa, 1991; Omeke and Onuora, 1992) have frequently been observed and seem to be associated with extravascular localization of trypanosomes. Leydig cells are however discernible even in advanced lesions (Ikede, 1979). Ikede and Akpavie (1982) studied the effect of drug therapy on the resolution of the genital lesions accompanying systemic *T. b. brucei* infection in male rabbits and found that regeneration of the seminiferous epithelium depended on the severity of the initial lesions and in severely affected testes normal seminiferous tubular recovery may not occur for many months.

Though *T. b. evansi* causes subclinical infections in sheep and goats (Rottcher *et al*, 1987) and in cattle (Losos, 1986) several reports have also stressed its capacity to induce testicular damage and infertility in these animals (Anosa, 1991; Ngeranwa *et al.*, 1991). *T. b. evansi*-induced gonadal lesions have also been observed in rabbits, mice and rats (Patel *et al.*, 1983; Avasthi *et al.*, 1985; Lemalatha *et al.*, 1986). Testicular changes induced by *T. b. evansi* infection are accompanied by extravascular localization of trypanosomes in the testicular parenchyma and surrounding tissues and closely resemble those caused by *T. b. brucei* infection (Ikede *et al.*, 1988; Anosa, 1991).

1.2.1.3. Changes in Extratesticular Tissues

Scrotal changes in *T. congolense* and *T. vivax* infections are usually mild and sometimes not present (Ikede *et al.*, 1988). Focal alopecia of the scrotal skin was described by Anosa and Isoun (1980) in sheep and goats chronically infected with *T. vivax* and recently Omeke and Onuora (1992) described scrotal dermatitis, alopecia and oedema in boars infected with *T. congolense*. Detailed studies conducted recently in cattle chronically infected with *T. congolense* (Sekoni 1990; Sekoni *et al.*, 1990b) failed to show any gross or microscopic changes on the scrotum and tunica vaginalis. In contrast, the effects of *T. brucei*-group on the scrotum are characterised by rapid reddening of scrotal skin which later becomes oedematous, dark, necrotic, haemorrhagic and ulcerated within 2 - 3 weeks after the onset of parasitaemia (Ikede, 1979; Ikede and Akpavie, 1982; Ngeranwa *et al.*, 1991; Omeke and Onuora, 1992). At necropsy, the scrotal skin is markedly thickened due to oedema and inflammation, there is accumulation of fibrinous exudate in the scrotal sac and adhesions of the tunica vaginalis (Moulton and Sollod, 1976; Ikede, 1979). Histological changes observed are associated with localization of trypanosomes in the scrotum and include marked scrotal dermatitis, oedema, hyperkeratosis and infiltration of macrophages, lymphocytes and plasma cells (Ikede, 1979; Omeke and Onuora, 1992).

Kaaya and Oduor-Okelo (1980) and Waindi *et al.* (1986) reported aspermic caput, corpus and cauda epididymis and an apparent increase in the connective tissues surrounding the epididymal tubules in goats infected with *T. congolense*. Similar changes have also been reported in *T. congolense*-infected bulls (Sekoni *et al.*, 1990b) and

T. vivax-infected sheep and goats (Anosa and Isoun, 1980). However in the latter animals, some of the epididymal tubules, which still contained scanty numbers of spermatozoa, were also found to contain exfoliated testicular germinal cells and round cells with pyknotic nuclei. In contrast, *T. b. brucei* has been reported to cause more severe epididymal changes than those induced by *T. congolense* or *T. vivax* infections (Ikede *et al.*, 1988; Anosa, 1991). *T. b. brucei* induces mild to severe epididymitis, atrophy of afferent ductules and epididymal tubules, pyknosis of epididymal epithelium and aspermic epididymides in severely affected testis (Losos and Ikede, 1970; Ikede, 1979; Ikede and Akpavie, 1982). Oedema and intensive mononuclear cell infiltration and extravascular localization of trypanosomes in the epididymal interstitial tissue have also been reported (Ikede, 1979).

Pathological changes in seminal vesicles, and prostate glands in *T. congolense* or *T. vivax* infections are usually mild or moderate (Sekoni *et al.*, 1990b). No lesions have been documented in the spermatic cord, Cowper's glands, urethra, penis, glans penis and prepuce. Ikede (1979) described marked lymphangiectasis and lymphangitis with the accumulation of lymphocytes and lymph in the spermatic cord of rams infected with *T. b. brucei*. The seminal vesicles had flattened cuboidal epithelium suggestive of low testosterone activity. Thrombosis of the pampiniform plexus has also been reported in dogs infected with *T. b. brucei* (Morrison *et al.*, 1981).

1.2.1.4. Changes in Semen Characteristics

Changes in seminal characteristics induced by trypanosome infections in several animal species and man have been well documented (reviewed by Ikede *et al.*, 1988). Decline in semen volume and sperm concentration and increase in primary and secondary sperm abnormalities seem to be consistent clinical features of all pathogenic trypanosome infections (Hornby, 1921; Stephen, 1966; Apted, 1970). Seminal changes induced by *T. congolense* infection have been described in goats (Kaaya and Oduor-Okelo, 1980; Waindi *et al.*, 1986; Gombe, 1989), bulls (Grundler and Djabakou, 1985; Sekoni *et al.*, 1988, 1990a) and boar (Omeke and Onuora, 1992). Seminal changes in sheep have been mainly studied in infections caused by *T. vivax* (Isoun and Anosa, 1974a, 1974b; Ikede,

1979; Anosa and Isoun, 1980; Agu *et al.*, 1986; Akpavie *et al.*, 1987; Sekoni, 1992, 1993) and *T. b. brucei* (Akpavie *et al.*, 1987).

The rate of development and severity of ejaculate pathology in *T. congolense* and *T. vivax*-infected animals depends on the degree of susceptibility and the virulence of infecting trypanosomes while, in *T. brucei*-group infections, they also depend on invasiveness of the parasites in the testicular interstitium (Ikede *et al.*, 1988). Clinically, the thick, opaque and creamy ejaculate found in normal mature males progressively becomes milky, then watery and transparent within 3-12 weeks of trypanosome infection. This occurs concomitantly with a decline in ejaculate volume and a progressive decrease in sperm motility and sperm concentration resulting in oligozoospermia and aspermia. Notably, Sekoni *et al.* (1988) reported 42% and 45% mean reductions in semen volume and 40% and 54% reductions in spermatozoa concentration within 12 weeks of *T. congolense* and *T. vivax* infection in bulls, respectively. Semen could not be obtained in 3 infected bulls within that period. Similar observations were reported in bulls infected with *T. congolense* for 6 weeks (Grundler and Djabakou, 1985) and in boars infected with *T. congolense* for 3 - 9 weeks (Omeke and Onuora, 1992). Recently, Sekoni (1992) has reported a 40% to 90% reduction in mean semen volume in rams within 3 - 9 weeks of infection with *T. vivax*. By week 9 post-infection, the mean sperm concentration, percentage live sperm and percent motile sperm had declined by 99%, 83% and 96%, respectively. Similar observations were reported by Agu *et al.* (1986) in rams infected with *T. vivax* for 9 weeks.

The effects of *T. b. brucei* and *T. b. evansi* infection on semen colour, consistency, sperm motility and concentration in sheep and goats (Akpavie and Ikede, 1987; Ngeranwa *et al.*, 1991) are essentially similar to those reported in haematic trypanosome infections. However the rate of development and severity of seminal changes is frequently exacerbated by the development of orchitis and epididymitis in infected animals (Ikede *et al.*, 1988).

The primary sperm abnormalities frequently described in trypanosome infections include head (narrow, small, swollen) and acrosome (detached and serrated) abnormalities, abnormal mid-piece (swollen, rough, abaxial) and an increase in proximal and distal droplets. Bent tails, coiled tails and detached heads are secondary abnormalities

frequently observed (Waindi *et al.*, 1986; Akpavie and Ikede, 1987; Sekoni *et al.*, 1991; Sekoni, 1992, 1993; Ngeranwa *et al.*, 1991). Akpavie and Ikede (1987) observed an 8% and 37% increase in primary and secondary sperm abnormalities respectively after 4 weeks of infection with *T. vivax*. Similar increases for *T. b. brucei* infection were 17.3% and 61.4%, respectively. In bulls infected with *T. congolense* or *T. vivax*, Sekoni *et al.* (1988) reported an increase of 5 - 95% and 5 - 100%, respectively in mean percentages of total sperm abnormalities after 12 weeks of infection. Treatment with Novidium(R) in bulls after 12 weeks of infection did not lead to a significant improvement in either testicular and epididymal morphology (Sekoni, 1990) or spermatozoa morphological abnormalities (Sekoni *et al.*, 1991) after 12 weeks of chemotherapy showing that the infected bulls were still sub-fertile. A delay in resolution of gonadal lesions in rabbits infected with *T. b. brucei* following diminazine aceturate chemotherapy (Ikede and Akpavie, 1982) has been mentioned earlier.

1.2.2. Reproductive Changes in the Female

Although there are several reports on the effects of African trypanosomiasis on gonadal function in the male, especially in domestic animals, relatively few comparable studies have been conducted in females animals and man. Menstrual disorders, high abortion rates and general infertility caused by human trypanosomiasis were reported by Macfie (1913), Ridet (1953) and Apted (1970). A review by Agwu and Nuru (1981) on transplacental transmission of trypanosomes in animals highlights the important role trypanosomiasis plays in terminating pregnancy and causing infertility in females. Roberts and Gray (1973) and O'Hara and Gombe (1985) emphasized the effect of trypanosomiasis in delaying sexual maturity in cattle and goats, respectively. Roberts and Gray (1973) reported that heifers dying from trypanosome infection or killed at 1 year of age had juvenile ovaries and at two years of age some of the heifers had underdeveloped uteri and had shown no signs of heat. A delay on the onset of puberty and suppression of ovarian activity in pre-pubertal goats infected with *T. congolense* were documented by O'Hara and Gombe (1985).

1.2.2.1. Changes in Ovarian Function

Effects of *T. congolense* infection on oestrous cycle lengths have been reported in goats (Luckins *et al.*, 1986; Llewelyn *et al.*, 1987; Mutayoba *et al.*, 1988a, 1988b) and cows (Llewelyn *et al.*, 1988). Similarly, ovarian changes induced by *T. vivax* in sheep have been reported by Ogwu *et al.* (1984). Of 10 boran cows infected with *T. congolense* (Llewelyn *et al.*, 1988) 5 cows (50%) became acyclic by day 60 post-infection and 3 cows (25%) had persistent corpora lutea between 60 - 90 days post-infection. Persistent corpora lutea extending for 48 - 94 days were also observed in British White does infected with *T. congolense* and maintained with sub-curative dose of diminazine aceturate (Llewelyn *et al.*, 1987). In both studies *T. congolense* infection was found not to affect the length of oestrous cycles. However, studies in goats show that chronic *T. congolense* infection may induce irregular short (6 - 16 days) and longer (28 day) oestrous cycles (Mutayoba *et al.*, 1988b) or longer cycles (53-97 days) (Luckins *et al.*, 1986) before infected animals becomes acyclic.

At necropsy, the ovaries of infected animals are small and atrophic or cystic (Isoun and Anosa, 1974a; Mutayoba *et al.*, 1988a) or contain large persistent corpora lutea (Llewelyn *et al.*, 1987, 1988). Histopathological changes which have been observed in the ovaries of *T. congolense*-infected goats include degeneration and atrophy of ovarian stroma and primordial germ cells, inhibition of follicle maturation, lack of corpora lutea and polycystic formation (O'Hara *et al.*, 1985; Mutayoba *et al.*, 1988a; 1988b) and extensive ovarian fibrosis (Isoun and Anosa, 1974a).

1.2.2.2. Effect of Trypanosomiasis on Pregnancy

The effect of trypanosomiasis on pregnancy is well documented for several species of pathogenic trypanosomes in man and animals. Apted (1970) noted that premature births, stillbirth, high abortion rates and sterility frequently accompanied trypanosomiasis in women. Abortion and infertility in buffalo and cattle infected with trypanosomiasis were later described by Parkne and Dhake (1972); Esuruoso (1974); Murray *et al.* (1981) and Ogwu *et al.* (1981, 1984). Kanyari *et al.* (1983) observed a high abortion rate, decline in kidding and twinning percentages as well as decline in mean weights of twins kids born from does infected with *T. congolense*. Increases in calving intervals ranging from 68

days (Trail *et al.*, 1991) to 169 days (Agyemang *et al.*, 1993) have been reported in N'dama cattle raised in trypanosome-endemic areas. Intra-uterine infections resulting in stillbirth or neonatal mortality have been observed in cattle infected with *T. vivax* (Ogwu *et al.*, 1986). Pregnant heifers infected with *T. vivax* have been observed to abort during the first trimester (Okech *et al.*, 1993) or deliver prematurely during the third trimester (Ogwu *et al.*, 1986). *T. vivax* infection in ewes during late pregnancy have also been reported to induce premature delivery to underweight lambs (Reynold and Okwuruke, 1988). Recently, Edeghere *et al.* (1992) reported that *T. b. brucei* infection in ewes has a devastating effect on pregnancy irrespective of the trimester of infection. The infection resulted in 16.5% abortions, 100% death of infected ewes and 33.3% neonatal deaths.

The review by Ogwu *et al.* (1981) highlights the possibilities of transplacental transmission of trypanosomes in animals and man and its role in the pathogenesis of abortion in trypanosomiasis. The first confirmed case involving maternal transmission of trypanosomes in animals was reported by Ikede and Losos (1972b) in a study where the ewe inoculated with *T. vivax* gave birth to a weak lamb 33 days later. The blood of the lamb contained large numbers of trypanosomes at birth and the lamb died 4 h later from severe anaemia. Although Isoun and Anosa (1974a) did report a similar finding, they were able to demonstrate the presence of *T. vivax* in the amniotic fluid of a pregnant sheep. Later, Ogaa *et al.* (1991) found trypanosomes in the allantoic fluid but not in the amniotic fluid of pregnant goats experimentally infected with *T. congolense*, providing evidence that *T. congolense* can possibly cross the placenta and cause abortion in goats.

Recently Trail *et al.* (1993) evaluated the influence of trypanotolerance on reproductive performance of the N'dama cattle under a medium tsetse challenge in Gabon. Trypanosomes detected were *T. congolense* and *T. vivax*. By using the changes in PCV as a repeatable trait and performance measured by cow calving rate, calf weaning weight and cow weight change over the lactation period they were able to show that there was a 3.3 $\pm$ 0.65% increase in calving rate, a 0.91 $\pm$ 0.4% increase in calf weaning weight and a 0.95 $\pm$ 0.39% increase in cow weight over the lactation period for 1% average increase in PCV. This study indicated that the effects of trypanosomiasis on pregnancy and the postpartal survival of the young and dam may depend on their ability to control anaemia.

1.2.3. Pituitary and Hypothalamic Changes

The role of the hypothalamus and anterior pituitary gland in regulating the reproductive function in animals and man is now well established. Factors which induce pathological or functional changes in the hypothalamo-pituitary axis are likely to cause adverse effects on reproduction. An increasing number of reports have indicated that reproductive changes observed in trypanosomiasis may be partly induced by changes in the hypothalamo-pituitary axis. Ridet (1953) and Apted (1970) observed endocrine dysfunctions, which were manifested as impotence, gynaecomastia, feminine distribution of fat, myxodaematous subcutaneous infiltration, infertility and sterility in human sleeping sickness. Edington and Gilles (1969) suggested that involvement of the pituitary gland in human trypanosomiasis could offer an explanation for the clinical signs of hypopituitarism seen in the terminal stages of the disease. However, the nature and extent of pituitary damage in humans is still poorly understood although Hawking and Greenfield (1941) noted fibrosis of the pituitary and atrophy of the thyroid in two cases of trypanosomiasis.

Experimental *T. b. brucei* infection has been shown to cause focal coagulative necrosis and interstitial mononuclear cell infiltration in the pituitary gland of sheep, goats, donkeys and dogs (Losos and Ikede 1970, 1972; Ikede and Losos 1972a, 1975; Moulton and Sollod, 1976; Morrison *et al.*, 1981). Similar changes were observed in natural *T. b. brucei* infection of horses (Ikede *et al.*, 1973). Lesions observed ranged from mild interstitial infiltration of lymphocytes, oedema and hyperaemia of the adenohypophysis to acute coagulative necrosis and fibrosis in infected animals. These lesions were associated with extravascular localization of trypanosomes in the gland. Mononuclear infiltration of the pars nervosa and surrounding meninges have also been reported in horses infected with *T. b. brucei* but trypanosomes were not observed in the lesions (McCully and Neitz, 1971; Ikede *et al.*, 1973). Thrombosis of the sinusoids in necrotic areas of the gland were later reported by Ikede and Losos (1975) in sheep infected with *T. b. brucei* indicating that necrosis could have resulted from ischaemia.

Although the occurrence of lesions in the adenohypophysis following *T. b. brucei* infection could be associated with extravascular localization of the parasites in the organ, changes in the pituitary have also been observed in sheep, goats and cattle infected with *T. congolense* or *T. vivax* (Losos and Ikede, 1972; O'Hara *et al.*, 1985; Mutayoba *et al.*,

1988a; Abebe, 1991). Mutayoba *et al.* (1988a) described reduced degranulation of the basophils and decreased basophilic acini secretion in goats chronically infected with *T. congolense.* Light microscopic studies have failed to show the presence of trypanosomes or overt degenerative lesions in the pituitary of *T. congolense or T. vivax*-infected animals. However, recent ultrastructural studies of the pituitaries of cattle infected with *T. congolense* (Abebe, 1991; Abebe *et al.*, 1993a) have confirmed that degenerative changes do occur in the pituitary as early as 6 - 7 weeks post-tsetse challenge and trypanosomes can be found in the microvasculature of the affected pituitaries but not in interstitial areas. The non-invasiveness of *T. congolense* into the pituitary parenchyma confirms that different mechanisms might be involved in the pathogenesis of pituitary lesions in animals infected with haematic trypanosomes. Although, *T. vivax* has been shown to localize extravascularly in the pituitary (Fiennes, 1950) and cerebrospinal fluid (Whitelaw *et al.*, 1988) overt histopathological changes in the pituitary of *T. vivax*-infected animals have not been reported.

Reports on the effects of trypanosomiasis on the function of the hypothalamus and other parts of the brain have been obtained from *T. b. brucei* infections (Stibbs, 1984; Schultzberg *et al.*, 1988; Amole *et al.*, 1989), *T. b. gambiense* (Marck *et al.*, 1985), *T. b. rhodesiense* (Schmidt and Bafort, 1987; Waitumbi, *et al.*, 1988) and *T. cruzi* (Pittella *et al.*, 1990) infections of rats, rabbits, dogs, monkeys, cattle and man. Soon after invasion of *T. b. brucei* into the blood stream of infected rat, trypanosomes rapidly penetrate the areas lacking blood-brain and blood-nerve barriers, namely the sensory ganglia and circumventricular organs including the area postrema, sensory ganglia, pineal gland and median eminence (Schultzberg *et al.*, 1988). This distribution of trypanosomes corresponds to the areas of the brain where some of the main clinical symptoms originate including pain (sensory ganglia), disturbed circadian rhythm (pineal gland) and neuroendocrine dysfunction (hypothalamus and pituitary). Invasion of the hypothalamus leads to local increases in several monoamines including 5-hydroxyindoleacetic acid (Amole *et al.*, 1989) and decline in serotonin (Stibbs, 1984). These neurochemical changes may affect hypothalamic activity (Stibbs, 1984). Light and electron microscopic studies in *T. b. gambiense*-infected rats (Marck, *et al.*, 1985) have shown that cerebral inflammation occurs, which may extend to the pituitary and other parts of the brain.

Generalized perivascular infiltration of lymphocytes and plasma cells, perivascular oedema and ischaemia have been reported in several parts of the brain, including the hypothalamus of cattle infected with the virulent strain of *T. b. rhodesiense* (Wellde *et al.*, 1989). Similar changes were described in dogs infected with *T. cruzi* (Pittella *et al.*, 1990).

Histopathological changes in the hypothalamus and brain in *T. congolense* and *T. vivax* infections are more mild than those induced by the *T. brucei*-group. Mwambu and Losos (1978) reported the dilation of blood vessels and mononuclear cell infiltrations in different areas of the brain in cattle infected with either *T. congolense* or *T. vivax* and Isoun (1975) described generalized fibrin formation in the blood vessels of the brain in mice infected with *T. vivax*. The cause of these changes in the brain in *T. congolense* infections seem to be associated with accumulation of trypanosomes in the microvasculature of the brain, including the hypothalamus and pituitary, causing local inflammation and ischaemia (Losos *et al.*, 1973; Abebe *et al.*, 1993).

1.2.4. Changes in Reproductive Hormones

There are few studies on reproductive endocrine changes induced by trypanosome infection in males. From plasma samples obtained three times a week, Waindi *et al.* (1986) observed a decline in plasma testosterone concentration in *T. congolense*-infected Toggernburg goats. Daily plasma testosterone concentrations were reduced significantly in infected goats (range 0.76 - 4.33 nmol l^{-1}) when compared to uninfected control goats (range 1.5 - 14.57 nmol l^{-1}). Although they associated the decline of testosterone concentrations in infected goats with the reduction in amplitude and frequency of testosterone peaks, the authors did not explain how testosterone peaks were assessed in samples obtained so infrequently. Testosterone is well known to be secreted in a pulsatile manner with its secretion dependent on pulsatile secretion of luteinizing hormone (LH) and gonadotrophin-releasing hormone (GnRH) from the pituitary and hypothalamus, respectively (Setchell, 1978; Pelletier and Caraty, 1981; Messi *et al.*, 1988). Decline in mean weekly plasma testosterone concentrations in a mixed *T. congolense* and *T. b. brucei* infection in West African dwarf rams were reported recently by Adeyemo *et al.* (1990). They reported a significant progressive

decline in mean plasma testosterone concentrations after 5 weeks post-infection (pre-infection levels 2.6 ± 0.26 ng ml^{-1}) while by week 10 post-infection, mean weekly levels were 0.6 ± 0.1 ng ml^{-1} and these levels only rose slightly to 1.1 ± 0.1 ng ml^{-1} within 5 weeks after diminazene aceturate treatment in infected rams. Detailed studies aimed at establishing the changes in pulsatile pattern of plasma testosterone concentration in trypanosome infections have not been documented.

The evidence that a decline in plasma testosterone concentration might possibly be associated with reduced testicular steroidogenesis was reported by Waindi *et al.* (1984). They showed that *in vitro* testosterone secretory capacity of rat hemitestes previously exposed to *T. congolense* was significantly reduced (values 8.44 ± 0.69 nmol/gm-testis) when compared to the controls (values 20.29 ± 0.08 nmol/gm-testis). The decline in testicular steroidogenesis in *T. b. brucei*-infected rats has recently been associated with the reduced responsiveness to pituitary gonadotrophins, possibly due to the decline in the number of testicular LH receptors expressed per testis and an increase in the equilibrium association constant (Ka) of the remaining receptors (Soudan *et al.*, 1992). However, studies to confirm whether similar changes occur in infected domestic animals or humans have not been documented.

Alterations in gonadal steroid hormone secretion have also been described in the female. Changes in plasma progesterone concentrations (O'Hara *et al.*, 1985; Luckins *et al.*, 1986; Llewelyn *et al.*, 1987) and progesterone and oestradiol-17ß (Mutayoba *et al.*, 1988b, 1988c) have been described in *T. congolense*-infected female goats. Similarly, changes in plasma progesterone were described by Llewelyn *et al.*, (1988) in *T. congolense*-infected cows. In all the above studies significant decreases in basal and mean luteal plasma progesterone concentrations were observed in infected goats and cows. Absence of the preovulatory oestradiol-17ß surge in some infected goats (Mutayoba *et al.*, 1988b) was linked with impaired ovulation and subsequent abnormal corpus luteum formation. Goats chronically infected with *T. congolense* have also been found to have elevated concentrations of plasma 13, 14-dihydro-15-keto prostaglandin $F_{2\alpha}$ (PGFM) a lung metabolite of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Mutayoba *et al.*, 1989a). Increased secretion of $PGF_{2\alpha}$ was suggested by Mutayoba *et al.* (1989a) to interfere with normal oestrous cycles and luteal function in goats and probably in other animals.

Studies on the effects of trypanosomiasis on pituitary gonadotrophin secretion have been reported in man (Emeh and Nduka, 1983; Boersma *et al.*, 1989) and rats (Hublart *et al.*, 1990; Soudan *et al.*, 1992). Emeh and Nduka (1983) showed that serum gonadotrophins (follicle stimulating hormone (FSH) and LH) were significantly depressed in 11 cases of advanced Gambian sleeping sickness. Values returned to near normal within a year after antitrypanosomal treatment. However single, rather than sequential samples were taken before and after treatment so this study did not allow for the pulsatile nature of gonadotrophin secretion. In another study, Boersma *et al.* (1989) observed no changes in serum gonadotrophins in humans infected with *T. b. gambiense.* The information available on the effects of trypanosomiasis on gonadotrophin levels in rats is also conflicting. Plasma LH and FSH were found not to be affected by acute *T. b. brucei* infection (Hublart *et al.*, 1990) despite the observed increases in pituitary LH and FSH content and a significant decline in serum testosterone concentration. In contrast, Soudan *et al.* (1992) have recently reported a significant decline in serum LH and testosterone concentrations in rats infected with the same strain of *T. b. brucei.* In both studies however, a single serum sample was obtained after 3 days of infection following either decapitation or terminal anaesthesia of the infected rats. As the pulsatile nature of gonadotrophin hormone release is well known, more studies need to be carried out, especially in domestic animals, by collecting more frequent samples at different stages of trypanosome infection to assess the dynamic changes in pituitary endocrine function and the role of the hypothalamus in inducing these changes.

1.2.5. Changes in the Adrenal Function

The adrenal gland plays a central role in most of the physiological adaptations that constitute the stress response and its role in altering the functions of the reproductive system is well established (Moberg, 1985; Armstrong, 1986; Olster and Ferin, 1987). Both the adrenal gland and the gonads depend on appropriate trophic hormones from the anterior pituitary gland the secretion of which is dependent on appropriate hypothalamic releasing hormones. Several factors which influence the function of either the hypothalamo-pituitary axis or the adrenal gland also cause marked effects in reproductive activity (Moberg, 1985). For example, administration of corticotropin

releasing hormone (CRH) or adrenocorticotrophin (ACTH) cause activation of the hypothalamo-pituitary-adrenal (HPA) axis and this suppresses reproductive activity in male rats (Charpenet *et al.*, 1981; Rivier *et al.*, 1986), bulls (Johnson *et al.*, 1982), boars (Liptrap and Raeside, 1975) and rams (Naylor *et al.*, 1990).

The adrenal gland is invariably affected in cases of trypanosome infections. Fiennes (1970) reported the infiltration by mononuclear cells below the capsule and penetrating the zona fasculata in cattle infected with *T. congolense*. Areas of necrosis and fibrosis were observed in the cortex and medulla. Increased adrenal weight and marked hypertrophy of the zona fasciculata and zona reticularis and an increase in the zona fasculata-reticularis ratio have also been reported in goats infected with *T. congolense* (Mutayoba *et al.*, 1988a). Valli and Forseberg (1979) reported adrenal hyperplasia and moderate cytoplasmic atrophy of the zona fasculata in calves infected with *T. congolense*, whereas recently, Ogwu *et al.* (1992) described hyperemia, haemorrhage, hyperplasia and severe mononuclear cell infiltration in the adrenal subcapsular and corticoid layers in *T. congolense*-infected zebu cattle.

An increase in total adrenal weights was observed in *Microtus montanus* (Voles) infected with *T. b. gambiense* (Seed *et al.*, 1976) and in sheep infected with *T. b. brucei* (Ikede and Losos, 1975). The adrenal cortex of infected sheep was hypertrophied and contained vacuolated cells and abundant fat. Subcapsular mononuclear cell infiltration was also evident. Moulton and Sollod (1976) further described the infiltration of lymphocytes and plasma cells in the zona fasculata and in perivascular spaces in *T. b. brucei*-infected calves.

The effect of trypanosomiasis on adrenal steroidogenic activity is still equivocal. For example, Mutayoba and Gombe (1989) reported a significant increase in plasma cortisol concentration during the acute and chronic phases of *T. congolense* infection in goats. Plasma cortisol levels increased significantly following infection from pre-infection values (range 10 - 25 nmol l^{-1}) to reach a peak of 59.0 $\pm$ 8.9 nmol l^{-1} on week 4 post-infection. Although cortisol levels declined slightly thereafter, mean values remained significantly higher (>30nmol l^{-1}) than control values up to 24 weeks post-infection. In contrast, Ogwu *et al.* (1992) reported non-significant increases in serum cortisol concentrations in infected cattle between week 2 - 6 post-infection followed by a

non-significant decline up to week 12, before increasing slightly thereafter. Detailed studies on the integrity of the hypothalamo-pituitary-adrenal (HPA) axis in Zebu cattle during the acute phase of *T. congolense* infection have been reported by Abebe (1991). Infected Zebu cattle showed a slight increase in cortisol concentration during the prepatent period followed by a decrease during the patent period. The author associated the decrease in cortisol concentrations with a low basal circulating concentration of ACTH which was observed in infected animals, and further suggested that low ACTH could have occurred as a result of a defect in pituitary function. Pituitary defects in infected animals were confirmed by low plasma ACTH and cortisol responses to exogenous CRH stimulation (Abebe, 1991; Abebe *et al.*, 1993b) and by failure of these animals to respond to insulin-induced hypoglycaemia stress (Abebe and Eley, 1992). The cause of the differences in adrenal response between goats (Mutayoba and Gombe, 1989) and cattle (Abebe, 1991; Abebe and Eley, 1992; Abebe *et al.*, 1993b; Ogwu *et al.*, 1992) to *T. congolense* infection is debatable. Detailed studies similar to those performed recently in cattle will be needed in small ruminants before the effects of *T. congolense* infection on the HPA axis in these animals is clearly defined.

1.3. PATHOGENESIS OF TRYPANOSOME-INDUCED INFERTILITY

In seeking to provide a mechanism by which trypanosomes affect the reproductive function of the host, two main hypotheses have been proposed. One whereby disease factors cause direct effects and the other in which the disease has indirect effects. Direct trypanosome effects on reproductive function are probably mediated via one or more of the wide variety of biologically active molecules secreted by the invading trypanosomes or may result from direct damage of the invaded tissue by trypanosomes *per se* or due to anoxia resulting from anaemia, thrombosis and infarction. Indirect trypanosome effects could be mediated by interaction of the responses of the neural, endocrine and immune systems of the host to infection. The immune system is highly activated in trypanosome infection with the production of several cytokines by activated macrophages and other immune cells during infection. Of these Interleukin-1 (IL-1) and Interleukin-6 (IL-6) are known to activate the hypothalamo-pituitary-gonadal

axis (Dinarello, 1988; Kalra *et al.*, 1990a; Gorospe and Spangelo, 1993) as a part of their pleiotropic action in stimulating the acute phase response.

1.3.1. Direct Trypanosome Effects on Reproductive Function

Several biologically active substances are now known to be produced by African trypanosomes and their possible role in the pathogenesis of trypanosomiasis has been reviewed by Tizard *et al.* (1978b) and further investigated in subsequent studies (Kaaya *et al.*, 1980; Hambrey *et al.*, 1980, 1984; Knowles *et al.*, 1987; 1989). The 64kD glycoprotein present on the surface coat of trypanosomes is known to be highly antigenic and when the surface coat is shed in the circulation it combines with antibody to form immune complexes (Tizard *et al.*, 1978b). Deposition of these immune complexes in the microvasculature of several organs may contribute to the immune-complex-derived lesions observed in trypanosomiasis (Boreham and Kimber, 1970). Morrison *et al.* (1981) and Jubb and Kennedy (1985) postulated that these immune complexes may be involved in circulatory disturbances resulting in for example anoxia and degeneration of the gonads. *T. b. gambiense* has been shown to metabolize tryptophan to several toxic products including indole-3-ethanol (tryptophol, TOL), indole-3-acetic acid (IAA), indole-3-lactic acid (ILA) and 5-hydroxy-trytophan to 5-hydroxytryptophol (Stibbs and Seed, 1975). TOL is known to suppress the immune system, interact with cell membranes and cause haemolysis, alter nerve impulse transmission producing sleeplike behaviour and lower body temperature (Tizard *et al.*, 1978b). The toxic effect of trypanosome by-products might play an important role in the alteration of the reproductive function of infected animals (Heisch *et al.*, 1970).

Several enzymes produced by dead or dying trypanosomes in the blood circulation are known to induce lesions in the host. Trypanosome-derived proteases, including cathepsin D, have been shown to convert plasma plasminogen to plasmin increasing the amount of fibrinogen degradation products in plasma (Boreham and Facer, 1973) and cathepsin D has also been shown to act on kininogens generating large amounts of kinins (Boreham and Kimber, 1970; Greenbaum, 1973). The active proteolytic activity of trypanosome-derived enzymes in the microcirculation of organs, including the reproductive organs, is potentially capable of causing local endothelial

damage and interfering with vascular permeability. Recently, Hublart *et al.* (1990) suggested that proteases of parasite origin may cause gonadotrophic dysfunction in rats infected with *T. b. brucei.* The second group of trypanosome derived enzymes are the phospholipases and lysophospholipases (Tizard *et al.*, 1978b, 1979). *T. congolense* and *T. b. brucei* have been shown to produce active phospholipase A1 upon *in vitro* autolysis (Tizard *et al.*, 1979; Hambrey *et al.*, 1980, 1984) and *in vivo* it is possible that phospholipase A1 acts on endogenous phosphatidylcholine to generate free fatty acids (FFA) especially linoleic and palmitic acid. Phospholipase A1 in conjunction with FFAs are capable of acting on cell membranes in several body organs and in the circulation may cause haemolysis. Linoleic acid and palmitic acid are potent immunosuppressants and a B-lymphocyte stimulants, respectively (Tizard *et al.*, 1979). Since *T. congolense* tends to localise within the microvasculature, phospholipase A1 may provoke endothelial damage in several organs including the gonads (Carne and Onon, 1978) and in case of *T. b. brucei*, autolysis within connective tissue may occur stimulating local inflammatory changes (Hambrey *et al.*, 1984). Other enzymes of trypanosomal origin which have been reported and may contribute to the pathogenesis of the lesions induced by trypanosome infections include peptidases (Knowles *et al.*, 1987, 1989) and alanine transaminase (Boid *et al.*, 1980). Host peptidases have restricted specificities but are known to inactivate hypothalamic releasing hormones (Griffin, 1976).

Direct tissue damage by invading trypanosomes is probably one of the most important mechanisms by which trypanosomes of the *T. brucei*-group cause deleterious effects on reproductive function. As reviewed earlier, upon entry into the blood circulation of the host, *brucei*-trypanosomes leave the vascular system and localize in large numbers in interstitial spaces of several body organs including the gonads, pituitary and hypothalamus where they provoke marked inflammatory changes (Ikede *et al.*, 1988). The direct effects induced by *T. congolense* and *T. vivax* infections on the reproductive system are probably associated with their tendency to localise in large numbers in the microvasculature causing generalised dilation, local endothelial damage, fibrin degeneration and thrombosis and hence interfering with local nutrient supply (Ashman and Seed, 1974; Banks, 1978; Valli and Forsberg, 1979; Abebe *et al.*, 1993a). Thrombosis and subsequent infarction and coagulative necrosis in the gonads, pituitary

and other organs has been reported in *T. congolense* (Masake, 1980; Waindi *et al.*, 1986; Sekoni *et al.*, 1990b), *T. vivax* (Anosa and Isoun, 1980) and *T. b. brucei* (Losos and Ikede, 1970, 1972; Ikede, 1979; Morrison *et al.*, 1981) infections.

Anaemia is well recognised to be an inevitable consequence of trypanosome infections and it plays a central role in the pathogenesis of this disease in several animal species (Hornby, 1921; Murray, 1979; Murray and Dexter, 1988; Katunguka-Rwakishaya, 1992). The pathogenesis of anaemia in trypanosomiasis has been extensively investigated. In cattle, anaemia is associated with increased red cell destruction (Mamo and Holmes, 1975), dyshaemopoiesis (Preston *et al.*, 1979) and haemodilution (Holmes, 1976) whereas in sheep it is probable that anaemia is caused mainly by increased erythrocyte destruction and haemodilution (Katunguka-Rwakishaya, 1992; Katunguka-Rwakishaya *et al.*, 1992). The possible involvement of anaemia in inducing reproductive lesions in trypanosomiasis has been emphasized by several workers (reviewed by Ikede *et al.*, 1988). Most degenerative lesions are associated with anoxia induced by chronic anaemia observed in chronic trypanosomiasis.

1.3.2. Indirect Trypanosome Effects on Reproductive Function

Indirect effects induced by trypanosome infection on reproductive function include those effects thought to be mediated by the host inflammatory mediators produced by a variety of cells including macrophages and monocytes in the infected host. Among these factors are cytokines released as soluble polypeptides mainly from cells of the immune system during acute illness in response to antigen, bacterial toxins and tissue injury (Evans and Whicher, 1992). A number of cytokines mediate diverse effects on a wide range of tissues with combined effects known as the acute phase response (Stadnyk and Gauldie, 1991). The production of cytokines in endocrine glands and their actions on hormone-responsive cells is currently a subject of intensive research (reviewed by Kennedy and Jones, 1991). It is now known that the secretion of several cytokines is increased during the course of trypanosome infection. Tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) secretion by activated macrophages is increased in *T. cruzi* infections (De Titto *et al.*, 1986; Silva *et al.*, 1992) and *T. congolense* (Mitchell *et al.*, 1986; Sileghem *et al.*, 1993). *T. congolense* has been

shown to induce an increase in TNF-α receptor expression on peripheral blood leucocytes in sheep (Winstanley *et al.*, 1993). Similarly, an enhanced spontaneous secretion of IL-1 from activated macrophages and parasitaemia-associated release of IFN-α, IFN-ß and IFN-γ by various host cells has been reported in mice infected with *T. b. brucei* (Bancroft *et al.*, 1983; Askonas and Bancroft, 1984; Sileghem *et al.*, 1989). Splenocytes from mice infected with *T. b. equiperdum* were also found to produce high amounts of IL-6, IFN-γ and TNF-α (Perito *et al.*, 1992).

The possible involvement of these cytokines in the pathogenesis of trypanosome-induced reproductive dysfunction has not been documented. However, several cytokines activated during trypanosome infections are known to affect endocrine functions (Kennedy and Jones, 1991). IL-1 administered systemically increases the plasma concentrations of ACTH and corticosteroids (Woloski *et al.*, 1985; Dinarello, 1988) via the stimulation of CRH (Uehara *et al.*, 1987). IL-1 effects on secretion of other pituitary hormones are less well documented and inconsistent in nature, with reports of both inhibitory and stimulatory effects (Rettori *et al.*, 1987; Bernton *et al.*, 1987; Beach *et al.*, 1989). The effects of IL-1 on the hypothalamo-pituitary-gonadal axis are also complex. *In vitro*, IL-1 enhances LH release from pituitary cells (Bernton *et al.*, 1987). In castrated male rats (Rivier and Vale, 1989), and in oestrogen-primed ovariectomised female rats (Kalra *et al.*, 1990a) systemic administration of IL-1 inhibits the release of LH via inhibition of hypothalamic GnRH secretion (Kalra *et al.*, 1990a; 1990b). Immunoreactive IL-1ß is present in human hypothalamus (Breder *et al.*, 1988), and specific, high-affinity IL-1 receptors have been characterised in the rat hypothalamus (Farrar *et al.*, 1987), pituitary cells (Tracey and de Souza, 1988) and epididymis and testis (Takao *et al.*, 1990). IL-1 has also been shown to inhibit the synthesis of testosterone by Leydig cells (Calkins *et al.*, 1988; Sun *et al.*, 1993; Tortorella *et al.*, 1993) and inhibit luteinization of granulosa cells (Adashi, 1990).

TNF-α produces similar biological effects to those of IL-1, but its effects on the hypothalamo-pituitary-gonadal axis are less documented. TNF-α has been shown to be produced by granulosa cells where it acts in an autocrine manner to attenuate the sensitivity of granulosa cells to follicle stimulating hormone (FSH) (Kennedy and Jones, 1991). It may also act as a paracrine factor increasing progesterone secretion by theca

cells, acting by influencing the conversion of 25-hydroxycholesterol to pregnenolone (Roby and Terranova, 1990). TNF-α also stimulates prolactin release from the anterior pituitary cells, a process which involves intracellular calcium mobilization (Koike *et al.*, 1991). Recently, TNF-α has been shown to antagonise FSH action in cultured Sertoli cells (Mauduit *et al.*, 1993) and to regulate the steroidogenic function of mouse Leydig cells (Xiong and Hales, 1993).

IL-6 has been shown to act in a paracrine manner to stimulate the release of prolactin, growth hormone and LH from rat anterior pituitary cells *in vitro* (Spangelo *et al.*, 1989). IL-6 may be released from activated pituitary cells (Spangelo *et al.*, 1990) and pituitary adenoma cells (Jones *et al.*, 1990) in concentrations capable of stimulating pituitary hormone release. IL-6 also affects the function of other endocrine glands including the ovary (Gorospe and Spangelo, 1993), adrenal (Evans *et al.*, 1990), thyroid (Grubeck-Loebenstein *et al.*, 1986) and the pancreas (Campbell *et al.*, 1989).

The regulation of steroidogenesis in the testis involves a complex interaction of hormones such as LH and FSH and intracellular signaling pathways (Leung and Steele, 1992). The hypothalamic neurohormone CRH is stimulated by several cytokines including IL-1, IL-6 and TNF-α during the acute phase response (Uehara *et al.*, 1987; Whicher and Westacott, 1992). During trypanosome infections, secretion of CRH is probably increased in response to stress induced by the infection (Mutayoba and Gombe, 1989; Soudan *et al.*, 1992). However, CRH has also been shown to act in an antireproductive manner at the level of the brain by inhibiting GnRH action (Rivier *et al.*, 1986). Immunoreactive CRF has been found to be present in the testis of sheep (Audhya *et al.*, 1987, 1989), rats (Fabbi *et al.*, 1990) and man (Leung and Steele, 1992) and it is secreted locally by several testicular cells including the Leydig cells, germ cells and epididymal cells (Yoon *et al.*, 1983; Fabbri *et al.*, 1990). CRF secreted from the Leydig cells is known to act in an autocrine manner through high-affinity receptors at the Leydig cell membrane (Ulisse *et al.*, 1989; Dufau *et al.*, 1993) to inhibit LH action by inhibiting gonadotrophin-induced cAMP generation and androgen production (Dufau *et al.*, 1993). Serotonin which is a major regulator of CRF production in the brain (Holmes *et al.*, 1982) is also present in the testis (Campos *et al.*, 1990). Stress-induced serotonin release from central and peripheral nerve terminals (Adell *et al.*, 1988) could

affect gonadal receptors and might be involved in stress-induced testosterone inhibition through CRF (Dufau *et al.*, 1993). Similarly, stimulation of serotonin release from the Leydig cells and intertubular macrophages and mast cells (Askenase *et al.*, 1980; Tinajero *et al.*, 1992), under the influence of macrophage-derived interleukins (Maddocks *et al.*, 1990) during testicular inflammation as that seen in trypanosome infection, could possibly impair Leydig cell steroidogenic activity.

1.3.3. Effect of Trypanosome-induced Pyrexia on the Function of the Testis

In most mammalian species, the male gonads are located outside the body cavity in a highly specialized skin pouch, the scrotum, where they are kept at about 2°C below body temperature in rhesus monkey, 6.2 - 7.1°C below body temperature in rams and 8.3 - 8.5°C below body temperature in rats and mice (Harrison and Weiner, 1948). The lower temperature is of great importance for the maintenance of normal testicular functions, particularly spermatogenesis (Kendell and Swerdloff, 1988; Steinberger, 1991; Clarke and Tilbrook, 1992). Effective control of stable testicular temperature is achieved by a combination of several mechanisms such as the presence of powerful scrotal themoregulatory reflexes (Waites, 1976) and the presence of a highly specialised thermal exchange between the testicular arterial and venous blood streams formed by extensive coiling of the testicular veins along the incoming internal spermatic artery (Harrison, 1949; Kirby, 1953; Waites and Moule, 1961).

It has been recognised for a long time that factors which induce elevation of testicular temperature interfere with normal spermatogenic activity. Damage of spermatogenic function of the testis by the way of artificial cryptorchidism has been produced in the rat (Nelson, 1951; Swerdloff *et al.*, 1971), rabbit (Asdell and Salisbury, 1941; Glover, 1959) and ram (Moore and Oslund, 1924) and by way of scrotal insulation in the ram (Glover, 1955; Dutt and Hamm, 1957) and bull (Moore and Aslund, 1924; Gunn *et al.*, 1942). Spermatogenesis is also disrupted as a result of acute febrile conditions (Gunn *et al.*, 1942; Wilson, 1964), raised ambient temperature (Rathore and Yeates, 1967; Rhynes and Ewing, 1973; Wettemann and Desjardins, 1979) and in the varicocele condition (Kay *et al.*, 1979). Some of the characteristic responses noted after prolonged exposure of the testes to higher temperature include, lowered fertility,

ejaculation of a high proportion of sperm with morphological abnormalities, decreased semen volume and sperm numbers and interference with spermatocyte and/or spermatid maturation.

Development of fever (pyrexia) is a consistent feature of an acute phase response in several infectious conditions and is mediated through the activation and subsequent interaction of several endogenous pyrogenic cytokines including IL-1ß, IL-1α, IL-6, TNF-α, IFN-α, IFN-γ and IL-8 (Whicher and Westacott, 1992). Animals suffering from trypanosomiasis develop chronic intermittent pyrexia with rises in temperature of between 1 to 4^{o}C above normal rectal temperatures depending on the virulence of infecting trypanosome strain and resistance of the host (Hornby, 1921; Losos, 1986; Stephen, 1986). Such a rise in body temperature has been repeatedly suggested to exert either a direct or an indirect effect on the function of testicular germ cells in infected animals (reviewed by Ikede *et al.*, 1988). This suggestion is supported by evidence that the changes in seminal characteristics and lesions observed in the testicular germ cells during trypanosome infections are closely similar to those induced by elevation of testicular temperature (Kaaya and Oduor-Okelo, 1980; Ikede *et al.*, 1988; Omeke and Onuora, 1992).

The mechanism by which a rise in temperature can induce damage to the spermatogenic process is not clear. Testicular spermatogenic cells show differing degrees of susceptibility to heat stress. Spermatocytes in late pachytene and round spermatids are known to be more heat sensitive than other spermatogenic cells (Blackshaw and Hamilton, 1971; Blackshaw *et al.*, 1973). This is possibly due to greater instability of their plasma membrane at increased testicular temperature (Blackshaw and Hamilton, 1971) leading to increasing lysosomal fragility and release of autolytic enzymes including acid phosphatases, ß-N-acethylhexosaminadase and arylsulfatase (Lee and Fritz, 1972). Elevated testicular temperature also interferes with spermatogenic activity by inducing a decline in enzyme activity involved in DNA synthesis in spermatogenic cells including DNA polymerase beta and DNA polymerase gamma (Fujisawa *et al.*, 1988).

Steroid biosynthesis and Leydig cell morphology are also affected by elevated testicular temperatures. Leydig cells from abdominal testes of adult rats made

unilaterally cryptorchid (Bergh *et al.*, 1984a) showed an increase in cytoplasmic lipid droplets, a sparse endoplasmic reticulum and an increase in the nuclear and cytoplasmic areas. Despite the increase in the organelles associated with steroidogenesis in the Leydig cells, serum testosterone (both basal and after LH stimulation) remains low to normal suggesting *in vivo* dysfunction of Leydig cells (de Kretser *et al.*, 1979; Wilton and de Krester, 1984; Bergh *et al.*, 1984b). Elevated testicular temperature is known to affect enzymes involved in both the Δ^4 testosterone biosynthetic pathway (pregnenolone to progesterone, 17α-hydroxyprogesterone, androstenedione and testosterone) and Δ^5 pathway (pregnenolone to 17α-hydroxypregnenolone, dehydroepiandrosterone, androstenedione and testosterone) (LeVier and Spaziani, 1968; Inano and Tamaoki, 1968; Farrer *et al.*, 1985). The Δ^5 pathway is thought to be more sensitive to elevated temperature than the Δ^4 pathway (LeVier and Spaziani, 1968). Since the Δ^4 pathway is more prominent than Δ^5 pathway in rats (Slaunwhite and Burgett, 1965), the effect of temperature on Δ^4 pathway has been studied more in these animals. Biosynthetic enzymes known to be more heat sensitive include those involved in the conversions mediated by 17α-hydroxylase and 17ß-hydroxysteroid dehydrogenase (de Krester *et al.*, 1979; Bergh *et al.*, 1984b; Steinberger, 1991), 3ß-hydroxysteroid hydrogenase (Inano and Tamaoki, 1968) and 17,20-desmolase (Farrer *et al.*, 1985). The changes in enzyme activity in the Δ^4 pathway are in agreement with previous reported observations on the impaired conversion of progesterone to testosterone by the testicular tissue from bilaterally cryptorchid adult rats (Ficher and Steinberger, 1982; Murphy and O'Shaughnessy, 1991). An increase in testicular temperature, on the other hand, has also been observed to increase the aromatase enzyme activity which converts testosterone to oestradiol (Munabi *et al.*, 1984).

Reduction in testosterone synthesis by Leydig cells exposed to high temperature is also supported by evidence from other animals which showed that the serum levels of testosterone in cryptorchid rams (Schanbacher and Ford, 1977) and bulls (Schanbacher, 1979) or in bulls exposed to high ambient temperature (Rhynes and Ewing, 1973), are normal or below normal, while serum levels of LH are significantly increased relative to that of animals with normal testis. Furthermore, the cryptorchid testes of the bull

(Schanbacher, 1979), ram (Schanbacher, 1980), dog (Eik-Nes, 1963) or rat (Risbridger *et al.*, 1981) respond poorly to exogenous gonadotrophins.

The underlying cause of the disturbed testicular steroid biosynthesis induced by increasing testicular temperature is still unknown but may be associated with alteration of paracrine influences by the damaged seminiferous tubules or changes in the microcirculation of the testis (Bergh *et al.*, 1984a). Furthermore there are a number of reports which have shown that an elevated testicular temperature induced a decline in the testicular content of several receptor types including receptors for LH (Huhtaniemi *et al.*, 1984; Hovatta *et al.*, 1986), FSH (Monet-Kuntz *et al.*, 1987), prolactin (Huhtaniemi *et al.*, 1984) and androgens (Monet-Kuntz *et al.*, 1987). Some of the changes in circulating and intratesticular testosterone concentrations might be due, partly, to altered blood flow at elevated temperature (Damber *et al.*, 1985). Studies by Galil and Setchell (1988b) showed that exposure of one or both rat testes to 43^{o}C for 30 min resulted in a significant reduction in testicular blood flow.

Elevated testicular temperature impaired the secretory activity of inhibin from the Sertoli cells (Au *et al.*, 1987; Demura *et al.*, 1987) and such changes are usually accompanied with an increase of circulating FSH levels presumably due to decline in the negative feedback mechanism induced by inhibin (Collins *et al.*, 1978; Au *et al.*, 1983). Other secretory products of the Sertoli cells are reduced by elevating testicular temperature, including seminiferous tubule fluid (Jegou *et al.*, 1984) and androgen binding protein (ABP) (Jegou *et al.*, 1984; Karpe *et al.*, 1984; Monet-Kuntz *et al.*, 1987).

However, despite the vast quantity of information available on testicular dysfunction induced by trypanosome infections, no information is available to link dysfunction with alteration of the testicular microenvironment causing changes to testicular thermoregulatory system. Availability of such information will provide useful evidence on the role of fever in the pathogenesis of trypanosome-induced testicular degeneration.

CHAPTER II
GENERAL MATERIALS AND METHODS

This chapter describes the experimental materials and methods which were common to most of the succeeding chapters.

2.1. Experimental Animals

The animals used in these studies were Scottish Blackface male pubertal rams obtained from a farm located in the West of Scotland. The rams at 6 months of age, were bought in September of both 1991 and 1992 for experiments commencing soon afterwards. They were housed in an open pen, ear tagged and subjected to thorough clinical examination. Their feet were trimmed and dipped in 10% w/v solution of zinc sulphate (Gold Hoop, Sheep Fair products, Brecon) to control footrot. They were vaccinated against pasteurella and clostridial infections using Ovivac-P$^{(R)}$ (Hoechst Animal Health, Milton Keynes). Faecal samples were taken and checked for nematode eggs and other parasites and all sheep were subsequently drenched with antihelmintic (Ivomec$^{(R)}$, 0.08% w/v ivermectin at 200 mg ivermectin kg^{-1} liveweight, MSB AGVET, Hertfordshire, UK).

The rams were allowed a 2 month acclimatisation period during which time they were closely examined and exposed to routine handling. They were bled twice a week for routine haematological analyses and plasma was obtained and stored at -20^{o}C to provide baseline endocrine data. They were weighed once a week using a sheep weighing scale (Poldenvale Ltd, Williton, Somerset). These procedures helped to acclimatise the rams and ensure that they were less excitable during the experimental period. Rams received approximately 500 gm day^{-1} concentrate food (306 Ewbol Store Lamb Pellets, BOCM Silcock) before and throughout the experimental period while hay and water were provided *ad libitum*.

Further management of the animals during the experimental period varied from one study to another and the details are given in the relevant sections.

2.2. Trypanosomes used in Experimental Infections

Trypanosoma congolense stabilates 57/10 and 57/11 were used in these experiments. These stabilates were derived from clone IL 1180, originally imported from ILRAD, Kenya by the Department of Veterinary Physiology, University of

Glasgow Veterinary School. The stabilates were maintained in microcapillary tubes stored in liquid nitrogen between serial passages in mice. The preparation of trypanosome innoculum and the infection of sheep were done by Miss D. M^{c}Kechnie of the Department of Veterinary Physiology, University of Glasgow Veterinary School. Essentially, the trypanosomes were inoculated intraperitoneally into irradiated mice (dose 6.5 Gys) and, at the first peak of parasitaemia, which occurred 7 days later, the mice were bled out by cardiac puncture under chloroform anaesthesia using heparin as anticoagulant. The trypanosome concentration in blood pooled from the infected mice was determined with a haemocytometer and subsequently the blood was diluted in phosphate buffered saline (PBS) at pH 8.0 containing 1.5% glucose to give a titre of 2 x 10^5 trypanosome ml^{-1}. The methods and trypanosome titres used for infecting sheep and numbers of animals infected are described in relevant chapters. As a routine during each infection, three irradiated mice were each injected with 0.3 ml of the inoculum intraperitoneally to confirm the infectivity of trypanosomes used.

2.3. History of *T. congolense* Stabilates

The stabilate IL 1180 is a cloned derivative of STIB 212, which was prepared after passage in rats of an isolate collected from a lion (*Panthera leo*) in the Serengeti region of Tanzania in 1971 (Geigy and Kauffmann, 1973). Stabilate STIB 212 was passaged twice in mice, preserved as stabilate IL 20E-8 and then cloned twice and preserved as stabilate IL 968. This was passaged once in mice and preserved as stabilate IL 1180 (Dwinger *et al.*, 1987). When stabilate IL 1180 was imported it was passaged twice in rats and once in mice to give stabilate GRVPS 57/1. Stabilate GRVPS 57/1 was passaged once in rats and twice in mice to yield stabilate GRVPS 57/10. Stabilate GRVPS 57/10 was again passaged once in mice and preserved as stabilate GRVPS 57/11.

2.4. Clinical Observations.

Starting from the day of infection, all experimental animals were closely observed for changes in demeanour. Jugular blood samples were examined daily using the buffy coat method (section 2.6) until the onset of parasitaemia. Once parasitaemia

was detected, blood samples were examined every two days in infected rams and once a week in control rams throughout the experimental period. Rectal temperatures (^{o}C) were also recorded three times a week starting on the day of infection.

Collection of blood samples

Blood samples were obtained from the jugular vein twice a week commencing one month prior to infection and three times a week during the experimental period. Samples were routinely taken between 9.00 and 10.00 am before the morning feed with every effort taken to minimise excitement during handling. Five ml of blood was collected in heparinised vacutainers using sterile 19 x 1" gauge needles. Blood collected before infection was used for the estimation of packed cell volume (PCV) and that collected during the infection period was used for estimation of PCV and parasitaemia. After PCV estimation, remaining blood was centrifuged at 1500 g and $4^{o}C$ for 15 min, then plasma was removed and stored at $-20^{o}C$ until needed for hormone assays. Intensive blood sampling was performed during the course of experiments for investigations of sequential changes of hormone concentration over a short time periods and the details of these procedures are provided in respective chapters.

2.5. Haematological Techniques

Estimation of PCV

The PCV was determined using the microcapillary method. Duplicate microcapillary tubes were filled with blood up to two third of their length. One end was sealed with plasticine and spun in a Hawksley microhaematocrit centrifuge for 8 min. The tubes were read with a microhaematocrit reader and the mean of the two duplicates was recorded to the nearest percentage point.

2.6 Parasitological Techniques

Trypanosome titre in the infected animals were determined three times a week in jugular blood samples. The trypanosomes were detected using the buffy coat/darkground microscopy method described by Murray *et al.* (1977) and the number of trypanosomes was estimated by the modified scoring method described by Paris *et al.*

(1982) as shown in Table (2.1). After the estimation of PCV, the microcapillary tube was cut, using a diamond tipped pencil about 1 mm below the buffy coat to include the top layer of red cells and 1 cm above the buffy coat to include the plasma. The contents of the capillary tubes were expressed onto a clean microscope slide, mixed gently and covered with a 22 x 22 mm coverslip. The entire preparation was examined using a phase contrast Leitz SM Lux microscope at 400x magnification.

Table 2.1: Darkground/ phase contrast buffy coat scoring method for estimation of parasitaemia

Scores	Trypanosomes per field	Estimated parasitaemia (trypanosome ml^{-1})
0	None	$<10^2$
1+	1 - 3 per preparation.	10^2 - 10^3
2+	4 - 10 per preparation	10^3 - 10^4
3+	1 per field	5×10^3 - 5×10^4
4+	2 -10 per field	5×10^4 - 4×10^5
5+	10 - 100 per field	$>5 \times 10^5$
6+	>100 per field	$>5 \times 10^6$

2.7. Hormone Measurements

The concentrations of testosterone, luteinizing hormone (LH), cortisol and adrenocorticotropin (ACTH) in plasma were measured. Similarly, testosterone and progesterone concentrations were measured in testicular tissue extracts and samples obtained during *in vitro* studies. The details of hormonal techniques used are provided in respective chapters.

2.8 Assessment of Puberty

Dyrundsson (1973) described the anatomical changes that take place in the reproductive organs of ram-lambs around puberty. Similar features were used to assess puberty in the rams used in these experiments. These included: both testes fully descended into the scrotum; detachment of all adhesions on the urethral process, glans penis and penile shaft leaving the penis freely retractable; presence of "pink flushing" of the inguinal skin around the scrotum; ability of the rams to provide semen with viable spermatozoa upon ejaculation using an artificial vagina or by electroejaculation. Thorough examination of the testes, epididymides, penis and prepuce was performed once in all rams during the acclimatisation period. Semen was collected once before animals were allocated to the respective studies. All rams had reached puberty before the experiments began.

2.9. Semen Collection and Evaluation

Semen was collected by electroejaculation using the Medata Ram Ejaculator (Medata Systems Ltd, West Sussex, U.K.). The ram was restrained in a standing position by an assistant. The penis was grasped with one hand and withdrawn from the prepuce. The glans penis and urethral process were inserted into a clean 12 x 75 mm glass tube and secured by hand by grasping the prepuce. The lubricated rectal probe was then inserted into the rectum to a depth of about 15 - 20 cm, taking care to avoid injury. The probe was pressed towards the floor of the pelvis and short stimuli (3 sec) were applied until ejaculation occurred.

Collected semen was immediately transferred into a water bath at 32^{o}C. Semen consistency was noted and scored on a scale of 0 - 5 as described by Evans and Maxwell (1987) (see Table 2.2). Sperm motility was estimated by examining a drop of semen on a warm slide chamber (Kova slide II, Kova Systems. ICL Scientific, USA) at 40x magnification and assessment followed the wave motion method described by Evans and Maxwell (1987) and was scored on a scale of 0 - 5 as shown in Table 2.3. The assessments of semen consistency and sperm motility were completed within 15 - 20 min of individual semen collections.

For assessment of sperm morphology and percentage of live sperms, a drop of semen was taken immediately after collection and added to a glass tube containing 10 drops of nigrosin-eosin (Blom, 1950) in a water bath at 32°C. The tube was incubated for one minute at 32°C and then two thin smears were made on clean glass microscopic slides, left to dry in air and stored in slide boxes. The slides were subsequently mounted with DPX and examined under oil emulsion at 400x magnification. Sperm defects were classified as primary i.e. defects arising in the testes and secondary i.e. defects occurring after the sperm have left the testes (Laing and Hammond, 1955). Table 2.4 shows the parameters which were assessed.

For assessment of acrosome defects (Table 2.4) another drop of semen taken immediately after collection was diluted in two drops of 3% w/v Sodium citrate (CH_3COONa) prewarmed at 32°C in a water bath prior to smearing. A thin smear was made on a clean microscopic slide and left to dry in air for 3 - 5 min. Slides were fixed in Spermafix(R) (Stain Enterprises, P.O. Box 152, Wellington, 7655, Rep. S. Afr.) for 5 min then dried and stored in slide boxes until needed for analysis. At the time of analysis, slides were gently washed 5 - 6 times in tap water and stained with Spermac(R) stain (Stain Enterprises, P.O. Box 152, Wellington, 7655, Rep. S. Afr.). They were subsequently examined unmounted on the same day under oil emulsion at 400x magnification. With this stain the sperm acrosome stained green, the equatorial region stained pale green and the rest of the head stained red. Midpiece and tail stained green.

2.10. Histopathology

Several organs including the testis epididymis, prostate, adrenal and pituitary glands were carefully harvested from experimental animals killed at different stages of infection. Details are provided in respective chapters. Sections made from the cauda epididymis, prostate, adrenal and pituitary glands were fixed in 4% w/v buffered neutral formalin. Sections made from the testes were fixed in Bouins fluid. All sections were processed for routine histology and paraffin embedded sections were sectioned at 5μm and stained with haematoxylin/eosin (H&E) and Martius scarlet blue (MSB). Sections from the pituitary were stained with orange fuchsin green (OFG). With this stain the

basophils stain pink and acidophils stain orange. Further processing of histological sections is described in respective chapters.

Table 2.2. Scoring method for consistency of ram semen (Evans and Maxwell, 1987)

Score	Consistency	Approximate number of sperm ($x10^9$ ml^{-1})
5	Thick creamy	4.5 - 6.0
4	Creamy	3.5 - 4.5
3	Thin creamy	2.5 - 3.5
2	Milky	1.0 - 2.5
1	Cloudy	0.3 - 1.0
0	Clear (watery)	insignificant

Table 2.3 Scoring method for sperm wave motion (Evans and Maxwell, 1987)

Scores	Class	Description
5	Very good	Dense, very rapidly moving waves. Individual sperm cells cannot be observed, ≥90% of spermatozoa active
4	Good	Vigorous movement, but the waves and eddies are not so rapid as for score 5. About 70 - 85% of sperm cells are active
3	Fair	Only small, slow moving waves. Individual spermatozoa cannot be observed. 45 - 65% of sperm cells are active
2	Poor	No waves are forming, but some movement of spermatozoa in visible. Only 20 - 40% of sperm cells are alive and their motility is poor.
1	Very poor	Very few spermatozoa (about 10%) show any sign of life, with weak movement only
0	Dead	All spermatozoa are motionless

Table 2.4. Morphological examination for ram semen (Laing and Hammond, 1955, Barth and Oko, 1989)

Sperm defects	Description
Primary defects	
Head defects	
Pyriform	narrow in the postacrosomal region (pèar shaped)
Tapered	narrow both in the acrosome and postacrosome region
Microcephalic	excessive small head
Macrocephalic	excessive large head
Duplicate	double or multiple heads
Abnormal acrosome	missing or present but flattened dented or beaded giving a knobbed appearance
Midpieces	
Amorphous	swollen midpieces
Abaxial	attached on one side of the head base
Duplicate	double or multiple midpieces
Tail defects	
Duplicate	double or multiple tails
Stumpy tails	very short tails attached to the base of the head
Secondary defects	
Detached heads	heads separated from tails
Proximal droplets	rounded cytoplasmic droplet retained in a proximal midpiece position
Distal droplets	rounded cytoplasmic droplet in the distal midpiece position
Bent tails	loops and bends affecting mainly the principal midpieces
Distal coiled tails	coils affecting the distal portion of the principal piece and end piece
Bent midpieces	bowing and distal midpiece reflex abnormality
Others parameters	
Live/dead ratio (%)	
Total primary defects (%)	
Total secondary defects (%)	

CHAPTER III

EFFECTS OF *TRYPANOSOMA CONGOLENSE* INFECTION ON THE PITUITARY, TESTIS, EPIDIDYMIS AND PROSTATE GLAND OF THE RAM

3.1 INTRODUCTION

The effects of trypanosomiasis on the reproductive function in the male have been extensively documented (reviewed by Ikede *et al.*, 1988). Both man and male domestic animals suffering from an acute or chronic trypanosomiasis develop varying degrees of pituitary gland damage and gonadal degeneration which are frequently more severe in infections caused by the *T. brucei*-group than in infections caused by *T. congolense* or *T. vivax* (Ikede, 1979; Losos and Ikede, 1972a; Ikede *et al.*, 1988; Omeke, 1991; Omeke and Onuora, 1992). This is due to the ability of the trypanosomes in the *T. brucei*-group to invade and multiply in the interstitial tissue where they cause marked inflammatory changes in the pituitary (Losos and Ikede, 1970, 1972; Ikede and Losos, 1975; Morrison *et al.*, 1981) and testes (Ikede and Losos, 1972a; Ikede *et al.*, 1973; Ikede, 1979; Van den Ingh and Van Dijk, 1975; Ikede and Akpavie, 1982; Anosa and Kaneko, 1984).

Pituitary and testicular dysfunctions induced by *T. congolense* infection have been documented in the bull (Glundler and Djabakou, 1985; Sekoni *et al.*, 1988, 1990a, 1990b, 1991), buck (Kaaya and Oduor-Okelo, 1980; O'Hara *et al.*, 1985; Waindi *et al.*, 1986; Gombe, 1989) and boar (Omeke, 1991; Omeke and Onuora, 1992). In these animals, *T. congolense* infection is associated with progressive, non-inflammatory, degenerative lesions characterised by a denudation of testicular germinal epithelium and production of poor semen quality leading to oligozoospermia and aspermia and a reduction in plasma testosterone concentration. Changes in the pituitary are mild, characterised by basophilic degranulation and slight acidophilic proliferation (Mutayoba *et al.*, 1988a); dilation of pituitary sinusoids and microvasculature and thickening of the extracellular matrix (Abebe, 1991; Abebe *et al.*, 1993a). In severely affected animals, necrosis and sclerosis of the hypophysis may occur (Ogwu and Njoku, 1991).

Pituitary-gonadal pathology in the ram has only been reported in infections caused by *T. vivax* (Losos and Ikede, 1970, 1972; Isoun and Anosa, 1974a, 1974b; Ikede, 1979; Anosa and Isoun, 1980; Agu *et al.*, 1986; Akpavie *et al.*, 1987). Recently, Sekoni (1992, 1993) described the semen characteristics and sperm morphological abnormalities in rams infected with *T. vivax*. The changes occurring in the pituitary and gonads of *T. vivax*-infected rams closely resemble the lesions induced by *T. congolense* in other

animals (reviewed by Ikede, *et al.*, 1988). However, unlike *T. congolense* which has not been associated with extravascular localization in the host tissues, *T. vivax* has been reported to localise extravascularly in the pituitary gland (Fiennes, 1950), the cerebrospinal fluid and aqueous humour (Whitelaw *et al.*, 1988) and other tissues including the lymph nodes (Emery, *et al.*, 1980) and cardiac muscle (Kimeto *et al.*, 1990). It is therefore possible that the mechanisms by which *T. vivax* and *T. congolense* infections induce pituitary-gonadal dysfunction might be different and this should be taken into account when studying the pathogenesis of *T. congolense*-induced reproductive dysfunction in animals.

The mechanisms by which *T. congolense* induce changes in reproductive function have not been studied but both direct and indirect factors might be involved. These factors were reviewed extensively in Chapter I (1.3.1, 1.3.2 and 1.3.3). Briefly, direct effects are likely to be induced either by damage associated with the localization of large numbers of trypanosomes in the microvasculature causing anoxia and thrombosis (Banks, 1978) or mediated via one or more of the wide variety of biologically active molecules secreted by invading trypanosomes (Tizard *et al.*, 1978b, 1979; Knowles *et al.*, 1987; Hublart, *et al.*, 1990). The indirect factors are probably mediated by interaction of the neural, endocrine and immune system responses of the host to infection. This is likely to involve the activation of the cytokine cascade which in turn activates or suppresses the hypothalamo-pituitary-gonadal axis as a part of their pleiotropic action in stimulating the acute phase response (Bancroft *et al.*, 1983; Stadnyk and Gauldie, 1991; Kennedy and Jones, 1991).

Development of fever is a consistent feature of an acute phase response (Whicher and Westacott, 1992) and animals suffering from an acute or chronic form of trypanosomiasis develop intermittent pyrexia with temperature rising between 1 - 4°C above normal rectal temperature (Hornby, 1921, Losos, 1986, Stephen, 1986). Such a rise in body temperatures might interfere with normal testicular spermatogenic and steroidogenic activities in infected animals as these testicular activities are very sensitive to elevated testicular temperature (see review Chapter I 1.3.3). Although pyrexia induced by trypanosomes during infection has been suggested to affect the function of the testis in infected animals (Kaaya and Odour-Okelo, 1980; Anosa and Isoun, 1980;

Ikede *et al.*, 1988, Omeke and Onuora, 1992), there are no reports which have shown that such intermittent low grade pyrexia in infected animals can cause damaging elevation of testicular temperature.

3.1.1. Objectives of the Study

Two *in vivo* studies designated as Study I and Study II were conducted on rams experimentally infected with *T. congolense*. The main objectives of these studies were as follows:

Study I

To identify the changes in seminal characteristics and gross and histopathology of the testis, epididymis, prostate and pituitary glands associated with *T. congolense* infection in the ram.

Study II

(i) **To measure scrotal temperature during infection and to assess whether it changed as pyrexia developed and whether this might play a role in inducing the observed semen and testicular pathology during infection.**

(ii) **To raise the scrotal temperature of normal rams by insulation to assess whether an increase in temperature can produce the gross and histopathological lesions to the same extent as those observed in rams infected with *T. congolense*.**

The animals used in both Study I and Study II were also the subject of investigation of reproductive endocrinology to be described in Chapter IV, while the animals used in Study I were subject of the investigation of the pituitary-adrenal axis to be described in Chapter VI. Furthermore, animals of Study II were the subject of the *in vitro* studies on Leydig cell function to be described in Chapter V.

3.2. MATERIALS AND METHODS

3.2.1. Experimental Animals

A total of nineteen pubertal Scottish Blackface rams were used during Study I and 26 rams were used during Study II. Their management before and during the

experimental period was described in Chapter II 2.1. Two days prior to infection in November, 1991, the rams used during Study I were transferred to a flyproof isolation unit where they were maintained under artificial light consisting of cycles of 8 h light, 16 h dark (lights on 8.00 - 16.00) which simulated the natural photoperiod during this time of the year. Rams used during Study II were transferred to the same isolation unit one week before the experiment began in November, 1992. The lighting regimen employed during Study II was similar to that used during Study I. Rams used in both studies were about 8 months old at the start of the infection period.

3.2.2. Animal Allocation and Infection

On the day of infection during Study I the animals were randomly matched into a group of 10 (infected) and a group of 9 (control) animals on the basis of their liveweight and haematocrit. Similarly during Study II matched groups of 10 (control) and 10 (infected) were used. Furthermore, to increase homogeneity, rams used during Study II were paired twins, one twin ram was allocated in the control group and the other twin ram was allocated in the infected group. A group of 6 rams (3 twin pairs) were used during Study II for the scrotal-insulation study. Table 3.1 provides the details of the number of animals used in each study and their respective pre-infection mean PCV and liveweight values at the start of the experiments.

Table 3.1: Number of uninfected (control), *T. congolense*-infected (infected) and scrotal-insulated (scrotal-insulated) animals and their preinfection mean PCV and liveweight values at the start of the experiments during Study I and II.

	STUDY 1		STUDY II		
	Control rams	Infected rams	Control rams	Infected rams	Scrotal-insulated rams
No of Animals	9	10	10	10	6
PCV (%)	31.20 ± 0.54	33.05 ± 0.79	31.45 ± 0.62	32.20 ± 0.72	30.17 ± 1.14
Liveweight (kg)	34.50 ± 1.23	34.95 ± 1.24	30.90 ± 1.02	30.55 ± 0.93	31.05 ± 1.22

Values are means ± s.e.m.

Each ram in the experimental (infected) group during both studies was infected, via the jugular vein, with approximately 4 x 10^5 trypanosomes contained in 2 ml PBS at pH 8.0 and containing 1.5% glucose. The stabilate used during Study I was *T. congolense* isolate 57/10 and during Study II was stabilate 57/11. Both stabilates were derived from stabilate IL 1180 as previously described in Chapter II 2.2 and 2.3.

3.2.3. Assessment of Clinical Parameters

Following infection the infected animals in both studies were closely observed for changes in general health. Jugular blood samples were taken daily as previously described (Chapter II 2.4) starting on day 1 of infection and were examined for presence of trypanosomes. Once parasitaemia was detected, blood samples were taken three times a week in the infected and control rams until the end of the study period. These blood samples were used for estimation of parasitaemia in infected rams and once a week blood from the control rams was also checked as a precautionary measure to monitor accidental infections. Parasitaemia levels were measured using the microcapillary buffy coat scoring method (Murray *et al.*, 1977; Paris *et al.*, 1982) as previously described in Chapter II 2.6.

The PCV was determined in all blood samples collected before the experiment (Chapter II 2.1) and during the infection period (Chapter II 2.4) using the microcapillary method described in Chapter II 2.5. Liveweights (kg) were measured once a week as previously described in Chapter II 2.1 and rectal temperatures (^{o}C) were measured three times a week starting on day 5 post-infection during Study I and the day of infection (day 0) during Study II.

The post-infection experimental period lasted 79 days during Study I and 58 days during Study II. Five infected and 4 control rams from Study I were killed on day 28, and all the remaining control and surviving infected rams were killed at the end of the study period. During Study II, five control and 4 infected rams were castrated on day 28 post-infection as described in (Chapter IV 4.2.3). All rams in Study II were killed at the end of the study period (day 58).

3.2.4. Estimation of Scrotal Circumference

The scrotal circumference was measured at two week intervals in rams used during Study I. Rams were restrained in a sitting position by one assistant while another assistant squeezed the testes together and aligned them in the distal end of the scrotum. Scrotal circumference was measured in the midline region of the two testes using a thin cotton tape and a centimetre ruler.

3.2.5. Measurement of Scrotal Temperature

The scrotal temperature was measured twice weekly in all rams used during Study II commencing from the first week of the infection. Measurements were made with a 38 s.w.g. copper - 40 s.w.g. constantan thermocouple mounted in a polythene probe and recorded by a 2 channel potentiometric recorder (Smiths Industries Ltd, UK, Type RE524.20, Model Servoscribe 2; 20 mV full-scale deflection). Recordings were always taken from a 1.0 cm diameter mid-anterior region of scrotal skin which had been shaved and marked at the start of the experiment. At the same time rectal temperature and room ambient temperature were also taken with a clinical thermometer and a Max-Min electronic thermometer (Model Diplex Electronics, Merck, UK), respectively.

3.2.6. Scrotal Insulation

Scrotal insulation was performed on 6 non-infected rams during Study II on the equivalent of day 14 post-infection. The insulation consisted of a pouch fashioned to fit the scrotum and made of 0.5 - 1.0 cm thick cotton wool laid over a cotton gauze. The pouch was wrapped with an elastic adhesive bandage (Elastoplast, Smith & Nephew Ltd, Hull, UK) and was held around the neck of the scrotum with the same bandage. The scrotal temperature was measured by introducing the thermocouple probe through the insulation at the scrotal neck.

3.2.7. Semen Collection and Evaluation

During Study I, semen from control and infected rams was collected by electroejaculation as previously described (Chapter II 2.9), on day 7 prior to infection and on days 27 and 69 after infection. During study II, semen was collected in a similar

manner from the control and infected rams on day 7 prior to infection and on days 21 and 49 after infection. The semen in the scrotal-insulated rams was collected on day 7 prior to insulation and on days 22 and 49 after insulation. Semen was analysed for consistency, sperm motility and morphology as previously described in Chapter II 2.9.

3.2.8. Gross and Histopathological Examination

A post mortem (PM) examination was conducted on infected and control rams killed on day 28 and 79 during Study I and on those dying during the course of infection (two infected rams in Study I died on days 70 and 77). Similarly, a PM was conducted on one infected ram which died on day 15 post-infection and in 10 control and 9 infected rams killed on day 58 post-infection during Study II. During Study I, both testes and epididymides were removed at PM and separated from the tunica layers and were subsequently weighed together (with the exception of the rams killed on day 28). Sections of the testis and cauda epididymis were taken during both studies, fixed in Bouins and processed for routine histology as described previously (Chapter II. 2.10). Similarly, the pituitaries and prostate glands were removed and trimmed and sections were fixed in 4% buffered neutral formalin and processed for routine histology. Slides were examined by light microscopy and the seminiferous tubular diameter was measured in rams used during Study I using a binocular micrometer (Graticules LTD, Tonbridge, Kent, U.K.). Seminiferous tubular diameter was estimated from two measurements taken perpendicular to each other. About 50 randomly selected seminiferous tubules cut transversely were measured per animal.

3.2.9. Statistical Analysis

Results are presented as means plus and minus the standard error of the mean (S.E.M.). The PCV and liveweight data were analysed on the basis of change from their respective pre-infection values. The difference in the scrotal and rectal temperatures measured at each occasion in each animal were calculated and were used to estimate the scrotal-rectal temperature gradients in infected and control rams. Comparison of data obtained in infected and control animals over time (PCV, liveweight, scrotal circumference, rectal and scrotal temperature) were subjected to analysis of variance

with repeated measures design using a computer statistical package ANIMAL DESIGNS 1, V 1.21 5/6 (Data International Service, Glasgow). The analysis partitioned variability between and within animal groups and group x time interaction. Differences between group means were analysed using either the Two-Sample t-test or where appropriate with the nonparametric Mann-Whitney test. In experiments involving more than two treatment groups, comparisons were analysed by one way analysis of variance followed by a *post hoc* between-groups comparison test (Newman-Keuls Multiple Range Test (MINITAB, Minitab Inc, State College, PA, USA). Comparisons were considered significantly different at $P<0.05$.

3.3. RESULTS

The results in this chapter are divided into two main sections. Section I provides the clinical-pathological results on the reproductive organs obtained during Study I, whereas, section II provides the results from Study II.

SECTION I

3.3.1 EFFECTS OF *TRYPANOSOMA CONGOLENSE* ON THE PITUITARY, TESTIS, EPIDIDYMIS, AND PROSTATE GLAND IN RAMS DURING STUDY I

3.3.1.1. Clinical Observations

Trypanosomes were observed in the blood of all infected sheep in Study I within 7 to 9 days post-infection. The first peak of parasitaemia (approx. 5×10^5 trypanosome ml^{-1}) occurred between 9 and 15 days post-infection and was followed by fluctuating levels of parasitaemia (Fig. 3.1a). Infected rams progressively became weak and dull with roughened fleeces. They also rapidly became anaemic with pale mucous membranes and the superficial lymph nodes were enlarged. All surviving infected rams remained parasitaemic up to the end of the study period.

Infected rams developed intermittent pyrexia during the post-patency period (Fig. 3.1b) with their mean body temperature fluctuating between $39.2 \pm 0.1^{o}C$ and 40.1

$\pm$ 0.6°C. These body temperatures were significantly higher ($P<0.01$) than those from the control rams which remained within the normal range throughout the infection period (range 38.4 $\pm$ 0.1°C to 39.3 $\pm$ 0.3°C). Statistical analysis of body temperature revealed a significant treatment effect between infected and control groups ($P<0.01$) with no significant group x time interaction.

Anaemia developed rapidly in the infected rams and a significant drop in PCV from pre-infection levels of 33.1 $\pm$ 0.8% ($P<0.001$) was observed following patency from day 12 onwards (Fig 3.1c). Changes in PCV showed a significant group treatment ($P<0.001$) and group x time interaction ($P<0.001$). Mean PCV values in control rams remained within the normal range of 30.5 $\pm$ 0.1% (Fig. 3.1c).

Changes in liveweight in infected and control rams are shown in Fig. 3.1d. The infection reduced the liveweight gain in infected rams to 13 gm day^{-1} when compared with the growth rates of 130 gm day^{-1} in the controls ($P<0.001$) during the study period.

Four control and 5 infected rams were killed on day 28. The remaining infected rams showed some loss in body condition and 2 rams died on day 70 and 77, from secondary *Pasteurella haemolytica* infection. The remaining 3 infected and 5 control rams were euthanized at the end of the study period.

3.3.1.2. Changes in Testicular Circumference

Changes in mean testicular circumference (TC) in the control and infected groups before and during the infection period are shown in Fig. 3.2a and corresponding mean testicular circumference/liveweight (TC/LW) ratios are shown in Fig. 3.2b. The mean TC values at the onset of the infection were not significantly different between the two groups. The TC values in the infected rams started declining within 12 days post-infection and from day 37 - 79 mean TC values were significantly lower ($P<0.05$) than in control rams. TC/LW ratios followed a similar trend. Following infection, changes in TC showed a significant treatment effect between groups ($P<0.05$) and a group x time interaction ($P<0.01$).

Fig. 3.1. Mean (± s.e.m.) parasitaemia scores (Fig. 3.1a), rectal temperatures (Fig. 3.1b), changes in packed cell volume (PCV) (Fig. 3.1c) and liveweight (Fig. 3.1d) in uninfected (n = 9, control) and *T. congolense*-infected rams (n = 10, infected) during Study I. Four control and 5 infected rams were killed on day 28 post-infection.

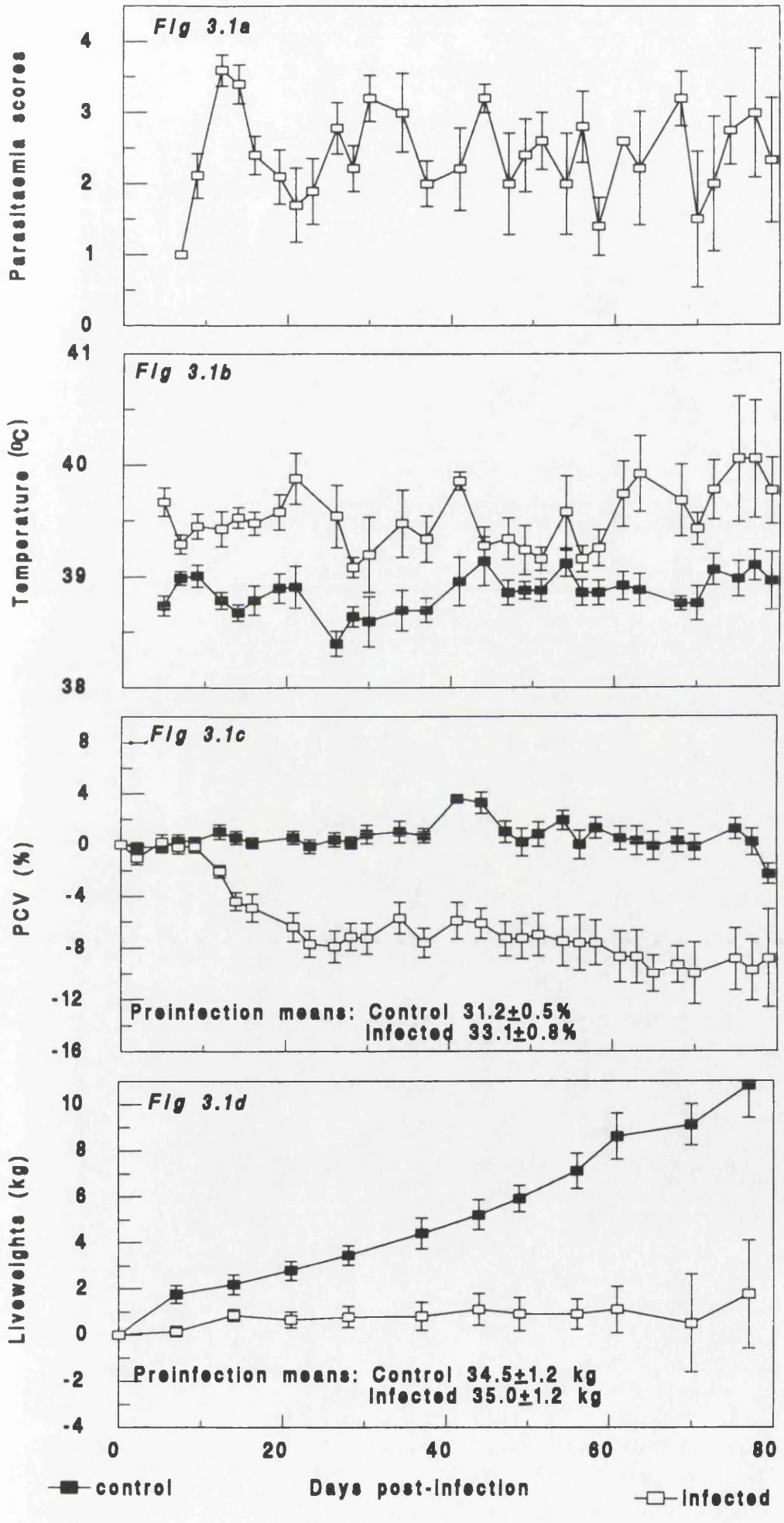


Fig 3.1a
Parasitaemia scores
4
3
2
1
0
Fig 3.1b
Temperature (ºC)
41
40
39
38
Fig 3.1c
PCV (%)
8
4
0
-4
-8
-12
-16
Preinfection means: Control 31.2±0.5%
Infected 33.1±0.8%
Fig 3.1d
Liveweights (kg)
10
8
6
4
2
0
-2
-4
Preinfection means: Control 34.5±1.2 kg
Infected 35.0±1.2 kg
0
20
40
60
80
control
Days post-infection
Infected

Fig. 3.2. Mean (± s.e.m) changes in testicular circumference (Fig. 3.2a) and testes circumference/liveweight ratio (Fig. 3.2b) in uninfected (n = 9, control) and *T. congolense*-infected (n = 10, infected) rams during study I. Four control and 5 infected rams were killed on day 28 post-infection.

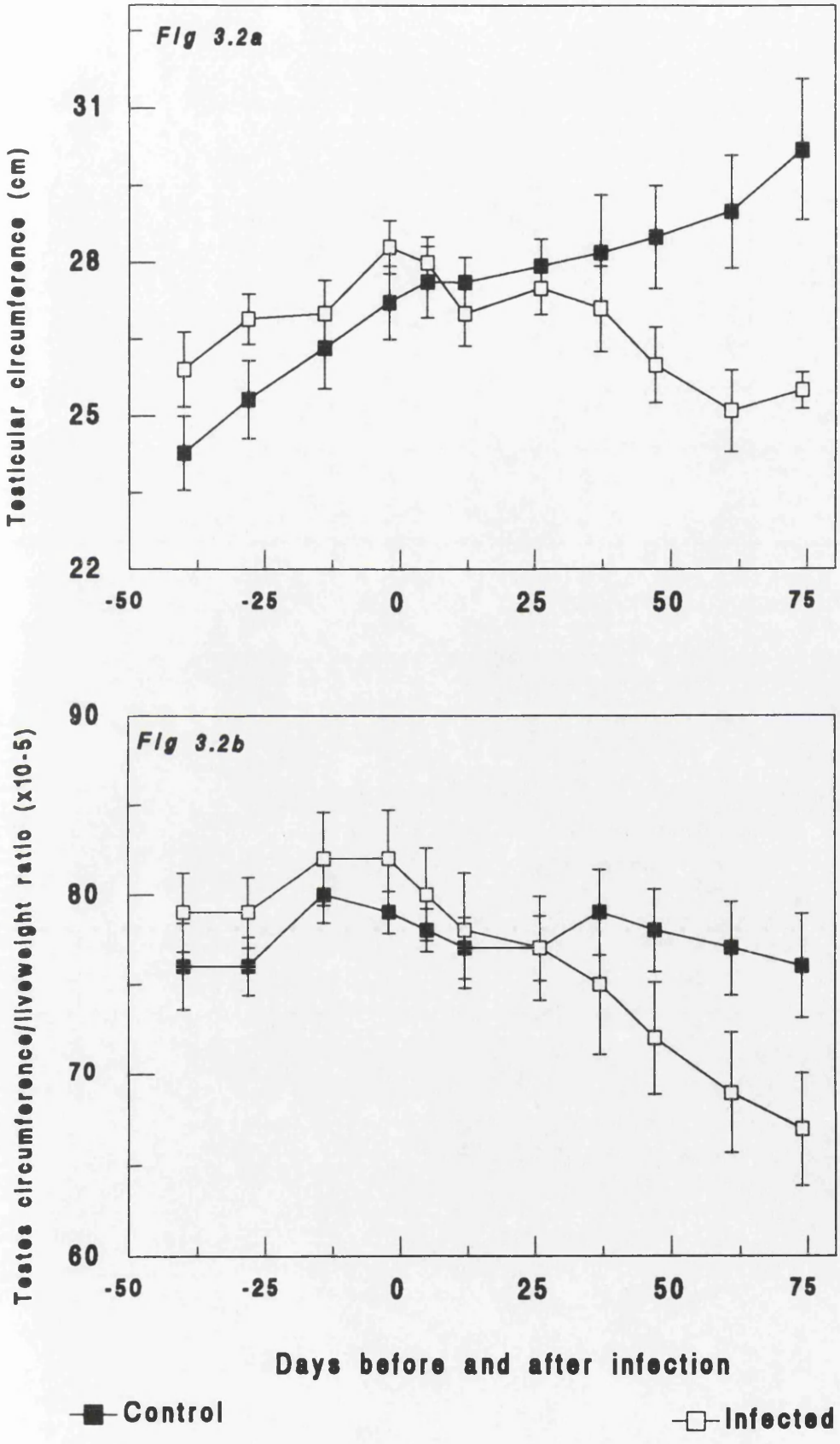


Fig 3.2a
Testicular circumference (cm)
31
28
25
22
-50
-25
0
25
50
75
Fig 3.2b
Testes circumference/liveweight ratio (x10-5)
90
80
70
60
-50
-25
0
25
50
75
Days before and after infection
Control
Infected

3.3.1.3. Changes in Semen Characteristics

The ejaculate volume and sperm concentration were not determined in semen collected from control and infected rams due to inconsistencies in the amount of semen obtained during electroejaculation. Instead, several parameters associated with semen quality were assessed. Table 3.2 shows the changes in mean percentage values for the seminal and sperm morphology abnormalities in control and *T. congolense*-infected rams before infection (day -7) and after infection (days 27 and 69) during Study I. The mean scores for semen consistency and sperm motion did not change significantly in control rams over time although the mean values for both parameters tended to increase in subsequent semen collections. All infected rams showed progressive and significant deterioration in semen quality as observed by marked declines in both semen consistency and sperm wave motion mean scores on days 27 and 69 post-infection ($P<0.05$ and $P<0.01$), respectively.

The mean total percentage of sperm showing primary sperm defects associated with the acrosome, head, midpieces and tails was less than 4% in control rams from the semen collected on 7 days prior to infection and on days 27 and 69 post-infection. However, the mean total primary sperm abnormalities was significantly higher ($P<0.01$) in infected rams on both days 27 (mean 6.64 $\pm$ 0.36%) and 67 (mean 13.06 $\pm$ 2.24%) post-infection when compared to the respective control values (mean 1.62 $\pm$ 0.20% and 1.96 $\pm$ 0.51%, respectively). The infection had no significant effect on primary tail defects in infected rams although a slight increase was observed from day 27 (mean 0.56 $\pm$ 0.18%) to 69 (mean 1.25 $\pm$ 0.59%) post-infection compared to control values. All other defects (acrosome, head and midpiece) increased markedly with time in infected rams. Significantly increased percentages ($P<0.001$) of primary acrosome and head defects were observed on days 27 and 69 post-infection in the infected rams. The acrosome defects which were encountered included detached and serrated acrosomes and flattened, dented and beaded acrosomes giving a knobbed appearance. Primary sperm head defects were associated mainly with an increase in pyriform, tapered and macrocephalic forms. Sperm with macrocephalic heads were found dead in nigrosin-eosin smears and they had either abaxial tail attachment or sometimes double tails. Significant increases in primary sperm midpiece abnormalities were observed in infected

rams on day 27 ($P<0.001$) and day 69 ($P<0.05$) post-infection, the defects observed being mainly swollen, rough and abaxial midpieces and occasionally double forms.

The mean percentage of sperm showing secondary defects in control rams did not change significantly with time and was less than 10% over the 3 semen collection occasions. Following infection the mean total secondary sperm defects increased to $21.73 \pm 4.14\%$ ($P<0.01$) in 10 infected rams on day 27 post infection and then to $66.12 \pm 8.39\%$ ($P<0.01$) in 5 surviving infected rams on day 69 post-infection. Changes in secondary sperm abnormalities on day 27 post-infection were associated with significant increases in the number of sperm with detached heads and proximal droplets ($P<0.001$); bent, swollen and coiled midpieces ($P<0.01$); bent and coiled tails ($P<0.01$) and sperm with distal droplets ($P<0.05$). Most of the sperms with detached heads were found dead in the nigrosin-eosin smears. The percentage of sperm showing secondary defects increased significantly ($P<0.01$) in 5 infected rams on day 69 post-infection. Out of $66.12 \pm 8.39\%$ total secondary defects encountered, the detached head accounted for $24.30 \pm 9.51\%$, abnormal midpieces $16.1 \pm 2.10\%$, tails $15.31 \pm 3.05\%$ and the proximal and distal droplet defects accounted for $1.79 \pm 0.31\%$ and $6.61 \pm 0.51\%$, respectively.

A progressive increase in the number of dead sperm was observed in the ejaculate of infected rams over the infection period. This increase was significant ($P<0.01$, $P<0.001$) on days 27 and 69 post-infection respectively when compared to control values.

3.3.1.4. Gross and Histopathological Examination.

Experimental rams killed 28 days post-infection had enlarged and oedematous lymph nodes, swelling of the spleen, petechiations in the heart and irregular focal areas of haemorrhage on the surface of the liver, kidney and lungs. Carcasses of infected sheep killed 79 days post-infection showed general emaciation and paleness. Muscular atrophy, depletion of body fat and accumulation of oedematous fluid in the peritoneum, pericardium and scrotal sac were also observed.

Table 3.2. Mean (±s.e.m.) percentage values for seminal and sperm morphology abnormalities in uninfected (control) and *T. congolense*-infected (infected) rams during Study I

	Days before and after infection				
	-7	27		69	
	(n = 19)	Control (n = 9)	Infected (n = 10)	Control (n = 5)	Infected (n = 5)
Semen consistency scores	3.50 ± 0.42	3.56 ± 0.29	2.10 ± 0.46[a]	4.00 ± 0.17	1.20 ± 0.37[b]
Sperm wave motion scores	3.25 ± 0.45	3.67 ± 0.24	1.90 ± 0.55[a]	3.80 ± 0.20	1.60 ± 0.25[b]
Primary defects (%)					
Acrosome defects	1.05 ± 0.44	0.71 ± 0.17	2.17 ± 0.30[c]	1.07 ± 0.10	4.48 ± 0.51[b]
Abnormal heads	0.81 ± 0.27	0.15 ± 0.06	1.84 ± 0.15[c]	0.22 ± 0.10	4.71 ± 1.72[c]
Abnormal midpieces	0.69 ± 0.11	0.57 ± 0.07	2.00 ± 0.23[c]	0.59 ± 0.39	2.62 ± 1.10[a]
Abnormal tails	0.22 ± 0.07	0.20 ± 0.07	0.56 ± 0.18	0.08 ± 0.05	1.25 ± 0.59
Secondary defects (%)					
Detached heads	0.81 ± 0.28	0.44 ± 0.10	3.61 ± 0.70[c]	1.27 ± 0.17	24.30 ± 9.51[b]
Proximal droplets	0.59 ± 0.12	0.27 ± 0.81	1.38 ± 0.25[c]	0.14 ± 0.06	1.79 ± 0.31[b]
Distal droplets	0.53 ± 0.26	1.03 ± 0.26	4.20 ± 2.32[a]	1.33 ± 0.56	6.61 ± 0.51[b]
Bent and coiled midpieces	3.24 ± 0.28	2.06 ± 0.29	5.26 ± 0.81[b]	2.12 ± 0.30	16.10 ± 2.01[b]
Bent and coiled tails	3.89 ± 0.38	3.66 ± 0.40	7.29 ± 0.97[b]	2.86 ± 0.49	15.31 ± 3.05[a]
Others					
Dead/live ratio (%)	13.17 ± 1.11	10.44 ± 1.06	38.97 ± 5.62[b]	6.90 ± 2.15	62.39 ± 2.66[c]
Total primary defects (%)	3.04 ± 0.60	1.62 ± 0.20	6.64 ± 0.36[b]	1.96 ± 0.51	13.06 ± 2.24[b]
Total secondary defects (%)	8.97 ± 0.37	7.42 ± 0.74	21.73 ± 4.14[b]	7.63 ± 0.87	66.12 ± 8.39[b]

Differences between the infected and corresponding control group in the same row are denoted by superscripts a,b,c ([a]P<0.05; [b]P<0.01; [c]P<0.001)

Changes in the pituitary gland

No overt pathological changes were observed in the pituitary gland of infected rams killed on day 28 post-infection but in rams killed on day 79 post-infection, various gradations of cytoplasmic changes were observed in the basophils. For example slight degranulation as evidenced by reduced staining of the cytoplasm (Fig. 3.3b) and marked degranulation and slightly increased connective tissue (Fig. 3.3c) were observed when compared to control pituitary cells (Fig. 3.3a). No changes were observed in the acidophils.

Changes in the testes

No overt gross testicular lesions were observed in rams killed on day 28 but the testes of rams dying on days 70 and 77 and of those killed on day 79 were soft and smaller in size. Only small amounts of semen were observed on the cut surface of the testis. The mean weights of the paired testes (including epididymides) of infected and control rams are depicted in Table 3.3. A nonsignificant decrease in mean testicular-epididymal weight and an apparent increase in testes-epididymal/liveweight ratio was observed in infected rams dying on days 70 and 77 and in those killed on day 79 when compared with control values (Table 3.3).

Histologically, compared with the controls, the mean diameter of the seminiferous tubules (Table 3.4) of infected rams was significantly larger ($P<0.05$) on day 28 post-infection and significantly smaller ($P<0.001$) in infected rams dying on days 70 (one ram) and 77 (one ram) and in those killed on day 79 after infection (3 rams). The testes of infected rams killed on day 28 post-infection also showed marked intertubular oedema and dilation of lymphatic vessels (Fig. 3.4b), but in most of the animals no marked changes in seminiferous spermatogenic activity was evident. Infected rams killed on day 79 post-infection showed varying degrees of seminiferous tubular epithelial degeneration which included general tubular atrophy (Fig. 3.4c) reduction in the number of germinal cell layers (Fig. 3.4c, 3.4d) and in severely affected tubules, only spermatogonia and Sertoli cells were evident (Fig. 3.4e). Other changes included increased intertubular connective tissue and apparent reduction in intertubular volume (3.4c, 3.4d, 3.4e). But in all infected rams no overt changes were observed in

the Leydig cells and there was no inflammatory cell infiltration in the testes. The comparative normal testis from the control ram is shown in Fig. 3.4a.

Changes in the epididymis

No gross differences from control rams were observed in the epididymis of infected rams killed on day 28 post-infection. In rams killed on day 79 only small amounts of semen were observed on cut surface of the epididymis when compared with controls. Histologically, only the cauda epididymis was examined and no marked changes in the sperm reserve (amount of sperm stored in the cauda epididymis) were observed in infected rams killed on day 28 (Fig 3.5b) compared to controls (Fig 3.5a) but reduction in sperm reserve was evident in rams killed on day 79 post-infection (Fig 3.5c). No inflammatory cell infiltration in epididymal tissues or degeneration of tubular epithelium were evident.

Changes in the prostate gland

The prostate glands of infected rams dying on days 70 and 77 and in those killed on day 79 were generally smaller and firmer than corresponding control prostate glands. The prostate gland weights and prostate/liveweight ratio (Table 3.3) were significantly smaller ($P<0.01$, $P<0.05$, respectively) in the infected rams than in control rams.

Histologically, marked changes in the prostate glands were evident in rams killed on day 79 compared to control rams and to infected rams killed on day 28. Changes as a result of infection on day 79 after infection included proliferation of interfollicular connective tissue, low cuboidal follicular epithelium (Fig. 3.6b) when compared to the tall columnar epithelium observed in the controls (Fig. 3.6a). In severely affected ram prostate gland, epithelial degeneration and collapse of the follicles was evident (Fig. 3.6c).

Changes in other reproductive organs

No gross lesions were observed in the testicular tunica layers, scrotum, penis and prepuce in any infected ram.

Table 3.3. Mean (± s.e.m.) paired testes (including the epididymides) and prostate gland weights and organ/liveweight ratios in uninfected (control) and *T. congolense*-infected (infected) during Study I

	Control (day 79) (n=5)	[a]Infected (days 70(n=1), 77(n=1), 79(n=3)) (n=5)
Testes (including the epididymides) (gm)	235.50 ± 18.3	192.53 ± 9.39
Testes/liveweight ratio ($x10^{-3}$)	4.96 ± 0.09	5.15 ± 0.42
Prostate gland (gm)	5.34 ± 0.48	3.21 ± 0.26 (P<0.01)
Prostate/liveweight ratio ($x10^{-4}$)	1.13 ± 0.08	0.84 ± 0.08 (P<0.05)

[a] One infected ram died on day 70 and another on day 77 due to secondary *P. haemolytica* infection and the other infected rams (n = 3) were killed with the control rams on day 79 post-infection

Table 3.4 Mean (± s.e.m) seminiferous tubular diameter and tubular diameter/testes weight ratios in uninfected (control) and *T. congolense*-infected (infected) rams during Study I

	Seminiferous tubular diameter (μm)	Seminiferous tubular diameter (μm)/ testis weight (gm)
Day 28		
Control (n = 4)	78.35 ± 0.95	NC
Infected (n = 5)	83.19 ± 1.32 (P<0.05)	NC
Day 79		
Control (n = 5)	76.28 ± 1.85	0.70 ± 0.39
[a]Infected (n = 5)	64.39 ± 0.78 (P<0.001)	0.67 ± 0.21

[a]Three infected rams killed on day 79 and one died on day 70 and another on day 77 of infection.
NC = Not calculated because the testes were not weighed on day 28 post-infection.
Correlation (r) between seminiferous tubular diameter and testicular weight of days 70, 77, 79 post-infection in infected and control rams was 0.70 (P<0.05) (n =10)

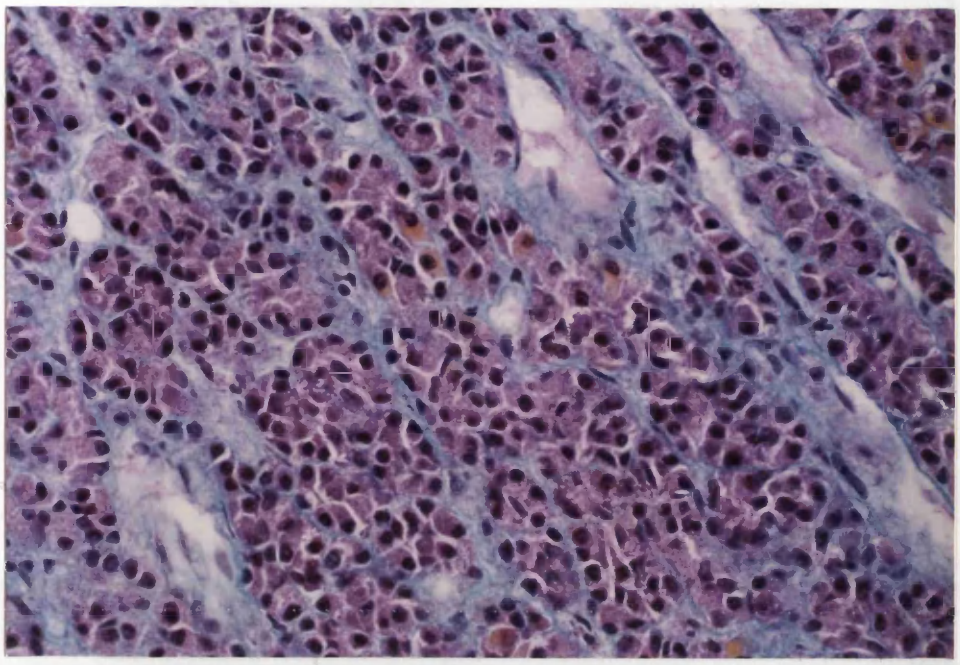


Fig. 3.3a Pituitary gland of a control animal showing intensely stained cytoplasm (appear pink when stained with Orange Fuchsin Green (OFG) stain) due to normal basophilic granulation of basophilic cells. x100 OFG.

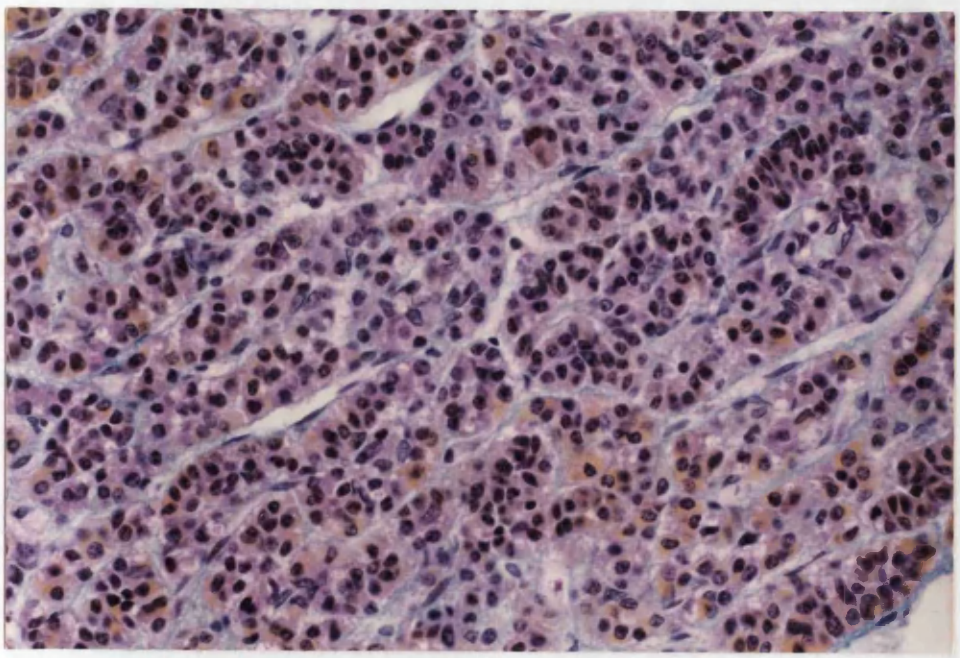


Fig. 3.3b: Pituitary gland of the *T. congolense*-infected ram killed on day 79 post-infection showing only slight reduction in basophilic granulation. Cytoplasmic staining is only slightly reduced compared to control. x100 OFG.

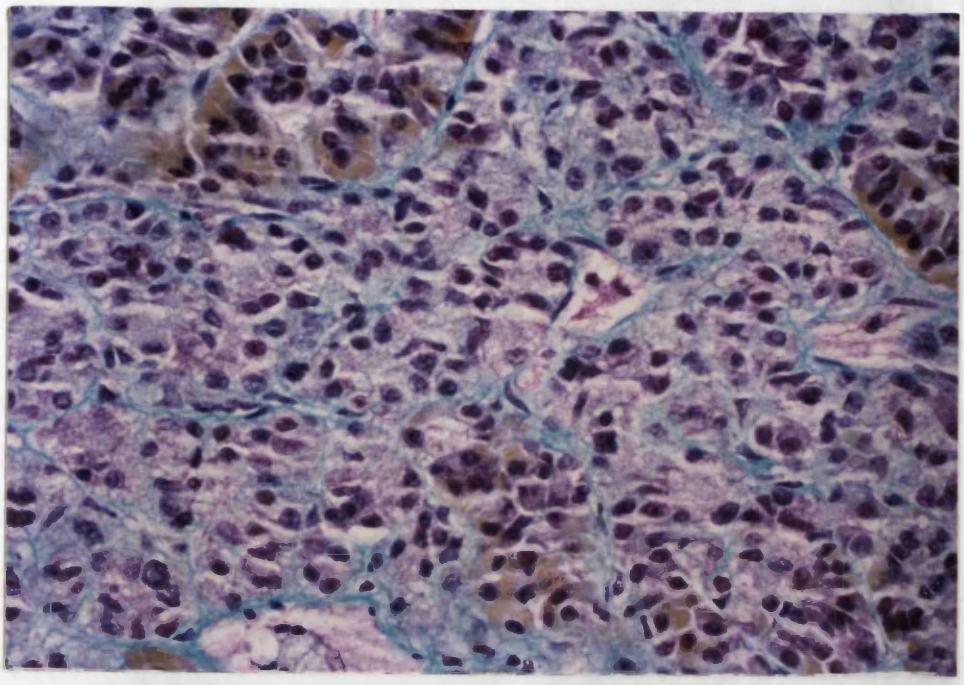


Fig. 3.3c: Pituitary gland of a *T. congolense*-infected ram killed on day 79 post-infecton showing marked reduction in basophilic granulation and a slight increase in interlobule connective tissue. Cytoplasmic staining of basophils is markedly reduced compared to control. x100 OFG.

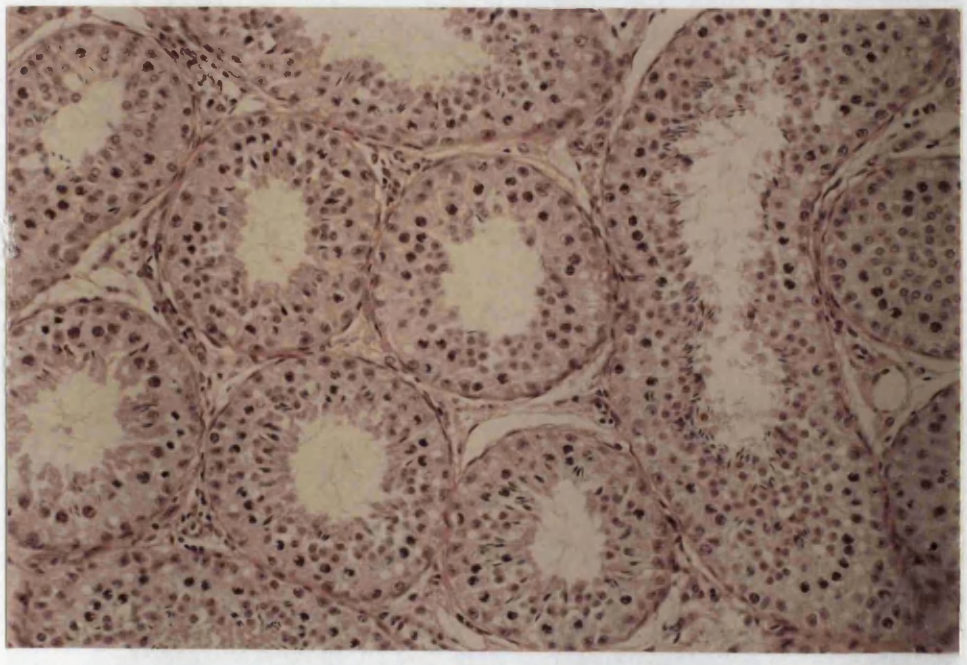


Fig. 3.4a: Testis of a control ram killed on day 79 post-infection showing normal seminiferous tubules at different spermatogenic cycles surrounded by sparse intertubular connective tissue x50 H&E.

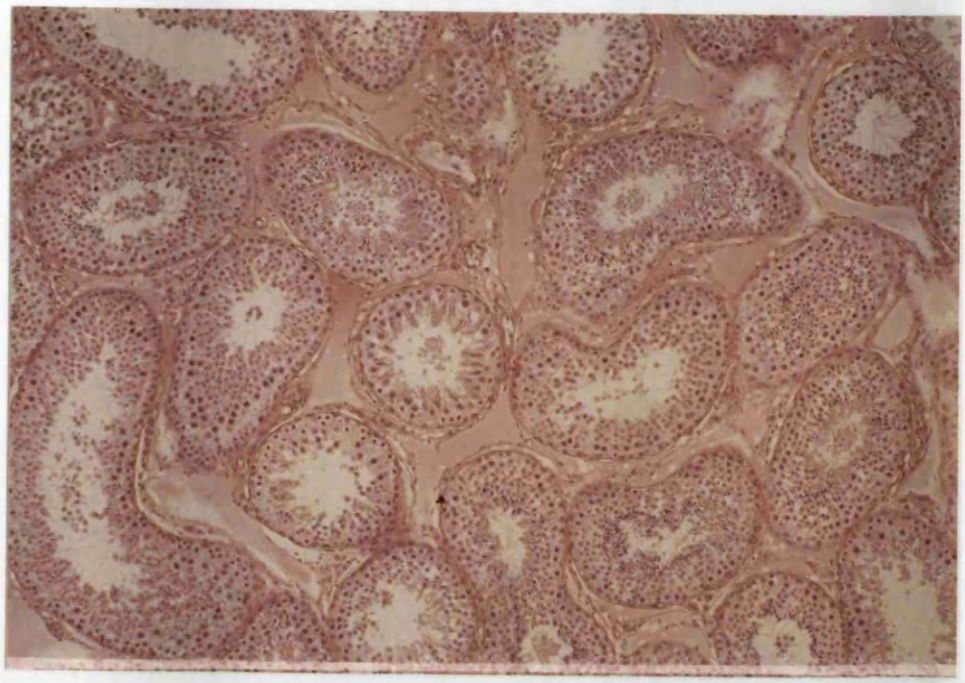


Fig. 3.4b: Testis of a *T. congolense*-infected ram killed on day 28 post-infection showing marked intertubular oedema and dilation of lymphatic vessels. Spermatogenic aciviy can still be seen in seminiferous tubules x25 H&E.

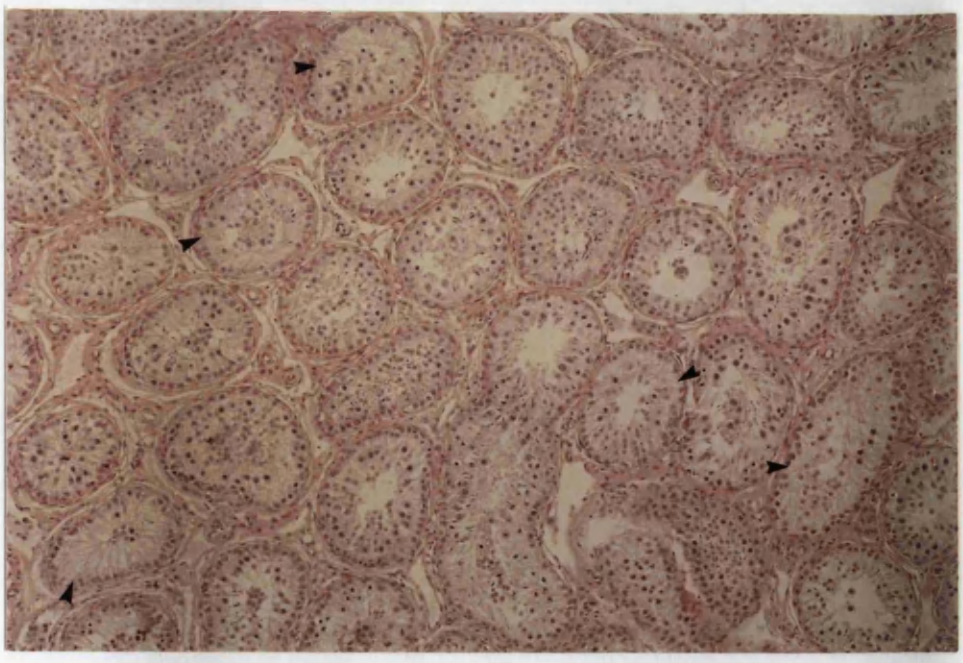


Fig. 3.4c: Testis of a *T. congolense*-infected ram killed on day 79 post-infection showing generalised atrophy of the seminiferous tubules and increased intertubular connective tissue. Note several tubules with 2 - 3 cell layer thickness (arrows) x25 H&E.

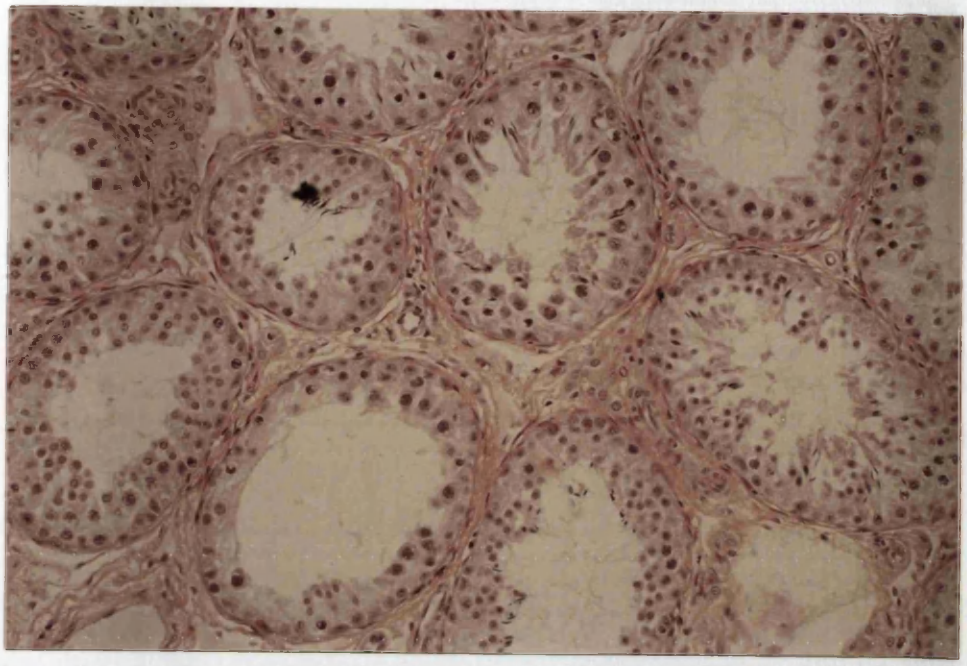


Fig. 3.4d: Testis of a *T. congolense*-infected ram killed on day 79 post-infection showing reductions in the layers of seminiferous spermatogenic cells, markedly decreased spermatogenic activity and increased intertubular connective tissue. x50 H&E.

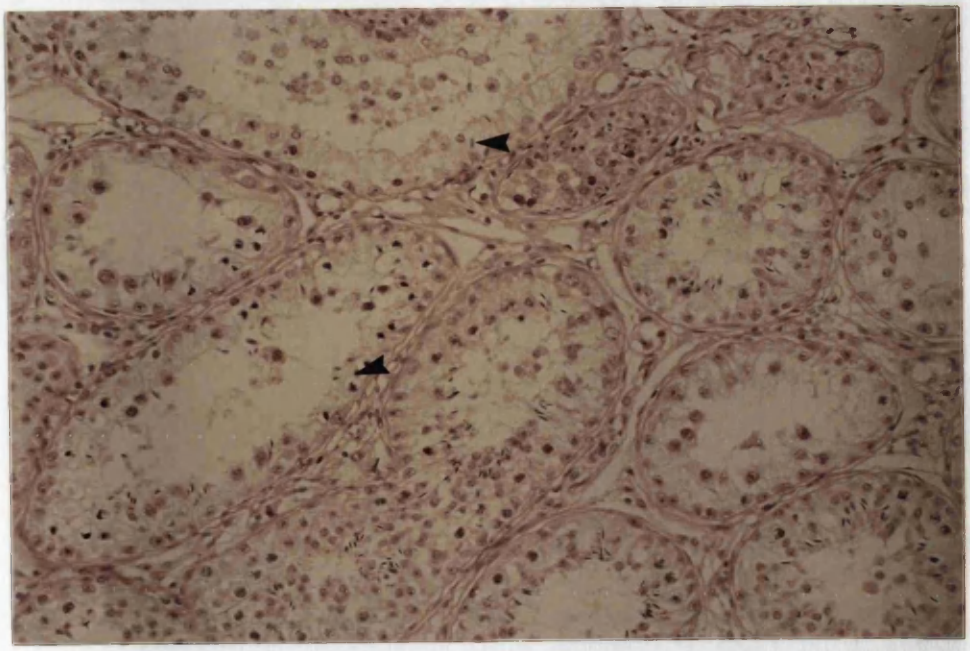


Fig. 3.4e: Testis of a *T. congolense*-infected ram killed on day 79 post-infection showing marked degeneration of seminiferous tubular epithelium and in some tubules only the spermatogonia and Sertoli cells are evident (arrows). x50 H&E.

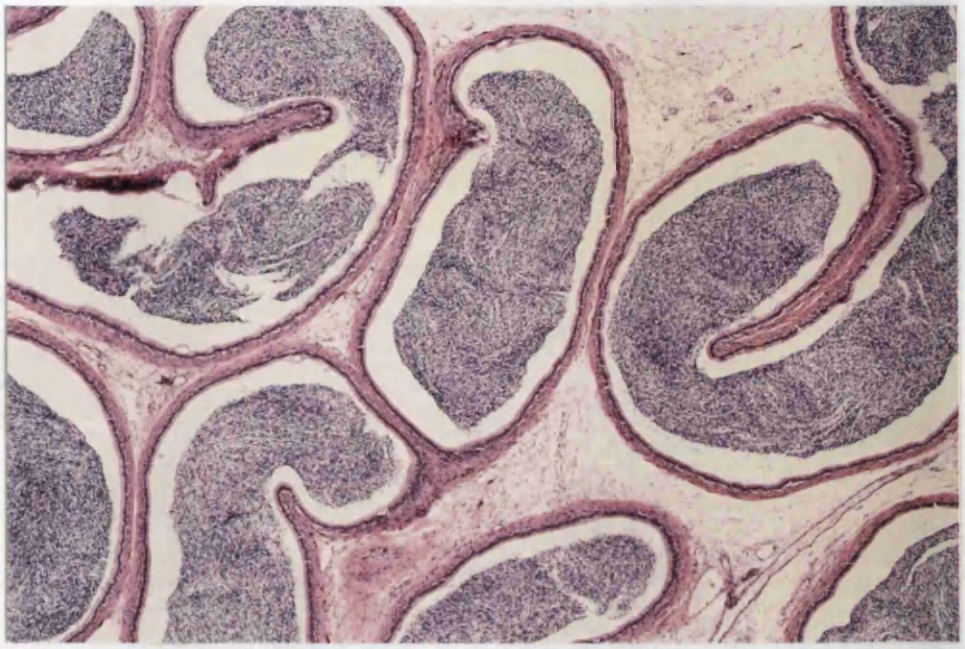


Fig. 3.5a: Cauda epididymis of a control ram killed on day 79 post-infection showing high concentration of spermatozoa stored (sperm reserve) in the tubules. x10 H&E.

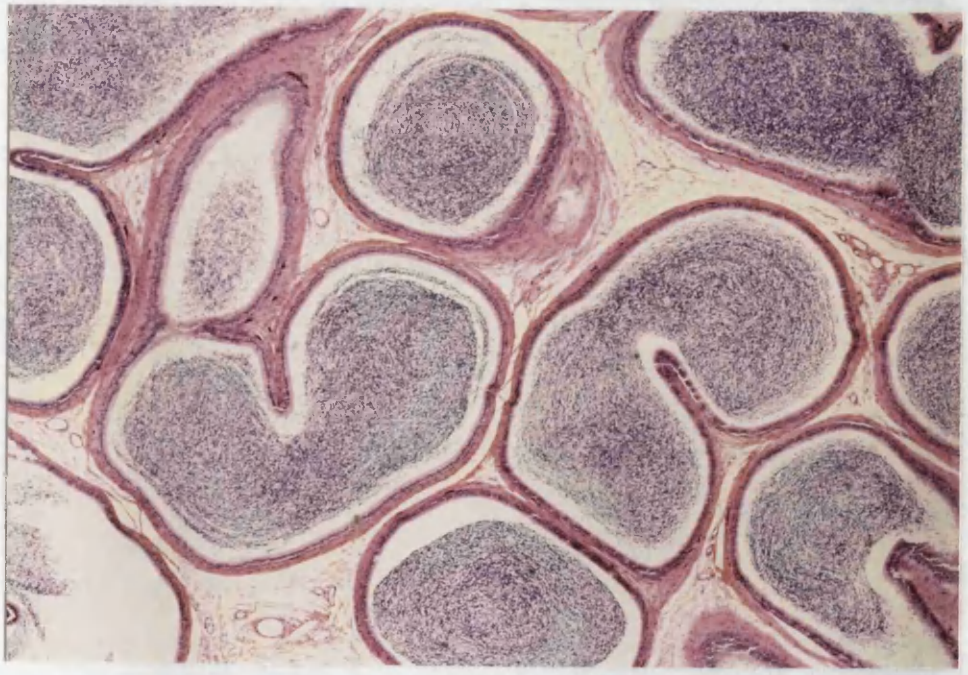


Fig. 3.5b: Cauda epididymis of a *T. congolense*-infected ram killed on day 28 post-infection showing a slight reduction in sperm concentration in the tubules compared to control (Fig 3.5a) x10 H&E.

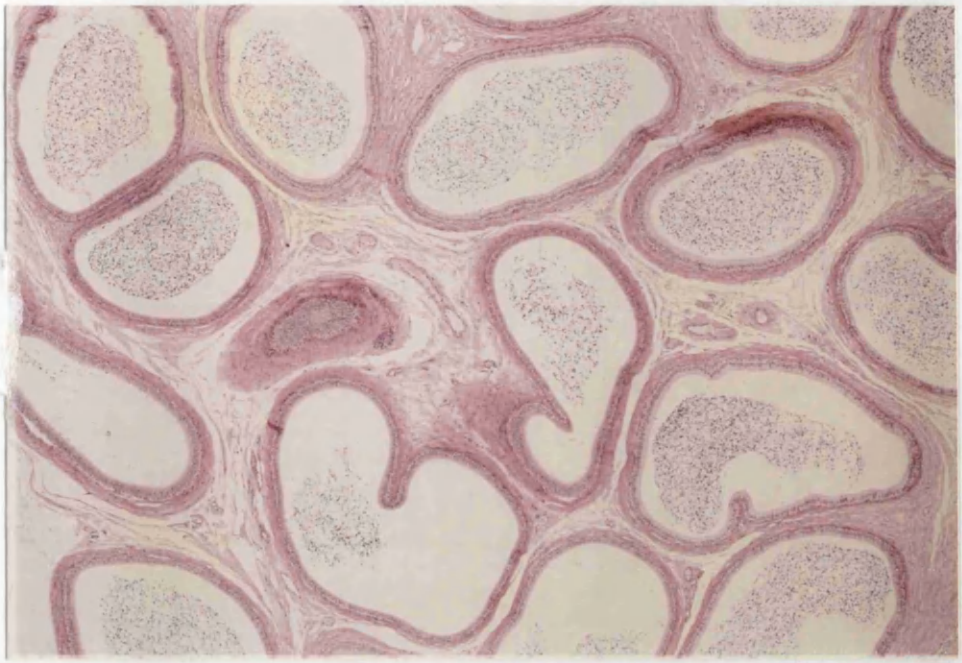


Fig. 3.5c: Cauda epididymis of a *T. congolense*-infected ram killed on day 79 post-infection showing marked reduction in sperm concentration in the tubules. x10 H&E.

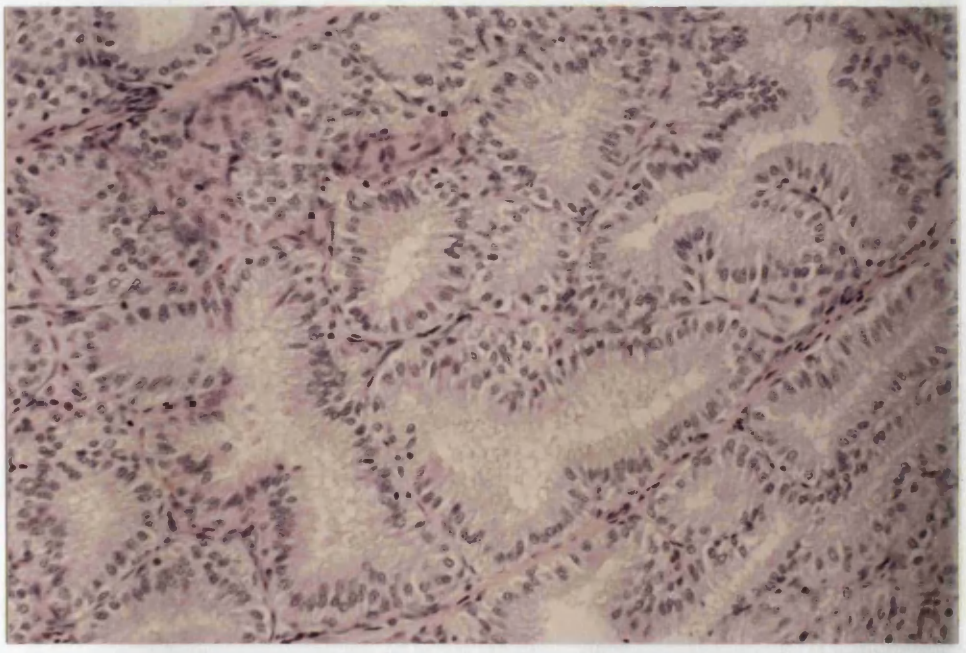


Fig. 3.6a: Prostate gland of a control ram killed on the equivalent of day 79 post-infection showing a normal gland with tall columnar stratified follicular epithelial cells and sparse interfollicular connective tissue x50 H&E.

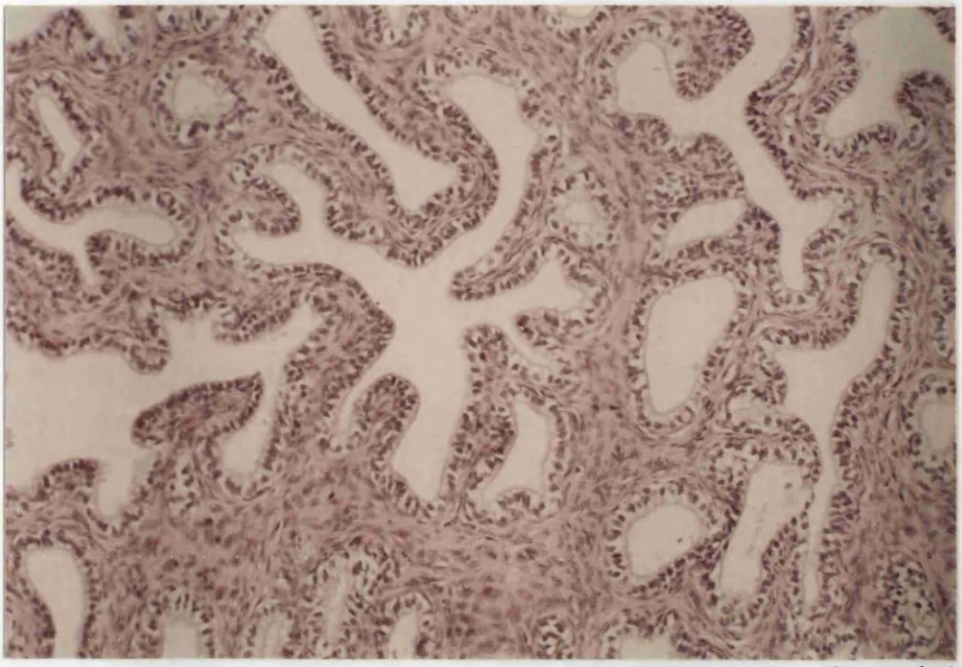


Fig. 3.6b: Prostate gland of a *T. congolense*-infected ram killed on day 79 post-infection showing reduction in the height of follicular epithelial cells (low cuboidal) when compare with control (Fig 3.16a) and marked increase in interfollicular connective tissue x50 H&E.

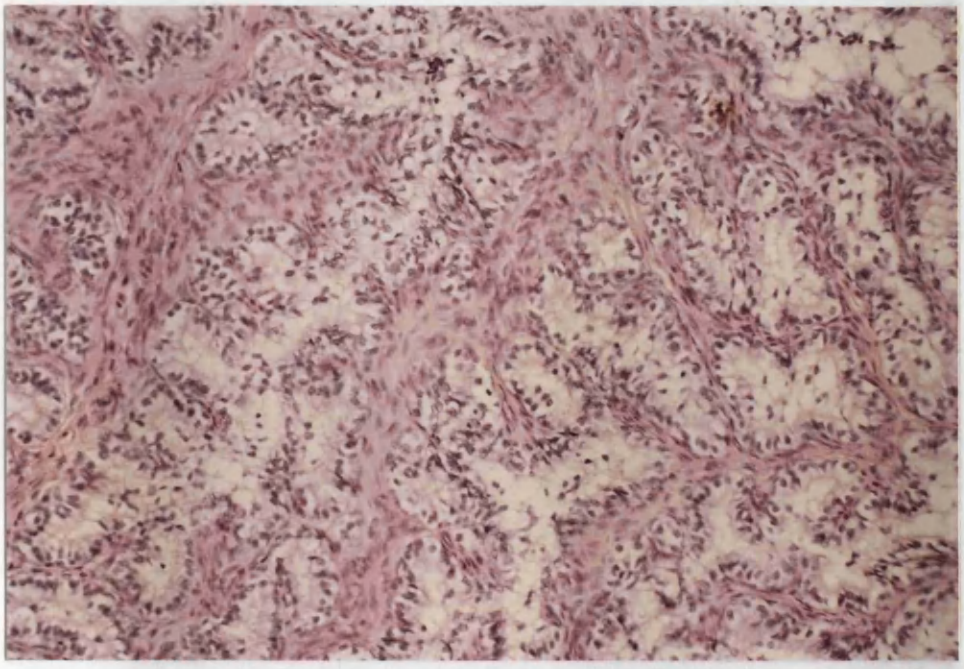


Fig. 3.6c: Prostate gland of a *T. congolense*-infected ram killed on day 79 post-infection showing atrophy and marked degeneration of follicular cells and increased interfollicular connective tissue x50 H&E.

SECTION II

3.3.2. COMPARATIVE CHANGES IN SEMINAL CHARACTERISTICS AND TESTICULAR PATHOLOGY INDUCED BY *TRYPANOSOMA CONGOLENSE* AND SCROTAL INSULATION DURING STUDY II

3.3.2.1. Clinical Observations

Trypanosomes were identified in the blood of infected sheep during Study II within 5 to 9 days post-infection. Infected rams exhibited similar clinical manifestations to those observed during Study I. The first mean peak of parasitaemia (mean scores 3.1 $\pm$ 0.4, approximately equivalent to 5×10^{-3} to 5×10^{-4} trypanosomes ml^{-1}) occurred on day 12 post-infection (Fig. 3.7a) and subsequently the levels of parasitaemia fluctuated throughout the study period. Infected rams developed low and fluctuating pyrexia (rectal temperature range 39.5 $\pm$ 0.1°C to 40.2 $\pm$ 0.2°C) (Fig 3.7b) from days 13 to 55 post-infection, temperatures being significantly higher ($P<0.05$ to $P<0.001$) than those measured in the control rams (range 38.9 $\pm$ 0.2°C to 39.4 $\pm$ 0.2°C).

The PCV values in the infected rams (Fig. 3.7c) declined rapidly from a preinfection mean value of 32.2 $\pm$ 0.7%, (Table 3.1) and by day 12 post-infection their PCV values (29.9 $\pm$ 0.9%) were significantly lower than control values ($P<0.05$). Thereafter, PCV values showed a progressive decline in infected rams and were maintained at significantly lower levels ($P<0.001$) from day 15 to day 58 when compared to control values (Fig. 3.7c). Statistical analysis of rectal temperature and PCV in infected and control rams between days 1 - 58 post-infection revealed a significant treatment effect ($P<0.01$, $P<0.001$, respectively) and a group x time interaction ($P<0.001$). Changes in liveweight in the infected and control rams are depicted in Fig. 3.7d. The liveweight gain in the infected rams was significantly reduced to 94 g day^{-1} ($P<0.01$) when compared to growth rates of 178 g day^{-1} in the controls during the study period.

One infected ram died on day 15 post-infection due to secondary *P. haemolytica* infection. All the remaining infected rams survived up to the end of the study period when they were killed together with the control rams.

Fig. 3.7 Mean (± s.e.m.) parasitaemia scores (Fig. 3.7a), rectal temperatures (Fig. 3.7b), changes in packed cell volume (PCV) (Fig. 3.7c) and liveweight (Fig. 3.7d) in uninfected rams (n = 10, control) and *T. congolense*-infected rams (n = 10, infected) during Study II. One infected ram died on day 15 post-infection due to secondary *P. haemolytica* infection.

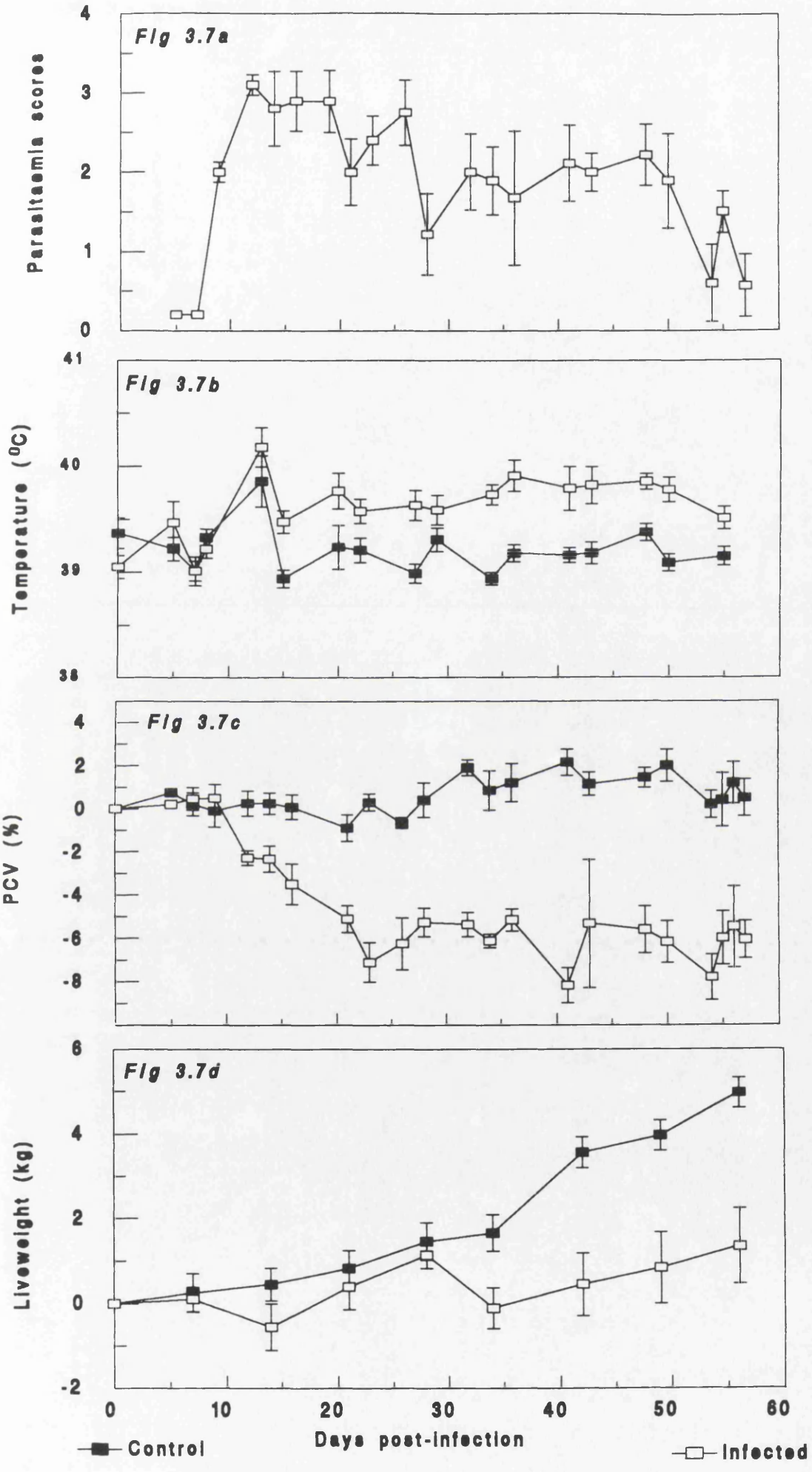


Fig 3.7a
Parasitaemia scores
4
3
2
1
0
Fig 3.7b
Temperature (°C)
41
40
39
38
Fig 3.7c
PCV (%)
4
2
0
-2
-4
-6
-8
Fig 3.7d
Liveweight (kg)
6
4
2
0
-2
0
10
20
30
40
50
60
Days post-infection
Control
Infected

3.3.2.2. Changes in Scrotal Temperature

The mean scrotal temperatures in the control, infected and scrotal-insulated rams are depicted in Fig. 3.8a. Corresponding scrotal-rectal temperature gradients are shown in Fig. 3.8b. The mean scrotal temperatures in the control rams varied between 32.5 ± 0.3°C to 34.5 ± 0.1°C giving a scrotal-rectal temperature gradient of -6.7 ± 0.3°C to -5.4 ± 0.3°C. The scrotal temperatures in the infected rams between days 13 - 58 post-infection increased significantly and varied between 33.2 ± 0.3°C ($P<0.05$) to 35.6 ± 0.30°C. ($P<0.003$). Infected rams showed a tendency of their mean scrotal-rectal temperature gradients to be lower than controls from day 13 onwards although on most occasions these values were not significantly different from control values. The mean scrotal-rectal temperature gradient of the infected rams during the same period varied between -6.2 ± 0.2°C (nonsignificant) to -4.7 ± 0.2°C ($P<0.05$). The scrotal temperature of scrotal-insulated rams varied between 32.6 ± 0.2°C and 34.8 ± 0.3°C and the scrotal-rectal temperature gradient varied between -6.7 ± 0.3°C and -5.0 ± 0.4°C. These values did not differ significantly from the control values.

3.3.2.3. Changes in Seminal Characteristics

Ejaculate volume and sperm concentration were not assessed in semen collected by electroejaculation in the rams used during Study II for the same reasons as in Study I. The same parameters used for assessing changes in semen quality during Study I were used during Study II and the results are presented in Table 3.5 for control, infected and scrotal-insulated rams. The pre-infection (day -7) semen values for control and infected rams as well as pre-insulation (day -7) semen values for the scrotal-insulated group were not different so they have been combined and are presented together. The results of semen analysis on day 21 post-infection in 10 control and 9 infected rams and on day 22 post-scrotal insulation in 6 insulated rams were analysed and compared with each other. Similarly seminal values for semen collections on day 49 post-infection in the control and infected groups and on day 49 post-insulation in the scrotal-insulated group were compared with each other (Table 3.5).

The mean scores for semen consistency and sperm wave motion in semen samples from the control rams showed a tendency to increase from day -7 to day 49

Fig 3.8. Mean (± s.e.m.) scrotal temperature (Fig. 3.8a) and scrotal-rectal temperature gradients (Fig. 3.8b) in uninfected (n = 10, control), *T. congolense*-infected (n = 9, infected) and scrotal-insulated (n = 6) rams during Study II.

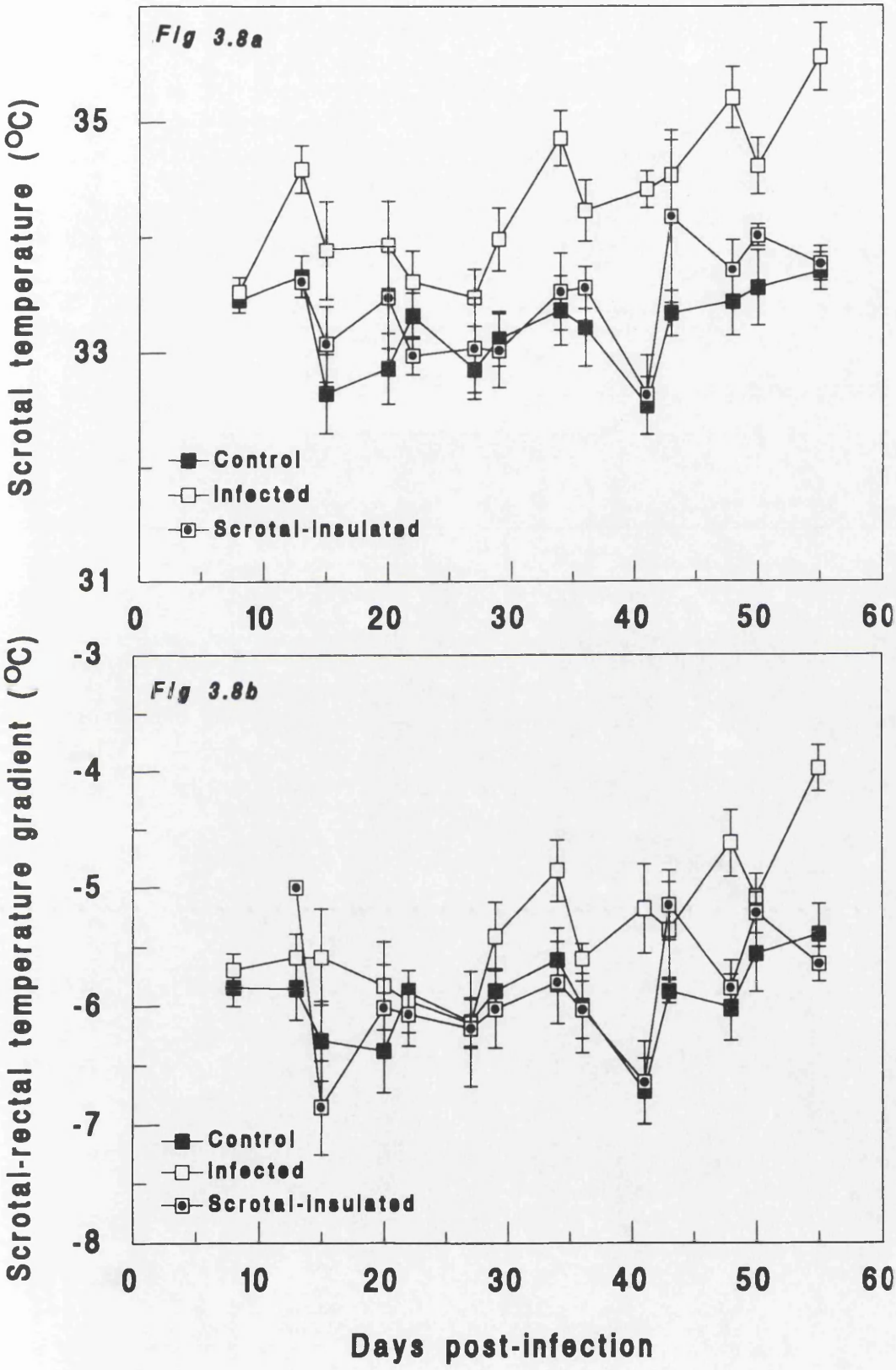


Fig 3.8a
Scrotal temperature (°C)
35
33
31
Control
Infected
Scrotal-insulated
0
10
20
30
40
50
60
Fig 3.8b
Scrotal-rectal temperature gradient (°C)
-3
-4
-5
-6
-7
-8
Control
Infected
Scrotal-insulated
0
10
20
30
40
50
60
Days post-infection

although this change was not significant over time. Semen consistency and sperm wave motion declined markedly in infected and scrotal-insulated rams over time. Mean semen consistency was significantly lower on day 49 in infected and scrotal-insulated rams ($P<0.01$) when compared to control values. Similarly, mean sperm wave motion was significantly lower ($P<0.05$) on days 21 (day 22 for scrotal-insulated group) and 49 in infected and scrotal-insulated groups when compared to control values. Mean values for semen consistency and sperm wave motion over the experimental period tended to be higher (non-significant) in infected than in scrotal-insulated groups.

The mean percentage values for total primary sperm abnormalities in control rams were less than 3% from semen collected on day -7 and on days 21 and 49 after infection, whereas a marked increase in total primary defects was observed in infected and scrotal-insulated rams during the same period. Total primary sperm defects were significantly higher ($P<0.001$) in the scrotal-insulated group on day 22 post-insulation than infected rams on day 21 post-infection and in controls at the equivalent time. However, total primary sperm abnormalities in the infected rams were also significantly higher ($P<0.01$) than in control rams on day 21 post-infection. Significant increase in primary sperm defects in the scrotal-insulated rams were mainly associated with acrosome, midpiece and tail defects whereas in infected rams the primary sperm defects were mainly associated with acrosome and head defects (Table 3.5). Acrosome and midpiece defects observed in infected and scrotal-insulated rams were essentially similar and matched those observed in infected rams during Study I (section 3.3.4). The semen of scrotal-insulated rams showed a preponderance of sperm with small ovoid microcephalic heads, pyriform and tapering heads and stumpy tails whereas the head defects in infected rams were mainly pyriform, tapering and macrocephalic heads.

No significant differences in mean total primary sperm defects were observed between the infected and scrotal-insulated rams on day 49 post-infection or post-insulation but in both groups, the mean total primary sperm abnormalities were significantly higher ($P<0.001$) than control values. Increased primary sperm defects in the infected rams on day 49 were mainly associated with head and midpiece defects whereas in scrotal-insulated rams at this time primary defects mainly involved acrosome and head defects (Table 3.5).

Table 3.5: Mean (± s.e.m.) percentage values for seminal and sperm morphology abnormalities in uninfected (control), scrotal-insulated (Scr-ins) and *T. congolense*-infected (infected) rams during Study II

	Days before (-) and after infection or scrotal insulation						
	-7	21			49		
	(n = 19)	Control (n = 9)	Infected (n = 10)	Scr-ins (n = 6)	Control (n = 5)	infected (n = 5)	Scr-ins (n = 6)
Semen consistency scores	3.53 ± 0.29	3.89 ± 0.33	2.33 ± 0.47	2.83 ± 0.48	4.6.0 ± 0.40	2.40 ± 0.51^{b}	2.67 ± 0.33^{b}
Sperm wave motion scores	3.35 ± 0.27	3.57 ± 0.29	2.22 ± 0.41^{a}	2.50 ± 0.34^{a}	4.40 ± 0.25	2.60 ± 0.51^{a}	3.17 ± 0.31^{a}
Primary defects (%)							
Acrosome defects	0.81 ± 0.31	0.62 ± 0.15	1.16 ± 0.11^{b}	2.03 ± 0.28^{c}	0.39 ± 0.11	1.02 ± 0.10	2.03 ± 0.28^{b}
Abnormal heads	0.83 ± 0.13	0.78 ± 0.16	2.87 ± 0.33^{a}	2.01 ± 1.17	0.67 ± 0.21	4.35 ± 0.85^{b}	4.68 ± 0.75^{b}
Abnormal midpieces	0.85 ± 0.17	0.40 ± 0.19	0.66 ± 0.13	1.98 ± 0.56^{c}	0.28 ± 0.12	2.72 ± 0.51^{a}	0.79 ± 0.42
Abnormal tails	0.14 ± 0.09	0.09 ± 0.02	0.06 ± 0.04	1.27 ± 0.60^{b}	0.11 ± 0.07	0.69 ± 0.31	0.23 ± 0.12
Secondary defects (%)							
Detached heads	0.85 ± 0.30	0.48 ± 0.16	1.93 ± 0.54^{a}	5.39 ± 2.32^{b}	0.35 ± 0.17	3.29 ± 0.34^{b}	0.85 ± 0.42
Proximal droplets	0.39 ± 0.25	0.56 ± 0.39	0.96 ± 0.31	0.77 ± 0.37	0.17 ± 0.10	1.50 ± 0.50	0.74 ± 0.54
Distal droplets	0.42 ± 0.13	0.35 ± 0.21	0.53 ± 0.17	4.90 ± 2.20^{a}	0.12 ± 0.07	2.50 ± 0.76	5.84 ± 2.46
Bent and coiled midpieces	3.48 ± 0.81	1.83 ± 0.37	4.46 ± 1.55	8.32 ± 1.29^{b}	1.93 ± 0.42	12.58 ± 4.35^{a}	3.86 ± 0.82
Bent and coiled tails	3.81 ± 0.49	3.05 ± 0.45	6.20 ± 0.87^{b}	7.27 ± 0.43^{b}	2.51 ± 0.19	13.99 ± 2.29	^{b} 4.57 ± 0.82^{a}
Others							
Dead/live ratio (%)	11.93 ± 2.60	8.43 ± 1.50	28.97 ± 3.22^{c}	21.61 ± 4.92^{c}	4.39 ± 0.69	37.58 ± 7.77^{b}	18.30 ± 3.02^{a}
Total primary defects (%)	2.60 ± 0.29	1.73 ± 0.29	4.74 ± 0.35^{b}	7.58 ± 1.00^{c}	1.25 ± 0.38	8.78 ± 0.57^{c}	7.64 ± 1.18^{c}
Total secondary defects (%)	9.41 ± 1.20	6.88 ± 0.80	14.07 ± 2.48^{b}	26.65 ± 4.00^{c}	5.01 ± 0.68	33.83 ± 5.76^{c}	15.37 ± 3.00

Differences between the infected and Scrotal-insulated groups and corresponding control group in the same row are denoted by superscripts a,b,c ($^{a}P<0.05$; $^{b}P<0.01$; $^{c}P<0.001$)

The mean total percentage of sperm showing secondary abnormalities in the control rams tended to decrease from 9.41 ± 1.20% on day -7 to 5.01 ± 0.68% on day 49. Following infection or scrotal-insulation a significant increase in sperm showing secondary defects was observed in infected rams (day 21 post-infection, $P<0.01$) and scrotal-insulated rams (day 22 post-insulation, $P<0.001$). A marked increase in mean total secondary sperm defects was observed on day 49 post-infection in infected rams when compared to control and scrotal-insulated rams ($P<0.001$) but the increase in scrotal-insulated rams was not significant when compared to control values on day 49 post-insulation. The significant increase in secondary sperm defects in infected rams on day 49 post-infection was mainly associated with increase in sperm with detached heads, bent and coiled tails, coiled and folded midpieces and to lesser extent sperm with distal droplets. In scrotal-insulated rams, secondary sperm defects on day 49-post-insulation were mainly associated with bent and coiled tails and to lesser extent sperm with distal droplets and abnormal midpieces.

An increase in the number of dead sperm in the ejaculate was observed in infected and scrotal-insulated rams with time. The increase was progressive in infected rams ($P<0.01$ on day 21 and $P<0.001$ on day 49 post-infection) but not in scrotal-insulated rams ($P<0.001$ on day 22 and $P<0.05$ on day 49 post-insulation).

3.3.2.4. Histopathological Examination.

Changes in the testis

The histology of the testis from control rams during Study II resembled those from control rams during Study I (Fig. 3.4a), hence no histological section is shown. Fig. 3.9a shows histopathological changes in the testis of an infected ram killed on day 58 post-infection while Fig 3.9b shows the testis of a scrotal-insulated ram killed after 58 days of insulation. Almost similar degenerative changes of seminiferous tubular epithelia are evident in both sections although they are slightly more marked in the infected ram. In both animals, there is almost similar increased intertubular space when compared to control testes (Fig. 3.4a) and an increased intertubular connective tissue is evident in the infected animal. No inflammatory cell infiltration is evident in both sections and was not observed in other infected and scrotal-insulated rams.

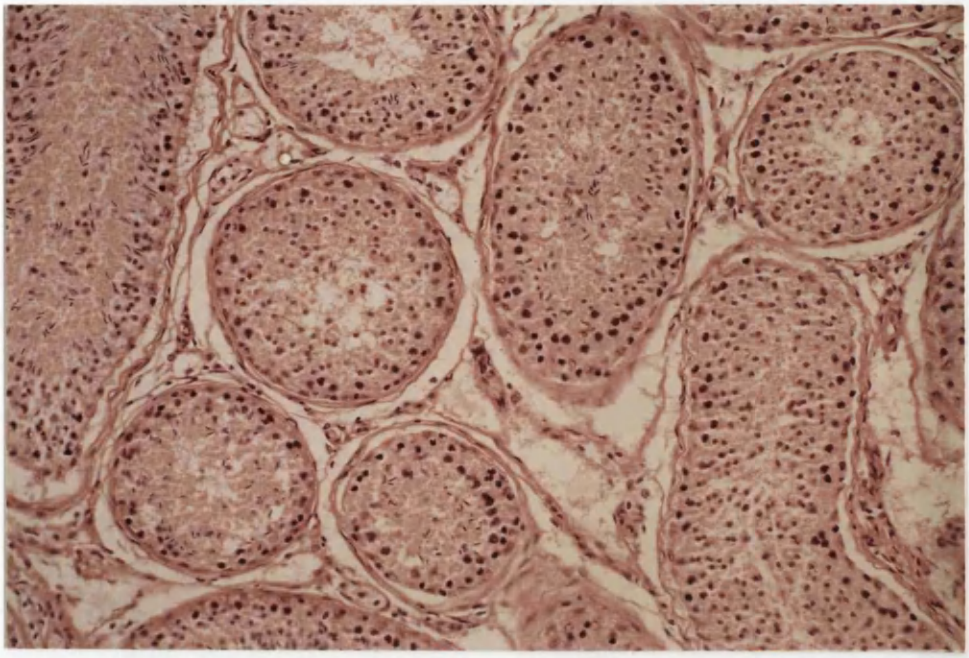


Fig. 3.9a. Testis of a *T. congolense* infected ram killed on day 58 of infection during Study II showing degeneration of the seminiferous germ cells, increased intertubular space and connective tissue x50 H&E.

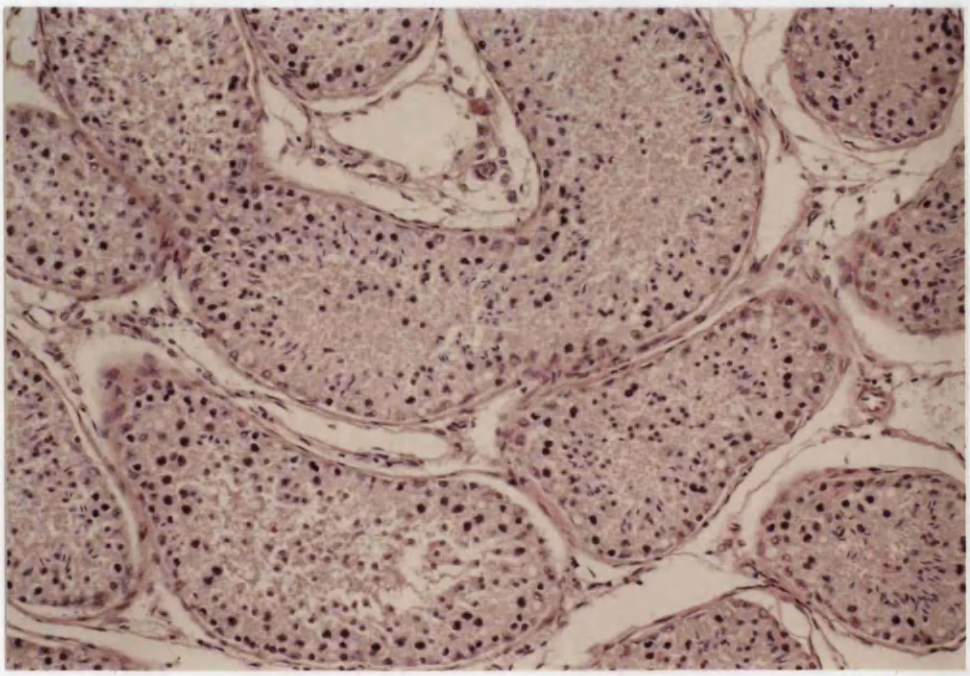


Fig. 3.9b. Testis of a scrotal-insulated ram killed on day 58 post-insulation showing degeneration of seminiferous tubular epithelium. Note the similarities in extent of degenerative change in the tubules and intertubular tissue with Fig. 3.9a. x50 H&E.

Changes in the prostate gland

The histopathological changes observed in the prostate gland of infected rams killed on day 58 post-infection resembled those observed during study I, hence no histological section is shown. The prostate glands of scrotal-insulated rams (Fig. 3.10) showed very similar changes to those observed in infected rams during Study I (Fig 3.6b - 3.6c). The follicular epithelium was low cuboidal but there was less proliferation of interfollicular connective tissue than that observed in the infected rams.

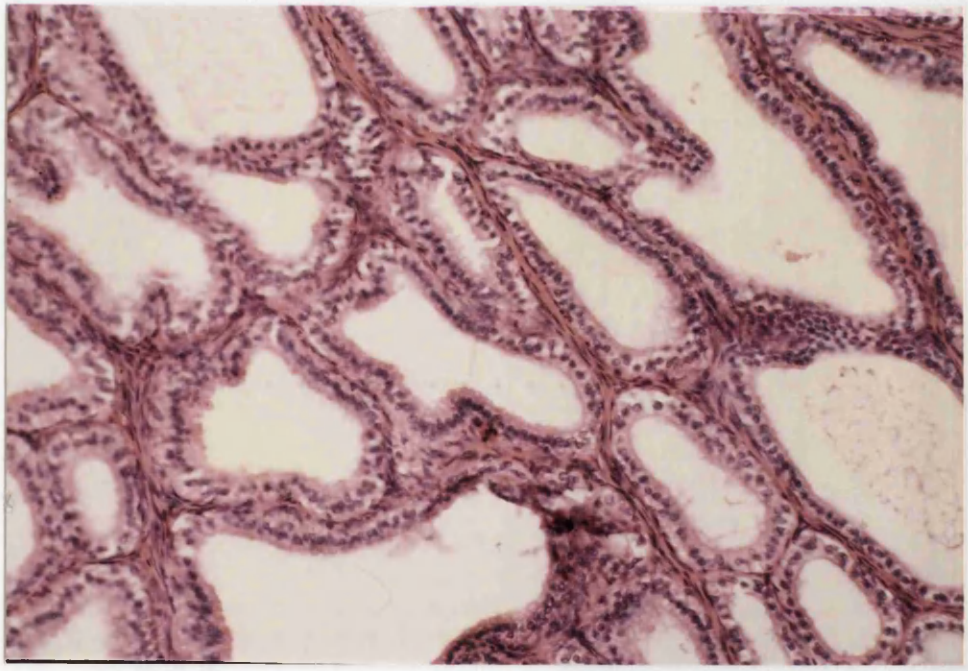


Fig. 3.10. Prostate gland of a scrotal-insulated ram killed on day 58 post-insulation showing marked reduction in the height of follicular epithelial cells when compared to control (Fig 3.6a). x50 H&E.

3.4. DISCUSSION.

Following experimental infection with *T. congolense* all infected rams in both Study I and Study II developed clinical manifestations of the disease within 1 - 2 weeks. Infected rams developed intermittent pyrexia and parasitaemia, rapidly became anaemic and had impaired growth rates. The clinical signs observed in infected rams resembled those recently reported in Scottish Blackface sheep infected with a similar trypanosome isolate (Katunguka-Rwakishaya, 1992) and also resembled those reported by other investigators (Hornby, 1921; Griffin and Allonby, 1979a; MacKenzie and Cruickshank, 1973; Stephen 1986; Losos, 1986). No death in infected rams was attributed to *T. congolense* infection *per se* and the three deaths which occurred in infected animals (two rams during Study I and one ram during Study II) where attributed to secondary *P. haemolytica* infection possibly resulting from lowered immunity (Losos, 1986). The high survival rate of infected rams might have been attributed to good management, provision of adequate nutrition and the low virulence of the *T. congolense* isolates used (Katunguka-Rwakishaya, 1992). Due to low virulence, the *T. congolense* isolates used were very suitable to produce chronic infection and allow long term effects of this disease on reproduction to be assessed. Under field conditions, it is chronic infections in which the effects of trypanosomiasis on the reproductive system assume a great importance (Luckins, 1992).

One of the objectives of this investigation was to identify the changes induced by *T. congolense*-infection in the reproductive organs of the ram. The results in this chapter show that *T. congolense* infection induced variable degrees of degenerative changes in the testis, epididymis and prostate gland and to a lesser extent in the pituitary gland of the ram. These lesions were progressive being more marked in infected rams which survived up to the end of the experimental period than in those killed at an earlier time during the study. *T. congolense* organisms remained within the blood vessels and none were found to localise extravasculary in these organs.

Changes in the pituitary gland observed by light microscopy in infected rams were mild and were characterised by evidence of a decrease in basophilic degranulation and slight increase in interstitial connective tissue between pituitary lobules. Similar pituitary changes have been described in chronic *T. congolense* infection of goats

(O'Hara *et al.*, 1985; Mutayoba *et al.*, 1988a) and cattle (Ogwu and Njoku, 1991). However, although no marked changes in the pituitary gland of *T. congolense*-infected animals are observed by light microscopy, electron microscopic studies in cattle 56 days after *T. congolense* infection have recently revealed swelling of the mitochondria, endoplasmic reticulum and Golgi apparatus within pituitary cells (Abebe, 1991; Abebe *et al.*, 1993a). Marked distension of the pituitary microvasculature with trypanosomes, activated macrophages, degranulated platelets, monocytes and other cellular debris were also observed.

Marked inflammatory changes in the pituitary gland such as coagulative necrosis, oedema, hyperemia, thrombosis, interstitial mononuclear cell infiltration and fibrosis usually accompany infections caused by *T. b. brucei* in sheep, goats, donkeys, horses and dogs (Losos and Ikede 1970, 1972; Ikede and Losos 1972a, 1975; Ikede *et al.*, 1973, 1977; Moulton and Sollod, 1976; Morrison *et al.*, 1981). Similar pituitary changes have been observed in man infected with *T. b. rhodesiense* (Hawking and Greenfield, 1941) and rats infected with *T. b. gambiense* (Marck *et al.*, 1985). In all cases, parasites were found in the pituitary lesions and their massive extravascular localization in the pituitary parenchyma was thought to contribute to the severity of pituitary lesions observed in infected animals. Similarly, there are reports that *T. vivax* may localise extravascularly in the pituitary gland (Fiennes, 1950) and cerebrospinal fluid (Whitelaw *et al.*, 1988). However, the predilection of *T. vivax* to invade and accumulate extravascularly in the pituitary gland is low when compared to *T. brucei* as no changes in the pituitary of *T. vivax*-infected animals have been reported.

Although less change was observed in the pituitary gland of infected animals in the present studies, *T. congolense* infection was associated with marked seminal and testicular pathology in rams. The quality of semen ejaculated by post-pubertal rams at the beginning of experiments during the present studies was lower and contained more primary and secondary sperm defects when compared to semen of mature rams (Dyrmundsson, 1973). Semen quality of pubescent ram lambs is, as a rule, relatively poor and the quality increases steadily with advancing age and more mature body development (Terrill, 1938; Dun, 1955; Skinner and Rowson, 1968; Skinner, 1971). Pubertal age was also associated with smaller seminiferous tubular diameter in control

rams (e.g. range 67.2 - 115.7 μm) compared to a diameter of >150 μm found in mature males (Anosa and Isoun, 1980; Wrobel and Dellmann, 1993). Increases in semen quality were observed in control rams as the two experiments progressed which could have been attributed to post-pubertal developmental changes in the rams.

T. congolense infection was associated with variable degrees of seminal and testicular pathology in the ram. These changes were progressive and tended to be more severe with the duration of infection. Changes in semen characteristics were associated with a progressive decline in semen consistency, sperm motility and an increase in the percentage of sperm with primary (arising from the testis) and secondary (arising from the epididymis) defects and in percentage of dead sperm. The primary and secondary sperm defects accounted for an equal share of abnormalities seen in semen obtained on day 27 post-infection during Study I and on day 21 post-infection during Study II. However, secondary sperm defects tended to out-weigh the primary defects in semen collected on days 49 and 69 post-infection during Study II and Study I, respectively. The completion of a normal spermatogenic cycle in the seminiferous tubule of the ram i.e. the time for progression from stem spermatogonia to immature spermatozoa is estimated to take about 50 days (Dyrmundsson, 1973; White, 1976; Russell *et al.*, 1990; Wrobel and Dellmann, 1993). This period is subdivided according to White (1976) into a mitotic (spermatocytogenesis) phase of about 15 - 18 days in which the stem spermatogonia undergoes a series of mitotic division to produce primary preleptotene spermatocytes. This is followed by a meiotic (spermatocyte) phase of about 15 - 17 days which results in the production of haploid spermatids and a maturational (spermiogenic phase) of 15 days for spermatids to undergo morphological transformation into immature spermatozoa. The time required for the immature spermatozoa released from the testis to reach maturity during their transport in the epididymis is estimated to take another 11 - 15 days in the ram (Ortavant *et al.*, 1969; Courot *et al.*, 1970). Hence, it is likely that the observed increase in sperm defects in infected animals on days 21 and 26 post-infection must have been due to the infection affecting sperm which were going through the spermiogenic phase at the time of infection. Similarly, sperm defects observed during Study II in infected and scrotal-insulated rams on day 49 post-infection and post-insulation and on day 69 post-infection during Study I could have arisen in sperm which

were doing through the meiotic and mitotic phases, respectively at the beginning of those experiments.

Primary sperm defects seen were mainly of the acrosome (detached and serrated and acrosome with knobbed defects), head (pyriform, tapered and macrocephalic) midpiece (abaxial, swollen rough and duplicate) and tail (stumpy). The secondary sperm defects seen were detached heads, bent, swollen and coiled midpieces and tails and cytoplasmic droplets. Similar sperm defects to those described in rams in the present studies have been described in goats (Kaaya and Oduor-Okelo, 1980; Waindi *et al.*, 1986; Gombe, 1989) and bulls (Grundler and Djabakou, 1985; Sekoni *et al.*, 1988, 1990a, 1991) infected with *T. congolense*. Furthermore, similar sperm defects have been described in rams infected with *T. vivax* (Anosa and Isoun, 1980; Agu *et al.*, 1986; Akpavie *et al.*, 1987; Sekoni, 1992, 1993) and *T. b. brucei* (Akpavie *et al.*, 1987).

As the infection progressed, the semen of infected rams in the present studies showed a progressive increase in the percentage of dead sperm and sperm showing secondary rather than primary abnormalities. Since the secondary sperm abnormalities are usually of epididymal origin (Setchell, 1984) it is likely that the epididymis is one of the main sites at which *T. congolense* affects sperm quality. The transit of sperm through the epididymis is usually associated with significant sperm maturation changes which include among others (1) gaining the capacity for progressive motility (2) final condensation of the nucleus and modification of the acrosome (3) migration of cytoplasmic droplet from the proximal to distal midpiece (4) resorption, phagocytosis and liquefaction of defective sperm (5) cross-linking of structural proteins by disulfide bonds and (6) absorption of seminiferous and rete testis fluid, thus concentrating the sperm (Barth and Oko, 1989). Changes in the epididymal function induced by *T. congolense* infection could have contributed to the observed decline in semen consistency and sperm motility and the marked increase in percentage of dead sperm and secondary structural sperm defects. No information is available on the effects of trypanosomiasis on the physiology of the epididymis and its influence on epididymal sperm maturation, transport and storage. This is one of the area which needs to be investigated especially during infections induced by trypanosomes which are less likely to invade epididymal tissues.

Changes in sperm quality occurred concurrently with other changes in the testis and epididymis of infected rams. The testicular degenerative changes were mainly of the simple type, consisting in most cases of changes in the numbers of seminiferous germ layers and a decrease in the amount of semen stored in the cauda epididymis. The testes of infected rams killed on day 28 post-infection during Study I had increased seminiferous tubular diameter and intertubular oedema when compared to testes of control rams. The increase in intertubular fluid in the testis suggests that the infection affected the testicular blood vascular and lymphatic dynamics. Changes in testicular fluid dynamics would partly explain the marked increase in intratesticular testosterone content which was observed in infected testis during this period (discussed in Chapter V). This increase in intratesticular testosterone may have caused an increase in seminiferous tubular diameter observed in the testis of infected rams. Testosterone plays a major role in the function of both Sertoli cells (Hansson *et al.*, 1974a, 1974b; Sanborn *et al.*, 1977, 1981) and germ cells (Sanborn *et al.*, 1975; Wright and Frankel, 1980). The dynamic processes involved in spermatogenesis, the meiotic conversion of primary spermatocytes to spermatids and the spermiogenic events of spermatid binding to Sertoli cells and the completion of spermiogenesis are androgen-dependent (Steinberger, 1971; Setchell, 1978; Cameron *et al.*, 1993). These processes are likely to be affected by the marked increase in intratesticular testosterone leading to the increased seminiferous tubular epithelial thickness and tubular diameter.

The changes in testicular morphology in *T. congolense*-infected rams were progressive since the testes of infected rams killed on day 58 post-infection during Study II showed more advanced degenerative changes than those killed on day 28 during Study I. Similarly, the infected rams killed on day 79 post-infection during Study I showed more advanced degenerative changes than those killed on days 28 (Study I) and 58 (Study II). The reductions in epithelial thickness and seminiferous tubular diameter and signs of testicular atrophy were more evident in rams killed on day 79 than in those killed on day 28 post-infection. Testicular atrophy (as measured by testicular weight) was significantly correlated with seminiferous tubular diameter. Testes from infected animals obtained on day 79 post-infection contained small seminiferous tubules and increased intertubular connective tissue leading to an apparent increase in the number of

Leydig cells in a given testicular cross-sectional area. Changes in the Leydig cells were not observed by light microscopy. This agrees with the findings of Ikede (1979); Kaaya and Oduor-Okelo (1980); Anosa and Isoun (1980) and Omeke and Onuora (1992). However, more in-depth studies are needed to confirm the lack of apparent changes in the Leydig cells as the available evidence from this and other studies has shown that *T. congolense* reduces plasma testosterone in infected animals (discussed in Chapter IV and V). The limited amount of sperm which was observed in the cauda epididymides of infected rams killed on day 79 compared to the amount found on day 28 post-infection also reflects the progressive decline in spermatogenic activity of infected rams. The changes in the testis and cauda epididymis of *T. congolense*-infected rams observed in the present studies have similarly been reported in other species of animals infected with either *T. congolense* or *T. vivax* (reviewed by Ikede *et al.*, 1988) and have recently been described in bulls (Sekoni *et al.*, 1990) and boars (Omeke and Onuora, 1992) after *T. congolense* infection.

It has been shown that the onset and severity of gonadal lesions depend on the virulence of the infecting trypanosome as well as susceptibility of the infected host (Ikede *et al.*, 1988; Sekoni *et al.*, 1990b). More severe degenerative testicular and epididymal changes than those described in the present studies have been described in infections induced by more virulent serotypes of *T. congolense* (Kaaya and Oduor-Okelo, 1980; Waindi *et al*, 1986; Sekoni *et al.*, 1990b) and *T. vivax* (Losos and Ikede, 1972; Isoun and Anosa, 1974a; 1974b; Anosa and Isoun, 1980). However, many of the animals affected more severely by trypanosome infections are likely to die and the effects of trypanosomiasis on the fertility of these animals is likely to be immaterial to the livestock farmer.

The prostate glands of *T. congolense*-infected rams had a flattened cuboidal epithelium and showed evidence of increased amounts of interfollicular connective tissue. Moderate prostate changes have been described in cattle infected with *T. congolense* or *T. vivax* (Sekoni *et al.*, 1990a). The changes in the prostate gland in infected rams in the present studies could have been induced by low plasma testosterone concentration (Chapter IV and V) since similar prostate changes are observed in castrated animals (Westin *et al.*, 1993). Under normal conditions the contribution of

prostate secretions to the total ejaculate volume in ruminants is about 4 - 6% but these secretions play an important role in neutralising the acidic seminal fluid and initiating activity of the ejaculated spermatozoa (Wrobel and Dellmann, 1993). Although the effects of *T. congolense* infection on prostate secretion was not investigated in the present studies, the histological changes observed were suggestive of decreased prostate secretory activity and this could possibly have contributed to the poor semen quality obtained in infected rams.

During *T. brucei* infections, the invasion of the gonadal tissue by the parasite probably plays a major role in inducing severe non-suppurative granulomatous periorchitis which leads to degeneration and atrophy of the testicular seminiferous tubules and changes in the epididymis and other testicular accessory tissues (reviewed by Ikede *et al.*, 1988). The mechanisms by which *T. congolense* induces infertility in animals is somewhat different and the evidence for involvement of direct trypanosome-borne and indirect trypanosome-induced factors was extensively reviewed in Chapter I. One of the factors which was investigated during Study II was the role of pyrexia induced by *T. congolense* infection on the seminal and gonadal changes of infected rams. Scrotal temperature of trypanosome-infected and control rams was measured during Study II and the changes in semen, testis and prostate gland were compared to those observed in rams whose scrotal temperature was locally elevated by scrotal insulation. It was evident in this study that *T. congolense* induced a significant increase in scrotal temperature in rams during infection. The changes in scrotal temperature almost parallelled similar increases in rectal temperature. The scrotal-rectal temperature gradient measured in control rams in the present study was within the normal range expected in the ram (Harrison and Weiner, 1948). Unfortunately, the scrotal insulation study was not completely successful as the scrotal pouch induced the scrotal skin to sweat which reduced the effectiveness of the pouch in elevating scrotal temperature. However, scrotal-insulation did induce varying degrees of seminal and testicular pathology, although the changes were small when compared to those observed in infected rams. Unfortunately the scrotal insulation study was carried out during the experiment from Nov, 1992 to Jan, 1993 and hence, this investigation could not be repeated. If this study were to be repeated in the future, it would be useful to try

different types of pouches using different insulating materials on rams during the non-breeding season. The pouch giving best results in terms of consistently raising the scrotal temperature could then be used in subsequent trypanosome studies.

Although several previous studies have postulated that trypanosome-induced pyrexia might contribute to the infertility observed in infected animals (Kaaya and Oduor-Okelo, 1980; Anosa and Isoun, 1980; Ikede *et al.*, 1988; Omeke and Onuora, 1992), there are no reports to-date which have shown that trypanosomiasis increases the testicular temperature of infected hosts. This is the first report to confirm that *T. congolense* infection increases the scrotal temperature of infected rams. It is therefore possible that some of the gonadal changes observed in infected rams in the present studies could have been induced by pyrexia which accompanied the disease. Evidence to support this hypothesis is available. Close similarities were observed in the nature of semen and gonadal pathology induced by the infection and scrotal insulation. The type of sperm defects observed were quite similar between the two studies and in both investigations, degeneration of the testes was not associated with any inflammatory response in the peritubular tissues. Furthermore, closely similar changes were observed in the prostate glands of trypanosome-infected and scrotal-insulated rams. In infections caused by the *T. brucei*-group, inflammation definitely plays a major role in the degeneration of the testicles and surrounding tissues but it would appear that a pyrexic effect may also contribute to gonadal damage. This is because the degenerative changes induced by these trypanosomes which include scrotal dermatitis, periorchiditis and epididymitis are likely to interfere with the cooling of the testis, as do other scrotal lesions such as eczema and ringworm (Arthur *et al.*, 1982).

Acute febrile conditions (Gunn *et al.*, 1942; Roberts, 1971; Arthur *et al.*, 1982) are known to induce testicular degeneration. Thus decreased sperm motility, increased percentage of dead sperm and marked non-inflammatory testicular degeneration occurs in tick-borne fever (Watson, 1964) and in several other conditions in which testicular temperature is raised, including cryptorchidism (Moore and Aslund, 1923; Asdell and Salisbury, 1941; Nelson, 1951; Swerdloff *et al.*, 1971), scrotal insulation (Gunn *et al.*, 1942; Glover, 1955), exposure to high environmental temperature (Dutt and Hamm, 1957) and varicocele condition (Kay *et al.*, 1979). In thermal testicular degeneration, the

spermatocytes in the late pachytene (meiotic) phase and spermatids undergoing the spermiogenic phase are known to be the most affected, followed by the spermatozoa, while the spermatogonia are the least affected (Blackshaw and Hamilton, 1971; Blackshaw *et al.*, 1973). In the present investigations the spermatogonia survived even in severely affected seminiferous tubules whereas the spermatocytes, spermatids and spermatozoa were severely affected. The vulnerability of the spermatocytes and spermatids to elevated testicular temperature is possibly associated with their plasma membrane instability at higher temperature leading to increasing lysosomal fragility and release of lysosomal enzymes (Blackshaw and Hamilton, 1971; Lee and Fritz, 1972). Furthermore, elevated testicular temperature may also damage immature spermatozoa undergoing morphological changes in the rete testes and caput epididymis (White, 1976; Arthur *et al.*, 1982) since young spermatozoa are more susceptible to the injurious effect of heat than mature spermatozoa present in the cauda epididymis (Kandeel and Swerdloff, 1988).

Pyrexia may also contribute to the reproductive endocrine changes frequently observed in trypanosomiasis (Ikede *et al.*, 1988). Changes in pituitary and testicular hormonal secretions were observed in infected ram in the present study, and the role of trypanosome-induced pyrexia on inducing these changes are discussed in Chapter IV and V which also include the effects of scrotal insulation on these secretions.

The development of fever (pyrexia) in *T. congolense* infection occurred at the onset of parasitaemia. The onset of pyrexia is usually mediated through the activation and subsequent interaction of several endogenous pyrogenic cytokines including IL-1ß, IL-1α, IL-6, TNF-α, IFN-α, IFn-γ and IL-8 (Whicher and Westacott, 1992). The secretion of most of these cytokines has been found to be stimulated during trypanosome-infections (Bancroft *et al.*, 1983; Wirth *et al.*, 1985; De Titto *et al.*, 1986; Silva *et al.*, 1992, Winstanley *et al.*, 1993; Sileghem, *et al.*, 1989; 1993). However, although an increasing body of evidence has recently shown that cytokines might affect the endocrine functions of several organs including the hypothalamo-pituitary-gonadal axis (discussed in Chapter IV), there are no reports which have proved that *T. congolense*-stimulated cytokines play a role in inducing the observed gonadal degenerative changes and such studies should be encouraged.

In conclusion, these studies shows that *T. congolense* induces progressive deterioration of sperm quality and non-inflammatory degenerative changes in the pituitary, testis, cauda epididymis and prostate of the ram. It is postulated that these changes are partly induced by trypanosome-induced pyrexia which results in the persistent elevation of testicular temperature of infected animals. The elevated testicular temperature possibly acts in conjunction with other trypanosome-borne and trypanosome-induced factors in inducing infertility in infected animals.

CHAPTER IV

REPRODUCTIVE ENDOCRINE CHANGES ASSOCIATED WITH *TRYPANOSOMA CONGOLENSE* INFECTION IN THE RAM

4.1. INTRODUCTION

During the course of infection, trypanosomes are known to cause damage to several organs including endocrine glands involved in reproductive processes in the male (reviewed in Chapter I and III). However, the sequence of events involved in the pathophysiological changes in endocrine function which affect the reproductive organs in animals suffering from trypanosomiasis is yet to be adequately studied. Alterations in gonadal steroid hormone secretion in the male have been described in goats infected with *T. congolense* (Waindi *et al.*, 1986; O'Hara *et al.*, 1985), in sheep infected with mixed *T. congolense* and *T. b. brucei* infections (Adeyemo *et al.*, 1990) and in rats infected with *T. b. brucei* (Soudan *et al.*, 1992). The common finding in these studies is a decline in plasma testosterone levels during infection. It has been suggested that the testicular degenerative changes which occur following trypanosome infection may partly be due to the depression of testicular steroidogenesis (Waindi *et al.*, 1984). However, plasma samples were collected infrequently in these studies at a rate of one sample (Adeyemo *et al.*, 1990) or 3 samples (Waindi *et al.*, 1986) per week and in those studies reported in the rat (Soudan *et al.*, 1992) plasma testosterone was only measured in a single plasma sample obtained from animals sacrificed on the third day after *T. b. brucei* infection. Since testosterone is secreted in a pulsatile fashion and plasma levels may change considerably in matter of hours, these studies did not allow for pulsatile alterations of plasma testosterone concentration.

The mechanism by which *T. congolense* induces the reproductive endocrine changes in the host is not completely understood and the possible involvement of direct trypanosome-borne and indirect trypanosome-induced factors has been reviewed in Chapter I. The regulation of steroidogenesis in the testis involves complex interactions of several hormones derived either from the pituitary gland or produced locally in the testes (Laung and Steele, 1992). As the function of the pituitary-gonadal axis depends on the trophic influence of releasing hormones secreted by the hypothalamus, factors induced by infective pathogenic trypanosomes which are capable of reducing hypothalamic-releasing hormone activity will impair reproduction.

Studies using the *T. brucei*-group have shown that the decline in gonadal steroidogenesis during infection might be associated with dysfunctions of the pituitary

gland manifested as a decline in serum concentration of the gonadotrophins LH and FSH in man (Emeh and Nduka, 1983) and rats (Soudan *et al.*, 1992). However, there are also conflicting reports which have shown that serum concentrations of gonadotrophins in man (Boersma *et al.*, 1989) and rats (Hublart *et al.*, 1990) remain stable during *T. b. brucei* infections despite an increase in pituitary LH content and a decline in plasma testosterone concentration in the latter (Hublart *et al.*, 1990). Unfortunately, in these studies single rather than sequential serum samples were taken and since gonadotrophins are secreted in a pulsatile fashion, the discrepancies in the findings might be associated with the failure to investigate properly the effects of trypanosome infection on the pulsatility of plasma LH and FSH secretion.

There are no comparable studies on gonadotrophic function which have been reported in animals infected with *T. congolense* or *T. vivax*. However, studies reported by Abebe (1991) have shown that cattle suffering from an acute form of *T. congolense* infection have impaired hypothalamo-pituitary activity as shown by a decline in the secretion of pituitary ACTH in response to CRH and insulin-induced hypoglycaemic stress. In order to understand the mechanisms involved in reproductive dysfunction caused by trypanosome infection, it is necessary to expand such studies to cover the hypothalamo-pituitary-gonadal axis. Furthermore it is important to take into account the changes in the pulsatile secretion of the pituitary hormones at different stages of trypanosome infection to fully understand the dynamic changes occurring in pituitary-gonadal function.

Fever is well recognised to be an inevitable consequence of trypanosome infections and it plays an important role in the pathogenesis of this disease in several animal species (Hornby, 1921; Losos, 1986; Stephen, 1986). As reviewed earlier in Chapter I, animals suffering from trypanosomiasis develop a highly fluctuating low grade pyrexia with temperature rising about 1 - 4^{o}C above normal rectal temperature. Several enzymes involved in Leydig cell Δ^4 and Δ^5 steroidogenic pathways are known to be highly sensitive to elevated testicular temperature (Steinberger, 1991). Furthermore, the function of the hypothalamo-pituitary axis is also known to be modified by several cytokines including IL-1, IL-6α and TNF-α which are activated during pyrexia (Kennedy and Jones, 1991) and are known to be stimulated during

trypanosome infections (Bancroft *et al.*, 1983; Askonas and Bancroft, 1984; Sileghem *et al.*, 1989, 1993; Mitchell *et al.*, 1986; Winstanley *et al.*, 1993).

Although it has been suggested that pyrexia induced by trypanosomiasis exerts a direct or indirect effect on testicular function in infected animals (Ikede *et al.*, 1988) no evidence is available which has shown that fluctuations in body temperature such as those which occur in trypanosomiasis can interfere with the function of the hypothalamo-pituitary-gonadal axis.

4.1.1. Objectives of the Study

Rams used in Study I and Study II, previously described in Chapter III 3.1.1., were also used to study the effects of *T. congolense* infection on the function of reproductive endocrine glands with the following objectives:

(i). To determine the long-term changes in plasma LH and testosterone concentrations induced by *T. congolense* infection.

This objective was intended to provide baseline information on changes in plasma LH and testosterone induced by *T. congolense* infection. This investigation was performed on the rams used in Study I (see chapter III) by measuring plasma LH and testosterone in blood samples collected three times a week throughout the infection period.

(ii) To determine the effect of infection on pituitary responsiveness to exogenous gonadotrophin-releasing hormone (GnRH) stimulation.

This objective was addressed by measurement of plasma LH and testosterone concentration in samples collected after GnRH injection at different stages of infection and was investigated in both Study I and Study II.

(iii) To assess the effects of *T. congolense* infection on the pulsatile secretion of plasma LH and testosterone at different stages of infection.

This was a follow-up investigation to Study I and was performed by measuring plasma LH and testosterone in samples collected frequently at different stages of infection during Study II.

(iv) **To compare the effects of elevation of testicular temperature by artificial means or by trypanosome infection on the pulsatile secretion of plasma LH and testosterone.**

This study was designed to compare plasma LH and testosterone secretion in rams subjected to scrotal insulation (see chapter III 3.2.6) and trypanosome infection (Objective iii).

(v) **To assess the effect of castration on pituitary function in trypanosome-infected rams.**

Testicular hormones regulate the function of the pituitary gland by a negative-feedback mechanism. Castration removes such inhibition and exaggerates pituitary gonadotrophin secretion providing an experimental model to investigate the effects of *T. congolense* infection on the hypothalamo-pituitary gonadotrophin secretion. These studies were conducted on half of the control and infected rams used in Study II (Table 3.1).

4.2. MATERIALS AND METHODS

4.2.1. Animals and Experimental Infection

The investigation of reproductive endocrine alteration during *T. congolense* infection was performed on the animals used during Study I and II as previously described in Chapter III 3.2.2. Animals were infected as described in Chapter II 2.2. and Chapter III 3.2.2. During Study II, 4 infected and 5 control rams were castrated on day 28 post-infection.

4.2.2. Bleeding Regimen

Routine blood sampling

Jugular blood samples were taken twice a week during Study I and II commencing one month prior to infection and three times a week up to day 79 post-infection during Study I and day 58 post-infection during Study II. Details of blood sampling techniques are given in Chapter II 2.5. Samples were used immediately for estimation of PCV and parasitaemia while plasma was stored at -20°C for subsequent hormonal studies.

Frequent blood sampling at various times during the infection

Frequent blood samples were collected using an indwelling cannula fitted in an external jugular vein as previously described by Jeffcoate (1992). Namely, the site to be cannulated was prepared by trimming the wool to a depth of about 2 mm above the skin, disinfected with Hibitane (ICI Pharmaceuticals, Macclesfield, Cheshire, U.K.) and left to dry in air. A polythene tube (i.d. 0.86 mm., o.d 1.27 mm, (Portex Ltd, Hythe, Kent, UK) was inserted into the external jugular vein to a depth of approximately 6 cm via a 14G hypodermic needle (Monoject, St. Louis, MO, USA). The free end of the cannula was passed through the centre of a 10 cm square of Elastoplast (Smith and Nephew Ltd., Hull, UK) whose reverse side was covered with contact adhesive. Another 10 cm square of Elastoplast was stuck over the first, thus sandwiching and securing the cannula. About 30 cm of free cannula was passed through the wool up to the top of the neck and fitted with a blunt 20G needle which was stoppered with the tip of 1 ml syringe. The cannula was kept patent with heparinized saline (200 I.U. ml^{-1}).

In Study I samples were collected intensively on day 22 and 62 post-infection; in study II on day 5 prior to infection and again on days 23 and 52 post-infection or on day 2 prior to infection and days 26 and 54 post-infection in rams which were castrated on day 28 post-infection. The scrotal-insulated rams were sampled intensively 51 days after insulation. The blood sampling protocol was to collect blood at 20 min intervals for 1 h before and 3 h after gonadotrophin-releasing hormone (GnRH) injection in rams used for study I or at 15 min intervals for 6 h before and 10, 20, 40, 60, 80, 100 and 120 min after GnRH injection in study II. Each ram was injected via the cannula with 20μg synthetic GnRH (L-7134, Sigma, Dorset, U.K.) dissolved in sterile saline. The blood was promptly centrifuged and frozen at -20°C until needed for hormonal measurements.

4.2.3. Castration

Castration was performed on 5 control and 4 infected rams during Study II on day 28 post-infection. Animals to be castrated were fasted overnight and weighed before castration. Castration was performed under general anaesthetic which was administered via an 18 gauge teflon trocart catheter inserted into the right cephalic vein and taped to the leg. Anaesthesia was induced by an intravenous injection of propofol (Rapinovet$^{(R)}$,

Coopers) at the dose rate of 4 mg/ml. Endotracheal intubation was performed with the aid of a laryngoscope with the animal in dorsal recumbency.

Following anaesthesia, the sheep was placed in dorsal recumbency on a cradle and the scrotum was shaved and disinfected. One testicle was squeezed as far distally into the scrotum as possible and a 10 cm skin incision was made on the anterior surface of the scrotum. The incision was continued distally, using scissors, such that there would be free drainage from the scrotal sac after castration. The tissues layers were blunt-dissected until the testicle with its tunica albuginea could be exteriorised. Once this was achieved, the testicle and the spermatic cord were pulled out of the scrotum as far as possible and two ligatures of catgut placed round the spermatic cord and held with artery forceps. The emasculator was placed round the cord with the bolt pointing distally, the jaws were closed and left for about 60 seconds. The cord was cut with the scalpel blade distal to the emasculator and the instrument was then released. A check was made that there was no haemorrhage from the cord before trimming the suture material and releasing the cord which then retracted towards the inguinal ring. The second testicle was removed in similar fashion through a separate incision. The scrotal wounds were not sutured and care was taken to ensure that the incision had indeed been continued through the distal part of the scrotum to allow free drainage of the blood. If this had not been achieved, the incision was extended.

Anaesthesia was maintained throughout the surgical procedure by the administration of incremental doses of propofol to effect. Following recovery of the swallowing reflex, the endotracheal tube was removed and the animal's head was left overhanging the cradle to permit the flow of saliva from the pharynx. Analgesia was provided by the intramuscular injection of buprenorphine (Temgesic$^{(R)}$, Reckitt and Colman, Hull, U.K.) 10 $\mu g\ kg^{-1}$, administered at the time of extubation. All castrated animals were allowed to recover from the anaesthesia in sternal recumbency and were separated from the uncastrated group for one week to allow the scrotal wound to heal. Scrotal wounds were examined three times a week. Recovery was uneventful and was complete within two weeks.

4.2.4. Hormone Measurements

LH radioimmunoassay

Plasma LH was determined by a second antibody RIA using the method recently described by Jeffcoate (1992). The primary antibody (R151) was raised in a rabbit against ovine LH (NIH-oLH-S25, National Hormone and Pituitary Program, NIADDK, Bethesda, MD, USA) and was used at 1:40,000 dilution to give binding of approximately 30%. Its crossreactivity has recently been described (Jeffcoate, 1992). Serial dilutions of LH standards (NIH-oLH-S25) were used at concentrations ranging from 0 to 100 ng ml^{-1}. oLH (LER-1056-C2) for iodination was donated by L.E. Reichert, Albany Medical College, NY, USA and ^{125}I-labelling was performed using the chloramine-T method. The tracer was used at 20 - 30,000 cpm per 100 µl. The second antibody reagents donkey anti-rabbit serum (DAR) and normal rabbit carrier serum (NRS) were obtained from the Scottish Antibody Production Unit (SAPU) (Carluke, Lanarkshire, Scotland) and were used at 1:15 and 1:300 dilution, respectively.

In the assay, 100 µl of the sample/standard were aliquoted into 3 ml polystyrene tubes (LP3) (Denley, Luckham Division, West Sussex, U.K) followed by addition of 200 µl of the primary antibody diluted in assay buffer (0.05M phosphate buffered saline , pH 7.5, Appendix 4). The tubes were incubated overnight at 4°C followed by addition of 100 µl of the ^{125}I-LH label. Tubes were further incubated overnight at 4°C and then 400 µl of freshly prepared DAR-NRS reagent was added. Further incubation continued for 8-h or overnight at 4°C. Separation of antibody-bound from the unbound fraction was performed by centrifugation at 1,500 g for 25 min at 4°C using a MSE Mistral 6L Centrifuge followed by aspiration of the supernatant using a finely drawn metal pipette connected on an aspirator pump attached on running water tap. The bound residue was counted on a Tri Carb gamma counter model 3255 (Packard) and results were computed using the S.A.S. immunoassay computer programme (P.R. Edwards, Molecular Endocrinology UCMSM, Winter 91).

The intra-assay CVs were 4.92% (n = 15) at 2.99 ng ml^{-1} and 3.86% (n = 15) at 10.71 ng ml^{-1}. The inter-assay CVs were 11.4% (n = 20) at 2.92 ng ml^{-1} and 7.4% (n = 20) at 10.22 ng ml^{-1}. The assay limit of detection at 2 x SD of the zero standard was 0.12 ng ml^{-1}.

Testosterone radioimmunoassay

Plasma testosterone concentration was determined by a double antibody ether extraction radioimmunoassay (RIA) as previously described by Cook and Beastall (1987). The ^{125}I-histamine-testosterone prepared by chloramine-T method (Cook and Beastall., 1987) was kindly provided by Dr. C. E. Gray of the Department of Pathological Biochemistry, Royal Infirmary, Glasgow, U.K. The testosterone standards were purchased from Steraloids (Croydon, U.K) and rabbit-anti-testosterone (AB-1030) from Bioclinical Services (Cardiff, U.K). According to the suppliers, the testosterone antibody was raised against a testosterone-3-(O-carboxy methyl)oxime-bovine serum albumin conjugate and shows the following cross-reactivity: testosterone, 100%; 5α-dihydrotestosterone, 16%; 5α-Androstane-3α 17-diol, 5.8%; 5α-Androstane-3ß, 17ß-diol, 3.7%; androstenedione, 2.1%; dihydroepiandrosterone, 0.04% and cortisol <0.01%. Testosterone standards ranging from 0 - 34.8 nmol l^{-1} were prepared by serial dilutions in donkey serum (S077-220) which was provided by SAPU. This donkey serum was obtained from a pool of blood obtained from a gelding and two female donkeys and contained non-detectable levels of testosterone in RIA. Donkey anti-rabbit (DAR) and normal rabbit carrier serum (NRS) were provided by SAPU

One hundred microlitre of sample or standard in duplicate was pipetted into 13 x 100 mm clean borosilicate glass tubes (Ciba-Corning, Essex, U.K.), 3 ml di-ethylether (analar grade, BDH, Poole, U.K.) was added and tubes were vortexed for 5 min in a Baird and Tatlock Multivortex Shaker (Searle Instrument, Harlow England). A methanol-dry ice bath was used to freeze the aqueous layer and the ether phase containing testosterone was carefully decanted into clean 10.5 x 70 mm borosilicate assay tubes. Ether was evaporated in a fume cupboard using a Tecam SC-3 sample concentrator (Techne, Cambridge, UK) connected to a vacuum pump.

The dried extraction residues were redissolved in 300 µl assay buffer (0.05M phosphate buffered saline, 0.25% w/v bovine serum albumin, pH 7.4, see Appendix 1), vortexed and 100 µl of the primary anti-testosterone antibody (1:5,000) and 100 µl of the tracer (containing approx. 10, 000 cpm) were added. The tubes were vortexed again and incubated at room temperature for 2 h. Thereafter, 500 µl of the double antibody reagent containing DAR and NRS (1:40 and 1:500 dilutions respectively) was added and tubes

were incubated overnight at 4°C. The antibody-bound fraction was separated from unbound by centrifugation at 1,500 g for 25 min at 4°C using a MSE Mistral 6L Centrifuge (Fisons Instruments, Sussex, U.K.) and the supernatant was aspirated using a finely drawn metal pipette connected to an aspirator pump attached to a running water tap. Counting and calculation of the results were performed in similar manner as for the LH RIA.

Using this technique, ether extraction efficiency for testosterone has been found to be over 96% by vortexing for 2 - 4 min (Cook and Beastall, 1987). To verify the efficiency of the extraction procedure employed in this assay 100 µl aliquots of donkey serum spiked with a known amount of standard testosterone (1.45, 12.5 and 32.6 nmol l^{-1}) were extracted using 3 and 10 ml di-ethylether for 4 and 10 min and run in a normal RIA (assay standards were extracted in the similar manner). The results obtained are shown in the Appendix 3. Extraction using 3 ml of ether and vortexing for 4 min produced similar testosterone recoveries (range 96.3 - 101.4%) as those obtained by using 10 ml of ether and extraction for 4 (range 95.2 - 102.9%) or 10 min (range 97.8 - 104.1%). In subsequent assays the volume of 3 ml ether and an extraction of 5 min was used and no recoveries were monitored.

The intra-assay coefficient of variation (CV) was determined by assaying 2 plasma samples containing different testosterone concentrations replicated 16 times in one assay and the CVs were 7.8% at 3.32 nmol l^{-1} and 5.9% at 12.36 nmol l^{-1}. Similarly, three quality control plasma pools containing low, medium and high testosterone concentrations were incorporated in each assay to monitor interassay variability. The inter-assay CV for 23 assays was 10.3% at 3.04 nmol l^{-1}, 6.7% at 14.95 nmol l^{-1} and 2.3% at 23.73 nmol l^{-1}. The assay limit of detection at 2 x standard deviation (SD) of zero standard was 0.13 nmol l^{-1}.

4.2.5. Statistical Analysis

The hormonal data in this chapter where applicable, are presented as mean ± SEM and differences between group data was regarded as significant at $P<0.05$. Changes in plasma LH and testosterone in 3 times weekly blood samples from Study I were grouped together on a weekly basis and analysed using a two-way analysis of

variance (animal groups and duration (time) of infection being the main two factors) followed by a *post hoc* analysis of group mean differences using the Newman-Keuls Multiple Range Test (ANIMAL DESIGNS 1, V 1.21 5/6, Data International Service, Glasgow). In samples collected frequently during Study II the pulsatility of hormonal profiles was analysed using the criteria of Canny *et al.*, (1990) that a pulse was present when (i) a value exceeded the previous value by at least three standard deviations of the estimate of the previous value calculated from duplicate sample results and (ii) when the peak value was followed by either a decline or no significant increment. The pulse amplitude was calculated by subtracting the concentration at the onset of the pulse from the peak concentration (Naylor *et al.*, 1990). Mean hormone concentration was calculated by averaging the concentrations and the pulse frequency by counting the number of peaks in the 6 h sampling period. (Naylor *et al.*, 1990). In addition, the area under the curve for plasma LH and testosterone concentrations over the 6 h sampling period was calculated using the Trapezoid rule (Schreiber *et al.*, 1988) and the integrated squared second derivative for log-transformed LH and testosterone concentrations in each sampling sequence was calculated using the same procedure as Jeffcoate, (1992) using MICROSOFT EXCEL 3.0 Computer Programme (Microsoft Corporation, U.S.A), This analysis provides a variability index between sequential samples and permits the fluctuation in hormone concentrations within a sampling window to be quantified and compared. The hormonal data obtained after GnRH injection were analysed using the analysis of variance with repeated measures designs (ANIMAL DESIGNS 1, V 1.21 5/6 (Data International Service, Glasgow) and by computing the area under the response curves using the Trapezoid rule. The effect of infection on several hormonal parameters during Study II was assessed statistically by analysis of variance followed by Newman-Keuls Multiple Range Test.

4.3. RESULTS

The results in this chapter are divided into 3 main sections. Section I describes results from Study I, detailing the effect of *T. congolense* on plasma LH and testosterone concentrations in rams in blood samples collected three times a week in 9 control and 10 infected rams and the results of injection of gonadotrophin-releasing hormone on

pituitary-testicular response in control and infected rams (Objective i and ii). Section II describes (a) the effect of *T. congolense* on the pulsatile secretion of LH and testosterone in 5 control and 5 infected rams during Study II (Objective iii) and the effects of GnRH stimulation on these hormones (Objective ii) and (b) comparative changes in plasma LH and testosterone concentrations induced by scrotal-insulation (Objective iv). Section III describes the changes in pulsatile secretion of LH and testosterone before infection, during infection and after castration of 5 control and 4 infected rams (Objective v) and also their response to GnRH.

4.3.1. SECTION I

LONG-TERM CHANGES IN PLASMA LUTEINIZING HORMONE AND TESTOSTERONE DURING *TRYPANOSOMA CONGOLENSE* INFECTION AND THE RESPONSE TO GONADOTROPHIN-RELEASING HORMONE INJECTION

4.3.1.1. Changes in Plasma LH and Testosterone Concentrations in Uninfected Control and *T. congolense*-infected Rams

Plasma LH and testosterone concentrations in blood samples collected twice weekly for 30 days prior to infection and three times per week up to day 79 post-infection in 10 infected and 9 control rams used during Study I are depicted in Figures 4.1 and 4.2. Fig. 4.1 depicts the results of rams killed on day 28 (see Chapter III 3.2.3) and Fig. 4.2 depicts the results of rams which survived up to day 79 post-infection and two infected rams (Nos 217 and 216) which died on day 70 and 77 respectively from secondary *P. haemolytica* infection.

Plasma LH concentrations fluctuated markedly between 0.2 to 3 ng ml^{-1} in all rams before infection and in control rams throughout the infection period. Following infection, there was no clear uniform pattern of change in plasma LH levels in infected rams because in some animals LH concentration tended to increase (No 210 and 212) but in others it decreased (rams 220, 216) and in others no change was obvious.

Plasma testosterone concentrations also fluctuated before infection in all rams with values varying between 0.1 to 25 nmol l^{-1}. A similar fluctuating pattern was

Fig. 4.1. Changes in plasma LH and testosterone concentrations in *T. congolense*-infected (n = 5) and uninfected control rams (n = 4). Blood samples were collected two times a week for 30 days before infection and three times a week during the infection period. All animals were killed on day 28 post-infection.

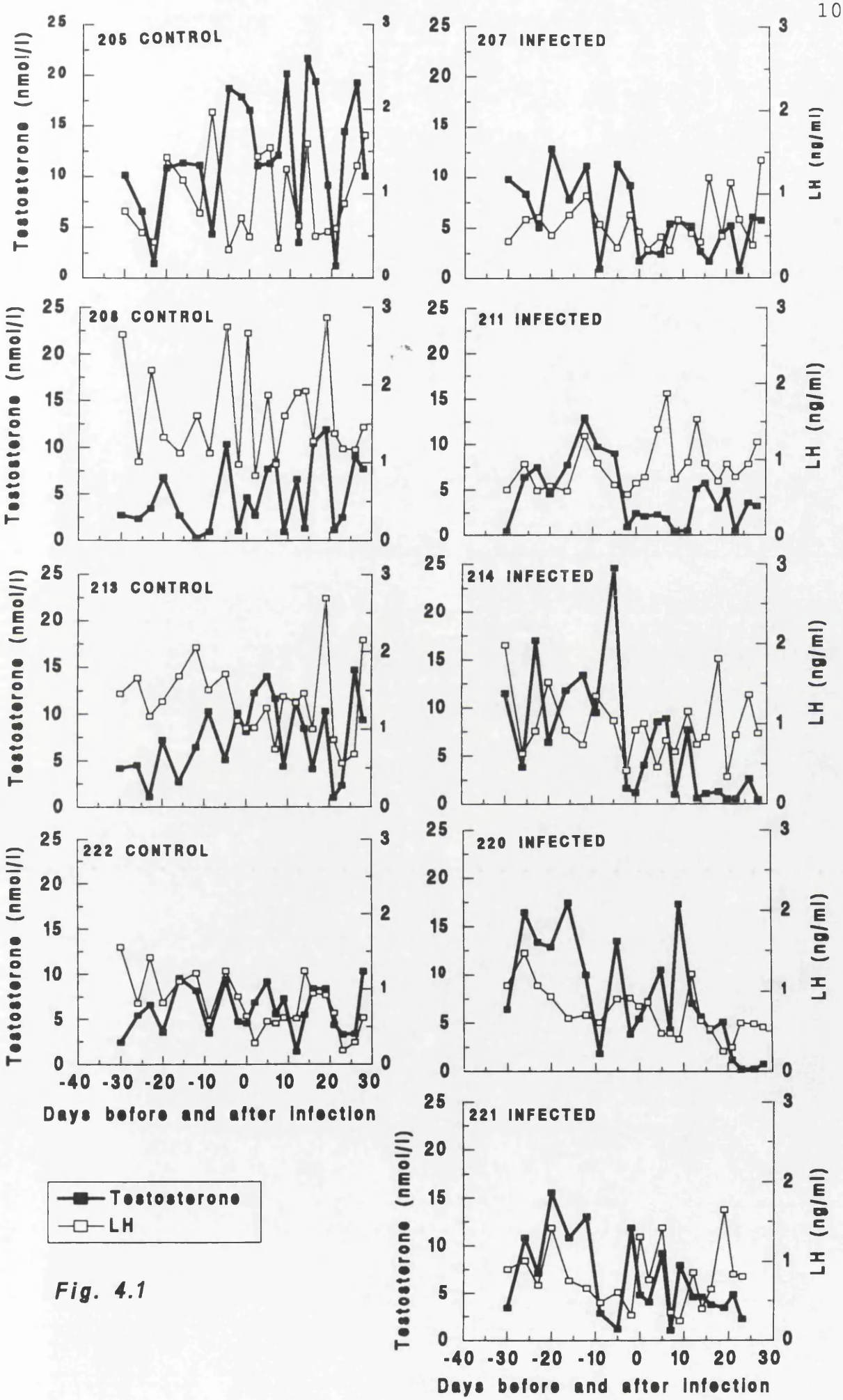

205 CONTROL
207 INFECTED
208 CONTROL
211 INFECTED
213 CONTROL
214 INFECTED
222 CONTROL
220 INFECTED
221 INFECTED
Testosterone (nmol/l)
LH (ng/ml)
-40 -30 -20 -10 0 10 20 30
Days before and after infection
Testosterone
LH

Fig. 4.1

Fig. 4.2. Changes in plasma LH and testosterone concentrations in *T. congolense*-infected (n = 5 infected) and control (uninfected) rams (n = 5 control). Blood samples were collected two times a week for 30 days before infection and three times a week during the infection period. One infected ram died on day 70 (No 217) and another on day 77 (No 216). All remaining infected animals (n = 3) and controls (n = 5) were killed on day 79 post-infection.

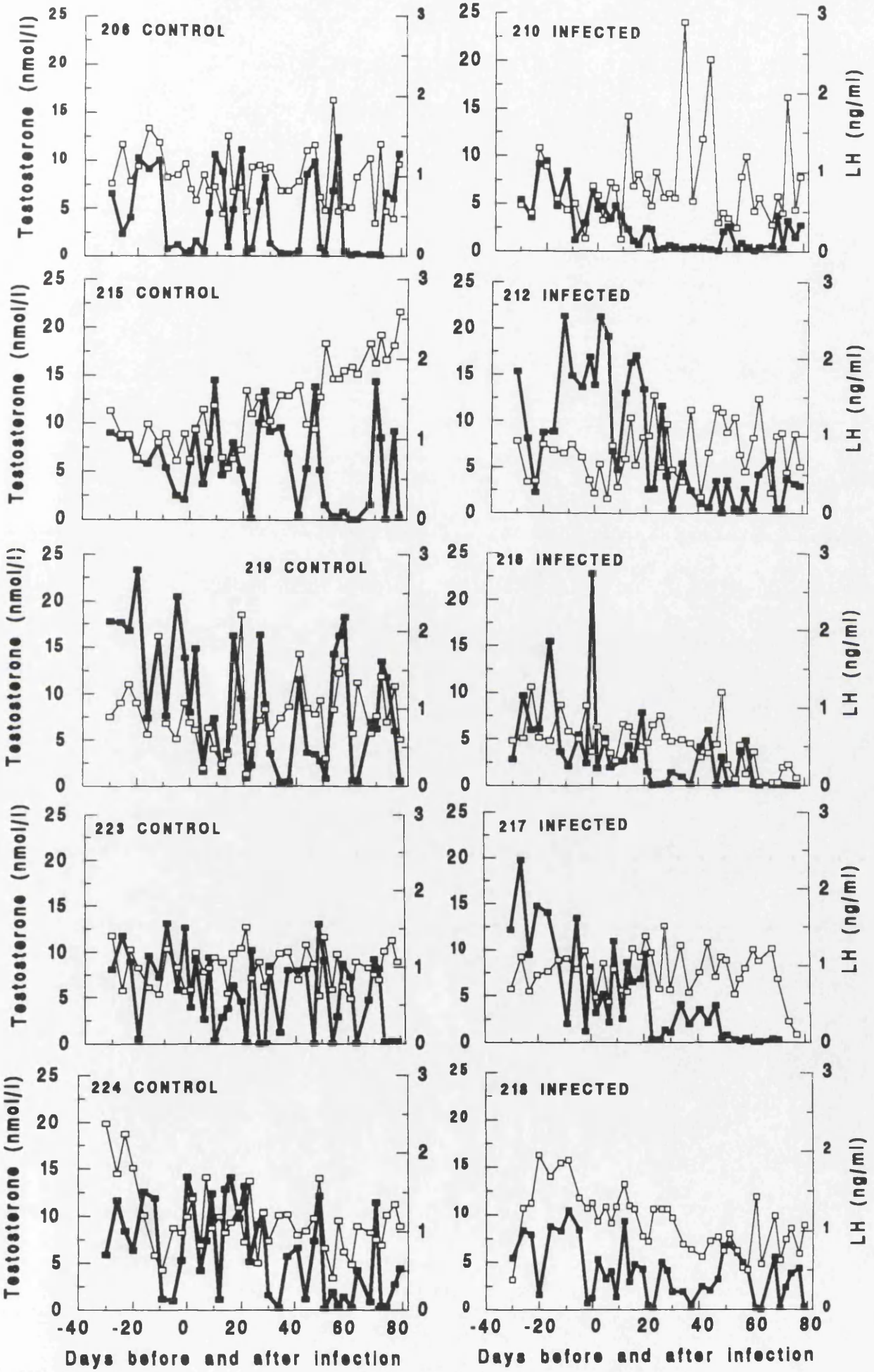

206 CONTROL
210 INFECTED
215 CONTROL
212 INFECTED
219 CONTROL
216 INFECTED
223 CONTROL
217 INFECTED
224 CONTROL
218 INFECTED
Testosterone (nmol/l)
LH (ng/ml)
Days before and after infection

Fig. 4.2

maintained in control rams throughout the infection period but plasma testosterone concentrations tended to decline in infected rams within 1 to 2 weeks post-infection. Plasma testosterone concentrations remained at low levels in infected rams from week 2 up to the end of the experiment with the exception of No 218 (Fig. 4.2).

For statistical purposes, the hormone data in control and infected rams were pooled into a weekly mean values and these results are presented in Fig. 4.3a (LH) and Fig 4.3b (testosterone). No significant differences in mean weekly plasma LH levels were observed between the infected and control rams before infection and up to week 8 post-infection. From weeks 9 - 12 post-infection, a significant decline in mean LH levels ($P<0.01$) was evident in the infected rams when compared to the control values. The mean plasma testosterone concentration tended to be higher in the infected rams over the 4-week period prior to infection. However, mean weekly plasma testosterone levels started to decline within the first week after infection in the infected rams and from weeks 4 - 12 post-infection, mean testosterone levels in infected rams were significantly lower ($P<0.01$) than in control rams. No significant changes in mean weekly plasma testosterone concentration were observed in the control rams over the experimental period.

4.3.1.2. Effect of GnRH on Plasma LH and Testosterone Concentration in Uninfected Control and *T. congolense*-infected Rams

Plasma LH response

Plasma LH concentration increased after GnRH injection on day 22 post-infection in both infected and control rams as shown in Fig. 4.4a. Table 4.1 gives the mean area under the LH response curve up to 3-h after GnRH injection. On day 22 post-infection, the increase in mean plasma LH concentration in response to GnRH injection showed a significant treatment effect ($P<0.001$) between infected and control groups and a significant ($P<0.05$) group x time interaction. A rapid increase ($P<0.001$) in mean plasma LH was observed in both groups within 20 min of GnRH injection and peak levels were observed at 40 min in the control group and 60 min in the infected group. Between 60 - 90 min post-GnRH injection, the mean LH levels in the infected rams were significantly higher ($P<0.05$) than corresponding control values. Thereafter, a rapid

Fig. 4.3. Mean (± s.e.m.) weekly plasma LH (Fig 4.3a) and testosterone (Fig. 4.3b) concentrations (from data shown in Fig 4.1 and Fig. 4.2.) in *T. congolense*-infected animals (n = 10, arrow 1 to 2 and n = 5, arrow 2 to 3) and uninfected control animals (n = 9, arrow 1 to 2 and n = 5, arrow 2 to 3).

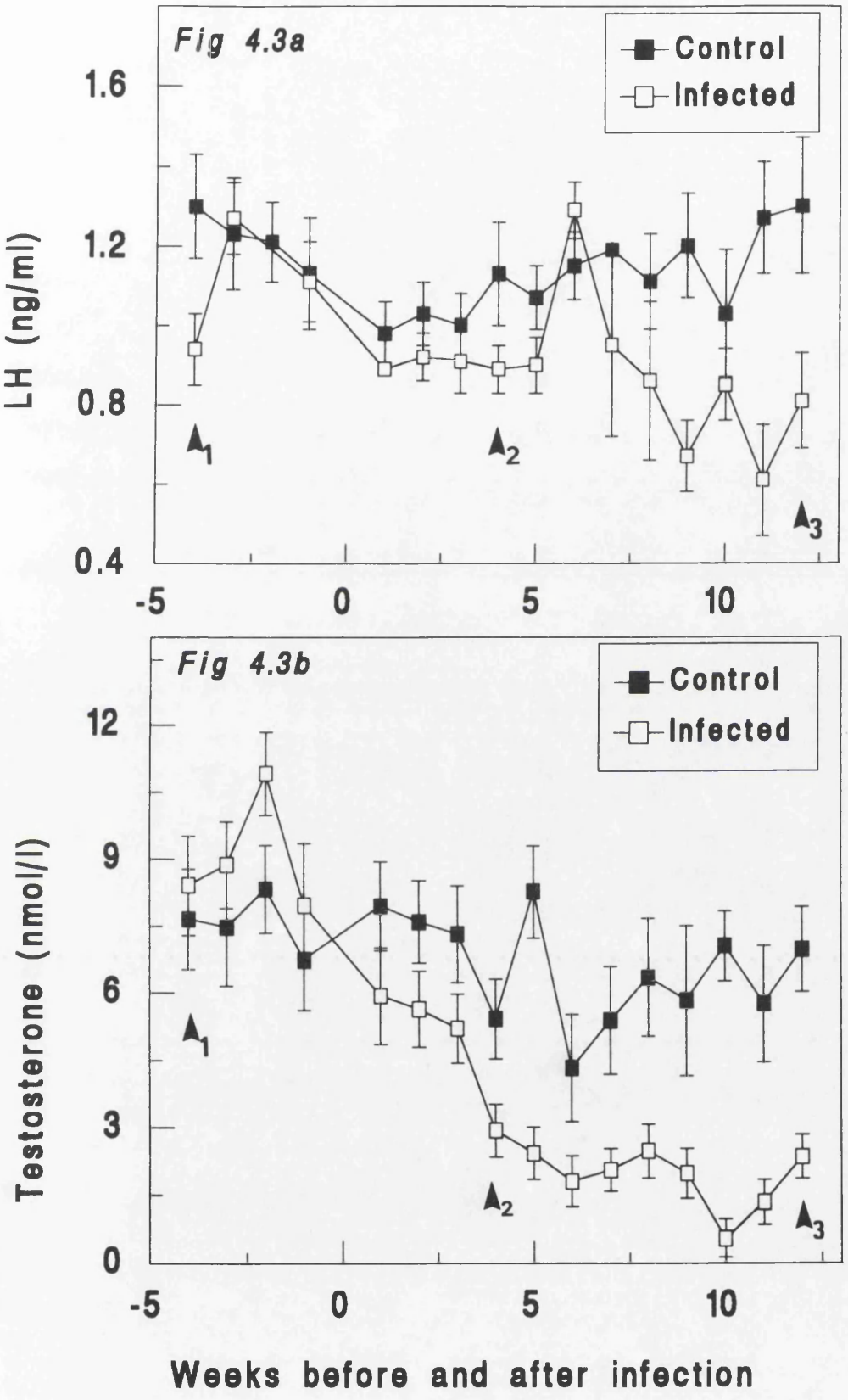


Fig 4.3a
Control
Infected
LH (ng/ml)
1.6
1.2
0.8
0.4
A1
A2
A3
-5
0
5
10
Fig 4.3b
Control
Infected
Testosterone (nmol/l)
12
9
6
3
0
A1
A2
A3
-5
0
5
10
Weeks before and after infection

decline in mean LH levels was observed in both groups and by 180 min post-GnRH injection, mean plasma LH levels were almost similar in the control and infected groups. The area under the response curve was significantly higher ($P<0.05$) in the infected than in control rams on day 22 post-infection.

Mean plasma LH response to GnRH on day 62 post-infection in infected and control groups are shown in Fig. 4.4c. A rapid significant rise ($P<0.001$) in plasma LH levels was seen within 20 min which was of similar magnitude in both control and infected groups. Thereafter, the rate of increase and decline in mean LH concentration was similar in both groups. The area under the response curve (Table 4.1) was similar between the two groups.

Plasma testosterone response

Changes in mean plasma testosterone concentration before and after GnRH injection in infected and control rams on day 22 post-infection are shown in Fig. 4.4b and the mean areas under the testosterone response curve after GnRH injection are depicted in Table 4.1. A rapid increase ($P<0.001$) in plasma testosterone levels was observed in control and infected rams within 40 min after GnRH injection. A peak occurred within 80 - 100 min in infected rams and 120 - 140 min in control rams. Mean plasma testosterone levels remained elevated up to 180 min in both groups. The increase in plasma testosterone in response to GnRH injection tended to be greater in control rams but this was not significantly different from infected values and no group x time interaction was observed. No significant difference was observed in the area under the testosterone response curves between the control and infected groups.

Mean plasma testosterone responses to GnRH on day 62 post-infection are depicted in Fig 4.4d with respective mean area under the response curves shown in Table 4.1. Mean basal testosterone levels in the infected rams from 0 - 20 min prior to GnRH injection were significantly lower ($P<0.05$) than control values. Following GnRH injection testosterone levels increased within 20 - 40 min until 140 min after which time a decline was observed in both groups. Mean testosterone response to GnRH injection showed a significant treatment effect ($P<0.01$) between groups (response being higher ($P<0.01$) in control rams than in infected rams) The area under the response curve

(Table 4.1.) was also significantly higher ($P<0.05$) in the control than in infected rams on day 62 post-infection.

Table 4.1: Index of plasma LH and testosterone during a 3-h period (area under curve) after GnRH injection (20 μg per animal) on day 22 and 62 of infection to a group of uninfected rams (control) and a group of *T. congolense*-infected rams (infected).

	Day 22		Day 62	
	Control (n = 5)	Infected (n = 5)	Control (n = 5)	Infected (n = 5)
LH				
Area under curve (ng-3h ml^{-1})	13.94 ± 1.43	29.20 ± 4.32*	15.90 ± 2.08	16.97 ± 2.77
Testosterone				
Area under curve (nmol-3h l^{-1})	33.36 ± 4.07	24.78 ± 3.61	36.65 ± 4.59	18.39 ± 2.79*

* Denote significant difference at $P<0.05$ compared with respective controls
Values are means (± s.e.m.)

Fig. 4.4. Mean (± s.e.m.) plasma LH and testosterone concentrations during a 3-h period after GnRH injection (20 μg per animal) on day 22 (Fig. 4.4a for LH and Fig. 4.4b for testosterone) and day 62 (Fig. 4.4c for LH and Fig. 4.4d for testosterone) after infection with *T. congolense* (n = 5, infected) or at the same time in uninfected control rams (n = 5, control).

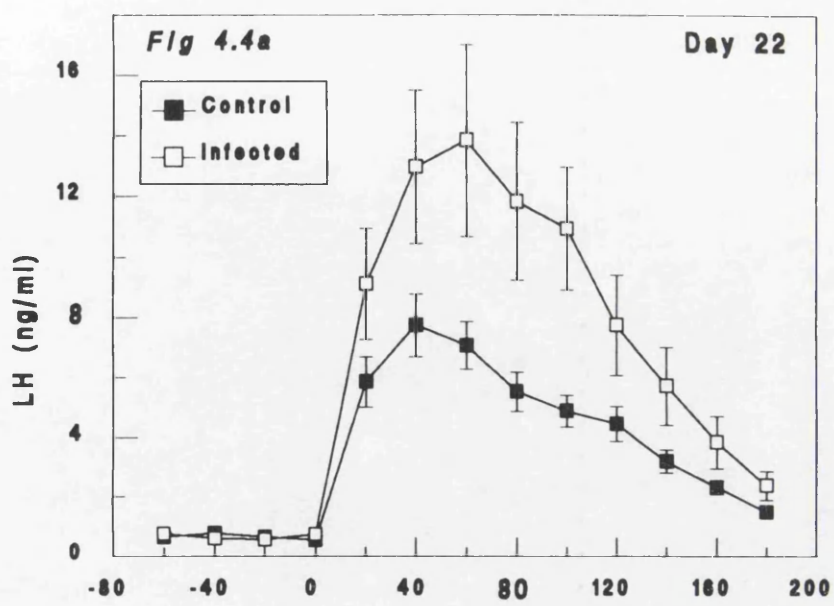


Fig 4.4a
Day 22
Control
Infected
LH (ng/ml)
0
4
8
12
16
-80
-40
0
40
80
120
160
200

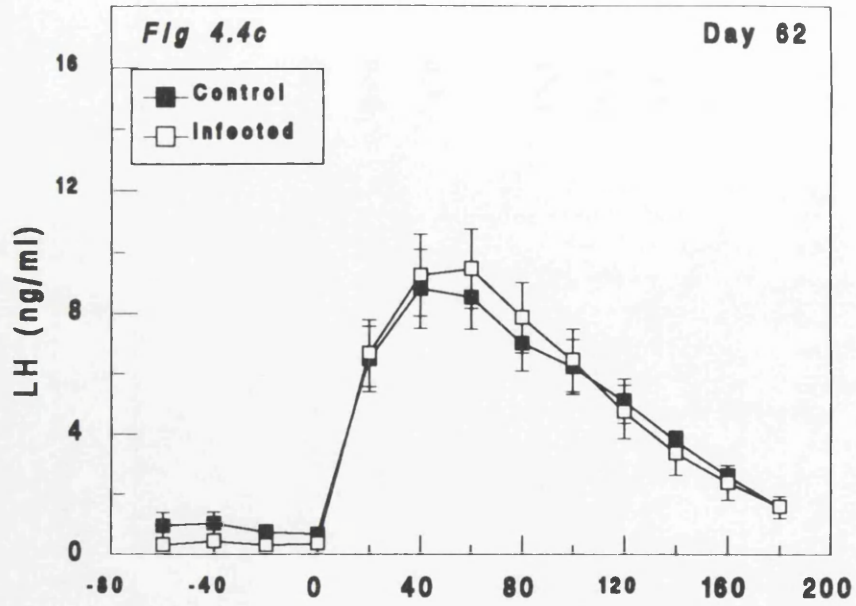


Fig 4.4c
Day 62
Control
Infected
LH (ng/ml)
0
4
8
12
16
-80
-40
0
40
80
120
160
200

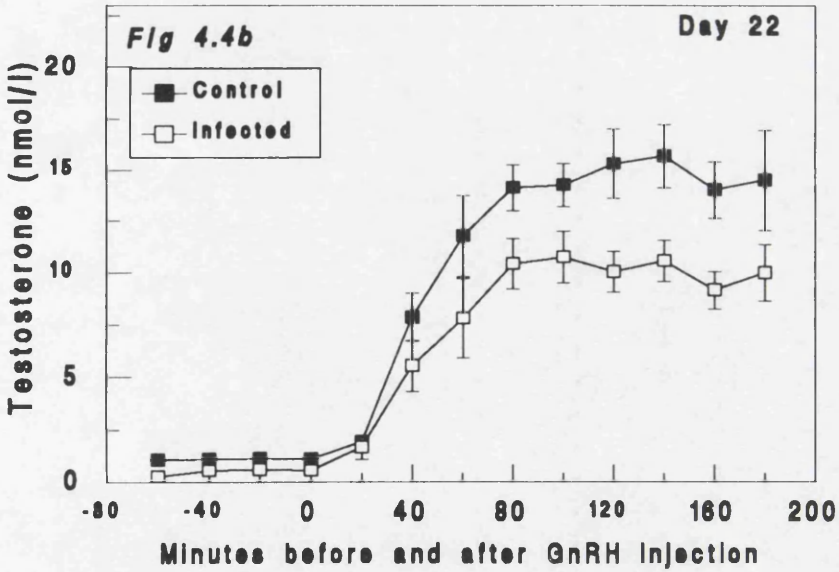


Fig 4.4b
Day 22
Control
Infected
Testosterone (nmol/l)
0
5
10
15
20
-80
-40
0
40
80
120
160
200
Minutes before and after GnRH injection

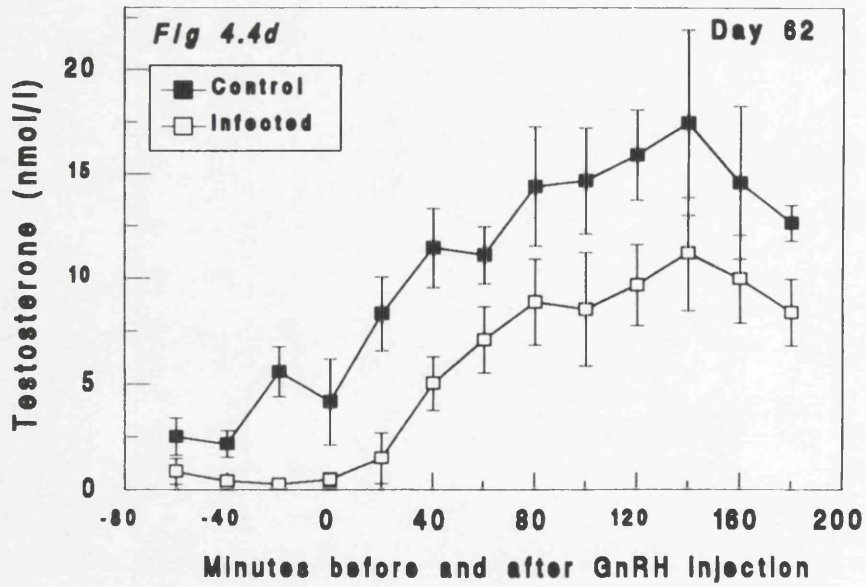


Fig 4.4d
Day 62
Control
Infected
Testosterone (nmol/l)
0
5
10
15
20
-80
-40
0
40
80
120
160
200
Minutes before and after GnRH injection

4.3.2. SECTION II

EFFECTS OF *TRYPANOSOMA CONGOLENSE* AND SCROTAL INSULATION ON THE PULSATILE SECRETION OF PLASMA LH AND TESTOSTERONE AND THE RESPONSE TO GONADOTROPHIN-RELEASING HORMONE INJECTION

4.3.2.1. Pulsatile Secretion of Plasma LH and Testosterone in Uninfected Control and *T. congolense*-infected Rams.

Pulsatile secretion of plasma LH

The LH concentration in blood samples collected every 15 min for 6 hours in control and trypanosome-infected rams on day 5 prior to infection (day -5) and on days 23 and 52 post-infection during Study II are shown in Fig. 4.5. (5 control rams) and Fig. 4.6 (5 infected rams). The results of the same animal on the 3 sampling occasions are presented in the same row. Table 4.2 summarises the changes in various parameters associated with pulsatile secretion of LH in the control and infected rams.

The concentration of LH in blood samples obtained every 15 min from control rams on all 3 sampling occasions exhibited the characteristic pulsatile secretion pattern during each 6-h sampling period (Fig 4.5). The mean LH pulse frequency, pulse amplitude and mean LH concentration in the control rams did not change significantly with time (Table 4.2). By averaging the LH data over the three sampling periods in the control rams, the calculated mean pulse amplitude, pulse frequency and overall mean plasma LH concentration were 2.28 ± 0.17 ng ml^{-1}, 4.73 ± 0.21 pulses per 6-h, and 2.00 ± 0.15 ng ml^{-1}, respectively. Similarly, there were no significant changes in either the mean area under the LH curve or LH variability indices within each sampling sequence in the controls (Table 4.2). The mean area under the LH curve for the three sampling sequences was 11.53 ± 0.83 ng-6h ml^{-1} and the corresponding LH variability index (log scale) was 5.18 ± 0.42.

None of the measured variables of the 6-h plasma LH profiles in infected rams on day 5 prior to *T. congolense* infection (Fig. 4.6), (i.e. mean pulse amplitude, pulse frequency, plasma LH concentration, area under the LH curve and LH variability indices (Table 4.2)) differed significantly from control values. On day 23 post-infection,

infected rams showed an increase in LH pulse amplitudes ($P<0.05$) when compared to respective day -5 values but were not significantly higher than control values on day 23 post-infection. No significant changes in mean pulse frequency and mean LH variability index where observed in infected rams when compared to the control values. However, the mean plasma LH concentration and area under the LH curve were significantly higher in the infected ($P<0.05$) than in control rams on day 23 post-infection.

On day 52 post-infection, infected rams had low basal plasma LH concentration and their mean LH concentration over 6-h sampling period was significantly lower ($P<0.05$) when compared to days -5 and 23 values; and when compared to mean LH values in control rams on the same day. The LH pulse amplitudes were too low to allow calculation of amplitudes or frequency of pulses by the criteria used on the LH results obtained on days -5 and 23. Instead, the area under the LH curve and LH variability indices were calculated in infected rams and results are shown in Table 4.2. The mean area under the LH curve and LH variability index were significantly lower ($P<0.05$) in the infected than in control rams on day 52 post-infection.

Pulsatile secretion of plasma testosterone.

The 6-h plasma testosterone profiles in samples obtained from control rams every 15 min on day -5 and on days 23 and 52 are depicted in Fig. 4.5. Only 1 or 2 plasma testosterone pulses were observed during the 6-h sampling period on each of these intensive blood sampling occasions. The onset of a plasma testosterone pulse was associated in all cases with an LH pulse amplitude of ≥ 2.5 ng ml^{-1} which occurred 20 - 40 min beforehand. The pulse amplitude and frequency for plasma testosterone could not be estimated accurately as on most occasions only one peak was recorded per animal and the majority of pulses tended to have uneven peaks. Instead, the change in plasma testosterone concentration over time was estimated using the area under the curve, mean concentration over the 6-h sampling period and by the estimation of testosterone variability indices per animal within each sampling period. Mean results are presented in Table 4.2. The mean testosterone concentration, area under the curve and testosterone variability indices (log scale) did not change significantly with time in the control rams. The calculated mean plasma testosterone concentration, area under the curve and

testosterone variability index over the three blood sampling occasions in the control rams were 7.23 ± 0.84 nmol l^{-1}, 40.83 ± 5.05 nmol-6h l^{-1} and 1.17 ± 0.2 (log scale), respectively.

Changes in pulsatile secretion of plasma testosterone in infected rams on day 5 prior to infection (Fig. 4.6.) were similar to the changes observed in control rams throughout the experimental period. The mean testosterone concentration (7.51 ± 1.40 nmol l^{-1}), area under the curve (42.94 ± 7.80 nmol-6h l^{-1}) and testosterone variability index (0.95 ± 0.37 log scale) (Table 4.2) did not differ significantly from values recorded in the control rams over the three bleeding occasions described in the previous paragraph. Following infection, a progressive significant decline in mean testosterone concentration and area under the curve was observed in infected rams on days 23 ($P<0.05$) and 52 ($P<0.001$) post-infection, respectively. Only one infected animal (Number 252, Fig 4.6) showed no significant changes in plasma testosterone concentration over the 3 sampling occasions. The testosterone variability index was significantly reduced ($P<0.05$) in infected rams on day 52 but not on day 23 when compared to control values.

4.3.2.2. Pulsatile Secretion of LH and Testosterone in Scrotal-insulated Rams.

Pulsatile secretion of LH

Pulsatile secretion of plasma LH in scrotal-insulated rams in blood samples collected intensively on day 51 post-insulation are shown in Fig. 4.7 and Table 4.2. The plasma LH profile was similar to that observed in control rams on days -5, and on days 23 and 52 post-infection. The mean LH pulse amplitude and pulse frequency were not significantly different from the control values on day 52. Similarly, the mean LH concentration, area under the LH curve and LH variability index did not differ significantly from the control values but were significantly higher ($P<0.05$) than values obtained in infected rams on day 52 post-infection.

Fig. 4.5. Pattern of plasma LH and testosterone concentrations in control rams (n = 5) in samples collected at 15 min intervals for 6-h on day 5 prior to infection (day -5) and on days 23 and 52 after infection. Results of the same animal on the 3 blood sampling occasions are presented in the same row.

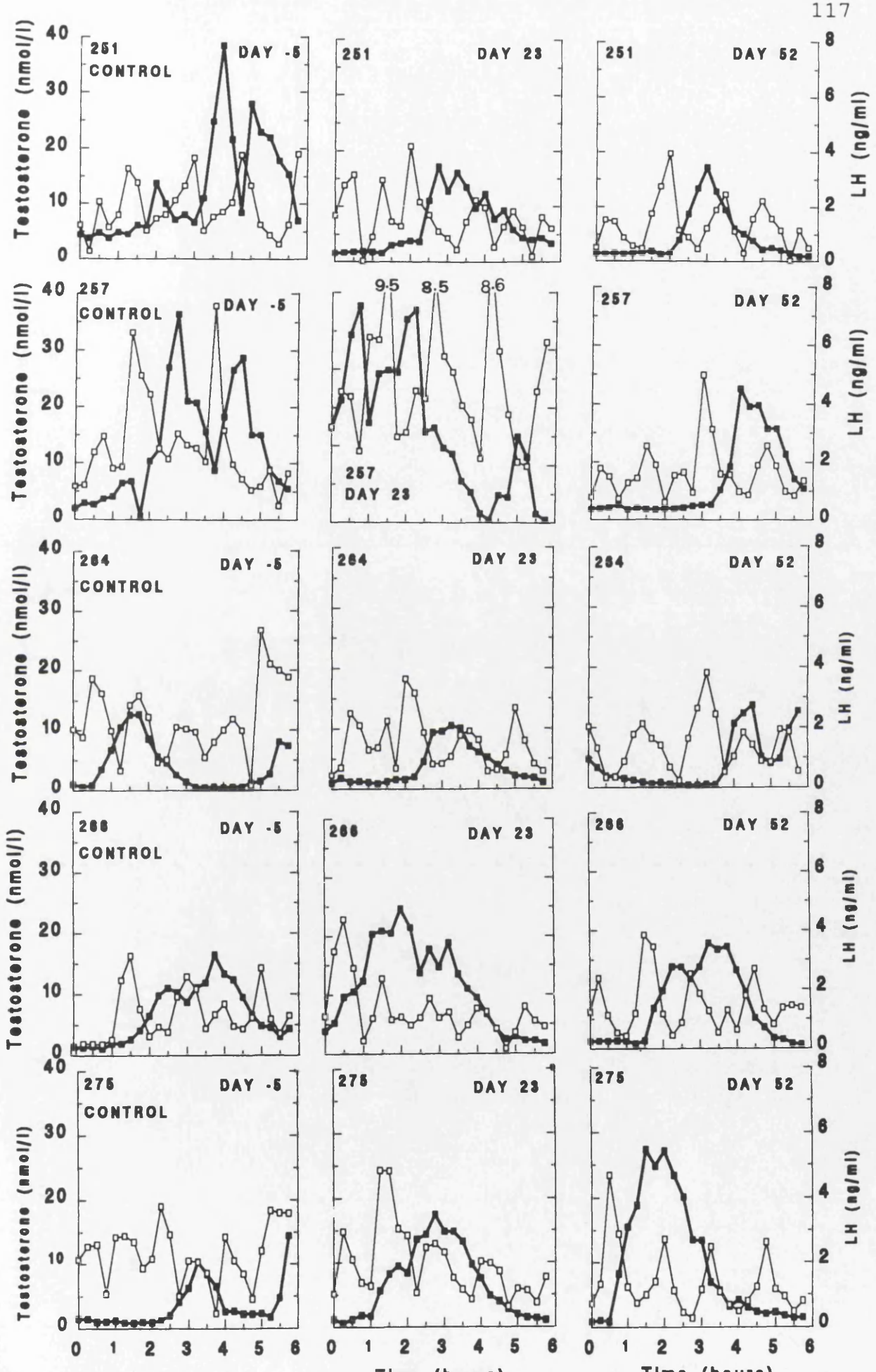

251 CONTROL DAY -5
251 DAY 23
251 DAY 52
257 CONTROL DAY -5
9.5 8.5 8.6
257 DAY 23
257 DAY 52
264 CONTROL DAY -5
264 DAY 23
264 DAY 52
266 CONTROL DAY -5
266 DAY 23
266 DAY 52
275 CONTROL DAY -5
275 DAY 23
275 DAY 52
Testosterone (nmol/l)
LH (ng/ml)
Time (hours)

Fig 4.5

Fig. 4.6. Pattern of plasma LH and testosterone concentrations in *T. congolense*-infected rams (n = 5, infected) in samples collected at 15 min intervals for 6-h on day 5 prior to infection (day -5) and on days 23 and 52 after infection. Results of the same animal on the 3 blood sampling occasions are presented in the same row.

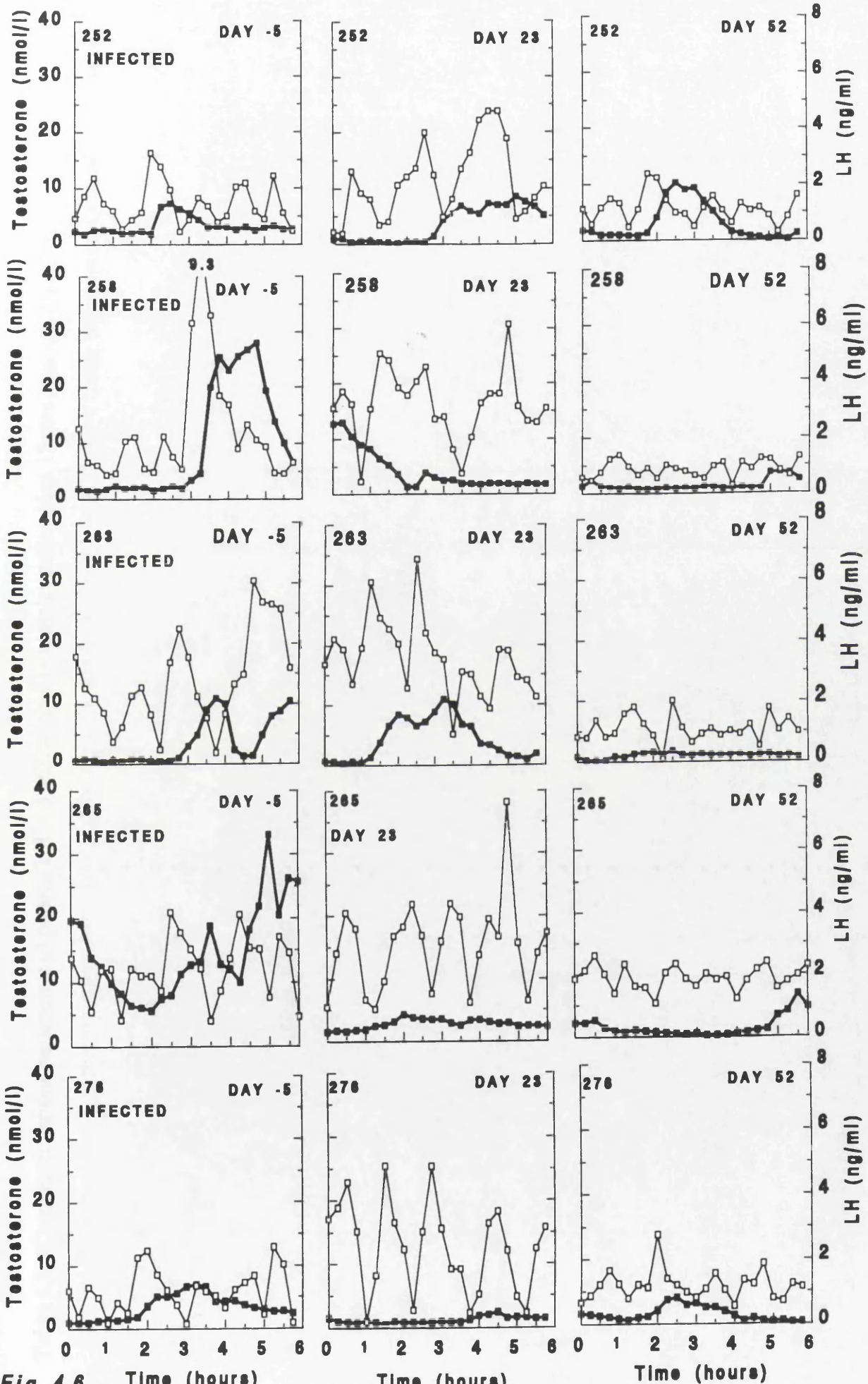

252 INFECTED
DAY -5
252
DAY 23
252
DAY 52
258 INFECTED
DAY -5
9.3
258
DAY 23
258
DAY 52
263 INFECTED
DAY -5
263
DAY 23
263
DAY 52
265 INFECTED
DAY -5
265
DAY 23
265
DAY 52
276 INFECTED
DAY -5
276
DAY 23
276
DAY 52
Testosterone (nmol/l)
LH (ng/ml)
Time (hours)

Fig 4.6

Table 4.2: Changes in various parameters of plasma LH and testosterone in samples collected at 15 min intervals for 6-h in uninfected (control) and *T. congolense*-infected (infected) rams on day 5 before infection (day -5) and days 23 and 52 after infection and in scrotal-insulated rams (scrotal-insulated) on day 51 post-insulation.

	Day -5		Day 23		Day 52		
	Control (n = 5)	Infected (n = 5)	Control (n = 5)	Infected (n = 5)	Control (n = 5)	Infected (n = 5)	[1]Scrotal-insulated (n = 6)
LH for 6h							
Pulse amplitudes	2.20 ± 0.27^{a}	2.27 ± 0.39^{ab}	2.53 ± 0.36^{ab}	3.41 ± 0.30^{b}	2.10 ± 0.23^{a}	NC	2.23 ± 0.16^{a}
Pulse frequencies	4.80 ± 0.37^{a}	4.60 ± 0.24^{a}	5.00 ± 0.37^{a}	4.20 ± 0.24^{a}	4.80 ± 0.20^{a}	NC	5.16 ± 0.32^{a}
Mean (ng ml^{-1})	2.10 ± 0.16^{a}	2.05 ± 0.21^{a}	2.17 ± 0.32^{a}	3.03 ± 0.17^{b}	1.82 ± 0.16^{a}	1.24 ± 0.17^{c}	1.72 ± 0.06^{a}
Area under response curve (ng-6h ml^{-1})	11.84 ± 0.84^{a}	11.64 ± 1.11^{a}	12.47 ± 1.85^{a}	17.43 ± 0.99^{b}	10.03 ± 0.22^{a}	6.59 ± 0.84^{c}	9.43 ± 0.30^{a}
Variability index	5.05 ± 0.71^{a}	5.54 ± 0.73^{a}	5.80 ± 0.77^{a}	4.61 ± 1.01^{a}	4.41 ± 0.09^{a}	2.84 ± 0.51^{b}	5.06 ± 0.27^{a}
Testosterone for 6h							
Mean (nmol l^{-1})	6.48 ± 1.05^{a}	7.51 ± 1.40^{a}	8.22 ± 1.83^{a}	3.55 ± 0.42^{b}	6.71 ± 0.56^{a}	1.73 ± 0.34^{c}	3.19 ± 0.66^{b}
Area under response curve (nmol-6h l^{-1})	37.40 ± 6.28^{a}	42.94 ± 7.80^{a}	49.29 ± 10.6^{a}	20.93 ± 2.2^{b}	30.78 ± 3.71^{a}	10.17 ± 2.20^{c}	18.9 ± 4.01^{cb}
Variability index	1.00 ± 0.24^{a}	0.95 ± 0.37^{a}	0.94 ± 0.08^{ab}	1.06 ± 0.22^{a}	1.22 ± 0.34^{a}	0.69 ± 0.10^{b}	1.22 ± 0.35^{a}

Means bearing different superscripts in control, infected and scrotal-insulated group in the same row differ significantly at $P<0.05$

[1]Blood sampling in scrotal-insulated rams done on day 51 post-insulation

NC = Not calculated due to absence of pulsatile secretion

Pulsatile secretion of plasma testosterone

Changes in plasma testosterone profiles in scrotal-insulated rams were intermediate between the control and infected rams (Fig 4.7). Only one ram (Number 272) did not show a testosterone pulse over the 6-h sampling window but the other 5 rams showed at least one pulse. The mean testosterone concentration and the area under the curve (Table 4.2) in these animals tended to be higher than values measured in infected rams but these values were still significantly lower ($P<0.05$) than control values.

4.3.2.3. Effect of GnRH on Plasma LH and Testosterone in Uninfected Control, *T. congolense*-infected and Scrotal-insulated Rams

Plasma LH and testosterone responses measured for 2 h after an intravenous injection of synthetic GnRH (20 μg per animal) on day -5 and on days 23 and 52 post-infection and day 51 post-insulation in 5 control, 5 infected and 6 scrotal-insulated rams during Study II are shown in Fig. 4.8a - 4.8f. Table 4.3 provides the mean area under the response curves for plasma LH and testosterone concentration on respective sampling occasions.

Plasma LH response

Five days before infection, plasma LH concentration increased significantly ($P<0.001$) in both groups within 10 min of GnRH injection and peak levels were observed within 10 - 40 min. Thereafter, a gradual decline was observed in all rams. Plasma LH response to GnRH injection did not differ significantly between infected and control rams and no group x time interaction was observed. Similarly the area under the LH response curve after GnRH injection was not significantly different between the control and infected animals (Table 4.3).

On day 23 post-infection a similar LH response to that on day -5 was seen in control and infected rams (Fig. 4.8c) was similar to the response observed on day -5. A significant increase ($P<0.001$) in plasma LH occurred within 10 min of GnRH injection in both groups. Peak plasma LH levels were observed within 10 - 40 min of injection in most animals. No significant differences in LH response was observed over time between the control and infected groups on day 23 post-infection.

Fig. 4.7. Pattern of plasma LH and testosterone concentrations in scrotal-insulated rams (n = 6) in samples collected at 15 min intervals for 6-h on day 51 post-insulation.

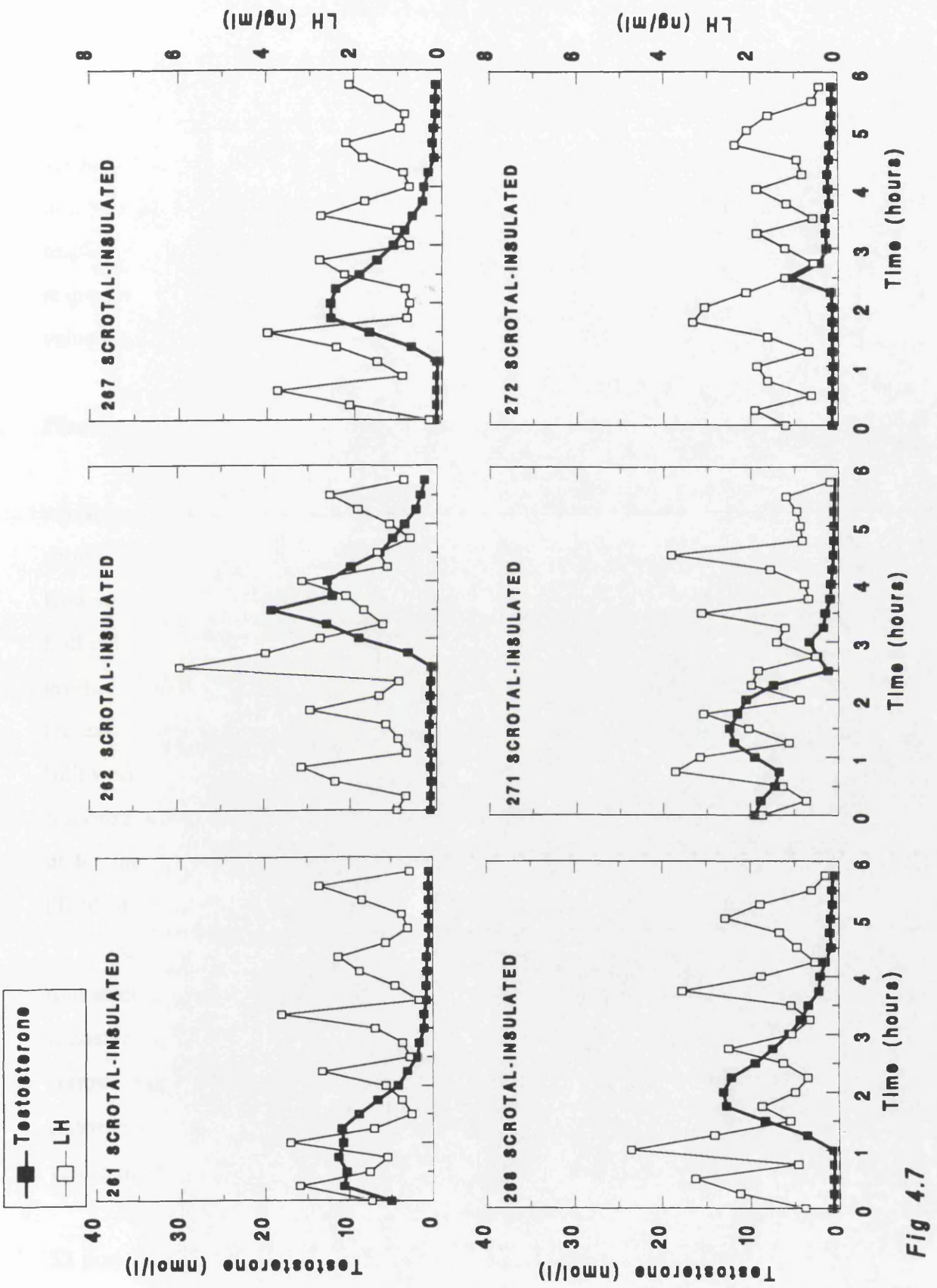

Testosterone
LH
261 SCROTAL-INSULATED
262 SCROTAL-INSULATED
267 SCROTAL-INSULATED
268 SCROTAL-INSULATED
271 SCROTAL-INSULATED
272 SCROTAL-INSULATED
Testosterone (nmol/l)
LH (ng/ml)
Time (hours)

Fig 4.7

Fig 4.8e shows the changes in mean plasma LH concentration in the control and infected rams after GnRH injection on day 52 post-infection. Superimposed with these results are the mean plasma LH response to GnRH in scrotal-insulated rams from samples collected on day 51 post-insulation. Plasma LH concentration increased rapidly in all three groups within 10 min of GnRH injection. No significant difference in LH response was observed over time between the three groups. However, the mean LH response in the infected rams tended to be low (nonsignificant) when compared with values measured in control rams on day 52 post-infection.

Plasma testosterone response

Five days prior to infection, experimental (infected group) rams had highly variable plasma testosterone concentration at the time of GnRH injection (time 0) as shown by a large standard error of the mean when compared to control rams (Fig 4.8b). However, the mean testosterone levels were not significantly different between groups. Following GnRH injection, a significant ($P<0.001$) increase in mean plasma testosterone levels was observed within 20 min (infected group) and 40 min (control group) and thereafter, plasma testosterone levels continued to increase gradually up to 80 - 100 min followed by a slight decline up to 120 min. No significant differences in testosterone response were observed over time between the two groups. Similarly, the mean area under the testosterone response curve was not significantly different between groups (Table 4.3).

On day 23 post-infection, plasma testosterone increased rapidly within 20 - 40 min after GnRH in both groups (Fig 4.8d) but from 60 - 120 min after GnRH injection testosterone response in the infected group was significantly lower ($P<0.05$) than in control rams. This accounted for a significantly lower ($P<0.05$) area under the testosterone response curve observed in the infected group when compared to the control values on day 23 post-infection (Table 4.3).

Changes in the plasma testosterone response for 2-h after GnRH injection on day 52 post-infection in the control and infected group and on day 51 post-insulation in the scrotal-insulated group are depicted in Fig 4.8f and Table 4.3. Plasma testosterone levels increased rapidly in all groups within 10 - 20 min after GnRH injection, reaching

Fig. 4.8. Mean (± s.e.m.) plasma LH and testosterone concentrations over a 2-h period in response to GnRH (20 μg per animal) administered on day 5 prior to infection (day -5, Fig. 4.8a for LH and Fig. 4.8b for testosterone); on day 23 post-infection (Fig. 4.8c for LH and Fig. 4.8d for testosterone) to uninfected control rams (n = 5, control) and *T. congolense*-infected rams (n = 5, infected); and on day 52 post-infection or 51 post-insulation in control (n = 5), infected (n = 5) and scrotal-insulated rams (n = 6) (Fig 4.8e for LH and Fig 4.8f for testosterone).

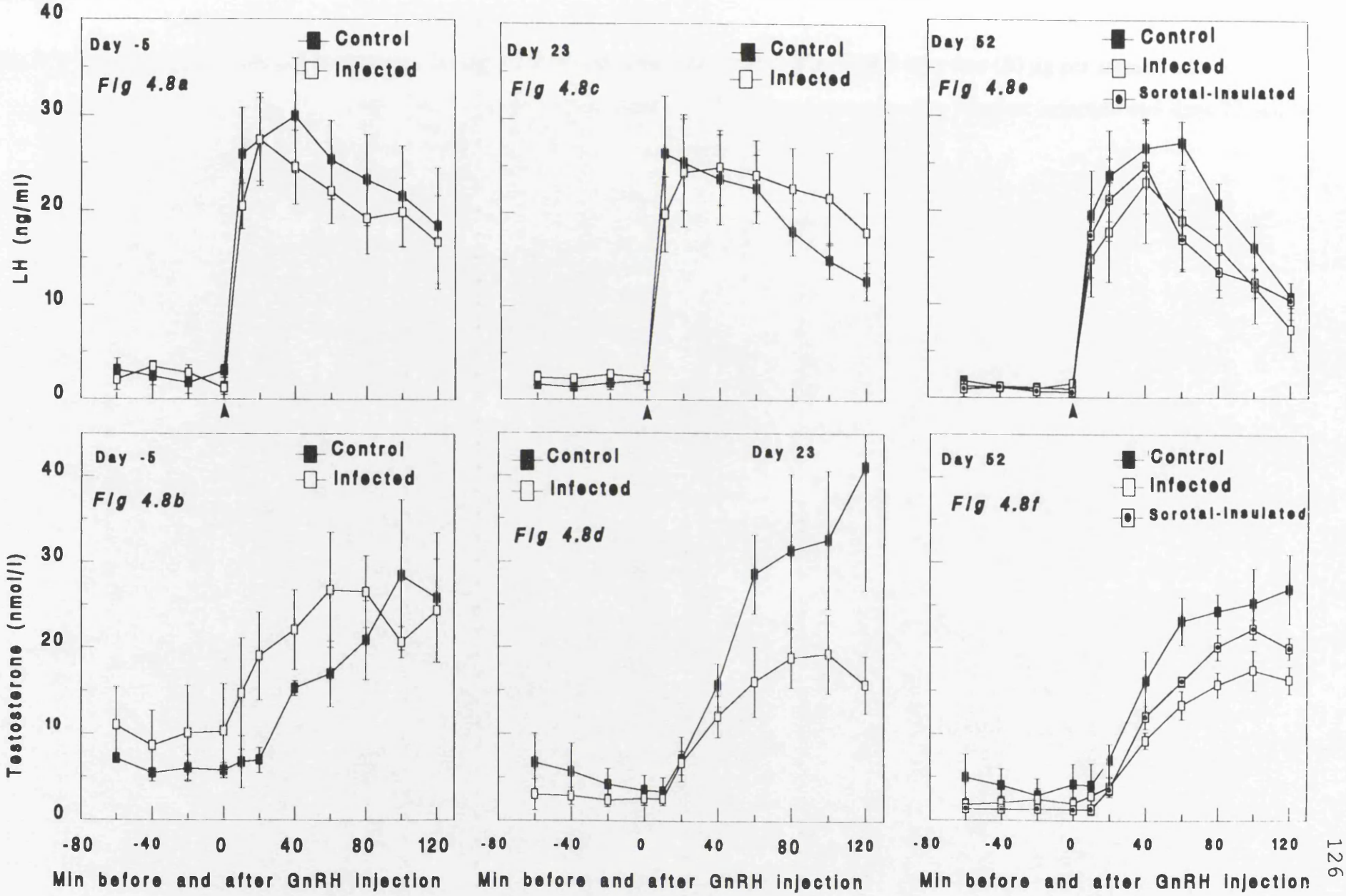

Day -5
Fig 4.8a
Control
Infected
Day 23
Fig 4.8c
Control
Infected
Day 52
Fig 4.8e
Control
Infected
Sorotal-Insulated
LH (ng/ml)
Day -5
Fig 4.8b
Control
Infected
Control
Infected
Day 23
Fig 4.8d
Day 52
Fig 4.8f
Control
Infected
Sorotal-Insulated
Testosterone (nmol/l)
0
10
20
30
40
-80
-40
0
40
80
120
Min before and after GnRH injection
Min before and after GnRH injection
Min before and after GnRH injection

Table 4.3: Index of plasma LH and testosterone during a 2-h period (area under curve) after GnRH injection (20 μg per animal) administered to a group of uninfected (n = 5 control) and *T. congolense*-infected (n = 5, infected) rams on day 5 before infection and days 22 and 54 post-infection) and to a group of scrotal-insulated (n = 6, scrotal-insulated) rams on day 51 post-insulation.

	Day -5		Day 22		Day 52		
	Control	Infected	Control	Infected	Control	Infected	[1]Scrotal-insulated
LH Area under curve (ng-2h ml^{-1})	44.87 ± 9.38	41.55 ± 6.1	39.50 ± 5.42	43.76 ± 7.51	41.08 ± 5.44[a]	31.41 ± 9.04[a]	32.38 ± 4.61[a]
Testosterone Area under curve (nmol-2h l^{-1})	36.76 ± 2.51	42.89 ± 7.44	44.65 ± 10.3	22.04 ± 1.52*	37.06 ± 4.44[a]	22.9 ± 1.16[b]	28.17 ± 0.92[b]

* = Denote significance at P<0.05 between control and infected groups on day 22 post-infection

[1]GnRH stimulation in scrotal-insulated rams performed on day 51 post-insulation

Means with different superscript on day 52 results are significant at (P<0.05)

Values are means (± s.e.m)

a peak within 40 - 60 min and followed by a gradual decline in all groups up to 120 min post-GnRH injection. Mean testosterone response over time was significantly lower ($P<0.05$) in infected and scrotal-insulated rams (infected vs scrotal-insulated - not significant) when compared to the control values. Similarly the mean area under the response curve (Table 4.3) was significantly higher ($P<0.05$) in the control when compared to the infected and scrotal-insulated group.

4.3.3. SECTION III

EFFECT OF CASTRATION ON THE PULSATILE SECRETION OF PLASMA LH AND THE RESPONSE TO GONADOTROPHIN-RELEASING HORMONE INJECTION IN RAMS INFECTED WITH *TRYPANOSOMA CONGOLENSE*

Five control and 5 infected rams used during Study II were assigned to study the effect of castration on the function of the pituitary gland following *T. congolense* infection. Unfortunately, one infected ram died on day 15 post-infection due to secondary *P. haemolytica* infection (Chapter III 3.3.6) reducing the infected group to 4 rams. The pre-infection hormonal data from this animal were excluded from the results. Castration was performed on day 28 post-infection. Intensive blood sampling and GnRH stimulation were performed 2 days prior to infection (day -2) and day 26 post-infection (2 days prior to castration) and day 54 post-infection (26 days after castration).

4.3.3.1. Pulsatile Secretion of Plasma LH and Testosterone before Castration

Pulsatile secretion of plasma LH

Plasma LH profiles in samples collected every 15 min for 6 h on day -2 and day 26 post-infection (2 days prior to castration) in 5 control and 4 infected rams used in this study are shown in Fig. 4.9 and Fig. 4.10, respectively. Pulsatile secretion of LH and testosterone was observed in both groups of rams, very similar to that already described in control and infected rams in those samples obtained 5 days before and 23 days after trypanosome infection (Section 4.3.2.1). Table 4.4 summarises the mean changes in various parameters associated with pulsatile secretion of LH in the same animals over the 3 blood sampling occasions.

Pulsatile secretion of Plasma testosterone

The concentration of testosterone in blood samples collected every 15 min for 6 h in the 5 control and 4 infected rams on day -2 and day 26 post-infection are depicted in Fig. 4.9 (control) and Fig. 4.10 (infected). Table 4.4 summarises the mean changes in various parameters associated with pulsatile secretion of testosterone in the same animals over the 3 blood sampling occasions. As for LH, these data are comparable to those described in control and infected rams in samples collected on day 5 before infection and on day 23 after infection (Section II 4.3.2.1).

4.3.3.2. Pulsatile Secretion of Plasma LH after Castration

The 6-h plasma LH profiles in 5 control and 4 infected rams in samples collected 26 days after castration are depicted in Fig 4.11 and mean data summarising changes in various parameters associated with pulsatile secretion of LH in the same animals is given in Table 4.4. Castration caused marked increase in plasma LH concentration following castration (note the 5 times increase in scale on the LH concentrations in Fig 4.11 compared to Fig 4.9 and 4.10). Many samples had LH concentration >10 ng ml^{-1} especially in the control rams. There were however, differences in the plasma LH profiles between the control and infected rams. Thus with the exception of one ram (Number 270) the other three infected rams showed LH pulses of low amplitude (<10 ng ml^{-1}) when compared to control castrated rams. The mean pulse amplitude, mean LH concentration over the 6-h window and area under the LH curve (Table 4.4) were all significantly lower ($P<0.01$) in the infected rams when compared to the control values (Table 4.4). No significant differences were however, observed in the mean pulse frequencies and LH variability index between the two groups.

Fig. 4.9. Pattern of plasma LH and testosterone concentrations in control rams (n = 5) in samples collected at 15 min intervals for 6-h on day 2 prior to (day -2) and 26 days after infection. Results of the same animal on the 2 blood sampling occasions are presented in the same row.

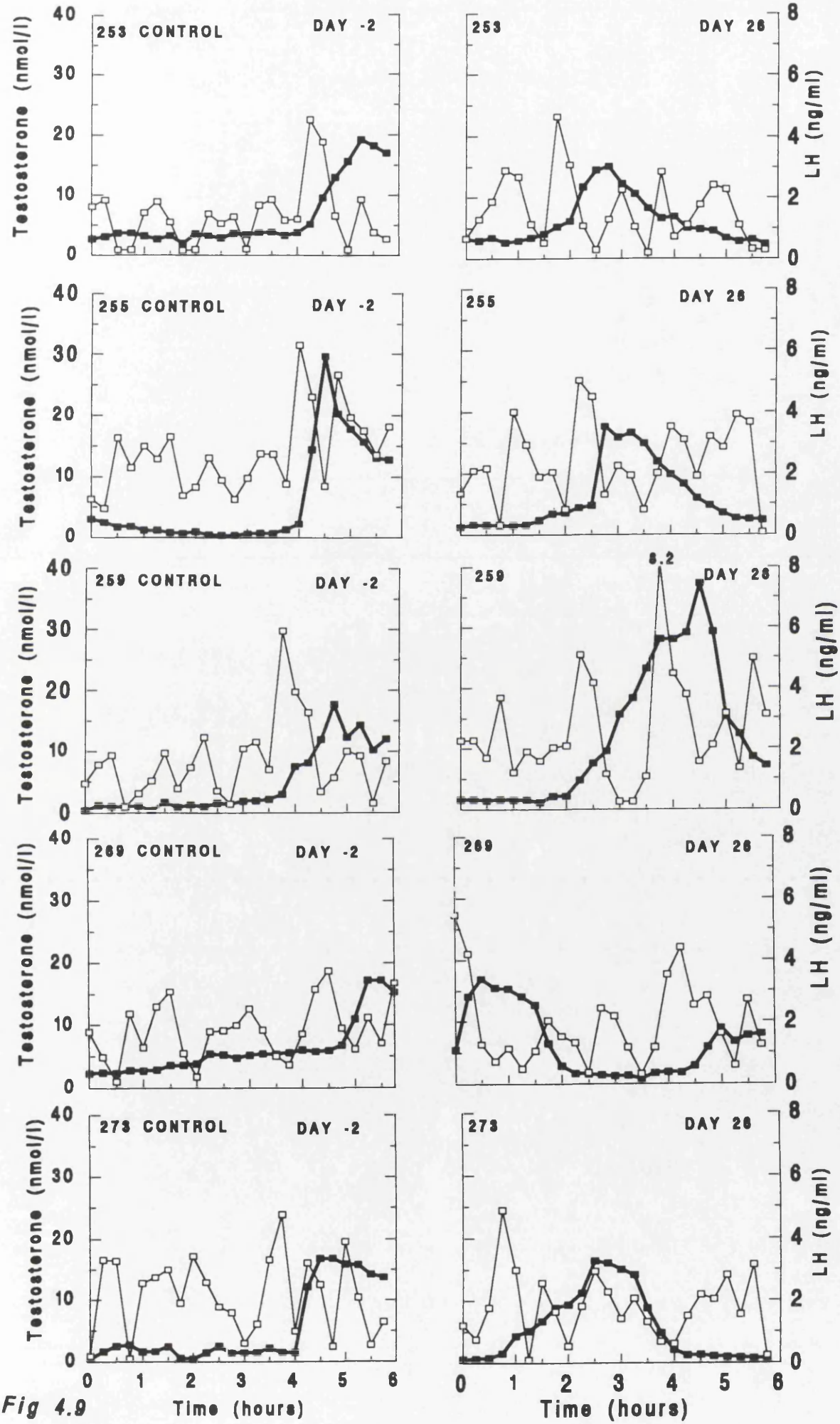

253 CONTROL
DAY -2
253
DAY 26
255 CONTROL
DAY -2
255
DAY 26
259 CONTROL
DAY -2
259
DAY 26
8.2
269 CONTROL
DAY -2
269
DAY 26
273 CONTROL
DAY -2
273
DAY 26
Testosterone (nmol/l)
LH (ng/ml)
Time (hours)
0
10
20
30
40
2
4
6
8
1
3
5

Fig 4.9

Fig. 4.10. Pattern of plasma LH and testosterone concentrations in *T. congolense*-infected rams (n = 4) in samples collected at 15 min intervals for 6-h on day 2 prior to (day -2) and 26 days after infection. Results of the same animal on the 2 blood sampling occasions are presented in the same row.

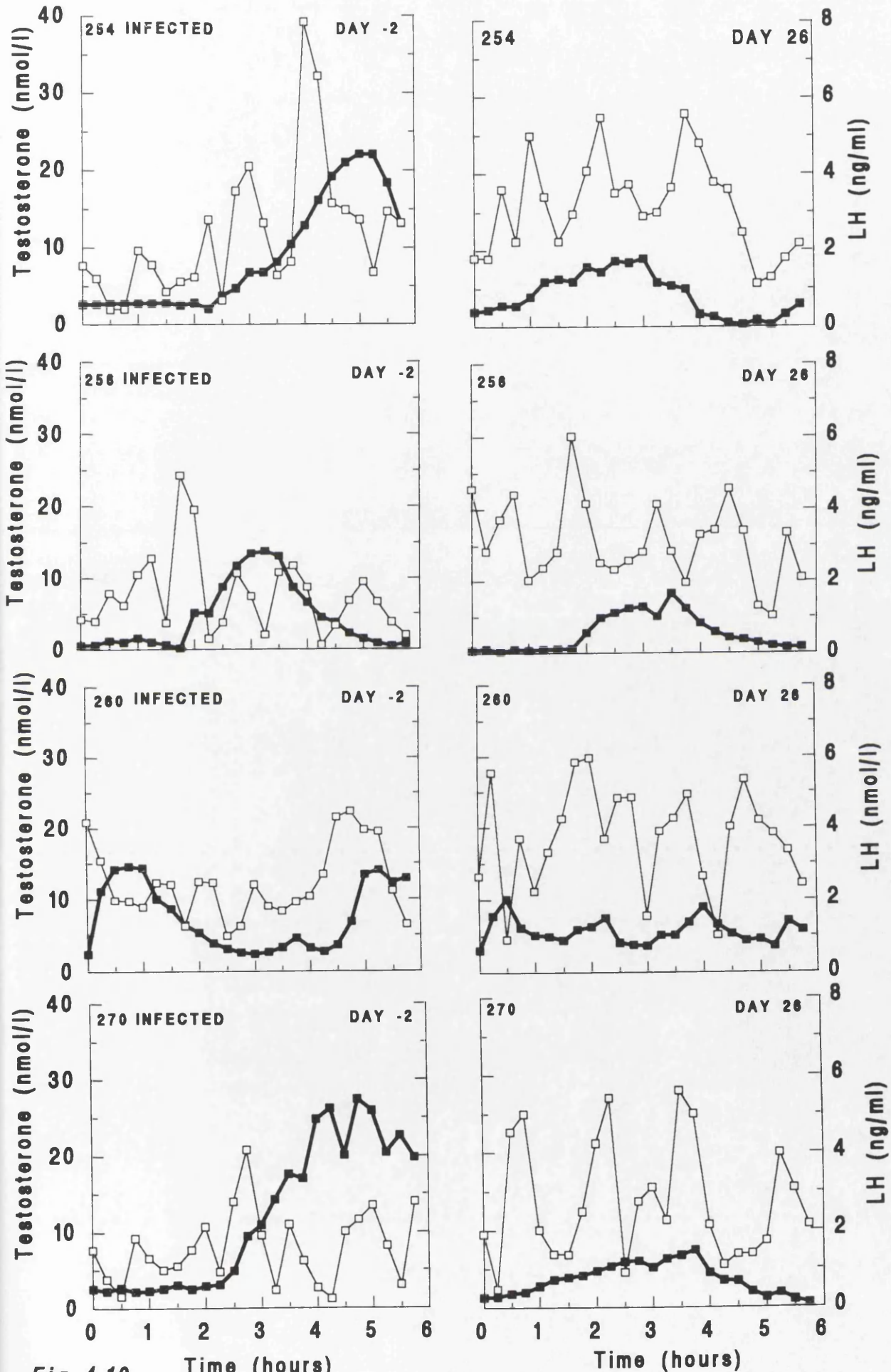

254 INFECTED
DAY -2
254
DAY 26
256 INFECTED
DAY -2
256
DAY 26
260 INFECTED
DAY -2
260
DAY 26
270 INFECTED
DAY -2
270
DAY 26
Testosterone (nmol/l)
LH (ng/ml)
LH (nmol/l)
Time (hours)
0
10
20
30
40
2
4
6
8
1
3
5

Fig 4.10

Fig. 4.11. Pattern of plasma LH concentration in uninfected (n = 5, control) and *T. congolense*-infected rams (n = 4, infected) in samples collected at 15 min intervals for 6-h on day 54 post-infection (26 days after castration).

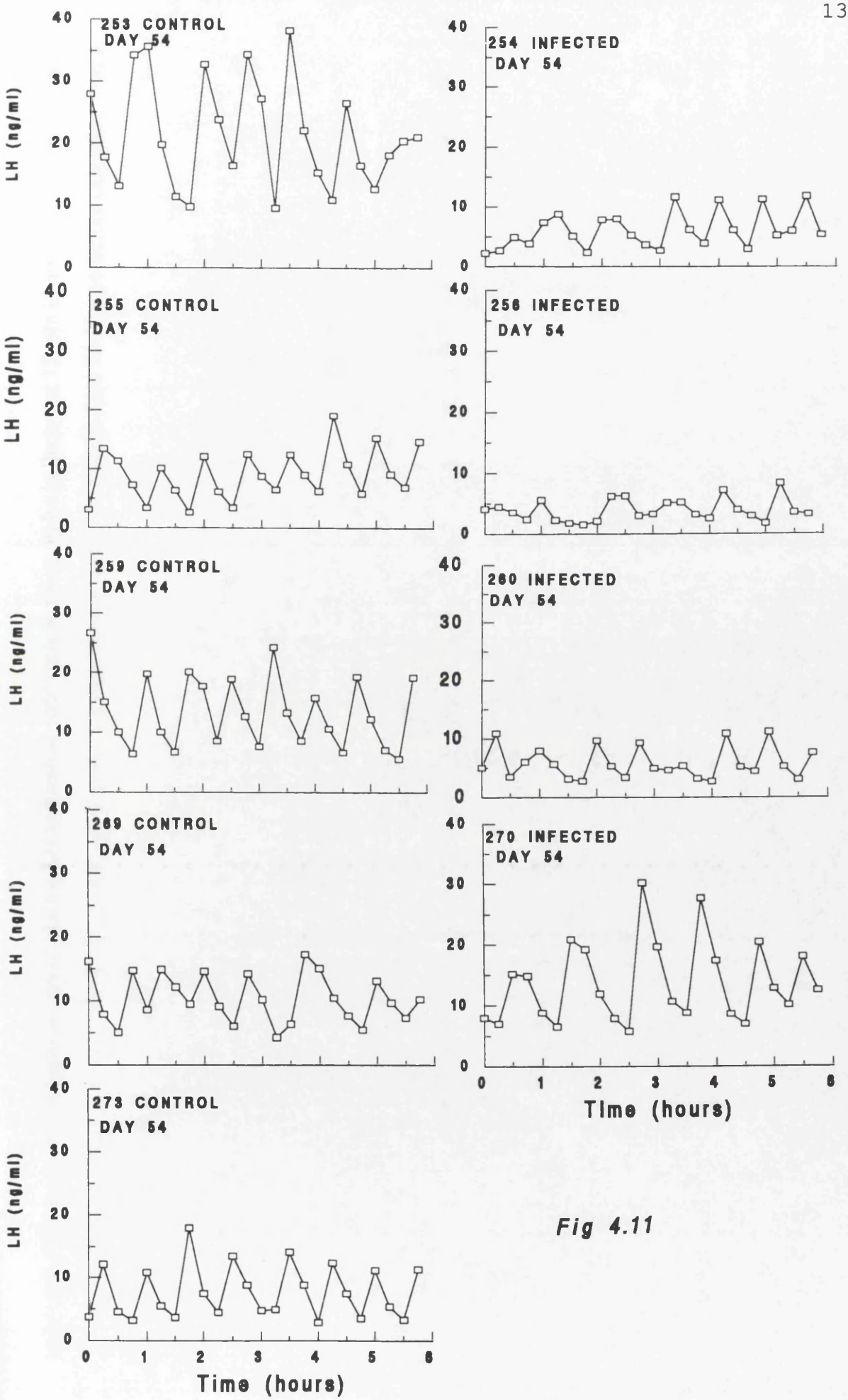

253 CONTROL
DAY 54
254 INFECTED
DAY 54
255 CONTROL
DAY 54
256 INFECTED
DAY 54
259 CONTROL
DAY 54
260 INFECTED
DAY 54
269 CONTROL
DAY 54
270 INFECTED
DAY 54
273 CONTROL
DAY 54
LH (ng/ml)
Time (hours)

Fig 4.11

Table 4.4: Changes in various parameters of plasma LH and testosterone secretion in samples collected at 15 min intervals for 6-h in uninfected (control) and *T. congolense*-infected rams (infected) on day 2 before infection (day -2) and on days 26 (2 days before castration) and 54 (26 days after castration) of infection during Study II.

	Day -2 Before infection		Day 26 2 days before castration		#Day 54 26 days after castration	
	Control (n = 5)	Infected (n = 4)	Control (n = 5)	Infected (n = 4)	Control (n = 5)	Infected (n = 4)
LH for 6h						
Pulse amplitudes	2.34 ± 0.20^{a}	2.15 ± 0.30^{a}	2.56 ± 0.25^{a}	3.27 ± 0.21^{b}	11.61 ± 0.85	7.73 ± 0.88***
Pulse frequencies	5.60 ± 0.25^{a}	4.75 ± 0.63^{a}	5.40 ± 0.25^{a}	5.50 ± 0.29^{a}	6.20 ± 0.37	6.00 ± 0.41
Mean (ng ml^{-1})	2.08 ± 0.23^{a}	1.95 ± 0.23^{a}	2.06 ± 0.22^{a}	3.20 ± 0.21^{b}	13.02 ± 2.22	6.64 ± 1.45**
Area under response curve (ng-6h ml^{-1})	11.84 ± 1.22^{a}	10.88 ± 1.23^{a}	11.86 ± 1.11^{a}	18.46 ± 1.26^{b}	73.00 ± 13.0	35.9 ± 7.3**
Variability index	6.34 ± 1.09^{a}	6.64 ± 2.05^{a}	7.59 ± 0.93^{a}	4.21 ± 0.71^{b}	6.64 ± 1.14	5.41 ± 0.51
Testosterone for 6h						
Mean (nmol l^{-1})	5.15 ± 0.27^{ab}	8.20 ± 1.56^{a}	7.55 ± 1.20^{a}	4.09 ± 0.62^{b}	--	--
Area under response curve (nmol-6h l^{-1})	30.56 ± 1.47^{a}	47.24 ± 8.29^{a}	42.71 ± 6.64^{a}	23.63 ± 3.23^{b}	--	--
Variability index	1.85 ± 0.75^{a}	1.23 ± 0.87^{a}	0.48 ± 0.08^{a}	1.20 ± 0.37^{a}	--	--

Means in the same row with the different superscripts on results of days -2 and 26 for testosterone are significantly different at $P<0.05$ and for LH at $P<0.01$.

, * = Denote significance at $P<0.01$, $P<0.001$, respectively, between respective control and infected groups on day 54 post-infection.

#Rams castrated on day 28 post-infection.

4.3.3.3. Effect of GnRH on Plasma LH and Testosterone in Uninfected Control and *T. congolense*-infected Rams before Castration

Plasma LH response

The mean plasma LH response for 2-h after GnRH injection (20 μg per animal) in the control and infected groups on days -2 and day 26 are depicted in Fig 4.12a and 4.12c, respectively. The mean areas under the LH response curve on both occasions are shown in Table 4.5. The plasma LH responses to GnRH injection in rams before castration was comparable to that described in control and infected rams in samples collected on day 5 before infection and on day 23 after infection (Section II 4.3.2.3).

Plasma testosterone response

Changes in plasma testosterone concentration for 2-h after GnRH injection in the control and infected groups on day -2 and day 26 after infection are depicted in Fig 4.12b and 4.12d, respectively. The plasma testosterone responses to GnRH injection in rams before castration was comparable to that described in control and infected rams in samples collected on day 5 before infection and on day 23 after infection (Section II 4.3.2.3).

4.3.3.4. Effect of GnRH on Plasma LH in Uninfected Control and *T. congolense*-infected Rams after Castration

Plasma LH response

Changes in the plasma LH concentration for 2-h after GnRH injection (20 μg per animal) on day 54 post-infection (26 days after castration) in control and infected rams is shown in Fig 4.12e and the mean areas under the LH response curves for the two groups are shown in Table 4.5. The plasma LH response to GnRH in the castrates (control and infected) was significantly higher ($P<0.01$) than that seen before castration (note the differences in the y-axis in the castrates) . Following GnRH injection (time 0) plasma LH levels rose rapidly from a mean value of 15.30 ± 2.11 ng ml^{-1} in the control rams and 8.54 ± 3.28 ng ml^{-1} in infected rams, peaking within 10 min in the control group (mean values 48.24 ± 5.69 ng ml^{-1}) and within 20 min in the infected group (mean vales 40.55 ± 2.30 ng ml^{-1}). A gradual decline in plasma LH concentration was observed in

both groups thereafter up to 120 min. These responses (i.e. LH peak amplitude and area under the LH response curve) were not significantly different between control and infected rams.

Fig. 4.12. Mean (± s.e.m.) plasma LH and testosterone concentrations during a 2-h period after GnRH injection (20 μg per animal) 2 days prior to infection (Fig. 4.12a for LH and Fig. 4.12b for testosterone) and on day 26 post-infection (Fig. 4.12c for LH and Fig. 4.12d for testosterone) in uninfected (n = 5, control) and *T. congolense*-infected (n = 4, infected) rams.

Fig 4.12e Mean (± s.e.m.) plasma LH concentration during a 2-h period after GnRH injection (20 μg per animal) on day 54 post-infection to uninfected (n = 5, control) and *T. congolense*-infected (n = 4, infected) castrated rams. Castration was performed 28 days after infection.

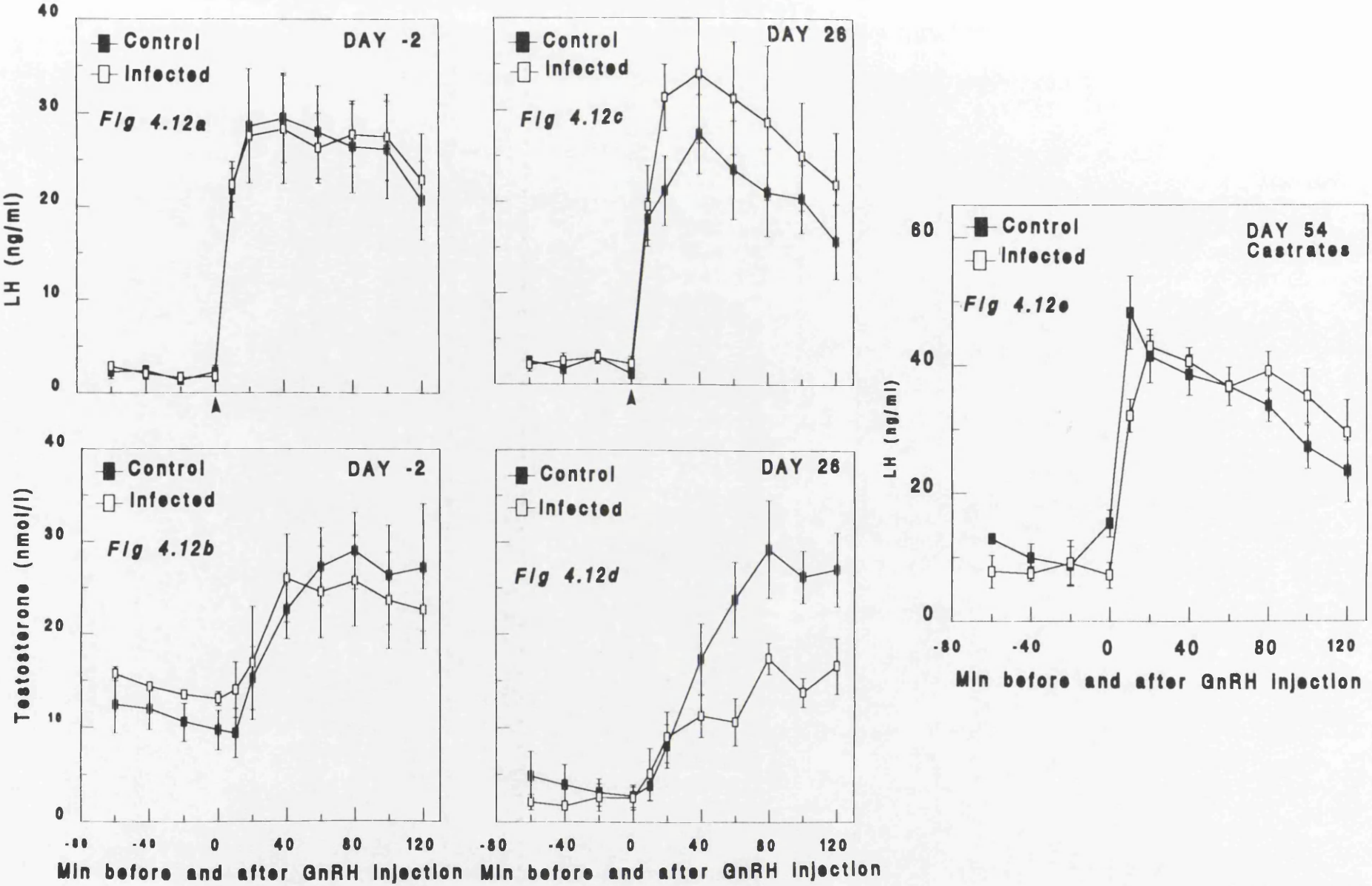

Control
Infected
DAY -2
Fig 4.12a
DAY 28
Fig 4.12c
DAY 54
Castrates
Fig 4.12e
Fig 4.12b
Fig 4.12d
LH (ng/ml)
Testosterone (nmol/l)
Min before and after GnRH injection

Table 4.5: Index of plasma LH and testosterone secreted over a 2-h period (area under curve) in response to GnRH (20 μg per animal) administered on day 2 prior to infection (day -2) and on days 26 (2 days before castration) and 54 (26 days after castration) of infection to a group of uninfected rams (control) and a group of *T. congolense*-infected rams (infected).

	Day -2		**Day 26**		**Day 54**	
	Before infection		**2 days before castration**		**26 days after castration**	
	Control (n = 5)	Infected (n = 4)	Control (n = 5)	Infected (n = 4)	Control (n = 5)	Infected (n = 4)
LH						
Area under curve (ng-2h ml^{-1})	50.48 ± 9.06	51.98 ± 6.33	41.81 ± 7.57	53.82 ± 9.64	68.96 ± 5.41	72.57 ± 4.38
Testosterone						
Area under curve (nmol-2h l^{-1})	47.49 ± 5.99	42.12 ± 4.04	40.34 ± 6.38	24.67 ± 2.62	--	--

Values are means (± s.e.m.).

4.4. DISCUSSION

The present studies investigated the changes in the endocrine function of the testis and pituitary gland during an experimental *T. congolense* infection in the ram. Several aspects of the secretory pattern of plasma testosterone and LH were investigated at different periods during the course of infection. Results obtained showed that soon after the onset of parasitaemia, plasma testosterone concentration declined and levels remained low throughout the infection period. The reduction in plasma testosterone concentration was associated with a progressive decline in testosterone pulse amplitudes leading to loss of testosterone pulses. Testosterone secretion in response to exogenous GnRH-induced LH secretion was also depressed throughout the infection period. Within 4 weeks of infection plasma LH pulse amplitudes increased significantly and this was accompanied by a tendency for increased responsiveness of the pituitary gland to exogenous GnRH. As the infection progressed up to 8 weeks, the plasma LH concentration declined. The decline in LH at this stage was not associated with the feedback influence of gonadal steroids or due to the failure of the pituitary gland to secrete and release LH as a similar LH change was observed in infected rams which had been castrated 4 weeks after infection when no gonadal influence could have been present. Also pituitary LH secretion in response to exogenous GnRH stimulation was not impaired throughout the infection period.

For discussion purposes, hormonal changes observed in both experiments are discussed together and are arranged starting with plasma LH and testosterone changes during Study I, followed by Study II, and then the hormonal responses to GnRH during both studies.

In the first experimental infection where plasma samples were collected infrequently to provide baseline information on changes in plasma LH and testosterone (Section I), plasma LH fluctuated markedly with values varying between 0.2 to 3 ng ml^{-1} in the control and infected rams throughout the experiment. Following infection, changes in plasma LH in infected rams did not show any clear uniform pattern of change during the first 8 weeks after infection as LH concentration tended to increase in some animals and decreased or remained unchanged in others. A decline in mean weekly LH values was observed from week 9 onwards. The episodic secretion of plasma LH could

not be determined in this study due to the long intervals between blood sampling. This is the first report describing LH changes during *T. congolense* infection in animals. One possibility why no clear pattern in plasma LH changes was observed in infected rams could be associated with the inherent secretory pattern of LH in plasma. Plasma LH is usually secreted episodically so that the concentration in plasma shows a sudden rise, rapidly followed by a return to baseline concentrations (Katongole *et al*, 1971; Schanbacher and Ford, 1976; Setchell, 1978). Hence, samples taken frequently are needed to detect plasma LH changes. This would explain the discrepancies in previous reports which have either shown no change (Boersma *et al.*, 1989; Hublart *et al.*, 1990) or a decline (Emeh and Nduka, 1983; Soudan *et al.*, 1992) in plasma LH and FSH concentrations measured in single samples during infections caused by *T. brucei*-group.

In these thrice weekly samples, plasma testosterone fluctuated as for LH in the control and infected rams before infection and in control rams throughout the study period. And for the same reasons as given above, the episodic secretion of plasma testosterone could not be determined due to the long interval between blood sampling. However, fluctuating values were obtained in control and infected rams varying between 0.1 to 25 nmol l^{-1} before the infection period. This pattern was maintained in control rams throughout the study period but *T. congolense* infection was associated with a rapid and progressive decline in plasma testosterone concentration. The changes in plasma testosterone occurred within the first or second week of infection subsequent to the onset of parasitaemia and pyrexia but before changes were noted in other clinical parameters such as PCV and liveweight. The mean plasma testosterone levels in infected rams remained significantly lower than in control rams from week 4 onwards. These findings agree with the previous observation of Waindi *et al.* (1986) in *T. congolense*-infected goats and Adeyemo *et al.* (1990) in rams with a mixed *T. congolense* and *T. b. brucei* infection. The rate at which plasma testosterone declined in infected rams seemed to depend on the susceptibility of individual rams to infection as measured by parasitaemia and PCV changes. Minimal changes in plasma testosterone were observed in ram 218 (Fig. 4.2) and this animal had lower parasitaemia levels than the other infected rams and its PCV values changed only slightly during the infection period. The ability of the trypanosome-infected animals to control parasitaemia and anaemia are usually used as

indicators of trypanotolerance (Murray *et al.*, 1990; Trail *et al.*, 1991) and the effects of trypanosomiasis on reproductive performance of trypanotolerant animals have been shown to be relatively mild when compared to trypanosusceptible ones (Mutayoba *et al.*, 1988c; Trail *et al.*, 1993; Okech *et al.*, 1993).

It seems possible that *T. congolense* infection may exert a time-dependent effect on pituitary LH secretion so that changes in plasma LH concentration are only observed several weeks after infection compared to the rapid decline in plasma testosterone concentration which was observed within 1 - 2 weeks of infection during the first study. This was investigated by measuring LH in plasma samples collected more frequently at different periods of infection during the second experiment (Section II). One of the objectives of the second experimental infection was to assess the effects of *T. congolense* on the pulsatile secretion of plasma LH and testosterone at different stages of infection. The evidence that was subsequently obtained suggests that *T. congolense*-induced changes in the pulsatile secretion of plasma LH in rams which could be divided into two phases (phase I and II). These will be used to aid the discussion in this chapter. Phase I is characterised by a period when infected rams showed increased amplitudes of pulsatile LH secretion. This was observed in the frequent blood samples obtained in infected rams on days 23 and 26 post-infection (see Fig 4.6 and 4.9). Phase II is characterised by a period when infected rams showed reduced amplitudes of LH secretion. This was evident in frequent blood samples obtained on day 52 post-infection in infected rams and also more clearly 26 days after castration in infected rams. However during both phase I and II, plasma testosterone concentration declined in infected rams. This was associated with reduced pulse amplitudes and later an absence of testosterone pulses (see Fig 4.6).

This is the first study to have measured plasma LH levels in frequent samples during trypanosome infection. During phase I, increased LH pulse amplitudes could have resulted from increased pituitary LH secretion triggered by low plasma testosterone concentration observed in the infected rams during this period. Lowered plasma testosterone would reduce the negative feedback constraints on the pituitary and hypothalamus (Setchell, 1978; Pelletier and Caraty, 1981, Authur *et al.*, 1982; Messi *et al.*, 1988) either alone or in conjunction with trypanosome-borne or -induced factors

(Hublart *et al.*, 1990). Phase II is the period when reduced pituitary LH secretion occurred concurrently with reduced plasma testosterone secretion. This was demonstrated in the infected rams on day 52 after infection (Fig. 4.6). The secretion of LH in a normal male animal is regulated by a series of feedback systems, which are either localized within the hypothalamo-pituitary complex or require input from the testes or other endocrine glands e.g. adrenal gland (Charpenet, 1981; Evans *et al.*, 1991). Decline in plasma testosterone to levels observed in infected rams in the present studies or as reported previously in intact rams immunised against testosterone (Schanbacher, 1982) and in castrated rams (Lee *et al.*, 1978; D'Occhio *et al.*, 1982a; Caraty, 1983) would be expected to enhance pituitary LH secretion and increase plasma LH concentrations. Evidence from these studies therefore suggests that the reduction in LH secretion in infected rams during phase II which occurred concomitantly with reduced plasma testosterone could be associated with the down-regulation of the hypothalamo-pituitary function by invading trypanosomes.

This is also the first study to have measured plasma testosterone levels in frequent samples during trypanosome infection. Plasma testosterone concentration in infected rams progressively decreased with time after infection. Thus mean plasma testosterone concentration in samples collected in infected rams on day 52 post-infection was lower than day 23 post-infection values which were also lower than day -7 pre-infection values. Results obtained in this study indicate that *T. congolense* induced a rapid and progressive impairment of testicular steroidogenesis. As testosterone plays an important trophic role in the function of the testis during spermatogenesis (discussed in Chapter III), it is likely that the rapid decline in testicular steroidogenesis induced by the infection may contribute to the degenerative changes of the spermatozoa which accompanied this disease. Several testicular germ cells (primary and secondary spermatocytes and spermatids) which were found to degenerate rapidly during the present and previous studies (Anosa and Isoun, 1980; Kaaya and Oduor-Okelo, 1980; Anosa, 1983) are dependent on normal testicular androgen secretion for their function (Steinberger, 1971; Setchell, 1978; Cameron *et al.*, 1993).

The detection of disease-associated changes in pituitary function in intact adult male animals is complicated by the low concentrations of LH normally present in plasma

(Katongole *et al.*, 1971; D'Occhio *et al.*, 1982a; Caraty, 1983). One method of increasing plasma LH concentrations is the removal of both testes as this significantly increases mean plasma LH levels (Hutchison and Goldman, 1975), frequency of LH rhythmic pulses (Gay and Sheth, 1972; Riggs and Malven, 1974) and LH response to exogenous GnRH (D'Occhio *et al.*, 1982b). This would in turn allow the changes in the functional response of the hypothalamo-pituitary axis to infection to be assessed. During the present investigations, four *T. congolense*-infected and five control rams were castrated on day 28 post-infection (Section III). Marked increases in plasma LH amplitude and in the LH response to GnRH were observed after castration in control and infected rams in samples obtained 26 days later (Fig. 4.11 and Fig. 4.12e). These findings were expected in respect of the generally held view on the role of testosterone in limiting gonadotrophin secretion (Hutchison and Goldman, 1975); Gay and Sheth, 1972; Riggs and Malven, 1974; D'Occhio *et al.*, 1982b). Infected castrated rams however, had lower mean LH concentration and LH amplitudes than control rams in the frequent 6-h samples, which supports the hypothesis that low plasma LH, that had been observed in infected non-castrated rams at this time (Fig 4.6), was associated with the impairment of the hypothalamo-pituitary axis and is not the result of negative feedback of testicular steroids.

The control of pituitary LH secretion is under the control of the hypothalamus and LH pulses occur in response to discrete pulses of GnRH released from the hypothalamus (Clarke and Cummins, 1982; Levine *et al.*, 1982; Caraty and Locatelli, 1988). The quantity of LH released is dependent on the amount of GnRH secreted by the hypothalamus and the sensitivity of the pituitary gland to GnRH (Evans *et al.*, 1991). The effects of *T. congolense* infection on the pituitary sensitivity to GnRH as well as testicular sensitivity to GnRH-induced LH secretion (pituitary-gonadal response test) in rams were both investigated at various stages of infection during Study I and II by measuring plasma LH and testosterone responses after intravenous injection of 20 μg of GnRH (Sections I to III). The dose used was selected to induce a pituitary LH response lasting 2 - 3 hours in sheep (Siddall and Crighton, 1977). *T. congolense* did not reduce the pituitary responsiveness to GnRH (secretion of LH) in infected rams on any occasion. Instead, it seemed to enhance the pituitary responsiveness to GnRH on day 22

post-infection as the infected animals during the first study (Section I) had a significantly higher LH response than control rams (see Fig. 4.4a). In addition in the second study, although the differences in LH response were not significantly different between infected and control rams on day 23 and 26 post-infection after GnRH challenge, a similar trend was observed (refer to Fig 4.8c and 4.12c). However, on all occasions when GnRH was injected during the post-infection period, infected rams had a reduced testosterone response in comparison to control rams. The observed tendency for the pituitary sensitivity to GnRH to increase in infected rams during phase I (increased LH pulse amplitude) during both studies could have been associated with up-regulation of pituitary GnRH receptor activity by low plasma androgen levels since a similar phenomenon is observed in castrated animals (Clayton and Catt, 1981; Conne *et al.*, 1982; Limonta *et al.*, 1986). Such an enhancement of the pituitary GnRH receptor activity could at least partly account for the observed increase in plasma LH amplitudes in infected rams during this period.

On the other hand, no change in the pituitary responsiveness to GnRH was observed in infected rams during phase II (reduced LH pulse amplitude) as infected and control rams had similar plasma LH responses when stimulated by GnRH (particularly apparent in castrates). However, the fact that infected rams had low plasma LH and testosterone concentrations prior to stimulation (LH effect more evident from castrates) suggests the hypothalamus was the site at which *T. congolense*-infection impaired pituitary LH secretion during this period. Although dynamic changes in GnRH secretion were not investigated in the present studies, simultaneous reductions in plasma LH and testosterone concentration are more likely to occur when there is a reduction in the secretion and/or release of hypothalamic GnRH. Similar observations have been observed in sheep immunized against GnRH (Schanbacher, 1982; Jeffcoate *et al.*, 1982).

Taken together, the results from these studies suggest that *T. congolense* infection is associated with a rapid decline in plasma testosterone following the onset of parasitaemia. During phase I of the infection reduction in plasma testosterone causes an increase in pituitary secretion of LH (i.e day 23 (Fig 4.6) and day 26 (Fig 4.10) study II) by the normal feedback mechanism. However, the increased LH production is ineffective at stimulating further testosterone secretion possibly because of the decreased

responsiveness of the testis to LH or decreased testosterone synthesis by Leydig cells (which is a subject of investigation in Chapter V). By phase II the pituitary gland is no longer able to respond to the low plasma testosterone concentration (i.e. day 52 (Fig 4.6) and day 54 (Fig 4.11 study II), possibly because of a reduction in hypothalamic GnRH secretion rather than the pituitary being unresponsive to GnRH or being unable to secrete LH, as the latter can still respond to exogenous GnRH stimulation and secrete LH in a manner similar to that observed in normal animals.

The hypothalamic secretion of GnRH is controlled by the activity of neurones present in the hypothalamus and higher centres (Everitt and Hokfelt, 1986) which turn involves the release of several monoamines including noradrenergic and dopaminergic agonists and serotonin (Donoso *et al*, 1971; Iversen, 1975; Jackson, 1975, 1977). Studies using mice chronically infected with *T. b. brucei* (Amole *et al.*, 1989), and rabbits (Stibbs, 1984), voles and mice (Stibbs and Curtis, 1987) chronically infected with *T. b. gambiense* have shown marked perturbations of several monoamine neurotransmitters including dopamine, serotonin, noradrenaline, and their acid metabolites homovanillic acid, 3,4-dihydroxyphenylacetic acid and 5-hydroxyindoleacetic acid in several parts of the brain including the midbrain-thalamus-hypothalamus areas. These neurochemical changes are likely to alter the hypothalamic GnRH secretory pattern and might also be involved in the behavioural symptoms that frequently accompany this disease in man and animals (Stibbs, 1984). Although such studies have not been reported in *T. congolense*-infected animals, it is likely that changes in brain neurotransmitters might occur during chronic infections as a result of the localization of *T. congolense* in large numbers in the brain microvasculature (Banks, 1978). This usually leads to vasodilatation of brain capillaries and development of pressure on surrounding brain parenchyma (Mwambu and Losos, 1978; Mills *et al.*, 1980). The findings obtained in the present studies along with those summarised above (Stibbs, 1984; Stibbs and Curtis, 1987; Amole *et al.*, 1989) indicate that the central (central nervous system and hypothalamus) control mechanisms, which regulate GnRH secretion, could have been affected during phase II of the infection.

GnRH secretion is also under peripheral gonadal negative-feedback control (Pelletier and Caraty, 1981; Pelletier, 1982; Thieulant and Pelletier, 1979; Messi *et al*,

1991). The findings obtained in the present studies also indicate that the peripheral (testis) control mechanism on GnRH secretion could have been affected during phase II of the infection. Impairment of the peripheral testicular feedback control mechanisms on hypothalamic GnRH secretion (i.e insensitivity to reduced plasma testosterone) by *T. congolense* on days 52 and 54 after infection could also be associated with the down-regulation of androgen and oestrogen receptors present in the hypothalamus (Pelletier and Caraty, 1981; Thiery and Pelletier, 1981; Pelletier, 1982). This may involve the desensitization of these receptors as hypothalamic androgen as well as oestrogen receptor concentrations are usually increased by reduction of peripheral testosterone concentration (Pelletier, 1982). The androgen and oestrogen receptors present in the hypothamalus are important in the negative-feedback control of GnRH by androgens, especially testosterone (Messi *et al.*, 1991). Similarly, androgen and oestrogen receptors are important in the feedback control of pituitary LH secretion by both androgens, especially 5α-dihydrotestosterone formed from testosterone by 5α-reduction process in the brain and anterior pituitary and oestrogens, especially oestradiol-17β formed by aromatization of testosterone in the hypothalamus, limbic structures and other parts of the central nervous system (Martini, 1982). The dynamic changes in hypothalamic GnRH production as well as hypothalamic and pituitary androgen and oestrogen receptor activities were not investigated in the present studies, but in the wake of the endocrine changes observed, such investigations should be planned in the future to verify the above speculations on the mechanisms behind the reported results.

Care should, however, be taken in interpreting the findings of the GnRH-pituitary-gonadal response obtained in the present studies since the differences in pituitary sensitivity to GnRH between infected and control rams could have been masked by the pharmacological dose of GnRH used. GnRH injection causes the release of LH in several animal species in a dose response fashion (Golter *et al.*, 1973; Zolman *et al.*, 1973; McCarthy and Swanson, 1976; Siddall and Crighton, 1977) and GnRH dose-LH response curves are usually useful when an accurate measure of a change in the sensitivity of the pituitary gland needs to be evaluated (Evans *et al.*, 1991). Since pituitary responsiveness to various doses of GnRH was not assessed in the present

investigations this possibility could not be eliminated and further studies on pituitary response to GnRH in trypanosome infection should examine this possibility.

It was observed during the present studies that the response of the pituitary gland and testis to similar GnRH doses was over 2 times higher in rams used during Study II than in Study I. The discrepancy between these findings remains unexplained and could be due to a difference in potency of the GnRH batches used possibly associated with deterioration on storage. The peptide hormones are sold on the basis of their total weight and according to the manufacturer (Sigma Chemical Company) the GnRH batch used in the first study was about 4 years old and the GnRH batch used in the second study was 2 years old. Since their biological activity was not tested prior to use, this possibility could not be eliminated. Rams used during both studies were of almost similar weight and age and both experiments were conducted during the same time of the year and in similar managemental conditions. GnRH doses used were prepared by the same person and care was taken to confirm that the dose used was similar during both studies. The different LH concentrations were verified by analysing samples from both studies in the same LH RIA to eliminate the possibility of interassay variability.

As discussed earlier, the testis was another site at which *T. congolense* appeared to induce profound hormonal perturbation. This was characterised by a rapid decrease in the plasma testosterone concentration and a progressive loss of testicular responsiveness to GnRH-induced LH secretion in infected rams. These findings were consistent with the recent report by Soudan *et al.* (1992) who noted a decline in testicular responsiveness to exogenous human chorionic gonadotrophin (hCG) in rats infected with *T. b. brucei.* Soudan *et al.* (1992) attributed the progressive loss of testicular responsiveness to hCG in rats to the decrease in the number of testicular LH receptors and desensitization of the remaining receptors as depicted by increase in their equilibrium association constant (Ka). Although changes in testicular LH receptor activity were not investigated in this work, it seems likely that similar changes could have occurred in the infected rams in view of the similarities in the testicular responsiveness results obtained in the present studies and in the rats described by Soudan *et al.* (1992). Further investigations in this area are needed to confirm this as a cause of decline in hormonal responsiveness. However, further *in vitro* investigations which

were conducted during Study II and which are described in Chapter V indicate that changes in testicular testosterone secretion in infected rams could also be associated with a decrease in Leydig cell steroidogenic activity.

It is clear that the changes in plasma LH and testosterone concentrations during *T. congolense* infection in rams in the present studies are related to perturbation of the pituitary and testicular functions. There are a number of means whereby endocrine systems can be affected by disease. Processes such as (i) pyrexia and associated host defence mechanisms, (ii) parasite intervention (inducing degenerative lesions), (iii) hormonal receptor inactivation and (iv) stress can possibly explain the hormonal imbalances which were found in the present studies.

The possible involvement of pyrexia induced by *T. congolense* infection in inducing the changes in the seminal and testicular pathology observed in infected rams was discussed in chapter III. However, as the development of fever involves the activation of numerous endogenous pyrogenic cytokines (IL-1ß, IL-α, IL-6, TNF-α, IL-8, IFN-α and IFN-γ) (Whicher and Westacott, 1992) with pleiotropic effects on several body tissues including the hypothalamo-pituitary-gonadal axis (see recent reviews by Kennedy and Jones (1991) and Gorospe and Spangelo (1993) and recent reports by Xiong and Hales (1993); Mauduit *et al.* (1993); Tortorella *et al.* (1993) and Sun *et al.* (1993)), it is possible that these cytokines might be involved in the endocrine disturbances observed in *T. congolense*-infected rams. Several reports have confirmed the activation of the cytokines described above during infections induced by *T. congolense* (Mitchell *et al.*, 1986, Winstanley, *et al.*, 1993; Sileghem *et al*, 1993) and *T. b. brucei* (Sileghem *et al.*, 1989; Mathias *et al.*, 1990; Wiesenfield-Hallin *et al.*, 1991) and at least one cytokine, IL-1, has been shown to inhibit GnRH release (Kalra *et al.*, 1990a). However, there are no reports which have linked the activation of cytokine cascades during trypanosome-infections with reproductive endocrine changes observed during this disease. Hence, investigations on trypanosome-induced cytokines and their effects at the hypothalamic, pituitary and gonadal level might be a fruitful area of research.

Investigations which were performed in rams during the second experiment to compare the endocrine changes induced by elevation of testicular temperature by

insulation and the changes induced by *T. congolense* infection (Section II) were not completely successful. Although scrotal insulation induced noticeable changes in spermatogenic activity, less marked endocrine changes compared to those observed in *T. congolense*-infected rams. For example plasma testosterone concentration was intermediate between values seen in control and infected rams (see Table 4.2). By measuring the scrotal temperatures of rams during Study II, it was apparent that *T. congolense* induced a significant rise in scrotal and most probably therefore in testicular temperature of infected rams. Leydig cells are known to be more resistant to thermal degeneration than are several stages of germ cells (Wettmann and Desjardins, 1979), and they are easily discernible by light microscopy in testis sections from animals in which marked testicular damage had been caused by trypanosome infection (Ikede, 1979; Kaaya and Oduor-Okelo, 1980; Anosa and Isoun, 1980; Omeke and Onuora, 1992), however, elevated testicular temperature can induce more subtle cytoplasmic and nuclear degenerative changes in Leydig cells (Kerr *et al.*, 1979; Bergh *et al.*, 1984a; Ezeasor, 1985). This leads to slow but progressive reduction in the total number of Leydig cells per testis (Lunstra and Schanbacher, 1988) and alteration in steroid biosynthesis (Le Vier and Spaziani, 1968; Inano and Tamaoki, 1968; Farrer *et al.*, 1985; Bergh *et al.*, 1984b; Munabi *et al.*, 1984; Steinberger, 1991).

In most cases where testicular temperature is elevated either by cryptorchidism (Schanbacher and Ford, 1977; Setchell *et al.*, 1977; Blanc *et al.* 1978; Schanbacher, 1980), or by shortening the scrotum (Thun *et al.*, 1980) plasma testosterone levels remain normal or only slightly below normal, while plasma LH levels are increased. The apparent disparity between normal plasma testosterone levels and reduced number of Leydig cells is thought to result from increased testosterone synthesis per cell in response to the increased LH levels (Schanbacher, 1979, 1980), increased rate of chemical reactions at elevated temperature (Hochereau-de Reviers *et al.*, 1979), hypertrophy of remaining Leydig cells (Kerr *et al.*, 1979; Lustra and Schanbacher, 1988), or a combination of these effects. Whereas these effects could help to account for the minimal alteration in plasma testosterone observed in the scrotal- insulated rams, it was apparent that plasma testosterone levels in *T. congolense*-infected rams continued to decline with the duration of the infection. It is possible that other trypanosome-borne

and/or induced factors (for example those discussed below) might be involved in inducing these hormonal changes.

The endocrine anomalies which were observed in the rams in the present studies could also have been induced by direct intervention of the parasite during infection. This is likely to be associated with the tendency of *T. congolense* to localise in large numbers in the microvasculature of the hypothalamo-pituitary-gonadal axis inducing local endothelial damage and interfering with local nutrient supply (Ashman and Seed, 1974; Banks, 1978; Abebe *et al.*, 1993a). During the last few years, however, increased emphasis has been placed on parasite components produced by dead trypanosomes in the host circulation, especially parasitic peptidases (Knowles *et al.*, 1987, 1989; Lonsdale-Eccles and Grab, 1987) and proteases (Hublart, *et al.*, 1990; Huet, *et al.*, 1992). For example, after inoculating rats with trypanosomal preparations of surface coat components and parasitic cellular pellet from *T. b. brucei* in the presence or the absence of protease inhibitors, Hublart *et al.* (1990) found that trypanosomal pellet obtained in the presence of antiproteases, induced a reduction in serum testosterone levels and increased pituitary LH and FSH content without causing a change in circulating LH and FSH. Similar endocrine changes were observed after 3 days of infection with live trypanosomes. Although these studies were carried out using *T. b. brucei.*, trypanosome-derived proteases are possibly produced by other species of trypanosomes including *T. congolense* (Boreham and Kimber, 1970; Boreham and Facer, 1973). In view of the large number of trypanosome-borne and -induced factors which have been documented (reviewed in Chapter I 1.3.1) there are many experiments to be performed before their role in inducing reproductive changes can be ascertained.

Soudan *et al.* (1992) have recently shown that the changes in plasma testosterone concentration in rats infected with *T. b. brucei* might involve desensitization of testicular LH receptors. In the present studies, the decreased testosterone response to GnRH-induced LH secretion observed in infected rams shows that such LH receptor inactivation might be occurring in *T. congolense* infection and this is further investigated in Chapter V.

Hormonal perturbations observed in the present studies could have been induced or exacerbated by trypanosome-induced stress. Trypanosome infections are known to

Fever acting alone or in combination with cytokines may cause detrimental effects on the hypothalamo-pituitary-gonadal axis. It many also involve production of several trypanosome-borne toxic factors as well as the effect of stress induced by the infection.

CHAPTER V

EFFECT OF EXPERIMENTAL *TRYPANOSOMA CONGOLENSE* INFECTION ON LEYDIG CELL STEROIDOGENESIS IN THE RAM

5.1. INTRODUCTION

Among the numerous clinical signs of *T. congolense* infection in the male, reproductive dysfunctions manifested by reduced libido and progressive production of poor quality semen leading to oligospermia and azospermia are well known (reviewed by Ikede *et al.*, 1988 and further reported by Sekoni, 1992, 1993; Sekoni *et al.*, 1988; Omeke and Onuora, 1992). *T. congolense* infection also induces progressive non-inflammatory degenerative lesions of the testicular germ cells, epididymides and accessory tissues (Sekoni, 1990; Sekoni *et al.*, 1990b; Anosa, 1991; Omeke and Onora, 1992). These gonadal changes are also induced by other pathogenic African trypanosomes which infect animals and man (reviewed in Chapter I).

To help to elucidate the mechanisms involved in the pathogenesis of trypanosome-induced infertility in different animal species, reproductive endocrine studies have shown that testicular changes are accompanied by a decline in plasma testosterone levels (Waindi *et al.*, 1986; Adeyemo *et al.*, 1990) which is possibly associated with impaired testosterone secretion by the testes during infection (Waindi *et al.*, 1984). Waindi *et al.* (1986) further suggested that a decline in testicular testosterone secretion might contribute to the observed testicular germ cell degeneration.

Recent studies by Soudan *et al.* (1992) have shown that a decrease in circulating testosterone concentration as well as a decline in testicular responsiveness to exogenous hCG stimulation in rats infected with *T. b. brucei* might, in part, be associated with the decline in the number of LH receptors expressed per testis and a decrease in the sensitivity of the remaining receptors associated with an increase in their equilibrium association constant (Ka). However, other factors are likely to be involved in reducing testicular steroidogenesis during *T. b. brucei* infection since loss of 30 - 40% of LH receptor numbers and partial desensitization of the remaining receptors reported by Soudan *et al.* (1992) would still probably not produce a profound effect on steroidogenesis as only 1% of the receptor population is usually sufficient to elicit a normal steroidogenic response (Chan *et al.*, 1981; Romerts and Brinkman, 1981; Dehajia *et al.*, 1982).

During *T. b. brucei* infection, changes in Leydig cell activity in the affected testis could partly be induced or exacerbated by the direct localization of the parasite in the

testicular interstitial spaces where it induces a marked inflammatory response (Ikede *et al.*, 1988). However, there are no reports of *T. congolense* being found in the testicular interstitium. One of the factors which is thought to contribute to the testicular degenerative changes and hormonal perturbations in trypanosome-infected animals is the thermal effect associated with the repeated bouts of hyperthermia (39.5 to 41.8°C) that accompany the peaks of parasitaemia (Isoun and Anosa, 1974b; Kaaya and Oduor-Okelo, 1980; Anosa and Isoun, 1980; Omeke and Onuora, 1992). Testicular degenerative changes almost similar to those observed during *T. congolense* infection occur in other febrile conditions (Gunn *et al.*, 1942; Watson, 1964) or when the testicular temperature is elevated in non-febrile conditions including cryptorchidism (Moore and Aslund, 1923; Wilton and de Krester, 1984; Lustra and Schanbacher, 1988), scrotal insulation (Glover, 1955; Dutt and Hamm, 1957) or after heat treatment (Moule and Waites, 1963; Gomes *et al*, 1971). Although no previous reports have shown that *T. congolense* infection causes an elevation of testicular temperature, the findings obtained in Study II Chapter III) during the present investigations, suggest that this does indeed occur.

Furthermore, results of Study I and II showed that *T. congolense*-infected rams had a reduced testosterone response to LH induced by exogenous GnRH and this was similar to the findings previously reported in rats infected with *T. b. brucei* following stimulation with hCG (Soudan *et al.*, 1992). Hence, to assess whether the reduction in plasma testosterone observed in infected rams during Study I and II could be caused by impaired function of the Leydig cells, *in vitro* studies were carried out on isolated Leydig cells obtained from uninfected control rams, *T. congolense*-infected rams and scrotal-insulated rams during Study II with the following objectives.

5.1.1 Objectives of the Study

(i) To determine whether *T. congolense* infection affects steroidogenesis by Leydig cells isolated from the testes of rams at different stages of infection.

To achieve this, production of testosterone and progesterone by isolated Leydig cells was measured *in vitro* under basal conditions and after stimulation by human chorionic gonadotrophin (hCG), dibutyryl cyclic adenosine monophosphate (dbcAMP) or 22R-

hydroxycholesterol (22ROHC). Testes were obtained from control and infected rams on 28 and 58 days post-infection during Study II.

(ii) To compare and contrast Leydig cell function in testes removed from scrotal-insulated rams and *T. congolense*-infected rams.

Steroid production by Leydig cells isolated from scrotal-insulated rams under basal conditions and after stimulation with hCG, dbcAMP or 22ROHC was compared with the steroid production by Leydig cells from infected and control rams (objective i) obtained on 58 days of infection.

5.2. MATERIALS AND METHODS

5.2.1. Animals

Rams used during Study II ((10 control, 9 experimental and 6 scrotal-insulated) and described in Chapter III 3.2.2) were also the subject of the present studies. One testis from each of these rams was obtained either after castration on day 28 post-infection (5 control and 4 experimental) or after the rams were killed at the end of the experiment (day 58 post-infection in 5 control and 5 infected rams and on day 58 post-insulation in 6 scrotal-insulated rams). The testes were collected with intact tunica albuginea and put into labelled plastic bags, immediately immersed into iced water and then moved within 30 min of collection, to the laboratory for *in vitro* studies. Before the testes were removed, 5 ml jugular blood was collected in heparin from each ram and the plasma obtained was used for hormone measurements.

5.2.2. Testicular Cell Dispersion

In the laboratory, the tunica albuginea and epididymis were carefully separated from each chilled testis using a scalpel blade and the testes were weighed. Thereafter, the testes were cut transversely into two halves to expose the testicular pyrenchyma. Deep testicular slices were cut from each testis, weighed (about 100 - 200 mg) and then cut into smaller pieces. The remaining testicular tissue was frozen in a methanol-dry ice bath and stored at -20°C. The testicular cells were dispersed using collagenase as described by Stalvey and Payne (1983). In this method, the testicular pieces were transferred into 50 ml sterile polystyrene tubes (Nunc, Roskilde, Denmark) containing

15 ml Medium 199 (M199) (Gibco, Life Laboratories, Paisely, Strathcylide, U.K.) pre-gassed with 5% CO_2 in air. The M199 contained 1 mg ml^{-1} crude collagenase (Worthington, type CLS, purchased from Lorne Diagnostics, Reading U.K.), 0.01% deoxyribonuclease II (D.8764, Sigma) and 1% (w/v) bovine serum albumin (BSA) (Sigma).

Collagenase digestion was accelerated by transferring the tubes to a water bath at 37°C and shaking for 10 - 15 min using a Gallenkamp shaker. After 15 min the tubes were transferred to an ice bath and undigested tissue clumps were left for about 5 min to settle to the bottom of the tubes. Supernatant fluid, containing dispersed cells, was carefully aspirated into another 50 ml sterile polystyrene tubes and diluted with 35 ml cold M199 (containing 0.1% BSA) to stop the collagenase activity. To the remaining tissue clumps, a further 15 ml of M199 containing 1% BSA was added and the digestion was continued for 15 min aided by intermittent agitation with a pipette at 5 min intervals. At the end of the second digestion, the undigested tissues were again left to settle in an ice bath for 5 min and the supernatant was aspirated. The first and second supernatants containing dispersed cells collected from the same tissue were then pooled. The dispersed cells were collected following 5 min centrifugation at 500 g at 4°C in an MSE Centrifuge and the supernatant was discarded by gently tipping. The cell pellets were washed once with 20 ml M199, containing 0.1% BSA followed by centrifugation.

The cells were re-suspended in 2 ml M199 containing 0.1% BSA and cell numbers were counted using a haemocytometer. The percentage of Leydig cells in each fraction was determined by histochemical staining for 3ß-hydroxysteroid dehydrogenase (3ß-HSD), a steroidogenic enzyme, present in steroid producing cells, which is involved in the transposition of the double bond present in Δ^5 - steroids (converted from Δ^5 pregnenolone) to Δ^4 - steroids which include testosterone (Ewald *et al.*, 1964). In the testis 3ß-HSD is mainly present in Leydig cells (O'Shaughnessy and Murphy, 1991). Staining of the Leydig cells was done according to Payne *et al.* (1980) using the nitro-blue tetrazolium (NBT) stain (9.4 ml PBS pH 7.2, 10 mg BSA, 10 mg nicotinamide adenine dinucleotide (Sigma N-1511), 2 mg NBT (Sigma) and 0.6 ml of 1 mg ml^{-1} pregnenolone (Sigma) in dimethylsulphoxide (Sigma)). The proportion of Leydig cells in the testis digests varied from 1.5% to 3.3%.

5.2.3. Cell Incubation

To measure steroid production in dispersed Leydig cells, a 100 µl fraction of each cell suspension was aliquoted into each of twelve 20 ml plastic scintillation vials (Packard). The 12 vials containing cells of the same tissue were divided into 3 batches of 4 vials. A maximally stimulating dose of hCG (200 mUI) (Sigma), dbcAMP (2mM) (Sigma) and 22ROHC (25 µM) (Sigma) in 900 µl M199 or M199 alone was then added to 3 of the tubes according to O'Shaughnessy *et al.* (1991). The tubes were gassed with 5% CO_2 for 5 min, capped and incubated at 32°C for 3 h. Incubated tubes were shaken gently at 30 min intervals. At the end of the incubation period the incubated cells were centrifuged at 1500 g for 5 min and the supernatants were removed and stored frozen at -20°C for hormone analyses.

5.2.4. Testicular Steroid Contents

To measure testicular steroid concentrations, 100 - 200 mg slices of testicular tissue were cut in duplicate from the frozen testes, weighed quickly and then cut into small pieces. The frozen, cut pieces were transferred into a 10 ml glass-teflon homogeniser containing 5 ml of absolute ethanol (Analar grade, BDH) and approximately 2000 cpm ^{14}C-testosterone (Amersham) was added to monitor recovery. The tissues were homogenised in iced water to extract the steroids. Once complete homogenization was achieved, the homogenates were transferred carefully into 13 x 100 mm borosilicate glass tubes (Ciba-Corning, Essex, U.K.) and centrifuged at 1500 g in an MSE centrifuge at 4°C for 25 min. Ethanol containing the extracted steroids was tipped into 12 x 75 mm borosilicate glass tubes and then dried under air using a Tecam SC-3 sample concentrator (Techne, Cambridge, UK) connected to a vacuum pump. The dried extraction residues were re-dissolved in 500 µl assay buffer (0.1M phosphate buffered saline , 0.1% gelatin, pH 7.0, see Appendix 2), vortexed and then left overnight at room temperature to ensure maximal dissolution in the assay buffer. The extraction mixture was assayed for testosterone and progesterone by radioimmunoassay and results are expressed as steroid concentration per gm of testis and steroid concentration per testis. The steroid recoveries varied between (95 - 110%) and hence no corrections for recoveries were made.

5.2.5. Steroid Radioimmunoassays

The concentration of testosterone in plasma samples was measured after extraction using ^{125}I-RIA and progesterone was measured without extraction using an enzyme immunoassay kit (Ridgeway Science Ltd, Alvington, U.K.). Both assays have been validated for use with ovine plasma, the former as described previously in Chapter IV 4.2.4, and the latter by the manufacturer. Testosterone and progesterone concentrations from frozen extracts obtained after *in vitro* cell incubation (Section 5.2.3) and from ethanol extracts of testicular tissues (Section 5.2.4) were measured directly without extraction using assays validated for use in such extracts. The assay were the ^{3}H-competitive RIAs described by Sheffield and O'Shaughnessy (1989) for testosterone and O'Shaughnessy *et al.* (1990) for progesterone. The primary antibodies, standards and ^{3}H-tracers for testosterone and progesterone were kindly provided by Dr. P.J. O'Shaughnessy of the Department of Veterinary Physiology, University of Glasgow Veterinary School. Originally, the respective ^{3}H-tracers were purchased from Amersham, standards from Sigma, testosterone antiserum from Guildhay Antisera, (Surrey, U.K.) and progesterone antiserum was donated by Dr. J.A. Foulkes of the Cattle Breeding Centre, Shinfield, U.K. The tracers were used at approximately 15,000 cpm per 100 µl, testosterone and progesterone standards were used in serial concentrations ranging from 0.2 - 25.6 pmol l^{-1} for testosterone and 0.5 - 33.0 pmol ml^{-1} for progesterone. The antisera were provided already pre-diluted and were further diluted by 1: 200 for the testosterone and 1:70 for the progesterone radioimmunoassays.

Both the testosterone and progesterone assays were run in a similar manner. Namely, 100 µl of the sample or standard was aliquoted into 10.5 x 70 mm borosilicate assay tubes followed by addition of 100 µl label and 100 µl antiserum. The tubes were vortexed and incubated overnight at 4°C. Separation of the bound from unbound fraction was done with addition of 0.25 ml of dextran-coated charcoal suspension (50 mg dextran + 0.5 g activated charcoal in 100 ml assay buffer) per tube and incubation at 4°C for 15 min. After incubation, tubes were spun at 1500 g at 4°C for 5 min and the supernatants were tipped into 10 ml scintillation vials containing 4 ml Ecoscint A (New Jersey, U.S.A.). Counting was performed using a liquid scintillation counter Tri-Carb

1600 TR (Packard) and results were computed using the computer programme described in Chapter IV 4.2.4.

Sample analysis was completed in two assays. The intra-assay CV in two samples each replicated 12 times in one assay for testosterone at 2.56 pmol l^{-1} and 18.37 pmol l^{-1} and progesterone at 123.6 pg ml^{-1} and 1668 pg ml^{-1}, (n = 12) were <10%. The assay limits of detection at 2 x standard deviation (SD) of zero standard were 100 fmol ml^{-1} for testosterone and 0.24 pmol ml^{-1} for progesterone RIA.

5.2.6. Statistical Analysis

Results were analysed using two sample t-test or analysis of variance followed by Newman-Keuls Multiple Range test using MINITAB (Minitab Inc, State College, PA, USA).

5.3. RESULTS

5.3.1. Plasma and Intratesticular Steroid Concentrations

Mean plasma testosterone concentration in 4 infected rams tended to be lower in infected than in control rams on day 28 of infection but these differences were not significantly different (Table 5.1). Similarly, mean plasma testosterone levels in 5 infected rams on day 58 post-infection and in 6 scrotal-insulated rams on day 58 post-insulation tended to be low, but were not significantly different from control values. Plasma progesterone concentration was undetectable in all rams at both occasions.

The testes from infected rams on day 28 post-infection had a 2-fold higher mean testosterone concentration than testes from control rams (Table 5.1). This difference was significant whether intratesticular testosterone content was expressed per gm testis ($P<0.01$) or per whole testis ($P<0.001$). On day 58 post-infection or post-insulation, scrotal-insulated rams had significantly higher ($P<0.001$) intratesticular testosterone content than controls, whereas testosterone levels were lower ($P<0.05$) in the 4 infected ram testes than controls (Table 5.1). One infected ram had a 4-fold higher testicular testosterone content on day 58 post-infection than the other 4 infected animals and its testosterone levels are presented separately. The intratesticular testosterone content in

Table 5.1. Plasma and intratesticular testosterone and progesterone levels in the uninfected (control), *T. congolense*-infected (infected) and scrotal-insulated (scr.-insulated) rams

		Testosterone			Progesterone		
		Control	Infected	Scr.-insulated	Control	Infected	Scr.-insulated
Day 28	**Plasma** (nmol l^{-1})	6.86 ± 1.75 (5)	3.37 ± 0.41 (4)	---	ND (5)	ND (4)	---
	Intratesticular (nmol/gm testis)	551.1 ± 50.4 (5)	1084.6 ± 52.2 (4)^{b}	---	ND (5)	ND (3)	--- 12.19 (1)
	(µmol testis^{-1})	44.95 ± 7.54 (5)^{x}	100.6 ± 10.3 (4)^{cx}	---	ND (5)	ND (3)	--- 0.88 (1)
Day 58	**Plasma** (nmol l^{-1})	4.57 ± 1.57 (5)	1.73 ± 0.55 (5)	3.31 ± 1.37 (6)	ND (5)	ND (4)	ND (6)
	Intratesticular (nmol/gm testis)	395.4 ± 40.4 (5)	261.2 ± 14.2 (4)^{a}	1134.0 ± 134.0 (6)^{c} 834.7 (1)	ND (5)	ND (4)	15.85 ±1.19 (6)
	(µmol testis^{-1})	37.08 ± 5.1 (5)^{x}	15.13 ± 2.8 (4)^{ay}	81.93 ± 7.93 (6)^{c} 55.33 (1)	ND (5)	ND (4)	1.11 ± 0.12 (6)

ND = Not detectable

Values are means ± s.e.m. for number of animals in parentheses

Differences between the infected , scrotal-insulated groups and corresponding control group in the same row are denoted by superscripts a,b,c ($^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$)

Differences between results of day 28 and 58 within each group (i.e control or infected) within the same column are denoted by superscript *x,y* (*xx* = not significant, *xy* = significant at P<0.01)

the control rams on day 28 and 58 was not significantly different but the mean values of day 58 in 4 infected rams was significantly lower ($P<0.01$) than the respective mean values of day 28.

The intratesticular progesterone content was undetectable in control rams on day 28 while a detectable amount (0.88 μmol per testis) was measured in the testes of one infected ram. Progesterone content was undetectable in infected and control testes on day 58 post-infection. Mean progesterone levels of 1.11 ± 0.12 μmol per testis were observed in scrotal-insulated testes.

5.3.2. Steroid Production by Isolated Leydig Cells

Testosterone production

Total testosterone production by isolated Leydig cells on days 28 and 58 post-infection are shown in Fig. 5.1 and Fig 5.2. Leydig cells isolated from control and infected testes produced almost similar basal levels of testosterone (control mean 39.03 ± 2.86 vs infected mean 39.11 ± 10.05 pmol per 10^5 Leydig cells) on day 28 post-infection. When cells were incubated with hCG, dbcAMP and 22OHC, testosterone production by Leydig cells from control testes increased about 7.2-fold after hCG, 3.5-fold after dbcAMP and 26.2 fold after 22ROHC treatment. The corresponding values for the infected group were 3.2-fold after hCG, 2.6-fold after dbcAMP and 15-fold after 22ROHC treatment. Testosterone production by Leydig cells from the infected group was significantly lower ($P<0.001$) after hCG and 22ROHC stimulation compared to control values.

On day 58 post-infection (Fig 5.2) basal testosterone production by Leydig cells from infected testes was significantly lower ($P<0.05$) (mean 17.89 ± 2.52 pmol per 10^5 Leydig cells) than cells from control testes (mean 32.29 ± 2.33 pmol per 10^5 Leydig cells) but not compared to cells from scrotal-insulated testes (mean 26.56 ± 2.43 pmol per 10^5 Leydig cells). Testosterone production by control and scrotal-insulated Leydig cells were not significantly different. Addition of hCG increased testosterone production by about 8-fold in the Leydig cells from control rams compared to a 5-fold increase in those from the scrotal-insulated rams and a 4.7-fold increase in infected rams ($P<0.001$), (scrotal-insulated vs infected groups not significant). There was no significant

difference between groups in testosterone production after addition of dbcAMP. Mean testosterone levels produced by the infected and scrotal-insulated Leydig cells after the addition of 22ROHC were not significantly different but both were significantly lower ($P<0.05$) than values from control cells. This is despite the responsiveness of the infected group being 37-fold compared to 28-fold and 24-fold response in the control and scrotal-insulated groups, respectively.

On comparing day 58 with 28 post-infection values, testosterone production by control Leydig cells under basal conditions or after stimulation by hCG, dbcAMP or 22ROHC was not significantly different, but testosterone production in infected cells on day 58 post-infection was significantly lower ($P<0.05$) under basal conditions but not after stimulation with hCG, dbcAMP or 22ROHC.

Progesterone production

Production of progesterone by Leydig cells *in vitro* under basal conditions or after addition of hCG or dbcAMP was very low or undetectable in all groups on day 28 post-infection (Fig 5.3) and day 58 post-infection (Fig 5.4). In contrast, progesterone production by cells from control and infected rams in response to 22ROHC was high and of similar magnitude on day 28 post-infection. On day 58 post-infection, progesterone production by the infected Leydig cells was 3.1-fold higher ($P<0.001$) than control while scrotal-insulated cells were 2.3-fold higher ($P<0.001$) than control in the presence of 22ROHC. Progesterone production from infected and scrotal-insulated cells was not significantly different. No detectable increase in progesterone was measured from Leydig cells collected from one control ram in the presence of 22ROHC and this data was excluded from the group progesterone values.

Comparing day 58 with 28 values in the presence of 22ROHC, there was no significant difference in progesterone production between control Leydig cells on both occasions or with infected cells on day 28 post-infection. Progesterone production by Leydig cells from infected rams was significantly higher ($P<0.001$) on day 58 post-infection than control (day 28 and 58) and infected (day 28) rams.

Fig. 5.1 Testosterone production by Leydig cells isolated from testes of uninfected (n = 5, control) and *T. congolense*-infected (n = 4, infected) rams on day 28 post-infection. Cells were incubated in M199 under basal conditions or in the presence of human chorionic gonadotrophin (hCG), dibutyryl cyclic adenosine monophosphate (dbcAMP) or 22R-hydroxycholesterol (22ROHC) for 3-h and the medium was collected for hormonal assay. Values are means (± s.e.m.) of triplicate incubations. Values with different superscripts within each group (i.e basal, hCG-, dbcAMP-, 22ROHC-stimulated) are significantly different $^{ab}(P<0.05)$, $^{ac}(P<0.001)$.

Fig. 5.2 Testosterone production by Leydig cells isolated from testes of uninfected (n = 5, control), *T. congolense*-infected (n = 5, infected) and scrotal-insulated (n = 6, scrotal-insulated) rams on day 58 post-infection or post-insulation. Cells were incubated in M199 under basal conditions or in the presence of human chorionic gonadotrophin (hCG), dibutyryl cyclic adenosine monophosphate (dbcAMP) or 22R-hydroxycholesterol (22ROHC) for 3-h and medium collected for hormonal assay. Values are means (± s.e.m.) of triplicate incubations. Values with different superscripts within each group (i.e basal, hCG-, dbcAMP-, 22ROHC-stimulated) are significantly different $^{ab}(P<0.05)$, $^{ac}(P<0.001)$.

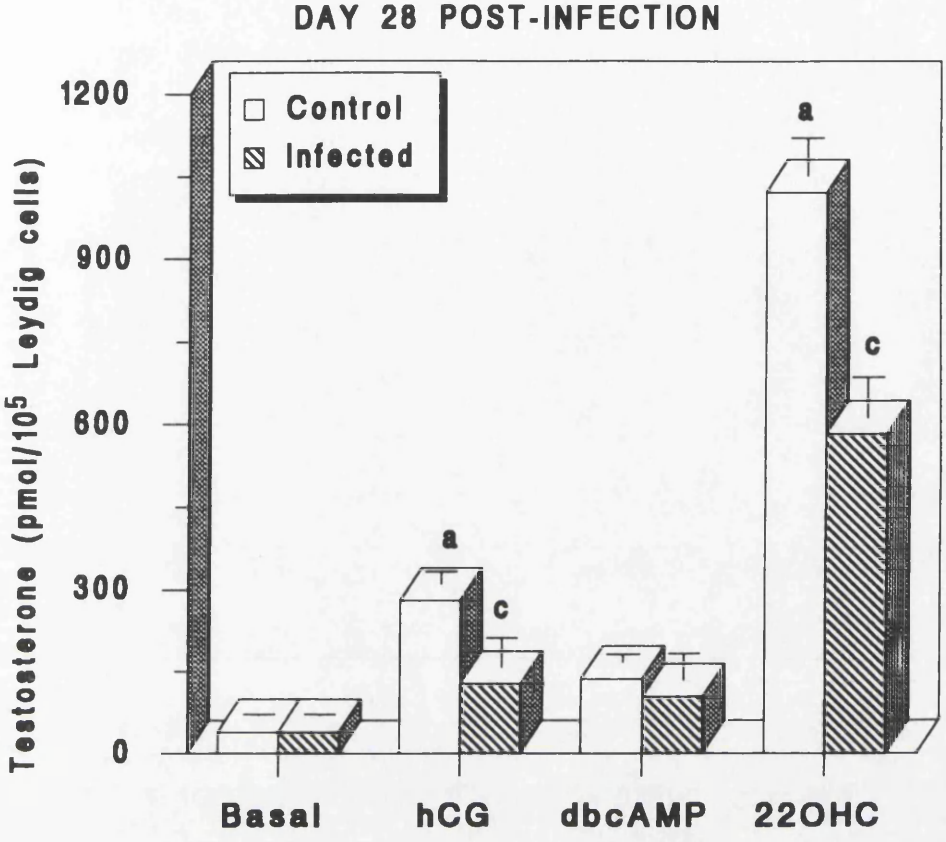

DAY 28 POST-INFECTION
Control
Infected
Testosterone (pmol/10⁵ Leydig cells)
1200
900
600
300
0
a
c
a
c
Basal
hCG
dbcAMP
22OHC

Fig 5.1

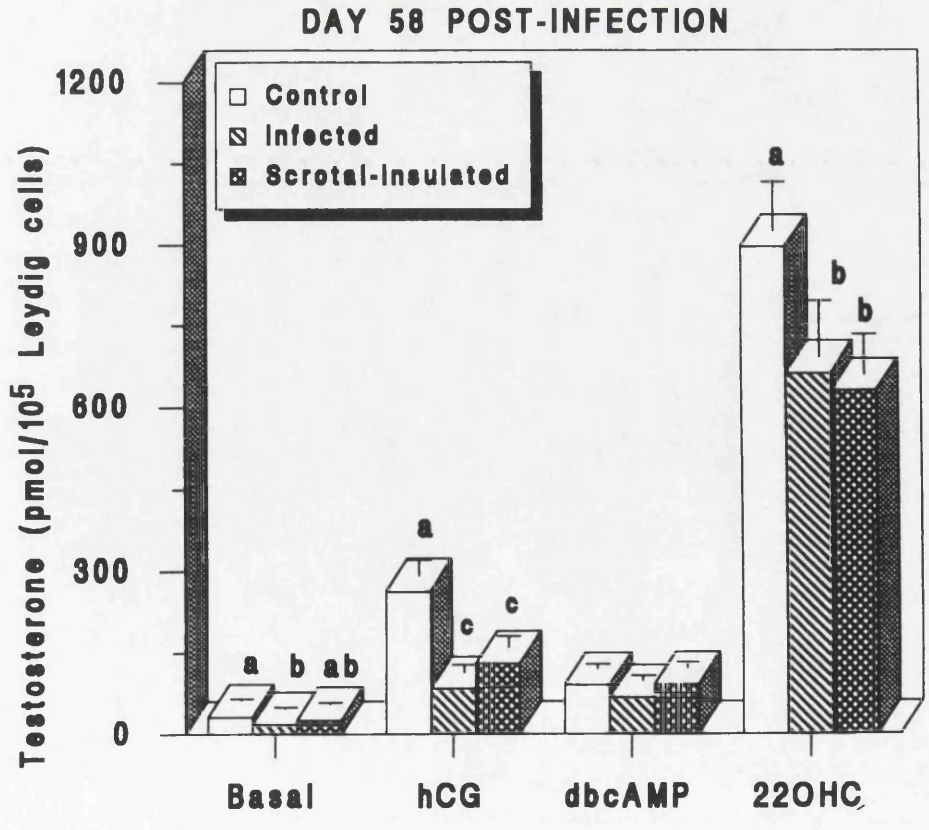

DAY 58 POST-INFECTION
Control
Infected
Scrotal-Insulated
Testosterone (pmol/10⁵ Leydig cells)
1200
900
600
300
0
a
b
b
a
b
ab
a
c
c
Basal
hCG
dbcAMP
22OHC

Fig 5.2

Fig. 5.3 Progesterone production by Leydig cells isolated from testes of uninfected (n = 5, control) and *T. congolense*-infected (n = 4, infected) rams on day 58 post-infection. Cells were incubated in M199 under basal conditions or in the presence of human chorionic gonadotrophin (hCG), dibutyryl cyclic adenosine monophosphate (dbcAMP) or 22R-hydroxycholesterol (22ROHC) for 3-h and medium collected for hormonal assay. Values are means (± s.e.m.) of triplicate incubations. ND = Not detectable.

Fig. 5.4 Progesterone production by Leydig cells isolated from testes of uninfected (n = 5, control), *T. congolense*-infected (n = 5, infected) and scrotal-insulated (n = 6, scrotal-insulated) rams on day 58 post-infection. Cells were incubated in M199 under basal conditions or in the presence of human chorionic gonadotrophin (hCG), dibutyryl cyclic adenosine monophosphate (dbcAMP) or 22R-hydroxycholesterol (22ROHC) for 3-h and medium was collected for hormonal assay. Values are means (± s.e.m.) of triplicate incubations. Values with different superscripts within each group (i.e basal, hCG-, dbcAMP-, 22ROHC-stimulated) are significantly different $^{ab}(P<0.05)$, $^{ac}(P<0.001)$. ND = Not detectable.

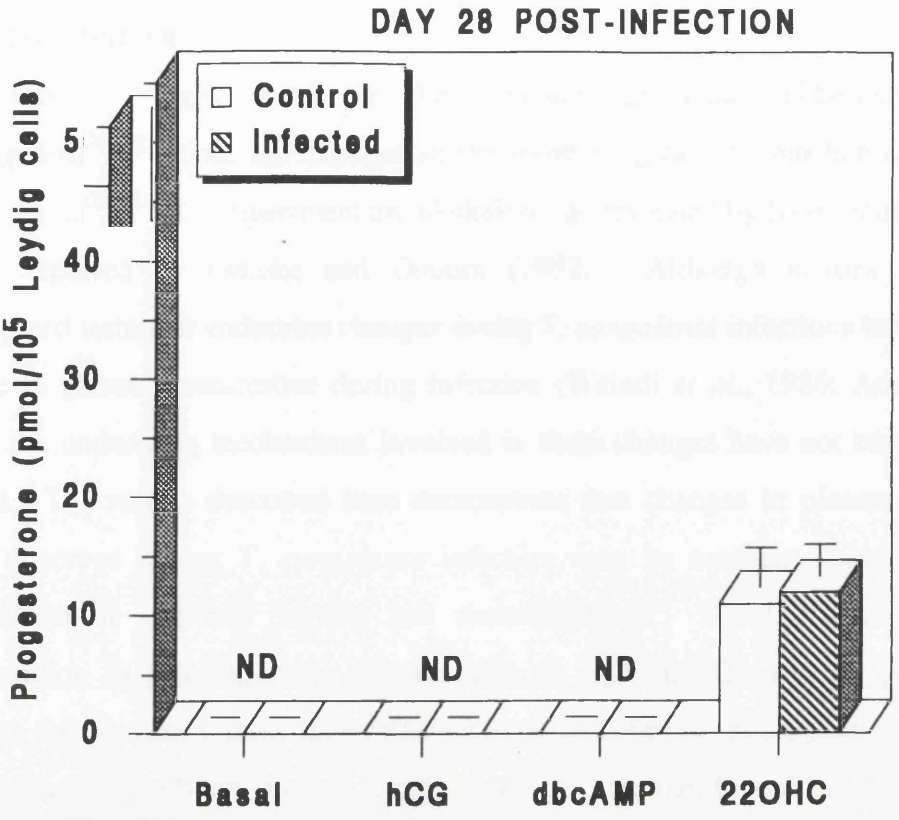

DAY 28 POST-INFECTION
Control
Infected
Progesterone (pmol/10⁵ Leydig cells)
0
10
20
30
40
5
ND
ND
ND
Basal
hCG
dbcAMP
22OHC

Fig 5.3

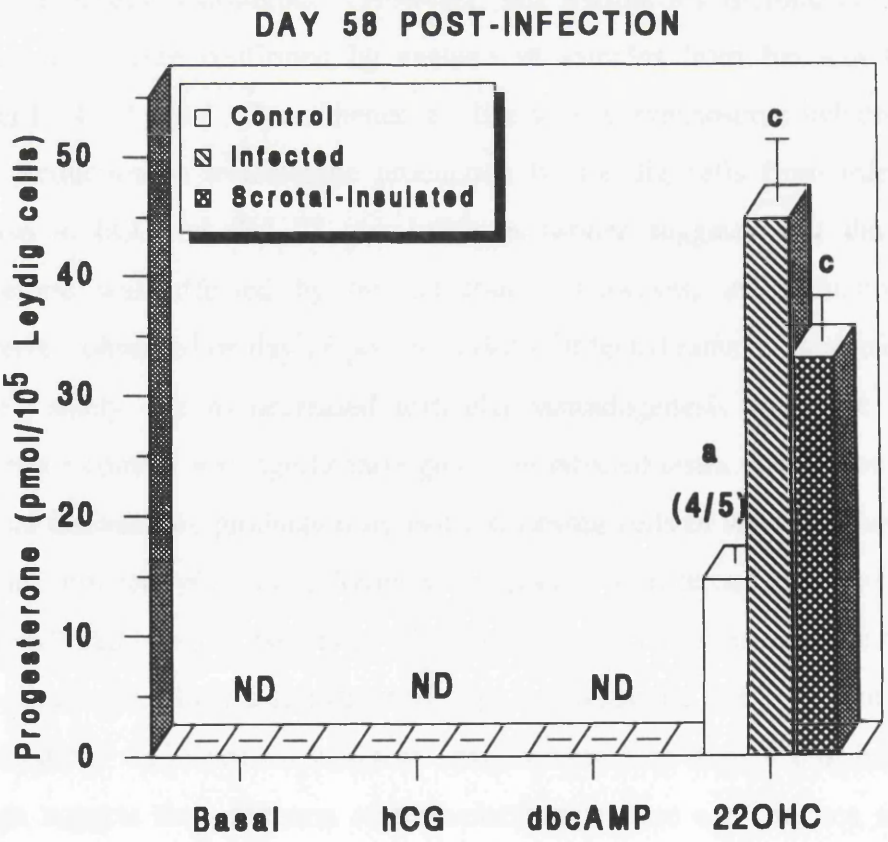

DAY 58 POST-INFECTION
Control
Infected
Scrotal-Insulated
Progesterone (pmol/10⁵ Leydig cells)
0
10
20
30
40
50
ND
ND
ND
a
(4/5)
c
c
Basal
hCG
dbcAMP
22OHC

Fig 5.4

5.4. DISCUSSION

Gross changes occur in the testicular germinal epithelium following *T. congolense* infection, but changes in the morphological and biochemical functions within the interstitial compartment are ill-defined as reviewed by Ikede *et al.* (1988) and further reported by Omeke and Onuora (1992). Although studies which have investigated testicular endocrine changes during *T. congolense* infections have reported a decline in plasma testosterone during infection (Waindi *et al.*, 1986; Adeyemo *et al*, 1990), the underlying mechanisms involved in these changes have not been previously studied. The results described here demonstrate that changes in plasma testosterone levels observed during *T. congolense* infection may be associated with progressive perturbation of testicular Leydig cell steroidogenesis. Basal plasma testosterone concentration in infected rams was reduced on both day 28 and 58 post-infection although this was not significantly different from the controls. Since plasma testosterone was measured in only one plasma sample from each animal, it could be argued that the low levels observed in the infected rams could be associated with the pulsatile secretory pattern of plasma testosterone. However, low plasma testosterone concentrations in infected rams were confirmed by analysis of samples from frequent bleeding (see Chapter IV 4.3.2 and 4.3.3) and hence, are likely to be trypanosome-induced.

Reduction in testosterone production by Leydig cells from infected rams in response to hCG on day 28 post-infection further suggests that the synthesis of testosterone was affected by the infection. However, the reduction in plasma testosterone observed on day 28 post-infection in infected rams in the present study may not be solely due to decreased testicular steroidogenesis since the intratesticular testosterone content was significantly greater in infected testes than in control testes and the basal testosterone production by isolated Leydig cells *in vitro* was not significantly different from controls values. Reduction in plasma testosterone in infected rams during this period might also be associated with impaired transfer of testosterone from the testes to the circulation. Since a similar trend (high intratesticular and low plasma testosterone concentration) was observed in scrotal-insulated rams on day 58 post-insulation, these findings suggest that elevation of testicular temperature can produce an increase in intratesticular testosterone along with a reduction in plasma testosterone concentration.

The observed plasma testosterone changes in infected rams on day 28 could therefore be explained by increased testicular temperature resulting from the infection (see Chapter III 3.3.7) affecting testicular blood flow. High testicular temperature is known to reduce testicular blood flow and interfere with testosterone secretion from the testis (Damber *et al.*, 1984; Galil and Setchell, 1988a, 1988b). However, there is also the possibility that the high intratesticular testosterone content observed in testes from infected sheep on day 28 post-infection could be associated with the increased plasma LH levels which were observed in these rams during this period (see Chapter IV 4.3.2. and 4.3.3). However, since high intratesticular testosterone content in infected rams was associated with low plasma testosterone on day 28 post-infection, the changes in plasma and intratesticular testosterone concentrations in these rams can be explained partly by decreased efflux of testosterone from the testis possibly caused by a decreased blood flow. Changes in testicular blood flow and its effects on testosterone secretion during trypanosome-infections were not investigated in the present studies and have not been reported elsewhere. Hence, further studies in this area will be required to assess the effects of *T. congolense* on testicular blood flow and its relationship with testosterone secretion in infected animals.

Changes in plasma testosterone on day 58 post-infection in infected rams appeared to be due to decreased testicular steroidogenesis since the intratesticular testosterone content was low in 4 out of 5 infected rams and the *in vitro* basal testosterone production by isolated Leydig cells from infected rams was also significantly lower than that in Leydig cells from controls animals. These results complement the findings reported in Chapter IV and reported by others (Waindi *et al.*, 1984; Adeyemo *et al.*, 1990) that testicular endocrine changes induced by *T. congolense* are progressive and are more marked with advancing duration of infection. Furthermore, the decreased response of the Leydig cells of infected rams to produce testosterone after *in vitro* hCG stimulation both on day 28 and 58 post-infection in the present studies were similar to those shown by Soudan *et al.* (1992) and also provide evidence to explain the low plasma testosterone response observed after GnRH stimulation in infected rams in this study (see Chapter IV 4.3.1.2, 4.3.2.2 and 4.4.3.3).

However, testosterone production by ram Leydig cells after stimulation with dbcAMP was reduced compared to the production after hCG stimulation and was not significantly different between groups. These results were in disagreement with the response observed in Leydig cells from mice where testosterone response to hCG and dbcAMP at the same doses as those used in the present study produced equivalent testosterone responses (Murphy and O'Shaughnessy, 1991; O'Shaughnessy *et al.*, 1991). The discrepancy between these results cannot be explained completely but might be related to differences in testicular steroidogenic activity in rams and mice because the doses of hCG and dbcAMP used in these investigations were based on the doses required to produce a maximum testosterone response in mice (Murphy and O'Shaughnessy, 1991; O'Shaughnessy *et al.*, 1991). However, the concentration of hCG used in the present study was more than 10 times that required to elicit a maximum testosterone response in mice and it therefore seems likely that it should have been sufficient to evoke maximum testosterone response in ram Leydig cells. It is probable that the dose of dbcAMP used might have been submaximal for ram Leydig cells. This discrepancy in Leydig cell testosterone response to hCG and dbcAMP in this study means that it was not possible to establish whether the decreased steroidogenic capacity of Leydig cells isolated from infected rams was induced by a decline in LH receptor activity as previously described by Soudan *et al.* (1992). An attempt to establish an LH receptor assay was not successful during the present studies, hence LH receptors could not be measured.

However, the present studies show that the decreased testosterone production by Leydig cells from infected rams could be related partly to the decreased steroidogenic activity since the defect was not overcome by incubation of the cells on day 28 and 58 post-infection with 22ROHC. Addition of 22ROHC to Leydig cells by-passes the LH receptor-cAMP system and supplies excess substrate to the mitochondria for pregnenolone synthesis. This process was shown to be LH dependent (Rommerts and Brinkman, 1981), and to be the rate-limiting step in steroid synthesis, catalysed by cytochrome P450scc (Halkerston *et al.*, 1961; Koriz and Kumar, 1970; Miller, 1988). Previous studies have shown that 22ROHC is more potent as a substrate for Leydig cell mitochondrial cholesterol side-chain cleavage than 25-hydroxycholesterol which was

commonly used for this type of study (Quinn *et al.*, 1985). The similarities in the *in vitro* testosterone synthesis findings obtained in infected and scrotal-insulated rams after hCG and 22ROHC stimulation on day 58 post-infection and post-insulation, suggest that the mechanisms of altered Leydig cell steroidogenesis in both groups might be similar and may be explained on the basis of elevation of testicular temperature. Elevation of testicular temperature is known to reduce the testicular LH receptor content (Huhtaniemi *et al.*, 1984; Hovatta *et al.*, 1986) and to affect several Leydig cell organelles associated with steroidogenesis (Kerr *et al.*, 1979; Bergh *et al.*, 1984a; Ezeasor, 1985). Furthermore several enzymes involved in the Δ^4 and Δ^5 testosterone biosynthetic pathways are affected by increased temperature (LeVier and Spaziani, 1968; Inano and Tamaoki, 1968; Munabi *et al.*, 1984; Farrer *et al.*, 1985; Steinberger, 1991). Steroid biosynthetic enzymes known to be particularly heat sensitive include 17α-hydroxylase and 17ß-dehydrogenase (Bergh *et al.*, 1984b), 3ß-hydroxysteroid hydrogenase (Inano and Tamaoki, 1968) and 17,20-lyase (Farrer *et al.*, 1985). Elevation of testicular temperature, whether by infecting trypanosomes or by scrotal-insulation, could have affected the activity of cytochrome P450c17α which mediates the activities of both 17α-hydroxylase and 17, 20-lyase, enzymes involved in the conversion of progesterone to 17α-hydroxyprogesterone and androstenedione (Miller, 1988) in the Δ^4 testosterone biosynthetic pathway. This supposition is supported by the presence of a high intratesticular progesterone content in scrotal-insulated rams and an increased progesterone: testosterone production ratio by Leydig cells of infected and scrotal-insulated rams on day 58 post-infection after stimulation with 22ROHC. Impairment of the conversion of progesterone to testosterone caused by reduced 17α-hydroxylase activity occurs in other conditions which induce elevation of testicular temperature, such as cryptorchidism (Ficher and Steinberger, 1982; Murphy and O'Shaughnessy, 1991).

However, during *T. congolense*-infection in rams, the elevation of testicular temperature occurred as part of a systemic febrile response which is usually activated by several endogenous pyrogenic cytokines including IL-1α; IL-1ß; IL-6, TNF-α, IFN-α, IFN-γ and IL-8 (Whicher and Westacott, 1992). The secretion of these cytokines is known to be activated during trypanosome infections (reviewed in Chapter I and discussed in Chapter III and IV). There is an increasing body of evidence to suggest that

cytokines alter the function of several endocrine glands including the reproductive system (reviewed by Kennedy and Jones, 1990) and recent studies have shown that TNF-α (Xiong *et al.*, 1993) and IL-1 (Sun *et al.*, 1993) may have a direct effect in regulating Leydig cell steroidogenesis. It thus seems possible that cytokine activation during *T. congolense* infection might have contributed to the observed Leydig cell dysfunction although further studies in this area are required.

In conclusion these results show that the androgen production by Leydig cells progressively declines with time after *T. congolense* infection. The underlying causes might be multifactorial but the elevation of testicular temperature which occurs as a result of trypanosome-induced pyrexia may contribute to these changes by altering the function of several enzymes involved in steroidogenesis.

CHAPTER VI

EFFECTS OF *TRYPANOSOMA CONGOLENSE* INFECTION ON THE PITUITARY-ADRENAL FUNCTION OF THE RAM

6.1 INTRODUCTION

Following the elucidation of the molecular structure of corticotrophin-releasing hormone (CRH) from sheep by Vale *et al.* (1981) its physiological significance in regulating the secretion of adrenocorticotrophin hormone (ACTH) and cortisol secretion has been recognised (Donald *et al.*, 1983; Plotzky and Vale, 1984; Rivier and Plotzky, 1986). Administering CRH centrally or intravenously elicits increases in plasma ACTH and cortisol. CRH has been used to test corticotrophic-adrenal responsiveness under various conditions (Pradier *et al.*, 1988; Shutt *et al.*, 1989; Parrot, 1990). It has been shown that through CRH, the hypothalamus plays a fundamental role in coordinating the body adaptations that constitute the stress response by modulating psychological, neuroendocrine and autonomic functions (Morbeg, 1985; Lenz *et al.*, 1987; Fisher, 1989).

Although the primary focus of most of the neuroendocrine studies has been on the stress response of the pituitary and adrenal gland, there is an increasing body of evidence which links stress-induced stimulation of ACTH and glucocorticoids with changes in other pituitary hormones including growth hormone, prolactin, thyroid stimulating hormone and the gonadotrophins (Moberg, 1985). Furthermore, evidence is now available which shows that stress induced-activation of the hypothalamo-pituitary-adrenal (HPA) axis might be responsible for subsequent infertility observed in male rats (Charpenet *et al.*, 1981; Rivier *et al.*, 1986), bulls (Johnson *et al.*, 1982), boars (Liptrap and Raeside, 1975) and rams (Naylor *et al.*, 1990).

Many disease processes are associated with neuroendocrine modulations which affect both the sympathetic adrenal-medullary and the pituitary-adrenocortical systems (Henry and Stephens-Larson, 1985). Trypanosome infections caused by the *T. brucei*-group have been shown to cause several psychoneuroendocrine anomalies in animals and man, possibly due to early parasitic invasion of the host central nervous system (CNS) including the sensory ganglia and circumventricular organs, area postrema, pineal gland, hypothalamus and median eminence (Stibbs, 1984; Stibbs and Curtis, 1987; Cosenza *et al.*, 1984; Schultzberg *et al.*, 1988; Amole *et al.*, 1989). There are also reports which have suggested that *T. congolense* infection may affect the function of CNS due to the tendency of the parasite to localise in large numbers in the

microvasculature of the CNS (Banks, 1978; Masake *et al.*, 1984). Reproductive disturbances have been described in infections caused by the *T. brucei*-group, *T. congolense* and *T. vivax* (reviewed in Chapter I and further discussed in Chapter III - V) which might be associated with altered functions of the hypothalamo-pituitary-gonadal and -adrenal axes. However, there no reports which have shown that modulation of the HPA axis by trypanosome infection may affect the function of the reproductive endocrine system in infected animals.

There are a few reports of adrenal abnormalities associated with pathogenic trypanosome infections. Increased adrenal weight, hypertrophy of the zona fasciculata-reticularis, focal necrosis and mononuclear cell infiltration have been described in *T. brucei*-infected sheep (Ikede and Losos, 1975) and *T. congolense*-infected goats (Mutayoba *et al.*, 1988a). Adrenocortical hyperplasia and moderate cytoplasmic atrophy of the zona fasciculata were described by Valli and Forsberg (1979) in calves infected with *T. congolense*. Ogwu *et al.* (1992) has recently described hyperaemia, haemorrhage and hyperplasia and severe mononuclear infiltration in the subcapsular and adrenocortical layers in *T. congolense*-infected zebu cattle. The information available on the effects of trypanosomiasis on adrenal steroidogenic activity is limited and also conflicting. Plasma cortisol levels were found to be significantly increased during the acute and chronic phases of *T. congolense* infection in goats (Mutayoba and Gombe, 1989). In contrast, only slight increases in plasma cortisol levels were observed during the prepatent period (Abebe, 1991) or at 2-6 weeks post infection (Ogwu *et al.*, 1992) in cattle infected with *T. congolense* and after this time the plasma cortisol concentration declined. The reasons for the differences in cortisol responses between goats and cattle during *T. congolense* infection have not been explained.

6.1.1. Objectives of the Study

The present studies were conducted on the rams used during Study I with the following objectives:

(i) **To determine the adrenal response to *T. congolense* infection in sheep giving particular attention to the relationship between changes in plasma cortisol levels and parasitaemia throughout the experimental period.**

This study was performed by measuring plasma cortisol levels in samples collected 2 times a week prior to infection and 3 times a week during the infection period. In addition the adrenal glands of all animals taken at post-mortem were used for histopathological studies.

(ii) **To determine whether the responsiveness of the HPA axis to intravenous injection of CRH is affected by trypanosome infection in rams, and by monitoring subsequent alteration in blood concentrations of reproductive hormones, to evaluate whether the infertility observed in trypanosomiasis could be associated with changes in the HPA axis.**

This study was performed by assessing the changes in plasma ACTH, cortisol, LH and testosterone in frequent blood samples collected after CRH injection at different stages of infection.

6.2. MATERIALS AND METHODS

6.2.1. Animals and Experimental Infection

The studies of the effects of *T. congolense* infection on pituitary-adrenal function were performed on the nineteen sheep (9 control and 10 infected rams) used during study I and previously described in Chapter III. 3.2.1. Experimental *T. congolense* infections were performed as described in Chapter II 2.2. During Study I, five infected and 4 control rams were killed on day 28 post-infection to provide samples for various histopathological studies described in this chapter.

6.2.2. Blood Sampling

Routine blood sampling

Blood samples used for monitoring cortisol concentrations throughout the experimental period were taken twice a week commencing one month prior to infection and three times a week during the infection period which ended 79 days after infection. Details of blood sampling techniques and storage of plasma samples for hormonal studies were described in Chapter II.2.4 and 2.5.

Frequent blood sampling and CRH injection

Ten rams (5 control and 5 infected) were used for CRH studies. On day 23 and day 65 post-infection, a jugular catheter was inserted into all rams as previously described in Chapter IV 4.2.2 between 8.00 - 10.00 am in the morning and kept patent with heparinized saline (200IU ml^{-1} in 0.9% w/v NaCl). Beginning 1 h later, 3 ml blood samples were taken every 20 min into heparinized tubes for a period of one hour. Each animal then received via the cannula a single dose of 25 μg synthetic ovine CRH (oCRH) (C-3167, Sigma, Poole, Dorset, UK) dissolved in 2 ml sterile saline. Subsequent blood samples were taken 5, 10, 20, 30, 40, 60, 90, 120 and 150 min after CRH injection. Blood samples were centrifuged immediately and the plasma snap frozen in dry ice and stored at -20°C for cortisol, adrenocorticotrophin (ACTH), luteinizing hormone (LH) and testosterone measurements. The CRH dose used was selected to give a pituitary-adrenal response lasting for 2 - 3 h in sheep (Pradier *et al.*, 1988).

6.2.3. Histopathology of the Adrenal Gland

Adrenal glands were removed at post mortem from control and infected rams killed on day 28 and 79 and on two infected rams which died on days 70 and 77 of the experiment (see Chapter III 3.2.8), weighed (with the exception of rams killed on day 28) and subsequently processed for routine histology as described in Chapter II 2.10. An average of six, 5μm sections per adrenal gland were either stained with Ehrlich's haematoxylin and eosin (H&E) stain or Martius Scarlet Blue (MSB) stain. Slides were examined by light microscopy and thickness of the adrenocortical layers was measured using a binocular micrometer (Graticules LTD, Tonbridge Kent, UK).

6.2.4 Hormonal Measurements.

Cortisol radioimmunoassay

A modified form of a solid phase competitive ^{125}I radioimmunoassay previously described by McConway and Chapman (1986) was used. This assay had previously been validated to measure cortisol in human serum/plasma. Extensive validation was performed and the limit of detection improved to permit accurate cortisol measurement

in ovine plasma. Cortisol for standard preparations was obtained from BDH, UK. ^{125}I-histamine-cortisol label was prepared using the Chloramine-T method according to Erlinger *et al.*, (1958) and was kindly provided by Dr. C. Gray of the Institute of Biochemistry, Royal Infirmary, Glasgow. Anti-cortisol serum (S004-201) was obtained from SAPU, (Carluke, Lanarkashire, UK). Its crossreactivity has previously been described (Naylor *et al.*, 1990). The preparation of steroid free ovine serum, standards and IgG-coated cellulose using the methods described by McConway and Chapman (1986) and the optimization of the cortisol standard curve were performed by Miss C. Seely of the Department of Clinical Veterinary Biochemistry, University of Glasgow Veterinary School. The antiserum was diluted as appropriate in 0.1M phosphate assay buffer pH 7.4 containing 0.05% sodium azide and 0.1% gelatin (see appendix Table 5). The cortisol label was diluted to give approximately 30,000 cpm in 100 µl of assay buffer containing 1 mg ml^{-1} of 8-anilino-naphthalen-1-sulphonic acid. In the assay, 50 µl duplicate aliquots of standards (ranging from 3.9 - 500 nmol l^{-1} prepared in steroid-free ovine serum) and samples were incubated for 1 h with 100 µl of labelled cortisol and 200 µl of cellulose-coated antibody diluted to give a maximum binding of 30 - 40%. Free and bound cortisol fractions were separated by centrifugation at 1500 g for 5 min and washing 3 times with saline. The bound fraction was counted on a Tri carb gamma counter model 3255 (Parkard) and the results were computed using the S.A.S. immunoassay computer programme (P.R. Edwards, Molecular Endocrinology, UCMSM, Winter 91). The recoveries of added cortisol (28.7, 36.6 and 97.8 nmol l^{-1}) in plasma samples containing different endogenous cortisol concentrations varied within an acceptably narrow range (96 - 110%). Assay limit of detection at 2 SD of maximum binding in the presence of steroid free ovine serum was 3.1 nmol l^{-1}. Intraassay coefficients of variation (CVs) were <10.8% (n = 13) for samples containing 14.6, 40.6 and 136.6 nmol l^{-1} and interassay CVs were <18.9% (n = 18) for samples containing 11.2, 31.7 and 77.15 nmol l^{-1}.

ACTH radioimmunoassay

Plasma ACTH was measured by a direct second antibody RIA standardised against NIBSC hACTH 74/555 (Holly Hill, Hampstead, UK) and using rabbit anti-

human ACTH (hACTH) serum described by Nicholson *et al.*, (1984). This antiserum is directed against the non-species specific amino-terminal (1-18) sequence of ACTH. ^{125}I-hACTH (1-39) prepared by the chloramine T method was used as a label. Normal rabbit serum and donkey anti-rabbit for all assays were provided by SAPU. The ACTH measurements were kindly performed by the staff at the Institute of Biochemistry, Royal Infirmary Glasgow with the assistance of Dr. C.E. Gray. The assay limit of detection of this assay is 3mU l^{-1} (1mU is approx. 4.03 ng ACTH) and intra- and interassay batch CVs are 8% and 16%, respectively.

LH and testosterone radioimmunoassay

Plasma LH and testosterone concentrations in frequent samples collected before and after CRH injection were determined by second antibody radioimmunoassays described by Jeffcoate (1992) and Cook and Beastall (1987), respectively. These assays were described in Chapter IV 4.2.4. For LH assay, the intraassay CV were <5% at 2.99 and 10.71 ng ml^{-1} and interassay CV were <13% (n = 5) at 2.86 and 11.32 ng ml^{-1}. For testosterone assay, the intraassay CV were <8% (n = 16) at 3.3 and 12.4 nmol l^{-1} and interassay CV were <12% (n = 9) at 3.0 and 22.6 nmol l^{-1}. The limits of detection of both assays were described in Chapter IV 4.2.4.

6.2.5 Statistics

The cortisol data obtained in samples collected three times a week and those obtained after CRH injection were analysed using the analysis of variance with a repeated measures design and followed, where applicable, with *post hoc* between group comparisons using the Newman-Keuls test (ANIMAL DESIGNS 1, V 1.21 5/6, Data International Service, Glasgow). The CRH-cortisol response data were also calculated as the area under the response curves of individual hormone concentrations against time using the Trapezoid rule according to Schreiber *et al.*, (1988) to test for differences between groups in the magnitude of hormonal response after CRH injection. The relationships between daily cortisol changes and parasitaemia levels were analysed by linear regression using MINITAB (Minitab Inc, State College, PA, USA). Unless specified, all values are presented as Mean $\pm$ SEM.

6.3. RESULTS

The results in this chapter are divided into 2 main sections. Section I describes results detailing the effect of *T. congolense* infection on changes in daily plasma cortisol levels and adrenal histopathological changes from all rams used during Study I. Section II describes the results of the pituitary-adrenal-gonadal response to CRH injection in 5 infected and 5 control rams used during Study I.

6.3.1. SECTION I

CORTISOL RESPONSE AND ADRENAL CHANGES ASSOCIATED WITH *TRYPANOSOMA CONGOLENSE* INFECTION IN RAMS

6.3.1.1. Changes in Plasma Cortisol Concentrations in Uninfected Control and *T. congolense*-infected Rams

Variations in mean plasma cortisol concentrations in blood samples collected two times a week from 28 days before the infection period and three times a week up to day 79 of the infection period in the control sheep are shown in Fig. 6.1. The mean cortisol and parasitaemia scores in the infected group are shown in Fig. 6.2. Variations in plasma cortisol and parasitaemia scores during the infection period in the individual infected rams which were killed on day 79 or died on day 70 and 77 are further depicted in Fig. 6.3a to Fig. 6.3e. There was little variation in plasma cortisol between control rams, so no individual control ram data is shown. Both control and experimental rams had similar pre-infection plasma cortisol mean values. Following infection changes in cortisol levels showed a significant group treatment effect ($P<0.01$) and group x time interaction ($P<0.001$). A significant rise in cortisol levels was observed in all infected animals between day 9 - 16 ($P<0.01$) which occurred concurrently with the onset of parasitaemia. A progressive nonsignificant tendency for plasma cortisol values to decline was observed from day 19 - 41 post-infection in all infected rams. Thereafter, all surviving infected rams showed increased but very variable adrenal activity from day 44 - 79 as reflected by highly fluctuating plasma cortisol values (Fig. 6.3a - Fig 6.3e). A significant increase in mean plasma cortisol concentrations was observed between day 51 - 56 ($P<0.01$) and day 77 - 79 ($P<0.05$) post-infection (Fig. 6.2). There was a

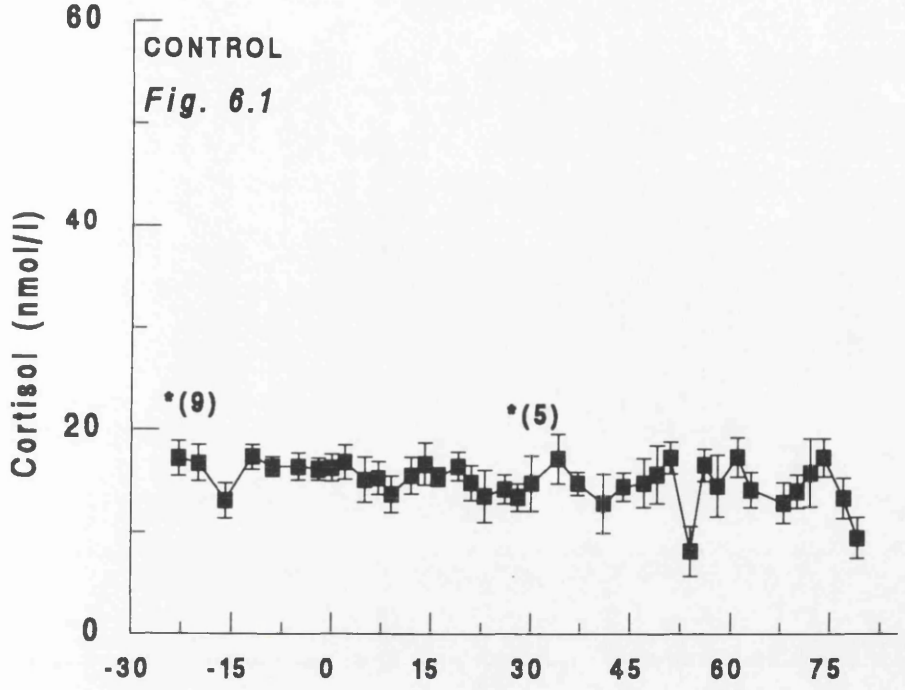
significant linear relationship between changes in parasitaemia and plasma cortisol levels in all infected rams from day 40 onwards; correlation varying from r = 0.52 ($P<0.05$) in ram 218 (Fig. 6.3e) to r = 0.63 ($P<0.01$) in ram 210 (Fig. 6.3a). The correlation coefficient values of other infected rams were intermediate between the latter two rams (r = 0.59 ($P<0.01$) for ram 212 (Fig. 6.3b), r = 0.53 ($P<0.05$) for ram 216 (Fig. 6.3c) and r = 0.59 ($P<0.02$) for ram 217 (Fig. 6.3d)).

6.3.1.2 Gross and Histopathology of the Adrenal Glands

No overt gross changes were observed in the adrenal glands of infected rams killed on day 28 post-infection but the adrenal glands of rams killed at the end of the study period appeared swollen and oedematous. The adrenal gland weights of the rams killed on day 28 post-infection were not measured. A nonsignificant increase in mean adrenal gland weight was observed in infected rams dying on days 70 and 77 and in those killed on day 79 when compared with control values (Table 6.1). However, the adrenal/liveweight ratios were significantly higher ($P<0.02$) in these infected rams in comparison to the controls. A nonsignificant increase in the thickness of the zona fasciculata-reticularis of the adrenal cortex was evident in infected rams killed on day 28. This effect was significant ($P<0.01$) in infected rams dying on day 70 and 77 and in those killed on day 79. Hypertrophy was not evident in the zona glomerulosa (Table 6.1) at any stage of infection. Apart from the hypertrophy of the zona fasciculata-reticularis, only mild histopathological changes were observed in the adrenals of infected rams killed on day 28. In contrast, adrenals from infected rams killed on day 79 showed marked changes including localised and diffuse subcapsular and cortical mononuclear cell infiltration (Fig. 6.4b); hypertrophy of the zona fasciculata-reticularis and accumulation of fat droplets in the zona glomerulosa (Fig. 6.4c) and focal coagulative necrosis in the zona fasciculata (Fig. 6.4d). Fig 6.4a shows a normal adrenal gland from uninfected control sheep.

Fig. 6.1 Mean (± s.e.m.) plasma cortisol concentration in uninfected control rams (n = 9) in samples collected twice a week before infection and three times a week during the infection period of 79 days. Four control rams were killed on day 28 post-infection. Numbers in parentheses indicate number of surviving rams in a group.

Fig. 6.2 Mean (± s.e.m.) plasma cortisol concentration and parasitaemia scores in *T. congolense*-infected rams (n = 10) in samples collected twice a week before infection and three times a week during the infection period of 79 days. Five infected rams were killed on day 28 post-infection, one died on day 70 and another on day 77 due to secondary *P. haemolytica* infection. Number in parentheses as above repeat.


CONTROL
Fig. 6.1
Cortisol (nmol/l)
60
40
20
0
*(9)
*(5)
-30
-15
0
15
30
45
60
75

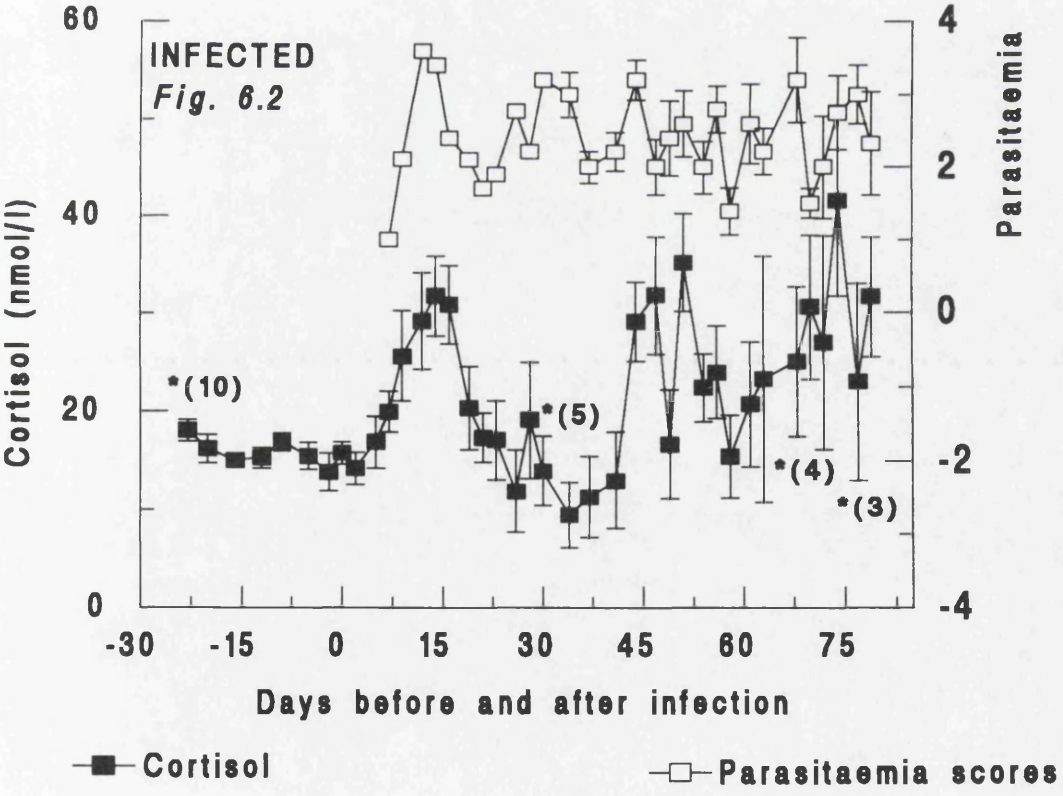

INFECTED
Fig. 6.2
Cortisol (nmol/l)
Parasitaemia
60
40
20
0
4
2
0
-2
-4
*(10)
*(5)
*(4)
*(3)
-30
-15
0
15
30
45
60
75
Days before and after infection
Cortisol
Parasitaemia scores

Fig. 6.3. Individual plasma cortisol concentrations and parasitaemia scores in *T. congolense*-infected rams (n = 5) in samples collected twice a week before infection and three times a week during the infection period of 79 days. Sheep No 217 died on day 70 and No 216 on day 77 post-infection due to secondary *P. haemolytica* infection.

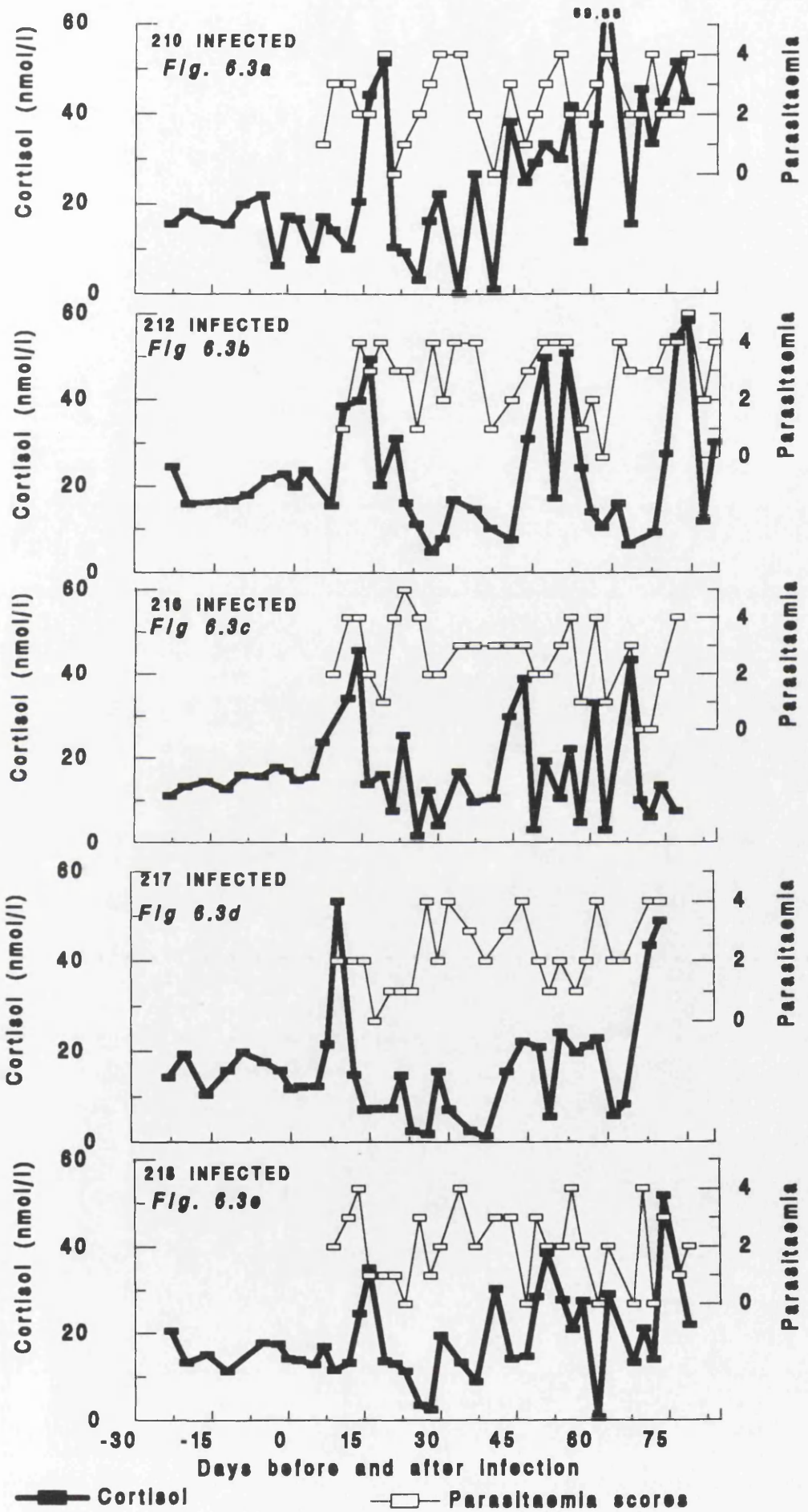

210 INFECTED
Fig. 6.3a
212 INFECTED
Fig 6.3b
216 INFECTED
Fig 6.3c
217 INFECTED
Fig 6.3d
218 INFECTED
Fig. 6.3e
Cortisol (nmol/l)
Parasitaemia
0
20
40
60
0
2
4
-30
-15
0
15
30
45
60
75
Days before and after infection
Cortisol
Parasitaemia scores

Table 6.1. Mean (± s.e.m.) adrenal weights and adrenal/bodyweight ratio and the thickness of the zonae glomerulosa and fasciculata/reticularis of rams infected with *T. congolense* (infected) and in uninfected control (control).

		Control		Infected		
Adrenal weights (gm)	(B)	2.28 ± 0.23	(5)	2.97 ± 0.21	(5)	
Adrenal/bodyweight ratio ($x10^{-5}$)	(B)	4.78 ± 0.18	(5)	7.73 ± 0.76	(5)	P<0.02
Zona glomerulosa	(A)	188.3 ± 19.6	(4)	173.6 ± 10.2	(4)	
(μm)	(B)	200.3 ± 10.8	(5)	207.8 ± 32.1	(5)	
Zona fasciculata/reticularis	(A)	1028.3 ± 50.2	(4)	1389.4 ± 96.0	(4)	
(μm)	(B)	1199.1 ± 99.6	(5)	1667.0 ±105.0	(5)	P<0.01

(A) mean values from rams killed on day 28 post-infection and (B) mean values from animals dying during the chronic phase or killed at the end of study period. Numbers in brackets denote sample number.

Fig. 6.4a Control

Section through the adrenal capsule (C), zona glomerulosa (Zg) and zona fasciculata (Zf) of the adrenal from uninfected control sheep (x25 H&E).

Fig. 6.4b. Infected

Section through the adrenal capsule (C), zona glomerulosa (Zg) and zona fasciculata (Zf). showing diffuse inflammatory cell infiltration in the subcapsular area and in cortical regions (arrows) in infected ram killed on day 79 post-infection, (80x H&E).

Fig. 6.4a

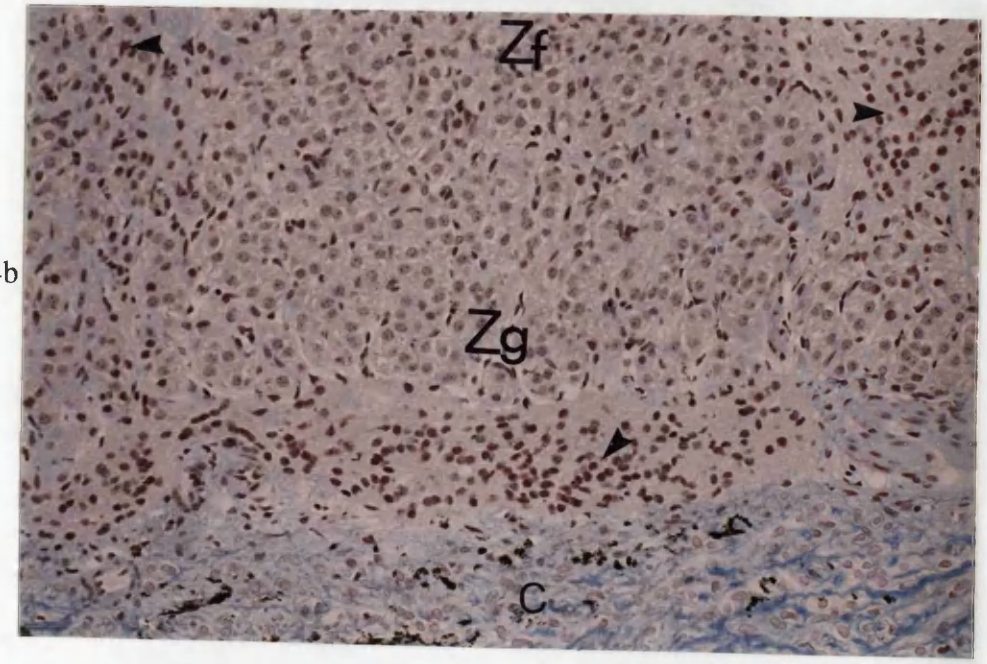


Fig. 6.4b

Fig. 6.4c. Infected

A section of the adrenal cortex showing fat deposition (arrows) in the zona glomerulosa (Zg) and extending in to the zona fasciculata (Zf) in infected ram killed on day 79 after infection. (x50 H&E)

Fig. 6.4d. Infected

A section through the zona fasciculata showing localised necrotic areas (arrows) in a ram killed on day 79 post-infection. (x100 H&E)

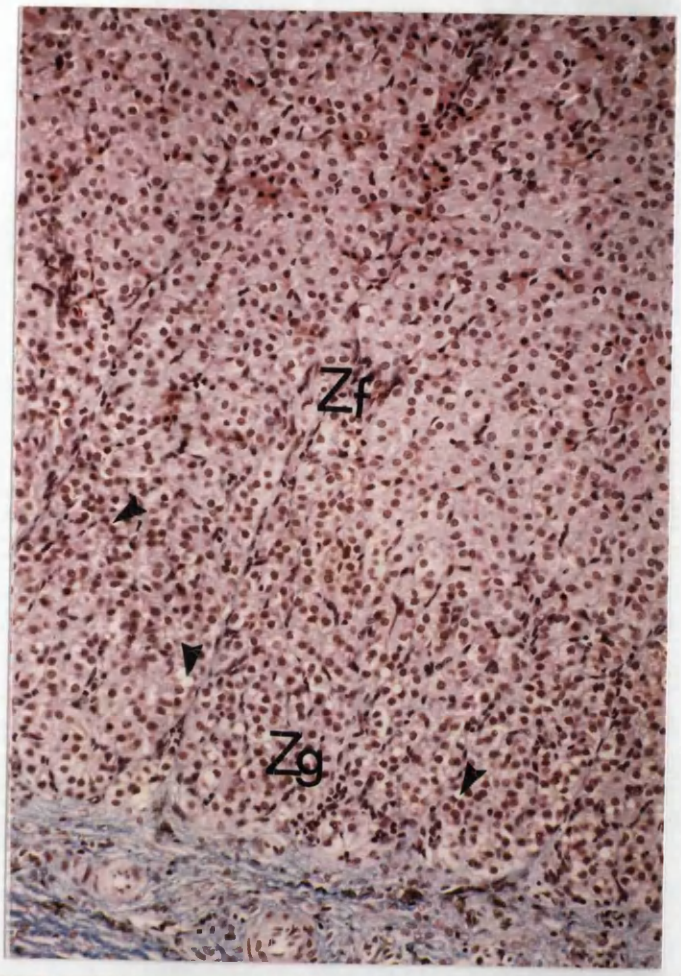


Fig. 6.4c

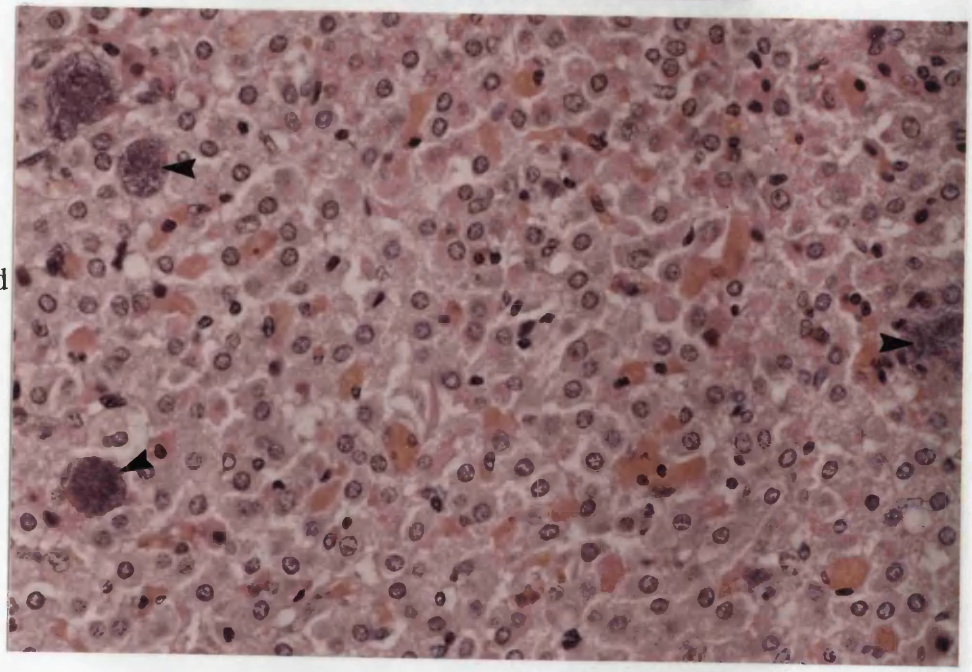


Fig. 6.4d

6.3.2 SECTION II

PLASMA ADRENOCORTICOTROPIN HORMONE, CORTISOL, LUTEINIZING HORMONE AND TESTOSTERONE RESPONSES IN RAMS INFECTED WITH *TRYPANOSOMA CONGOLENSE* TO EXOGENOUS CORTICOTROPHIN-RELEASING HORMONE

6.3.2.1. Plasma ACTH Response

Injection of CRH resulted in a marked ($P<0.001$) increase in plasma ACTH levels in infected and control rams on day 23 and day 65 post-infection. Statistical analysis of the ACTH response on day 23 and day 65 revealed a significant treatment effect ($P<0.001$) between groups and group x time interaction ($P<0.001$). ACTH response to CRH on day 23 was initially similar between the control and infected groups (Fig. 6.5a) and peaked at 20 min post-CRH injection (peak levels were not significantly different between groups). By 30 min post-CRH injection, mean ACTH levels had declined by 46% from peak values and by 68% at 150 min post-CRH injection in infected rams. Corresponding figures for control rams were 9% and 58%, respectively. This accounted for a significantly smaller ($P<0.05$) area under the response curve (Table 6.2) in the infected group when compared to the controls.

ACTH responses to CRH injection at day 65 are depicted in Fig. 6.6a. ACTH response in one infected ram was delayed up to 90 min post-CRH injection and its data were excluded but ACTH in the other four infected rams increased rapidly from a mean of 5.58 ± 1.62 mU l^{-1} at 0 min (time of CRH injection) peaking within 10 min at 38.6 ± 14.56 mU l^{-1}, whereas, control values increased from 4.58 ± 0.73 mU l^{-1} at 0 min to 21.3 ± 3.93 mU l^{-1} at 15 min post-CRH injection. Post-peak ACTH decline was more rapid in the infected rams than in controls. The area under the response curves (Table 6.2) were not significantly different between the infected and control groups at this stage (day 65) of infection.

6.3.2.2. Plasma Cortisol Response

Injection of CRH on day 23 post-infection produced a rapid rise in plasma cortisol levels within 5 - 10 min in all animals (Fig. 6.5b) coinciding with plasma ACTH rise. Mean peak levels were observed within 30 min in both groups and thereafter, a

similar gradual decline in plasma cortisol levels was observed in both groups. Statistical analysis of cortisol response to CRH revealed no significant treatment effect ($P>0.05$) between infected and control groups and the area under the response curves were not significantly different between infected and control groups (Table 6.2).

Mean plasma cortisol changes following CRH injection in infected and control groups on day 65 post-infection are depicted in Fig. 6.6b. The cortisol response revealed a significant treatment effect ($P<0.001$) between groups and a treatment x time interaction ($P<0.001$). The infected ram which showed a delayed ACTH response also had a slow cortisol response and these data were excluded. Cortisol levels increased rapidly in the other four infected animals and between 15 - 30 min post-CRH injection mean levels were significantly higher ($P<0.05$) than corresponding control values. A sharp decline in plasma cortisol concentration was observed from 40 - 80 min post-CRH injection and after which mean levels were similar in both groups. The area under the response curves were not significantly different between the infected and control rams.

6.3.2.3. Plasma LH Response

Changes in mean LH levels on day 23 in infected and control groups are depicted in Fig. 6.5c. Levels fluctuated between 0.15 - 1.0 ng ml^{-1} in both groups prior to CRH injection. Plasma LH concentrations tended to decline between 10 - 30 min after CRH injection in the controls and between 5-10 min in the infected group. There was no significant treatment effect on LH response between groups (Fig 6.5c., Table 6.2)

Mean plasma LH response to CRH injection on day 65 in both groups are shown in Fig. 6.6c. Control rams had nonsignificantly higher mean basal LH levels than infected rams prior to and after CRH injection on day 65. There was no significant treatment effect on plasma LH concentration between infected and control rams (Fig 6.6c, Table 6.2).

6.3.2.4. Plasma Testosterone Response

Changes in plasma testosterone concentrations on day 23 are shown in Fig. 6.5d. Plasma testosterone concentration remained basal in infected and control rams up to 40 min post-CRH injection and then increased sharply ($P<0.001$) in both groups. This

delayed increase was significantly higher ($P<0.05$) in the infected than in the control group of rams. There was a significant treatment effect on plasma testosterone concentration ($P<0.001$) and also a treatment x time interaction ($P<0.05$) between infected and control rams. The mean area under the testosterone response curves after CRH injection was significantly higher ($P<0.05$) in the infected than in control group (Table 6.2).

On day 65, mean plasma testosterone concentrations from 20 min before to 60 min after CRH injection were significantly lower ($P<0.05$) in infected than control rams; the overall mean testosterone concentration in the infected rams was <1.0 nmol l^{-1} compared to >3.5 nmol l^{-1} in the control rams (Fig 6.6d). In both groups the plasma testosterone concentration did not alter significantly from pre-CRH injection values during the 60 min period following CRH injection. Subsequently however, a rise in plasma testosterone was observed from 80 min to 120 min post-CRH injection in both infected and control groups. The area under the response curves was not significantly different between the two groups.

Fig. 6.5. Mean (± s.e.m.) plasma concentrations of ACTH (Fig. 6.5a), cortisol (Fig. 6.5b), LH (Fig. 6.5c) and testosterone (Fig. 6.5d) during a 2.5-h period after CRH injection (25 μg per animal) administered on day 23 post-infection to uninfected (n = 5, control) and *T. congolense*-infected (n = 5, infected) rams.

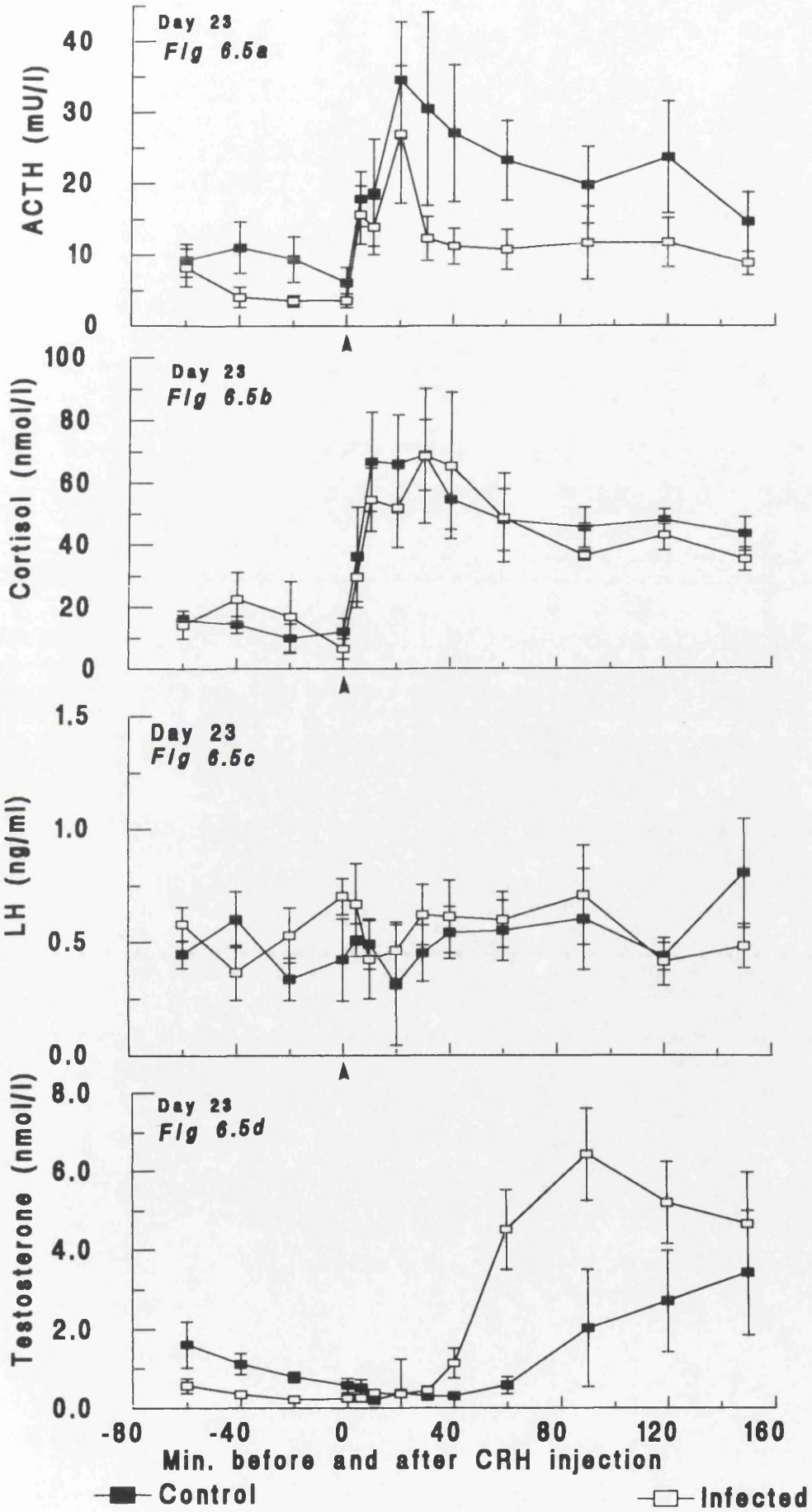

Day 23
Fig 6.5a
ACTH (mU/l)
40
30
20
10
0
Day 23
Fig 6.5b
Cortisol (nmol/l)
100
80
60
40
20
0
Day 23
Fig 6.5c
LH (ng/ml)
1.5
1.0
0.5
0.0
Day 23
Fig 6.5d
Testosterone (nmol/l)
8.0
6.0
4.0
2.0
0.0
-80
-40
0
40
80
120
160
Min. before and after CRH injection
Control
Infected

Fig. 6.6. Mean (± s.e.m.) plasma concentrations of ACTH (Fig. 6.6a), cortisol (Fig. 6.6b), LH (Fig. 6.6c) and testosterone (Fig. 6.6d) during a 2.5-h period after CRH injection (25 μg per animal) administered on day 65 post-infection to uninfected (n = 5, control) and *T. congolense*-infected (n = 5, infected) rams.

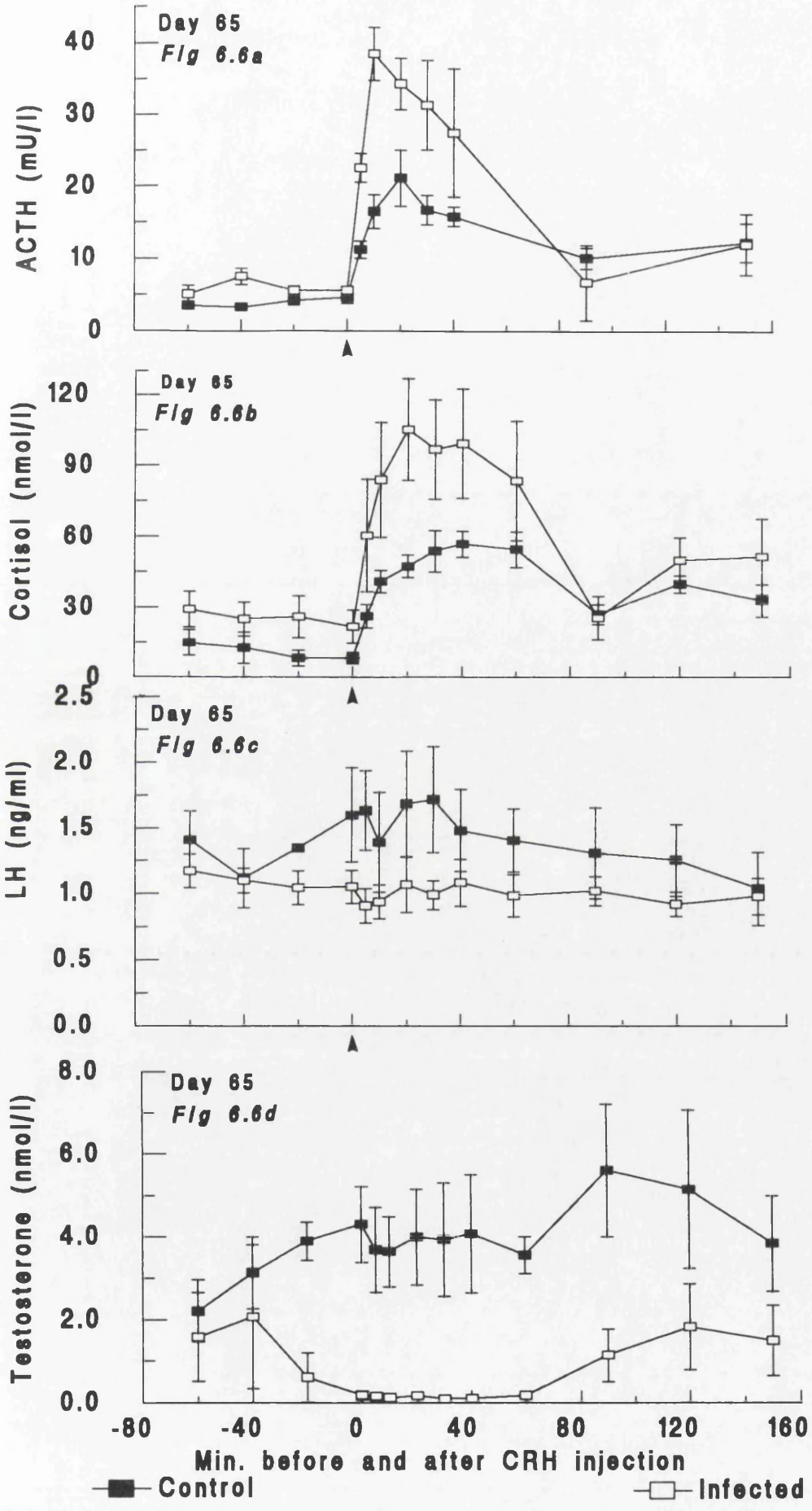

Day 65
Fig 6.6a
ACTH (mU/l)
40
30
20
10
0
Day 65
Fig 6.6b
Cortisol (nmol/l)
120
90
60
30
0
Day 65
Fig 6.6c
LH (ng/ml)
2.5
2.0
1.5
1.0
0.5
0.0
Day 65
Fig 6.6d
Testosterone (nmol/l)
8.0
6.0
4.0
2.0
0.0
-80
-40
0
40
80
120
160
Min. before and after CRH injection
Control
Infected

Table 6.2: Index of plasma ACTH, cortisol, LH and testosterone during a 2.5-h period (area under the curve) after CRH injection (25 μg per animal) administered 23 and 65 days after infection to groups of uninfected (control) and *T. congolense*-infected rams (infected).

Days post-infection	23		65	
	Control (n = 5)	Infected (n = 5)	Control (n = 5)	Infected (n = 4)
ACTH ($mU\text{-}2.5\ h\ l^{-1}$)	61.89 ± 8.67	29.85 ± 8.2*	24.87 ± 4.86	35.22 ± 8.0
Cortisol ($nmol\text{-}2.5\ h\ l^{-1}$)	125.6 ± 17.9	116.9 ± 1.7	85.81 ± 7.87	119.5 ± 26.4
LH ($ng\text{-}2.5\ h\ ml^{-1}$)	1.32 ± 0.13	1.41 ± 0.29	2.97 ± 0.76	2.13 ± 0.24
Testosterone ($nmol\text{-}2.5\ h\ l^{-1}$)	3.45 ± 1.56	9.45 ± 1.09*	7.78 ± 2.88	3.57 ± 1.48

*Values significantly different ($P<0.05$) from corresponding control values

6.4. DISCUSSION

One of the responses of an animal to infection, injury or other stressful conditions is the release from the adrenal cortex of glucocorticosteroids, predominantly hydrocortisone (cortisol) (Peers and Flower, 1992). Changes in plasma cortisol in this study show that *T. congolense* infection modulates adrenocortical activity in sheep. Infection enhanced cortisol secretion in rams at the onset of parasitaemia between day 9 - 16 (see Fig 6.2). This was succeeded by a period of fluctuating but reduced cortisol secretion between days 19 - 41 while from day 44 onward there were periods of time when plasma cortisol was higher in infected than control rams and these periods were significantly correlated with waves of parasitaemia. For discussion purposes in this chapter, the period between 1 - 6 weeks (about 40 days) post-infection in rams which was characterised by rapid decline in PCV, impaired growth, highly fluctuating rectal temperatures and rapid decline in body condition (see Chapter III 3.3.2), will be termed the acute phase of infection, while the period from 7 to 12 weeks (from 41 to 79 days) which was characterised by only slight changes in PCV and liveweight will be termed the chronic phase of infection. The acute and chronic phases of infection are not necessarily the same as phase I and II which were terms used in Chapter IV to define the alterations in LH and testosterone responsiveness during the infection. Increased plasma cortisol concentration has also been observed at the onset of parasitaemia in goats (Mutayoba and Gombe, 1989) and cattle (Abebe, 1991; Ogwu *et al.*, 1992) infected with *T. congolense.* However, unlike the situation in goats where an increase in cortisol secretion was observed during the entire acute phase (3 - 8 weeks), peaking at week 4 post-infection (Mutayoba and Gombe, 1989), it was evident from the present study that the plasma cortisol concentration declined in sheep after the initial peak (i.e. after 2 weeks) which was associated with the onset of parasitaemia. Similar findings were reported in cattle by Abebe (1991). Differences between the adrenal response during the acute phase observed in sheep in this study and that reported in goats (Mutayoba and Gombe, 1989) could be related to species differences in the host response to infection and in differences in susceptibility of animals to the serotype of trypanosome used for infection.

However during the chronic phase of infection the adrenocortical response in sheep resembled that of goats (Mutayoba and Gombe, 1989), in that increased but fluctuating cortisol levels were found. This differs from the situation in cattle (Ogwu *et al.,* 1992) where decreased cortisol levels were observed during this period of infection. Again species differences in the host and the serotype of the parasite may be responsible, but the occurrence of a biphasic cortisol response as found in the present study means that care must be taken in the selection of the periods for comparison of results between different experiments even in the same host species.

Histopathological changes observed in the adrenals of *T. congolense*-infected rams were comparable to those reported by others in trypanosome-infected sheep (Ikede and Losos, 1975), goats (Mutayoba *et al.,* 1988a) and cattle (Abebe, 1991; Ogwu *et al.,* 1992). Hypertrophy and deposition of large amounts of lipid droplets as that observed in the present study have also been seen in adrenal cortices of animals with trypanosomiasis (Ikede and Losos, 1975) and has been considered to be a sign of increased adrenocortical activity. It seems likely that the extensive hypertrophy of the adrenocortical layers which was observed in rams dying during the chronic phase or killed at the end of the study period occurred in response to increased adrenocortical activity and might explain the source of the enhanced cortisol secretion observed during the chronic phase of infection. The causes of adrenocortical pathology in *T. congolense* infection have not been investigated but may be associated with the tendency of trypanosomes to localise in large numbers in the microvasculature of organs such as the adrenal gland, causing local endothelial damage, fibrin degeneration, thrombosis and ischaemic necrosis. (Banks, 1978).

Following CRH injection, increases in plasma ACTH and cortisol concentrations were observed in both infected and control groups during the acute phase (day 23, see Fig 6.5a,b) and chronic phase (day 65, see Fig 6.6a,b) peaking within 10 - 20 min for ACTH and 30 - 40 min for cortisol. These results confirm the ability of CRH to induce ACTH and cortisol release as previously demonstrated in sheep (Pradier *et al.,* 1988) and cattle (Abebe, 1991; Abebe *et al.,* 1993b). However, the alteration in pattern of ACTH response that was observed between infected and control rams following CRH injection during the acute and chronic phases of infection (compare Fig 6.5a and 6.6a) suggests

that the biphasic changes in plasma cortisol levels observed in infected rams in the present studies could possibly be associated with reduced and then enhanced ACTH and cortisol secretion by pituitary and adrenal glands respectively during the acute and chronic phases of *T. congolense* infection.

The ACTH response to CRH was significantly lower in the infected rams than in the control rams on day 23 post-infection. This result accords with previous observations (Abebe 1991; Abebe *et al.*, 1993b; Abebe and Eley, 1992) in which a significant reduction in plasma ACTH response following intravenous injection of CRH or insulin within 4 to 5 weeks (during the acute phase) of *T. congolense* infection was observed in cattle. However the concomitant reduction in cortisol response to CRH injection which was reported in cattle (Abebe, 1991, Abebe *et al.*, 1993b) was not observed in infected rams at this stage of infection as the mean cortisol response was not different between groups. Hence, unlike the results in cattle, where reduction in plasma cortisol levels during the acute phase of *T. congolense* infection might be associated with both pituitary and adrenal defects (Abebe, 1991; Abebe *et al.*, 1993b; Ogwu *et al.*, 1992), the results from the present study show that infected sheep exhibit reduced pituitary responsiveness to CRH resulting in reduced pituitary ACTH secretion, but the adrenal response to the secreted ACTH was maintained at the same level as in the control sheep.

The responsiveness of the pituitary-adrenal axis to CRH in the infected rams was increased during the chronic phase. Thus plasma ACTH and cortisol concentrations increased rapidly and peaked earlier at higher levels in the infected than in the control rams when CRH was administered on day 65 post-infection (see Fig 6.6a,b). These findings agree with the high plasma cortisol concentrations (see Fig 6.2) and hypertrophy of the adrenocortical layers (see Table 6.1) observed in infected rams during this period and highlight the possible differences in adrenal responses to *T. congolense* infection during the chronic phase of infection between sheep (this study) and goats (Mutayoba *et al.*, 1989) on one hand, and cattle (Ogwu *et al.*, 1992) on the other. These findings further suggest that high plasma cortisol levels observed during the chronic phase in rams during the present studies might have been associated with a *T. congolense*-induced increase in ACTH secretion caused by an increased

responsiveness of the pituitary gland to CRH. Suppression of the HPA axis during the acute phase of infection might have been a component of a more widespread response to the onset of parasitaemia and fever which were observed during this stage and a rapid deterioration of other clinical parameters including haemogram, liveweight and general body condition. It seems, however, that sheep can partly recover from the changes associated with the acute phase of infection but that HPA activity becomes enhanced during the chronic phase.

Injection of CRH of day 23 and 65 post-infection had no significant effect on LH release in either infected or control rams. Although on both occasions in some animals there was a fall in LH concentration within 5 - 15 min post-CRH injection, there was too much individual animal variation for this to be taken as a response associated with increases in plasma ACTH and cortisol. In other animals, including rats and cattle, it has been established that stress-induced activation of the HPA axis impairs the pituitary secretion of LH (Johnson *et al.*, 1982; Rivier *et al.*, 1986). The current results confirm other studies which have shown that injection of CRH in sheep either intravenously (Parrot *et al.*, 1988) or intracerebroventricularly (Horton *et al.*, 1988) has no inhibitory effect on LH secretion. Indeed it has been reported that CRH may have a dose-dependent stimulatory effect on LH secretion (Naylor *et al.*, 1990).

The common feature of the response of plasma testosterone concentration to CRH administration was that at least for 40 min after CRH injection there were no significant alterations but after this period plasma testosterone concentration then increased in control rams and also in infected rams during both the acute and chronic phases of infection (see Fig. 6.5d and 6.6d). Elucidation of the mechanisms of this delayed response is beyond the scope of this investigation but could be related to previously reported effects of CRH (Dufau *et al.*, 1993) or cortisol (Bambino and Hsueh, 1981) on testicular steroidogenesis acting alone or in concert. CRH is known to inhibit Leydig cell gonadotrophin-induced cAMP generation and subsequent androgen production by acting on a specific high affinity receptor on the Leydig cell membrane (Ulisse *et al.*, 1990; Dufau *et al.*, 1993). Similarly, glucocorticoids are known to inhibit gonadal steroidogenesis by inhibition of gonadal LH receptors in male (Bambino and Hsueh, 1981) and female (Kawate *et al.*, 1993) animals. Whilst there was no significant

reduction in plasma testosterone in the period immediately following CRH injection, the delayed increase in plasma testosterone concentration therefore could be a result of CRH or cortisol initially limiting the secretion and release of androgens from the testis. The effect is to delay and therefore coordinate the following pulse of testosterone release.

This explanation of the results is supported by the observation that the delayed rise in plasma testosterone after CRH injection coincided with the fall in plasma cortisol concentrations implying that the latter could be the main *in vivo* mediator involved in this process. The difference in magnitude of the delayed rise in plasma testosterone in control and infected rams following CRH injection during the acute phase of infection can be explained by the presence of higher intratesticular testosterone reserves which were observed in rams during the acute phase of infection (see Chapter V). During the chronic phase, the testosterone response to CRH was the same in infected and control rams although the pre- and post-injection concentrations of testosterone were lower in the infected than control rams. Decreased plasma testosterone concentration was observed during the chronic phase in all infected rams (see Chapter IV) and chronic trypanosomiasis has been reported to induce a decline in plasma testosterone in sheep (Adeyemo *et al.*, 1990) and goats (Waindi *et al.*, 1986).

It was not possible in these studies to investigate how the observed changes in the HPA axis in infected rams affected the pulsatile secretion of plasma testosterone or LH since samples were taken too infrequently (three samples per week). Although LH and testosterone pulse analysis was done during study II the changes in the function of HPA axis were not investigated and hence future studies should determine how the present observations on ACTH and cortisol relate to the activity of the hypothalamo-pituitary-gonadal axis.

However, it was apparent from the present study that during the acute phase of infection, infected rams showed a decreased ACTH response to CRH whereas this group of rams showed an increased LH response to GnRH (see Chapter IV, Fig 4.4a). However, during the chronic phase of infection, the pituitary response to CRH was higher whereas the LH response to GnRH was comparable between infected and control rams (see Chapter IV, Fig 4.4c). At the same time, the gonadal responsiveness in terms of steroid production was also changing. Thus testosterone secretion in response to

GnRH-induced LH secretion was reduced during both the acute and chronic phases of the infection, whereas the adrenal response to CRH-induced ACTH secretion remained unchanged during the acute phase and increased during the chronic phase. These observations suggest that *T. congolense* infection exerts reciprocal effects on the functions of the hypothalamo-pituitary-adrenal axis on one hand and the gonadal axis on the other hand in rams and possibly in other animals. Similarly, increased adrenal function during *T. congolense* infection has been reported to occur concurrently with reciprocal decrease in the function of other endocrine glands including the thyroid gland (Mutayoba and Gombe, 1989; Abebe and Eley, 1992b). The possible mechanisms by which *T. congolense* induces reciprocal alterations in the functions of hypothalamo-pituitary-adrenal, -gonadal and thyroid axes in infected animals are equivocal but it is possible that these changes occur as part of a complex adaptive systemic response to stress of invading trypanosomes and thus to the altered "milieu interieur".

In conclusion, *T. congolense* infection was observed to induce a biphasic alterations in the function of the HPA axis in rams. The onset of parasitaemia (1 - 2 weeks after infection) was associated with increased cortisol secretion. This was succeeded within 3 - 7 weeks after infection by a period of fluctuating but reduced cortisol secretion. Thereafter, cortisol secretion was again markedly increased. The variation in the adrenal response during the course of *T. congolense* infection is likely to be related to the variations in responsiveness of the pituitary to CRH observed in the present study and previously reported in cattle by Abebe (1991) and Abebe *et al.* (1993b). CRH-induced ACTH secretion is impaired during the acute phase and markedly stimulated during the chronic phase of *T. congolense* infection in rams. Whether this biphasic response is related to the interaction of the pituitary with the several trypanosome-borne or -induced factors previously reviewed in Chapter I, is open to further investigation which will be helped by the increasing availability of reagents to monitor the mediators and reactants of the nonspecific defense mechanisms of sheep. A change in the pituitary-adrenal activity during infection leading to altered cortisol secretion could be involved in the changes in testicular steroidogenesis observed during trypanosome infections in sheep and also modulate other abnormalities including immunosuppression and changes in energy and protein metabolism.

CHAPTER VII

GENERAL DISCUSSION AND CONCLUSIONS

This thesis concerns the pathophysiology of the hypothalamo-pituitary-gonadal and adrenal axes during the course of *T. congolense* infection in rams. Previous investigations (reviewed in Chapter I) have shown that trypanosomiasis causes pathological changes in the testes and adrenal gland in animals and man leading to altered function of these organs. However, only a few studies have investigated the changes induced by *T. congolense* on the endocrine function of the testes (Waindi *et al.*, 1986; Adeyemo *et al.*, 1990) and the adrenal gland (Mutayoba and Gombe, 1989; Abebe, 1991; Omeke and Onuora, 1992). Endocrine changes in the pituitary gland have only been reported in infections induced by the *T. brucei*-group of trypanosomes (Emery and Nduka, 1983; Boersma *et al.*, 1989; Hublart *et al.*, 1990; Soudan *et al.*, 1992). Furthermore, little is known of the mechanisms underlying the pituitary-gonadal and adrenal endocrine changes observed during trypanosome infections and especially changes induced by haematic trypanosomes (*T. congolense* and *T. vivax*). As reproductive dysfunction induced by *T. congolense* infection causes great economic losses in trypanosome-endemic areas, it is important to understand the processes whereby infections with these organisms lead to reduced productivity.

The working hypothesis of the present investigations was that *T. congolense* infection can cause changes in the hypothalamo-pituitary axis which contributes to the gonadal and adrenal endocrine changes. The objectives of these studies were to assess the function of the hypothalamo-pituitary-gonadal axis during the course of *T. congolense* infection in rams and the role of pyrexia and stress (measured by changes in endocrine function of the hypothalamo-pituitary-adrenal (HPA) axis) caused by the infection in inducing these changes. These objectives were addressed in two major studies and the results obtained showed that *T. congolense* infection of rams induced a reduction in semen quality and caused non-inflammatory changes in the gonads and accessory tissues. In addition, the infection induced a rapid reduction in plasma testosterone concentration but a decline in plasma LH concentration occurred only after several weeks of infection. Testicular morphological and endocrine changes were probably in part induced by the elevation of testicular temperature associated with trypanosomiasis, whereas changes in pituitary LH secretion were associated with an impairment of the hypothalamus to secrete and/or release GnRH. Trypanosome-induced

changes in the function of the HPA axis (increase in cortisol) was found to depress testosterone release into the circulation.

Prior to these studies the only available information on the effects of trypanosomiasis on reproduction in rams was based on studies performed using *T. b. brucei* and *T. vivax*. To complement the present studies on reproductive endocrine changes associated with *T. congolense* infection in rams, clinical investigations were conducted on changes in semen characteristics and histopathology of the pituitary, testes, epididymis and prostate glands. These studies (Chapter III) showed that *T. congolense* infection induced progressive deterioration of semen quality and non-inflammatory degenerative changes in the testes, epididymis and prostate gland of rams similar to those reported previously in other animals. In addition, increased degranulation of basophilic cells was observed in the pituitary gland of infected rams.

Several previous reports had suggested that testicular dysfunction induced by trypanosome infection might be a thermal effect caused by the fluctuating pyrexia associated with this disease. This was the justification for investigating whether the low grade fluctuating pyrexia induced by *T. congolense* could impair the thermoregulatory mechanisms of the testis in infected animals and induce a rise in testicular temperature. Scrotal temperature measurements reported in Chapter III showed that this indeed occurs. A trial was also undertaken to assess whether changes in testicular function comparable to those observed during *T. congolense* infection could be induced by artificial elevation of testicular temperature using scrotal-insulation (Chapter III). However, this investigation was not totally successful as the scrotal pouch used was not ideal for this study and the scrotal skin of insulated rams tended to sweat and might therefore have reduced scrotal and testicular temperatures. Changes in testicular exocrine and endocrine function of scrotal-insulated animals were in most cases intermediate between the infected rams and normal control rams. Probably the most important findings of this part of the investigation were the close similarities between infected and scrotal-insulated rams in terms of observed sperm abnormalities, gonadal pathological changes (Chapter III) and altered Leydig cell steroidogenesis (Chapter V). This suggests that persistent pyrexia induced by trypanosome infection could contribute to the dysfunction of the exocrine and endocrine testes.

Subsequent investigations compared reproductive endocrine changes in infected and control animals under a variety of conditions. Measurement of plasma testosterone and LH concentrations in samples collected three times a week (Chapter IV 4.3.1) showed that there was a significant decline in plasma testosterone levels in infected rams within 1 - 2 weeks of infection which became progressively more marked during the study period. No significant changes in mean plasma LH concentration were observed up to week 8 of infection but thereafter a significant decline occurred. The present findings on the plasma testosterone changes induced by *T. congolense* infection in rams are in agreement with previous reports in goats infected with *T. congolense* (Waindi *et al.*, 1986) and in sheep infected with mixed *T. congolense* and *T. b. brucei* infections (Adeyemo *et al.*, 1990) but the changes in plasma LH in samples collected infrequently did not show any clear uniform pattern of change as LH concentration tended to increase in some rams and decreased in others, while in some rams no change was obvious. It was therefore necessary to re-assess plasma LH changes by collecting samples more frequently during the infection period because of the known pulsatile mode of LH secretion.

When plasma testosterone concentration was measured in samples collected at 15 min intervals for 6 h at different stages of infection (Chapter IV 4.3.2 and 4.3.3), the tendency of plasma testosterone to decline after infection was confirmed. In addition it was shown that the decline in plasma testosterone was associated with a progressive decline in pulse amplitude leading eventually to complete absence of testosterone peaks in some animals within 8 weeks of infection. Analysis of LH in frequent samples showed an increase in plasma LH pulse amplitude on day 23 and 26 post-infection in infected rams followed by a reduction on days 52 and 54 post-infection, suggesting that *T. congolense* induces a biphasic pituitary LH secretory response. Thus although plasma testosterone declined rapidly following the onset of parasitaemia, the changes in plasma LH were time dependent. This confirmed the earlier results that a decline in plasma LH is observed but only after several weeks of infection.

Furthermore, in an additional experiment to assess the effect of infection on pituitary LH secretion, rams were castrated on day 28 post-infection thus removing the negative feedback of testicular testosterone. Pulsatile secretion of LH was then assessed

in frequent samples collected three and half weeks afterwards. These results show that LH pulse amplitudes were reduced in castrated infected animals compared to castrated control rams, indicating that the pituitary secretion of LH in infected rams was defective at this stage of infection. These findings provide evidence that a decline in plasma LH pulses observed on days 52 and 54 post-infection in infected intact rams could not be associated with feedback influence of gonadal steroids. However, the observed alteration of pituitary LH secretion in infected castrated rams did not demonstrate whether this was caused directly by a defect in the ability of the pituitary gland to secrete LH or whether it was associated with decreased hypothalamic GnRH secretion and/or reduced pituitary responsiveness to GnRH.

To assess whether changes in plasma LH in infected rams were induced by defects in LH secretion occurring at the level of the pituitary alone or were mediated by reduced responsiveness of the pituitary to GnRH, a GnRH challenge (pituitary-gonadal response test) was conducted (Chapter IV). The results show that pituitary responsiveness to GnRH (as measured by LH response) was not impaired in infected rams at any stage of the experimental period. However, the testicular responsiveness (as measured by testosterone response) to GnRH-induced LH stimulation was decreased compared to controls. These results strongly suggest that the observed decline in pituitary LH secretion in infected animals is not due to the impairment of pituitary gland to secrete and release LH but is probably associated with defects in hypothalamic GnRH secretion.

Investigation of *in vitro* Leydig cell steroidogenesis (Chapter V) show that changes in testicular endocrine function are associated with impairment of Leydig cell steroidogenesis. This may be mediated by an increase in testicular temperature affecting one or more of the enzymes involved in testosterone biosynthesis. The ability of *T. congolense* to impair Leydig cell LH receptor activity which was previously reported in rats infected with *T. b. brucei* (Soudan *et al.*, 1992) was not confirmed in this study.

Taken together, these studies have shown that *T. congolense* may induce gonadal and pituitary endocrine dysfunction in rams by differing mechanisms. Thermal effects resulting from *T. congolense*-induced pyrexia probably contributed to the dysfunction of the exocrine and endocrine testes. It is known that pyrexia can also influence

hypothalamo-pituitary function and in trypanosome infection could either have been induced by pyrexia *per se* or have resulted from the action of several pyrogenic cytokines which are likely to be stimulated during infection. Alternatively, it is also probable that trypanosome-borne factors such as proteases (Hublart *et al.*, 1990) may have contributed to the impairment of the hypothalamic - GnRH secretion.

Depression of the hypothalamo-pituitary-gonadal axis in infected animals might have occurred as a secondary response due to trypanosome-induced stress so further studies were undertaken (Chapter VI) to assess the effect of infection on the HPA axis and the latter's involvement in the observed reproductive endocrine changes. These studies showed that *T. congolense* infection induced a significant increase in plasma cortisol levels at the onset of parasitaemia. However, after this initial rise, plasma cortisol levels in infected animals declined for a while (3 - 6 weeks post-infection in this study). During this period, the pituitary secretion of ACTH in response to CRH injection was reduced in infected rams whereas plasma LH secretion and pituitary responsiveness to GnRH had been shown to be increased (Chapter IV). However, as the infection progressed the level of HPA axis activity increased, whilst the function of the hypothalamo-pituitary-gonadal axis was depressed. Increases in ACTH and cortisol following CRH injection had no effect on plasma LH levels but did affect the testicular release of testosterone. Thus although the effect of trypanosome-induced stress (as measured by cortisol) on pituitary LH secretion was not clear from these studies, it is likely that activation of the HPA axis during the course of infection might have exacerbated the reduced secretion of testosterone.

In conclusion, the results demonstrated that the integrity of the hypothalamo-pituitary-gonadal axis is defective during *T. congolense* infection in rams. This is due to early testicular damage, possibly induced in part by the trypanosome-induced pyrexia which affects both the exocrine and endocrine testis. Changes in pituitary gland function which are characterised by a progressive reduction in LH secretion are probably associated with progressive impairment of hypothalamic GnRH secretion. These defects in hypothalamic-pituitary function may be due to a direct trypanosome effect and/or to trypanosome activation of the host defence system (an indirect mechanism).

The studies presented in this thesis were conducted in a controlled environment, with the animals well provided for in terms of feed, water, health and hygiene. However, since these factors comprise the most important constraints affecting livestock productivity in trypanosome-endemic regions, follow-up investigations from these studies need to assess the interaction of environmental and husbandry constraints on the function of the hypothalamo-pituitary-gonadal axis in infected animals.

The practical implications of *T. congolense*-induced gonadal changes to the livestock industry in trypanosome endemic areas are immense. In early stages of infection when sperm quality and testicular steroidogenesis are decreasing, it is likely that the fertility of infected animals will be reduced. In later stages, especially in chronically infected animals, progressive impairment of the hypothalamo-pituitary-gonadal axis undoubtedly leads to variable degrees of infertility associated with reduced libido and atrophy of the testes leading to oligozoospermia and eventually aspermia. Arthur *et al.* (1986) reported that while testicular degeneration occurs quickly, regeneration after removal of an insult, is usually a protracted process. Hence, the danger exists that even if trypanosome infection is terminated by chemotherapy, advanced testicular degenerative changes may take a very long time to recover and affected animals may remain infertile for a long period. Indeed, evidence to support this view is now available (Ikede and Akpavie, 1982; Sekoni, 1990; Sekoni *et al.*, 1990a, 1991).

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APPENDICES

APPENDIX 1:

Testosterone ^{125}I-Radioimmunoassay assay buffer

(0.05 M Phosphate buffered saline, 0.25% bovine serum albumin, pH 7.4)

Stock solutions:

(a) 0.25M Sodium dihydrogen orthophosphate- 9.8 gm $NaH_2PO_4.2H_2O$ (MW 156.01) dissolved in 250 ml distilled water.

(b) 0.5M Disodium hydrogen orthophosphate - 35.5 gm Na_2HPO_4 (MW 141.96) or 89.54 gm $Na_2HPO_4.12H_2O$ dissolved in 500 ml distilled water.

Assay buffer

To make 1 litre assay buffer

15 ml solution (a)

60 ml solution (b)

8.77 gm Sodium chloride

0.25 gm Merthiolate (Thiomersal, Sigma)

Adjust pH to 7.4 and store at 4^{0}C for 6 months.

Assay buffer was prepared fresh for each assay by adding 0.25% Bovine serum albumin.

APPENDIX 2:

Testosterone and Progesterone ^{3}H-Radioimmunoassay assay buffer.

(Phosphate buffered saline- gelatin buffer, pH 7.0)

Reagents

Sodium dihydrogen orthophosphate	$NaH_2PO_4.2H_2O$	6.08 gm l^{-1}
Disodium hydrogen orthophosphate	$Na_2HPO_4.2H_2O$	11.2 gm l^{-1}
Sodium azide (NaN_3)		1.0 gm l^{-1}
Sodium Chloride (NaCl)		8.77 gm l^{-1}

Adjust pH and store at 4^{0}C

1.0 gm l^{-1} gelatin was added in assay buffer before use.

APPENDIX 3:

Influence of Ether Volume and Extraction Time on Testosterone Recoveries in ^{125}I-Testosterone Radioimmunoassay.

	Testosterone added nmol l^{-1}	*Mean testosterone (nmol l^{-1}) assayed after ether extraction	
		4 min extraction	10 min extraction
3 ml ether	1.45	1.57 (101.4%)[a]	1.41 (97.4%)
	12.5	12.61 (100.9%)	12.39 (99.1%)
	32.6	31.39 (96.3%)	33.24 (102.0%)
10 ml ether	1.45	1.38 (95.2%)	1.51 (104.1%)
	12.5	12.36 (98.8%)	12.71 (101.75)
	32.6	33.54 (102.9%)	31.89 (97.8%)

*8 replicates of each sample

[a]Figures in parenthesis are parcentage recoveries

APPENDIX 4:

LH ^{125}I-Radioimmunoassay assay buffer

(0.05M Phosphate buffered saline, 0.25% Bovine serum albumin, 0.1% NaEDTA pH 7.5)

Stock solutions

(a) 0.5M Sodium dihydrogen orthophosphate - 59.99 gm NaH_2PO_4 dissolved in 1 litre distilled water, add 0.4 gm merthiolate.

(b) 0.5M Disodium hydrogen orthophosphate - 141.96 gm Na_2HPO_4 dissolved in 2 litres distilled water, add 0.4 gm merthiolate

(c) **0.5 M Phosphate Buffer** Made by titrating approximately 300 ml of solution (a) into 2 litres of solution (b) to pH 7.5., add 0.05 gm methiolate and store at 4°C.

(d) **0.05 M Phosphate buffered saline** (pH 7.5)

In 1 titre buffer

100 ml solution (c)

1 gm Disodium diaminoethanetetra-acetic acid (NaEDTA)

8.77 gm Sodium chloride (NaCl)

900 ml distilled water

Check pH and stored at 4°C

0.25% Bovine serum albumin is added in assay buffer before use.

APPENDIX 5:

Cortisol ^{125}I-Radioimmunoassay assay buffer.

(0.1 M Phosphate buffer, 0.1% gelatin, pH 7.4)

Reagents

Sodium dihydrogen orthophosphate	$NaH_2PO_4.2H_2O$	2.18 gm l^{-1}
Disodium hydrogen orthophosphate	Na_2HPO_4	12.21 gm l^{-1}
Sodium azide	(NaN_3)	0.50 gm l^{-1}

Adjust pH to 7.4

1.0 gm l^{-1} gelatin is added in the assay buffer before use.