EFFECTS OF EXPERIMENTAL TRYPANOSOMA VIVAX INFECTION ON THE OESTROUS CYCLE OF MATURE YANKASA EWES

BY

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A THESIS SUBMITTED TO THE POSTGRADUATE SCHOOL AHMADU BELLO UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE, DEPARTMENT OF VETERINARY PARASITOLOGY AND ENTOMOLOGY, AHMADU BELLO, UNIVERSITY, ZARIA KADUNA STATE, NIGERIA

DECEMBER, 1989

DECLARATION

I hereby declare that the work reported in this thesis was carried out by me in the Department of Veterinary Parasitology and Entomology, under the supervision of Dr. A. Sannusi, Professor C. 0. Njoku and Professor E. 0, Oyedipe.

No part of this thesis has been presented in any previous application for a higher degree. All sources of information are specifically acknowledged by means of references.

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CERTIFICATION

This thesis, "Effects of experimental *T. vivax* infection on the oestrous cycle of mature Yankasa ewes," by Dr. T. K. Adenowo meets the regulations governing the award of the degree of Master of Science of Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is partly dedicated to all men and women who had endured hardship in order to give their fellow creatures a better standard of living. It is equally dedicated to the past, present and the future generations, to Joseph Adefolarin Adenowo (of blessed memory), Mary Tanimowo Adenowo, Agnes Adetutu Adenowo, Joseph Adekunle Adenowo and Patrick Adewale Adenowo.

It is largely dedicated to God through Jesus Christ, the Light of Divine Wisdom, who did not come to be served but to serve and gave his life as a ransom for mankind.

ACKNOWLEDGEMENTS

My profound gratitude goes to God who provided the health and wisdom we needed for this investigation. To Jesus Christ His beloved Son who is the Light of Divine Wisdom. I gratefully acknowledge the tireless effort of Dr. A. Sannusi, my major supervisor. I am very much indebted to Professor C. 0. Njoku who is more of a professional father to me than a supervisor; his words of encouragement at odd times contributed to the success of this work. I am most grateful to the last but never the least of my supervisors; prof .E.O. Oyedipe whose "brain-child" this work is. His tireless contribution from the beginning to the end of this work is profoundly acknowledged.

I am equally indebted to Professors Saka Nuru and Michael Olayiwole, respective Director and Deputy Director of N.A.P.R.I. for their support. I am also grateful to the International Atomic Energy Agency, Vienna, Austria for equally supporting this study under a Technical Cooperation Project NIR/5/014. The words of wisdom of Professor Ogunsusi, Dr. and Dr. (Mrs,) Ogunkoya had been sources of inspiration to me. The technical assistance of all the members of staff of N.A.P*R.I. and Department of Veterinary Parasitology, Ahmadu Bello University, is highly acknowledged.

To those I cannot individualize, I must let you know that you are equally very important, in one way or the other, for the success of this work. I finally pray that the Lord in His mighty strength will continue to use you in His positive plan for man-kind through Jesus Christ our Lord.... Amen.

ABSTRACT

Twelve mature Yankasa sheep (6 infected and 6 control ewes) were used to investigate the effects of experimental _Trypanosoma vivax_ infection on selected reproductive organs and on oestrous cycle parameters. Daily rectal temperatures and weekly body weights were taken, and blood samples for haematology were also collected twice a week from all the animals before and after the infection.

Prior to infection, oestrus was synchronised using progestagen sponges, following which animals were observed for oestrus signs twice daily (A.M. arid P.M.) using a vasectomised ram. Heat observations were carried out daily and plasma for progesterone determination collected every other day for 2 cycles to assess oestrous cycle parameters prior to infection and subsequently till end of the experimental period.

Undulating parasitaemia, hyperthermia and anaemia were observed in infected ewes following T. _vivax_ infection. Three types of abnormal oestrus occurred based on progesterone profiles: 2 infected ewes showed silent oestrus, 2 others exhibited temporary anoestrus while the last 2 showed complete anoestrus. These oestrus anomalies were associated with various degrees of pathological observations such as mononuclear (mostly lymphocytes and macrophages) cellular infiltration and necrosis of the anterior pituitary gland, and hyalinization of the ovary with no evidence of follicular development. These results confirm that T. _vivax_ infection can adversely affect reproduction through interference with ovarian activity and other reproductive organs of Yankasa ewes.

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CHAPTER I

INTRODUCTION

Trypanosomiasis is a complex disease of livestock constituting a major constraint to the agricultural and socio-economic development of large areas of Nigeria. The disease in domestic animals is caused principally by three species of trypanosomes, namely *Trypanosoma vivax*, *T. congolense* and *T. brucei* (Saror, 1975; Ikede and Losos, 1975a, 1975b; Ilemobade, 1981). These trypanosomes are transmitted by several species of tsetse flies (Ilemobade, 1981; Bourn et al., 1984).

Trypanosomiasis occurs in all domesticated animals in tropical Africa, resulting in acute or chronic clinical manifestations characterized by fever, anaemia, emaciation, subcutaneous oedema and weakness. Severe reduction in animal productivity reflected in poor growth, low milk yield, reduced capacity for work in work oxen, anoestrus, irregular oestrous cycles, abortion and infertility are also consequences of the disease (Boyt, 1971; Nuru, 1974, 1986; Griffin, 1978; Kanyari et al., 1983). Death is very common in some cases during which gradual wasting are a dominant feature (Nuru, 1974, 1986; Ige and Amodu, 1975; Luckins et al., 1986; Ogwuet al., 1986; Sekoni, 1987). Recent field studies, have also shown that ovine and caprine trypanosomiasis could result in significant economic losses (Griffin, 1978; Griffin and Allonby, 1979a, 1979b; Kanyari et al., 1983).

There are indications that trypanosomiasis can result in reproductive failures such as anoestrus, irregular oestrous cycle, abortion and still birth in small ruminants (Boyt, 1971; Luckins et al., 1986; O'hara and Gombe, 1986; Llewelyn et al., 1987). Some workers have also investigated the reproductive pathophysiology of trypanosome infections of cattle (Vohradsky, 1971; Isoun et al., 1975; Ogwu et al», 1984, 1986), and West African dwarf sheep and goats (Isoun et al., 1974a, 1974b; Anosa, 1977; Anosa and Isoun, 1980a). The indications from these studies are that trypanosomiasis can result in infertility or even sterility. However, clinical heat is yet to be correlated with the progesterone profiles through sequential hormonal assays. Further information on the effects of trypanosomiasis on reproductive performance of Nigerian ewes (especially Yankasa) is very scanty.

This study was therefore designed to investigate the pathophysiology of the oestrous cycle of Yankasa ewes infected with T. vivax, by

(a) observations on oestrous cycle, and progesterone profiles;

(b) identification of gross and histopathological changes of the female reproductive organs.

CHAPTER 2

2. LITERATURE REVIEW

2.1 Introduction

Although a lot of work had been done on the immense problems posed by trypanosomiasis, there is remarkable deficiency in our current knowledge of the interaction of the disease with nutrition and reproduction in small ruminants. This deficiency exists because there are relatively few reports on the reproductive pathology and endocrinology of ovine or caprine trypanosomiasis.

This review evaluates the literature on the trypanosomiasis of small ruminants. A brief review of the normal reproductive cycle of ewes is given in order to assist us in evaluating deviations from the normal, following trypanosome infection.

2.2 Trypanosomiasis in ruminants with particular emphasis on small ruminants

Jasiorowski (1972) reported that the average daily per caput consumption of animal protein in the developed countries was 49 grammes, while it was only 11 grammes in the developing countries. The lower animal production output in the developing countries has been incriminated for this phenomenon.

Finelle (1974) enumerated the benefits resulting from effective trypanosomiasis control as an increase in livestock numbers, reduction in the mortality rate, improved carcass quality, an increase in growth rate and improved fertility. Small ruminants have a lower per capita value than cattle and are easily sold in times of need. They are highly prolific, often producing twins and triplets. Griffin (1978) observed that despite the fact that trypanosomiasis was given wide recognition for decades as being the greatest obstacle to increased productivity of livestock in Africa, preventing vast areas of potentially good land from being grazed, very little research had been done to determine the impact of the disease in sheep and goat production. However, recent surveys and epidemiological studies on trypanosomiasis in sheep and goats indicate that small ruminants can be, and often are, severely affected by the disease which may be of greater economic importance in such stock than suggested by earlier reports (Griffin, 1978; Griffin and Allonby, 1979a).

Mackenzie and Cruickshank (1973) reported an acute disease among naturally infected sheep, lasting up to 43 days after the appearance of trypanosomes, in which there was a rapid decrease in haematological indices. In their studies a more protracted form was also observed, lasting 11 and 12 months with an acute episode accompanied by a sudden fall in haemoglobin level just before death. The third group exhibited a protracted disease with a steady fall in haemoglobin level, but death occurred without the acute episode.

Similar syndromes were also described by Griffin and Allonby (1979a) in naturally infected sheep and goats. The subacute syndrome as well as the acute and chronic forms was described based on the period of detectable parasitaemia before death.

The acute form lasting up to 6 weeks was characterized by a rapid decrease in packed cell volume (P.C.V.) and widely fluctuating body temperature during the period of parasitaemia, resulting in death or self cure in some cases. In the latter case the host appears to make a complete recovery, showing normal P.C.V, levels and temperatures while parasites gradually disappear from the blood stream. The subacute form has been classified as lasting up to 12 weeks and is characterized by a slow but steady decline in P.C.V. and body weight, with fluctuating temperatures. Again, hosts so affected may either die or show an apparent self-cure.

Griffin and Allonby C1979b) assessed the visible losses (mortality), and the invisible losses (weight loss) in sheep and goats kept for 35 weeks and indicated that there were considerable economic losses in natural infections. They concluded that the disease may be of greater significance in sheep and goats than had hitherto been appreciated, and that control of trypanosomiasis was well justified in terms of weight gain and reduced mortality.

A lot of work had been reported on the clinical manifestation of animal trypanosomiasis. Following natural or experimental trypanosomiasis, there is undulating parasitaemia, accompanied by intermittent fever (Maclennan, 1970; Fiennes, 1970; Anosa and Isoun, 1974, 1980b; Ogwu et al., 1984; Uzoigwe, 1984; Arunsi et al.) 1985; Luckins et al,, 1986; Maikaje et al., 1986).

During the course of infection, changes in the surface antigens (surface coat) of the trypanosomes occur through the process of antigenic variation. This phenomenon represents one major device by which the parasites establish chronic infections, insp.it e of the immune responses mounted by the host (Doyle, 1977; Murray and Urquhart, 1977; Cross, 1978; DeGee, 1979; Pearson et al., 1981; Shapiro and Murray, 1982; Nantulya, 1986).

Anaemia has for long been regarded as the most consistent and important feature of trypanosomiasis in cattle (Fiennes, 1954; Saror, 1975, 1979; Luckins et al., 1986) and in sheep and goats (Clarkson, 1968; Losos & Ikede, 1972; Anosa & Isoun, 1976, 1980b; Griffin, 1978; Luckins et al., 1986).

2.3 Pathology of trypanosomiasis in ruminants with emphasis on small ruminants

Trypanosoma vivax infection of cattle, sheep and goats has been recognised for a long time as a chronic wasting disease (Hornby, 1937). In a general review of the pathology of the tsetse-transmitted trypanosomes, Losos & Ikede (1972) observed that the type of lesions in the disease was governed by different distributions of parasites in the host. The haematic group, T. vivax and T. congolense, cause injury mainly by anaemia withoutmajor histological changes in the solid tissues. Anaemia that occurs in infections caused by the solid tissue invaders (T. brucei) is of secondary importance. The

species of trypanosome in the solid tissue group causes a chronic disease characterized by extensive mononuclear cell infiltrations and degenerative changes of the tissues in sites of trypanosoma invasion.

Observations on numerous natural cases in Nigeria revealed that neither the clinical signs nor lesions are remarkable or pathognomonic. Gross lesions observed include emaciation, dehydration, oedema and enlargement of the lymph node/spleen; gelatinous atrophy of the body fat, palor of the organs, enlargement of haemolymph nodes and accumulation of straw-coloured fluid in the serosal cavities (Fiennes, 1953; Vohradsky, 1971; Isoun and Esuruoso, 1972; Saror, 1975; Van den Ingh et al., 1976a; Anosa, 1977, 1983). The testes were often atrophied and calcified (Isoun & Anosa, 1974b; Isoun et al., 1975; Anosa & Isoun, 1980a). Cystic ovaries have been described in sheep (Isoun and Anosa, 1974b; Anosa, 1977) and in cattle (Vohradsky, 1971).

Histologically, the formation of fibrin microthrombi in blood vessels of the testes, kidney and brain has also been reported in acute cases of T. vivax infection (Isoun and Esuruoso, 1972; Van den Ingh et al., 1976a; Anosa, 1977; Anosa, 1983). The follicles of the lymph nodes of T. vivax infected cattle were moderately reactive (Van den Ingh et al., 1976b; Mwongela et al., 1981). In T. vivax infected goats these follicles were hyperplastic with enlarged germinal centres; they were more numerous in the cortex and formed tertiary nodules in the medulla (Van den Ingh et al., 1976a).

Lesions in the male reproductive organs include degeneration of the seminiferous tubules, with disappearance of spermatozoa and spermatids and less commonly spermatocytes. The latter result in a reduction of the mean seminiferous tubular diameter and epithelial thickness. There were also spermatid giant cell formation and calcification during T. vivax infections (Isoun & Anosa, 1974b; Isoun et al., 1975; Anosa & Isoun, 1980a). These changes were accompanied by decreases in semen volume, sperm concentration and motility, and increases in the numbers of abnormal and dead spermatozoa (Isoun & Anosa, 1974a; Sekoni, 1987). Spermatozoa were absent or very scanty in the epididymis of sheep and goats with severe testicular degeneration (Anosa & Isoun, 1980a).

The adrenal and pituitary glands of infected sheep and goats were normal, although Mott cells were seen in the pituitary of one sheep (Anosa, 1983). Patchy necrosis was reported in the adrenals of cattle (Van den Ingh et al., 1976b). T. vivax is traditionally classified as an intravascular parasite (Losos and Ikede, 1972) and was not found extravascularly (Van den Ingh et al., 1976; Anosa, 1983). However, extravascular T. vivax has been found in lymphatic glands and pituitary but not in other organs (Fiennes, 1950). More recently, the organism has been found in lymph (Emery et al., 1980). Heisch et al. (1970) reported that organisms injected into the testis of a goat multiplied in the connective tissues for 2 weeks and subsequently disappeared.

The presence of *T. vivax* in ovarian smears and amniotic fluid of one pregnant ewe was noted (Isoun and Anosa, 1974a) and could have an effect on the fertility of infected stock. Ikede and Losos (1975c) observed pituitary lesions in ovine trypanosomiasis, but suggested that detailed studies of hormonal activities of infected animals would be necessary to correlate the observed lesions and their clinical manifestations. Thus, an understanding of the normal oestrous activity and its regulation in ewes will be vital in this respect.

2.4 The possible effect of trypanosomiasis on reproductive parameters

Oestrus (heat) is the period of sexual receptivity in domestic animals. It is determined clinically by behavioural manifestations of sexual activity and also by the endocrine events occurring during this stage of the reproductive cycle (Frandson, 1975; Pelletier *et al*., 1977). The mean duration of oestrus in Yankasa ewes as reported in the literature was 25 + 2.2 hours (Igono *et al*., 1982; Oyedipe et al., 1986a). Normal oestrous cycle had been found to range from 16 to 21 days in the ewe (Igono *et al*., 1982; Osinowo, 1982; Oyedipe *et al*., 1986a).

Endocrine profiles provide a clear understanding of the complex physiologic events that occur during normal and abnormal reproductive functions (Edqvist & Stabenfeldt, 1980; Oyedipe *et al*., 1986b). The development of radio-isotopic techniques for hormone analysis has been an important tool for studying reproductive functions in domestic animals. In this regard, progesterone assay has been found to be of greatest practical importance in assessing the reproductive status of the female (Perera & Abeyratne, 1979; Adeyemo, 1986; Choung et al., 1986). Thus, in non-pregnant female, the concentration of progesterone in the peripheral circulation is usually an accurate index of the presence or absence of corpus luteum (CL) and reproductive status (Oyedipe et al., 1985).

Progesterone levels in the blood normally follow a cyclic pattern being low on day of oestrus and high at dioestrus or mid-cycle (Kozaneck & Reklewska, 1982; Monty, 1984; Dobson, 1985; Oyedipe et al., 1986b). It has been shown however, that under normal conditions, regression of the corpus luteum (CL) and subsequent decline in plasma progesterone occurred at about 16 days after the previous oestrus i.e. 3 - 4 days before the onset of sexual receptivity (Roberts, 1971; Arthur, 1977; Oyedipe et al., 1986b). Closely following this decline in the level of peripheral plasma progesterone is a rise in the concentration of oestrogenic hormone. This acts via the hypothalamus to cause the behavioural oestrus; it also causes the release of an "ovulatory surge" of luteinizing hormone (LH) from the pituitary gland at the beginning of oestrus and progesterone increase at dioestrus.

Reproductive physiology of small ruminants is under the control of the hypothalamus and the pituitary. Hypothalanic gonadotropinn releasing hormone causes release of both follicle - stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary. The latter are responsible for follicular development and ovulation. Both (FSH) and (LH) are produced in the anterior pituitary gland (Arthur, 1977; Hansel and Convey, 1983; Junquira et al., 1985b).

LH and FSH are released coincidently at, or near the onset of oestrus in ewes (Pant et al., 1977). Follicular development due to FSH, results in increase in circulating oestropen. The increase in oestradiol that occurs during the preovulatory period appears to be the stimulus that triggers the gonadotrophin surge. Following ovulation, the CL develops and secretes progesterone until the end of an infertile cycle.

It is therefore possible that trypanosomiasis can affect one of the regulating organs of reproduction, such as hypothalamus, pituitary and ovary. While no pathological lesions of the reproductive organs were observed by Isoun & Anosa (1974a), it may be desirable to identify which axis of reproduction may be influenced. These are viz: the hypothalamus-pituitary-axis, the pituitary-gonadal axis and the utero-ovarian axis. Isoun and Anosa (1974a) however, showed the presence of T. vivax in ovarian smears and amniotic fluid of sheep and goats. The mechanism of action of T. vivax on the pathophysiology of reproduction therefore remains unclear.

It has long been recognised that trypanosomiasis influences the reproductive potential of animals. However, the literature is very scanty nn how the endocrine system is affected. We hope to utilize the available information to highlight the effect of trypanosomiasis on the endocrine system.

Pituitary hormones (gonadotropins) play a major role in the reproductive process. In particular, follicle stimulating growth hormone (GH) play an important part in the spermatogenic cycle in males and oestrous cycle in females. Consequently, absence of pituitary gonadotropins as a result of hypophysectomy, leads to a block in sperm maturation in rats (Cleraont and Morgentaler, 1955) or to marked testicular degeneration and abortion in man and animals (Steinberger & Duckett, 1967; Apted, 1970; Ikede & Losos, 1975a).

Evidence of specific damage of the pituitary gland had been reported .in infected domestic and laboratory animals. Extensive mononuclear inflammation of the gland had been described in sheep and goats experimentally infected with T. brucei (Losos & Ikede, 1970, 1972). In addition, 10 out of 19 sheep experimentally infected with T. brucei showed extensive coagulat.ive necrosis and fibrosis of the adenohypophysis (Ikede & Losos, 1975b). The cause of testicular degeneration observed in animal trypanosomiasis was believed to be due to the effect of prolonged fever, thrombosis of spermatic blood vessels and general wasting of body organs (Anosa and Isoun, 1980a; Anosa, 1983).

Emeh and Nduka (1983) reported a significant depression of serum gonadotropins (ESH and LH) in a study of cases of advanced Gambian sleeping sickness. A year after antitrypanosomal treatment, the values returned to normal. Vohradsky (1971) together with Isoun and Anosa (1974b) reported cystic ovaries in cattle and sheep infected with T. vivax respectively.

These changes may be related to hormonal imbalance from pituitary damage, to the stress of infection and to the effect of hyperthermia (Ikede et al., 1988); the sequential pathophysiology of which must be adequately studied in order to appraise the level of endocrine imbalance in trypanosomiasis. Waindi et al,, (1986) presented evidence associating testicular degeneration with trypanosome-induced endocrine dysfunction namely, depressed testosterone levels in goats and laboratory animals. Llewlyn et al., (1986); observed that goats exhibited reduced plasma progesterone concentrations in the cycle following T. congolense infection, leading to a severe reduction in breeding efficiency.

There is evidence that luteinizing hormone (LH) released from the pituitary gland plays a role in maitenance of luteal function (Roberts, 1971; Arthur, 1977). A reduction in the ability of the corpus luteum to produce progesterone might have been due to changes in the pattern of LH secretion by the pituitary gland. Alterations in body condition, live-weight, stress and pathological lesions arising from the infection may be important factors influencing the activity of the hypothalamic pituitary axis (Llewelyn et al., 1986). Emeh and Nduka (1983) reported a decrease in mean plasma LH concentration in 11 human patients during _T_. _gambiense_ infection. Griffin (1978) attributed the pathogenic effect of _T_. _congolense_ in sheep and goats to the resultant anaemia rather than pituitary lesions. Isoun and Anosa (1974) observed a 44% increase in plasma volume in goats 1 - 2 months after infection with _T_. _congolense_. This might partly explain the possible reduction in plasma progesterone concentrations found in similar investigations. A prolonged luteal phase with elevated progesterone levels was reported by Luckins _et al_. (1986), in 5 cows and basal progesterone levels in 4 others following _T_. _congolense_ infection.

CHAPTER 5

3. MATERIALS AND METHODS

3.1 EXPERIMENTAL ANIMALS, SOURCE AND MAINTENANCE

Twelve mature Yankasa ewes and one vasectomized Yankasa ram (1-3 years old) were obtained from National Animal Production Research Institute, Shika, and Department of Parasitology and Entomology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. The ages of the animals obtained from records were confirmed by the method of temporary and permanent incissors (Wilson & Durkin, 1984). All animals were accommodated in a flyproof house, where they were kept for 6 months before the commencement of the investigation. During this period of acclimatization, the animals were dosed with thiabendazole and were sprayed with Pfizona (Pfizer Nigerian Ltd.) to control ectoparasitosis.

The animals were fed 2% body weight of concentrates comprising 40% cotton seed cake and 60% wheat bran or brewers dried grain. Local gamba hay, water and mineral salt lick were provided ad libitum in the pen throughout the experiment. Prior to the commencement of the experiment the animals were free of trypanosomes based on weekly haematocrit centrifuge tests (Woo, 1969).

3.2 EXPERIMENTAL DESIGN

Following the period of acclimatization, the ewes were divided into 2 groups (control and infected) with 6 animals per group. This selection was done by stratified randomization of packed cell volume.

Prior to the commencement of the experiment, all the ewes were synchronized with intravaginal progesterone sponges containing 60mg of medroxyprogesterone acetate (Osinowo, 1982). Ewes were checked for standing heat at mornings (8-9 AM) and evenings (4-5 PM) for a period of 1 hour each using the vasectomised ram which was usually introduced at each time of heat detection. This was continued throughout the duration of the experiment.

Rectal temperatures, parasitaemias, haemoglobin concentration, packed cell volumes, body weights, clinical heat detection (oestrus detection) and plasma progesterone levels were monitored for about 32 days before infection and 50 days following *T. vivax* infection.

3.3 TRYPANOSOME STOCK

A stock of *T. vivax* (Kabam/84 - NITR - 14) , was used for this investigation. Stabilates were cryopreserved at -196°C in liquid nitrogen until used.

1 Supplied by Dr. Elizabeth Kaggwa Kyewalabye of the Department of Parasitology & Entomology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria.

3.4 INOCULATION OF ANIMALS

A donor ewe was inoculated with the trypanosome stabilate. On the second day of detectable parasitaemia blood from the doner ewe was sub-inoculated intravenously to each of the 6 experimental ewes. Each inoculum contained approximately 3.5×10^7 trypanosomes in 10ml of donor blood. The trypanosomes were enumerated with a haemocytometer (Sannusi, 1977), using trypanosome counting fluid.

3.5 BLOOD SAMPLING

About 5ml of jugular blood was collected into test tubes containing disodium salt of ethylene diamine tetra-acetic acid (EDTA) as anti-coagulant. The blood was centrifuged and plasma separated and preserved at - 20°C until analysed for progesterone.

3.6 OESTRUS DETECTION

Oestrus was monitored twice daily in the morning between 08.00 and 09.00 hours and in the evening between 16.00 and 17.00 hours. During each detection period, the behavioural responses of the ewes to the presence of the vasectomized ram were recorded. The length of the oestrous cycle was the period between two consecutive heats; the duration of heat was the period in hours of "ram-acceptance" by the ewe while heat-intensity was estimated by the number of mounts per oestrus (Roberts, 1971; Arthur, 1977).

3.7 HAEMATOLOGICAL PARAMETERS

The packed cell volume (PCV) was determined by the micro-haematocrit centrifuge technique and values were read using the Hawksley microhaematocrit reader (Gelman Hawksley Ltd., England). The haemoglobin (Hb) concentration was measured by the cyanmethaemoglobin method using an electronic haemoglobinometer (Coulter Electronics, Hearts, England). Parasitaemia was estimated daily using the haematocrit centrifuge technique, HCT (Woo, 1969) and scored as follows:

+ = Less than 10 trypanosomes seen per field

++ = 10-20 trypanosomes see per field

+++ = More than 20 trypanosomes see per field

++++ - Massive trypanosomes see per field

3.8 PLASMA PROGESTERONE DETERMINATION

Radio-immuno-assay (RIA) of progesterone was carried out using the FAO/IAEA solid phase progesterone direct assay kit which has been validated for our use (Pathiraja *et al*., 1986). The assay procedure was as follows: to antibody coated tubes 100ul of standard (0-4n mol/1) samples or quality controls and then 1 ml of buffered ^{125}I labelled progesterone solution

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1 Supplied by the Animal Production and Health Section, International Atomic Energy Agency, Vienna, Austria to the Department of Animal Reproduction, NAPRI, Shika -

Zaria on Technical Cooperation Project NIR/5/014.

were added and vortexed for 30 seconds. The mixture was incubated for 4 hours at room temperature and the liquid phase decanted vigorously; centrifugation was not required. The tubes were tapped on paper tissue and the radio activity bound to the antibody - coated tube counted for 1 minute in a gamma counter (Nuclear Enterprises 1612 Gamma Counter). Progesterone concentratioris were estimated using a logit-log transformation performed with an RlA data soft wear integrated in the gamma counter. The sensitivity of the assay defined as twice the standard deviations away from the zero standards was 0.06 ng/ml. The within and between assay coefficients of variation were 5.7% respectively.

3.9 GROSS PATHOLOGICAL EXAMINATION

Animals were slaughtered at the end of this study. The ovaries were weighed on an electronic chemical balance (Mettler - P. 163). The left and the right ovaries were examined grossly to identify any abnormalities. The pituitary gland of each animal was also examined.

3.10 HISTOLOGICAL EXAMINATION

Thin smears were made from the sections of each of these organs, stained with Giemsa and examined under the light microscope for the presence or absence of *T. vivax*. Tissues were fixed in 10% solution of formal-saline and well labelled.

Histological sections stained with haema-toxylin and eosin (H and E) (Junqueira et al., 1975a) were examined with the light microscope.

3.11 STATISTICAL ANALYSES

The values of individual parameters for each group of ewes were pooled together pre and post *T. vivax* infection. Mean data of infected and control animals were compared statistically by the students-t-test (Steel and Torrie, 1960; Winer, 1971).

CHAPTER 4

4. RESULTS

4.1 PARASITAEMIA

All the infected ewes developed parasitaemia 2 days post infection. Several peaks of parasitaemia occurred throughout the experiment (Fig. 3.2 - 3.4). Control ewes remained free of parasites throughout the experiment (Fig. 3.1).

4.2 RECTAL TEMPERATURE

Rectal temperatures showed periodic increases, often associated with peaks of parasitaemia (Figs. 3.2 - 3.4). There were fluctuations from normal values (taken to be 38.8°C) up to 42°C (Fig. 3.3). Control ewes had normal temperatures throughout the experiment.

4.3 HAEMATOLOGICAL OBSERVATIONS

Following the onset of parasitaemia, all the infected ewes developed anaemia. Their PCV and Hb dropped from respective preinfection values of 29 ± 2.9% and 10.3 ± 0.9g/dl .to 12.3 ± 2.U and 6 ± 1.4 g/dl at about 50 days following T. Vivax infection (Figs. 3.2 - 3.4). There were no significant changes in PCV and Hb of the control ewes throughout the experiment (Fig. 3.1).

4.4 BODY WEIGHT

All the infected ewes progressively lost weight during the experiment, 2 of which later began to gain weight (Figs. 3.2 - 3.4). The observed weight loss at day 46 was about 17% of the original weight (Appendix lb). The weights of the control ewes had increased by 4% at the end of the study period (Appendix la)

4.5 OESTROUS CYCLES

A total of 10 oestrous cycles were observed in the control ewes while 12 cycles occurred in the infected ewes during the pre-infection period. Oestrous cycles in the control ewes ranged between 16 and 21 days with a mean (± SE) of 18.3 ± 1.5 days (Table 1). During the pre-infection period the oestrous cycles in the infected group ranged between 16 and 21 days with a mean of 18.3 ± 1.5 days (Table 2). The pre-infection duration of oestrus ranged from 24 to 32 hours, a mean of 27.2 ± 4.4 hours, for the control ewes (Table 1), while it ranged between 24 and 33 hours with a mean of 28.3 ± 4.8 hours for the infected group of ewes (Table 2). Intensity of oestrus ranged between 14 and 18 mounts per oestrus with a mean of 15.4 ± 1.5 mounts per oestrus (Table 1) for the control ewes, and between 14 and 20 mounts per oestrus with a mean of 16.2 ± 2 mounts per oestrus for the experimental animals before T. vivax infection (Table 2).

Control ewes exhibited regular progesterone profiles and the basal values between these progesterone peaks coincided with the clinical heat. Following T. vivax infection, 3 patterns of progesterone profiles were obtained:

a) The first was marked by 1 regular well demarcated progesterone peak and a smaller, not well defined peak which was clinically recognised as silent heat (Fig. 3.2).

b) The second group of 2 ewes demonstrated 40 days of basal progesterone level post _T_. _vivax_ infection followed by a progesterone rise. This was recognised clinically as temporary anoestrus (Fig. 3.3).

c) The third group of 2 ewes exhibited basal progesterone levels post infection and clinically they were anoestrus throughout the study period (Fig. 3.4).

Each figure is a prototype of an animal in each group.

Table 1: PARAMETERS OF CLINICAL OESTRUS IN 5 CONTROL YANKASA EWES

Oestrous Parameters	Pre-Experimental	Post-Experimental
Oestrous cycle (days)	18.3 ± 1.5*	17.8 ± 1.0
Duration of oestrus (hours)	27.2 ± 4.4	27.4 ± 1.7
Intensity of oestrus (mounts/ oestrus)	15.4 ± 1.5	15.2 ± 0.8

* Data given as mean ± SE.

Table 2: PARAMETERS OF CLINICAL OESTROUS IN 6 T. VIVAX INFECTED YANKASA EWES.

Oestrous Parameters	Pre-Infection	Post-Infection
Oestrous cycle (days)	18.3 ± 1.5*	**
Duration of oestrus (hours)	28.3 ± 4.8	**
Intensity of oestrus (mounts/oestrus)	16.2 ± 2.0	**

* Data given as mean ± SE.

** Animals were clinically anoestrous.

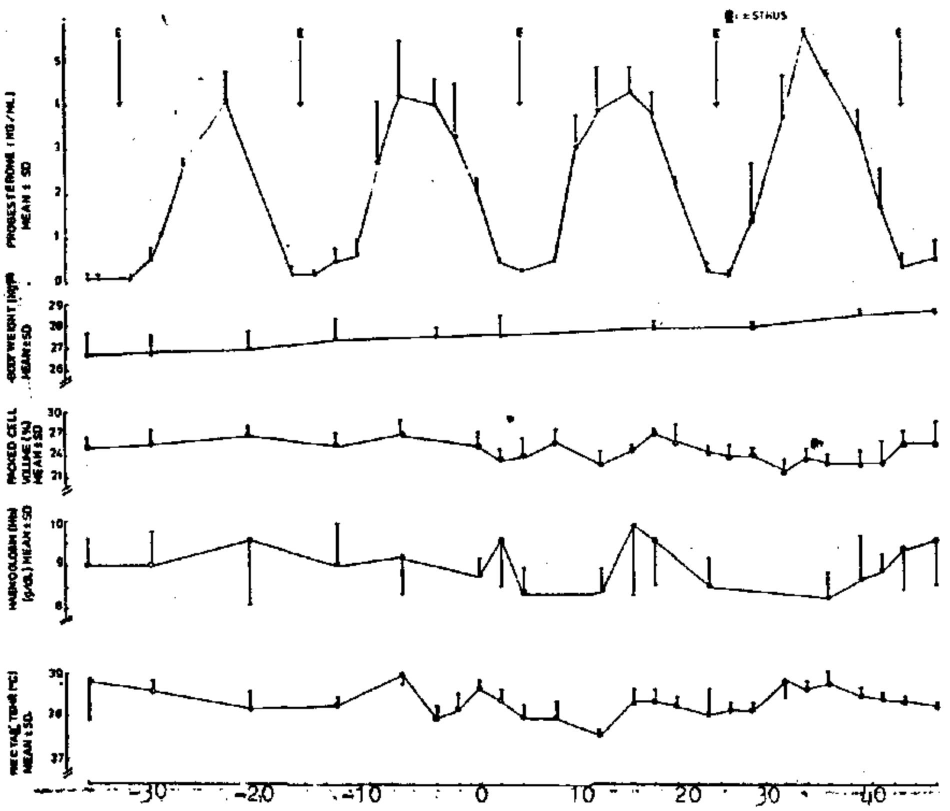


Figure 1: Mean progesterone levels, body weights, packed cell volumes, haemoglobin concentrations and rectal temperatures of five control Yankasa ewes.

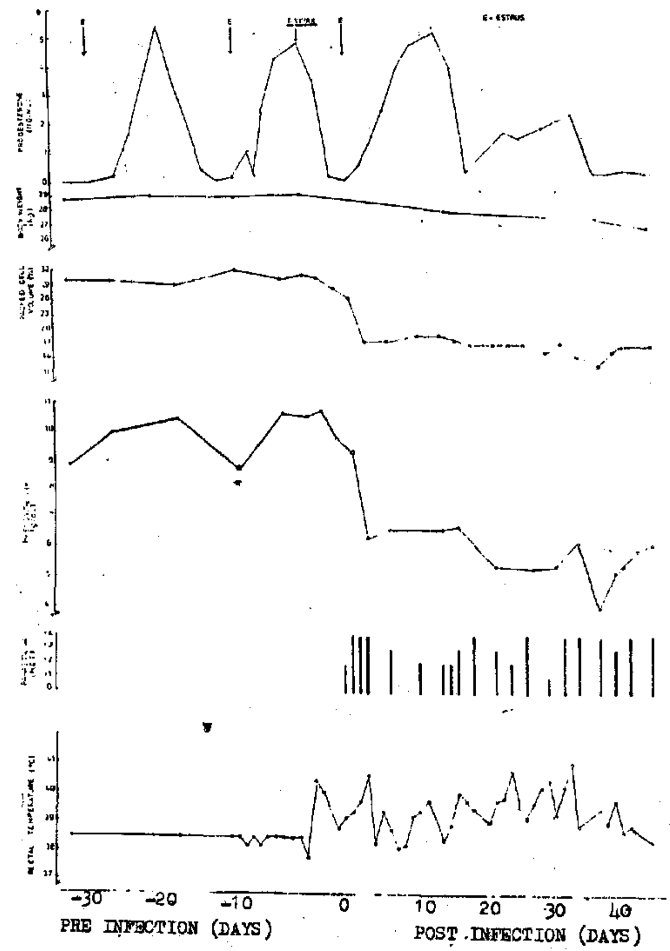


Figure 2: Progesterone levels, body weights, packed cell volumes, haemoglobin-concentrations, parasitaemias and temperatures of T. vivax infected Yankasa ewe No. 355 with silent oestrus.

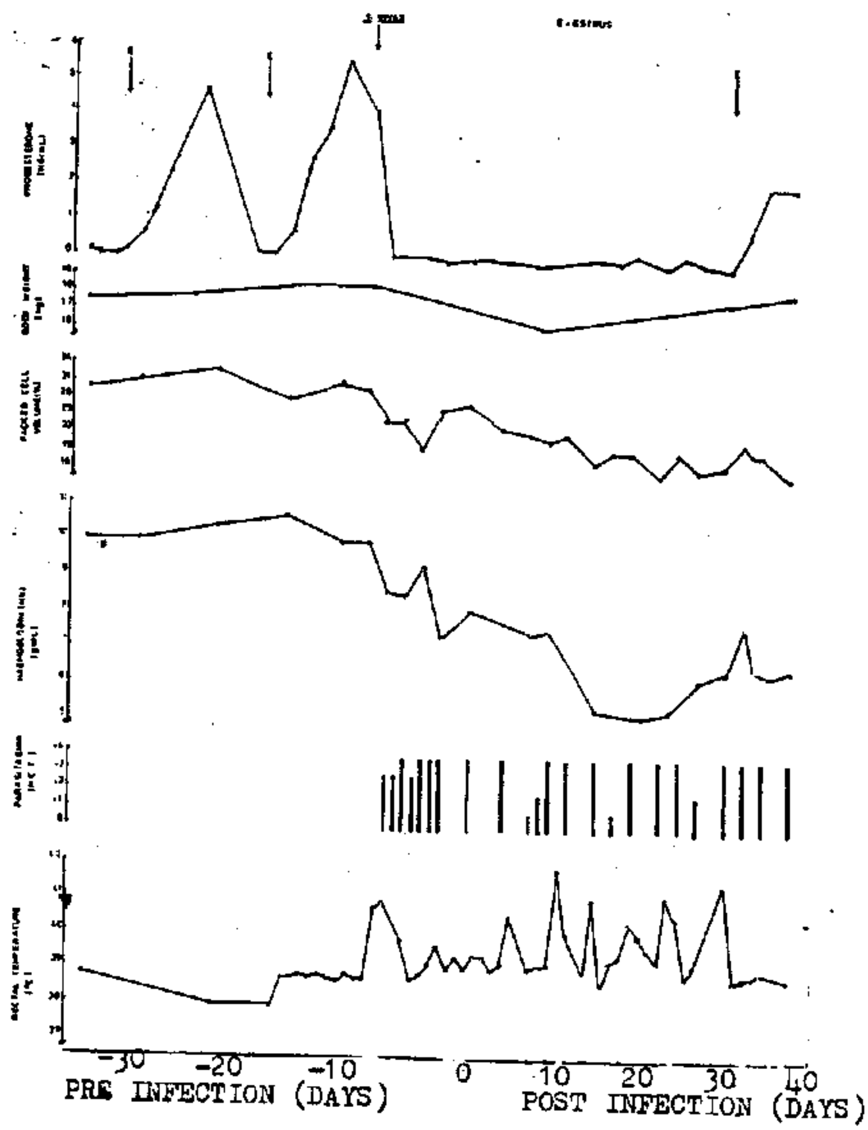


Figure 3: Progesterone levels, body weights, packed cell volumes, haemoglobin concentrations, parasitaemias and temperatures of T. vivax infected Yankasa ewe No. 379 with temporary anoestrus.

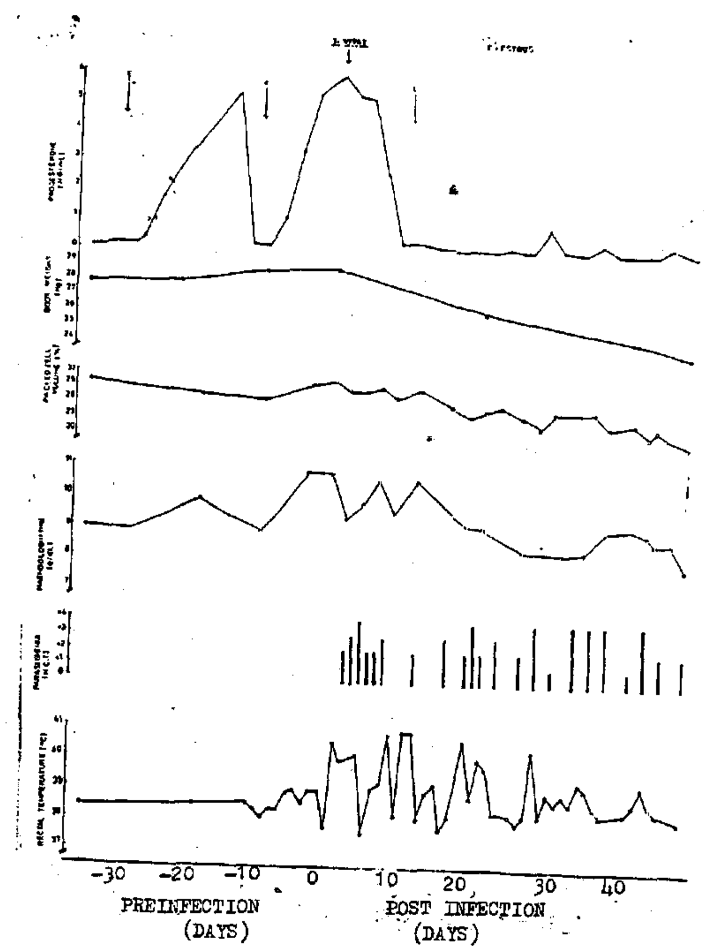


Figure 4: Progesterone levels, body weights, packed cell volumes, haemoglobin-concentrations, parasitaemias and temperatures of T. vivax infected Yankasa ewe No. 236 with complete anoestrus.

4.6 GROSS PATHOLOGICAL OBSERVATIONS

The ovaries of T. vivax infected ewes showed marked atrophy. The ovaries of the infected group weighed about half those of the control group. ($P<0.01$, Table 3). This was more apparent when the ovarian weights were expressed as percentages of the body weights (Table 3).

The ovaries of 4 infected animals were static with little or no trace of follicular development and functional corpusluteum CL; 2 other infected ewes had mildly functional ovaries with few primary follicles and atretic CL. All the ovaries of the control ewes were actively functional and showed the presence of functional CL and Graffian follicles.

There were no significant gross changes detectable in the hypothalamus and the pituitary glands of the infected animals.

4.7 HISTOPATHOLOGICAL OBSERVATIONS

Parasitaemia was demonstrated in the Giemsa-stained jugular blood of infected ewes (Plate 5A). Trypanosomes were also demonstrated in the Giemsa-stained smears made from the ovaries of the infected ewes. Histological changes in the hypothalamus, the pituitary glands and the ovaries of T. vivax infected ewes were similar although the lesions were more severe in some ewes than others. Generally the lesions in the hypothalamus of the infected ewes consisted of generalized haemorhage (Plate IB), while those of the pituitary glands were characterized by mononuclear (mostly lymphocytes and macrophages) cellular infiltration into the capsules and the parenchyma (Plate 2B). Most of the acidophilic and basophilic cells were nectrotic with dark pyknotic nuclei and faded cytoplasm. Few tissue structures and cellular outlines could still be seen in some areas (Plate 3A).

Histopathological lesions in the ovaries of the infected ewes ranged from mild fibrosis of the ovarian stroma characterized by the presence of few primary, secondary, atretic follicles and CL with no trace of tertiary and Graff.ian follicles, to extensive fibrosis of the ovarian stroma with no evidence of CL and follicular development (Plated4A & B).

Trypanosomes (whole or nuclei) were not observed in the blood vessels or elsewhere in histological sections. There were no histopathological lesions in the control ewes.

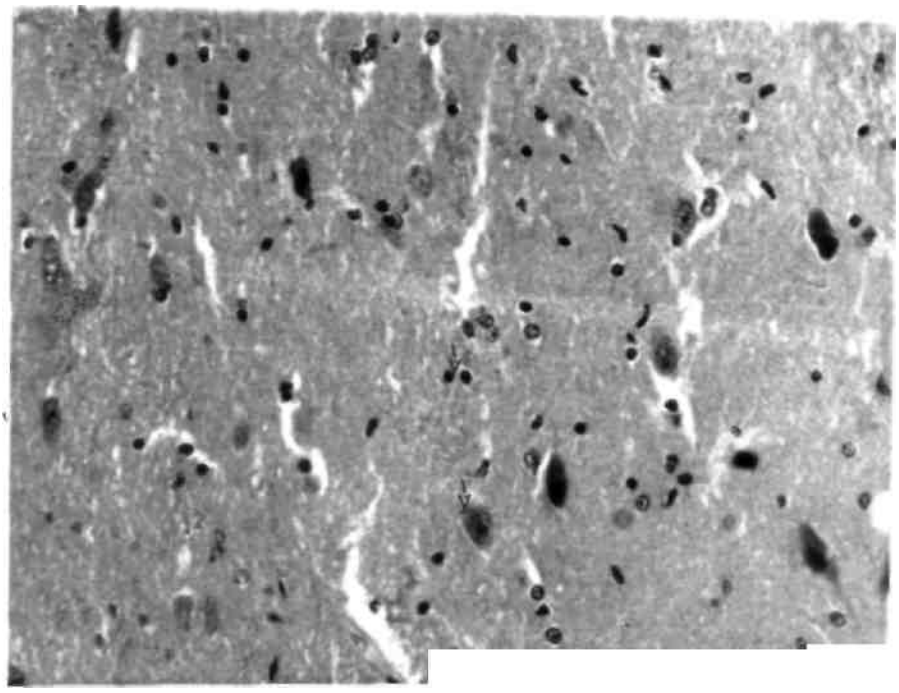
Table 3: OVARIAN WEIGHTS IN *T. VIVAX*-INFECTED AND CONTROL EWES

Group	Ovarian weights	
	Total (g)	Percent body weight
Control (n=3)	1.31 + 0.28[a]	0.009 ± 0.001
Infected (n=6)	0.65 ± 0.007[b]	0.005 ± 0.001

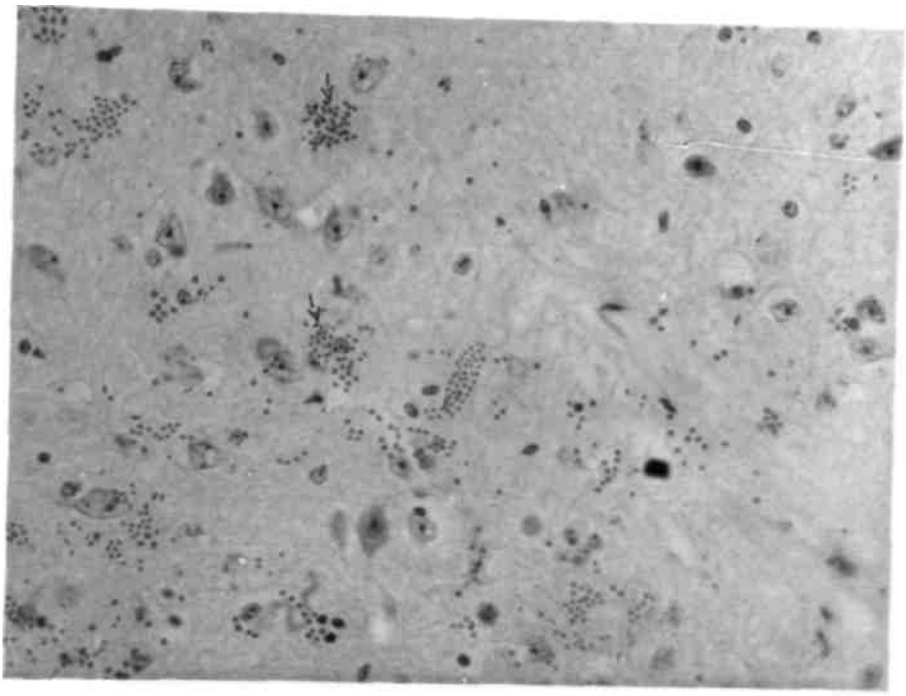
a = Mean ± SE

b = Significantly different from corresponding controls ($P < 0.01$)

LEGENDS TO PLATE 1

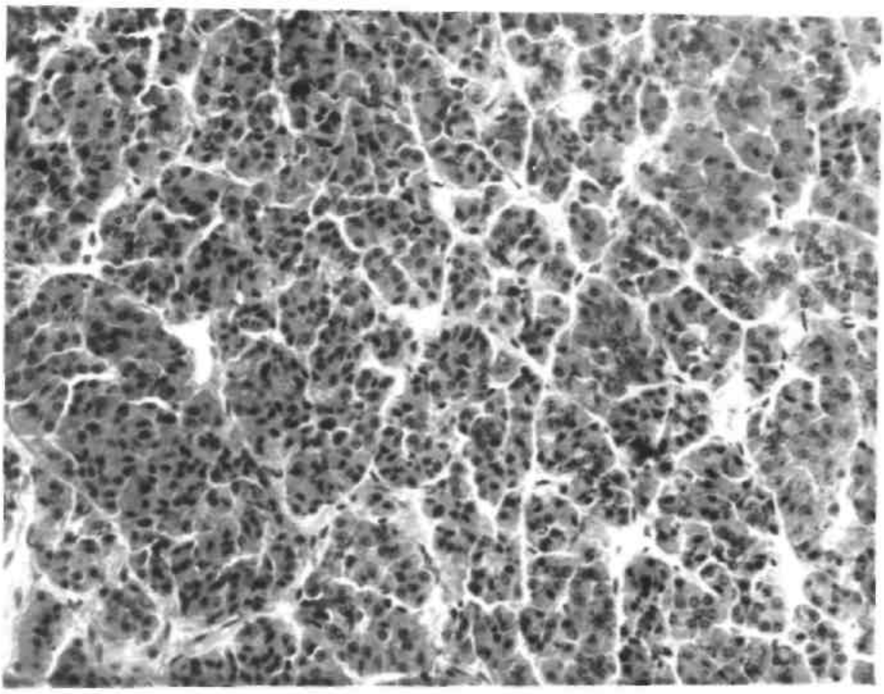


A. Hypothalamus of a control ewe showing normal nerve cells (arrowed) H$E x 400

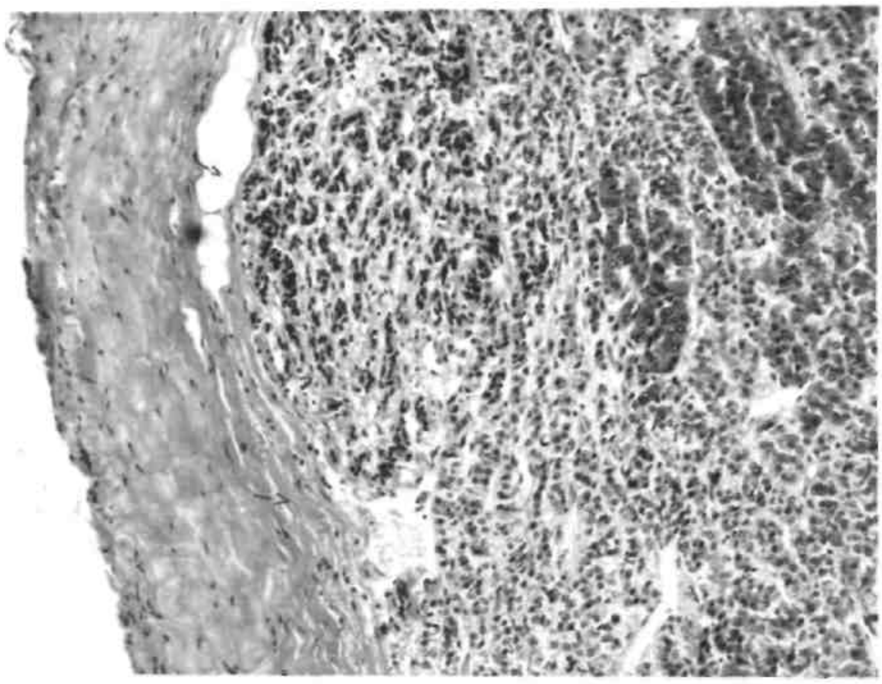


B. Hypothalamus of an infected ewe showing generalized haemorrhage (arrowed) H&E x 400

LEGENDS TO PLATE 2

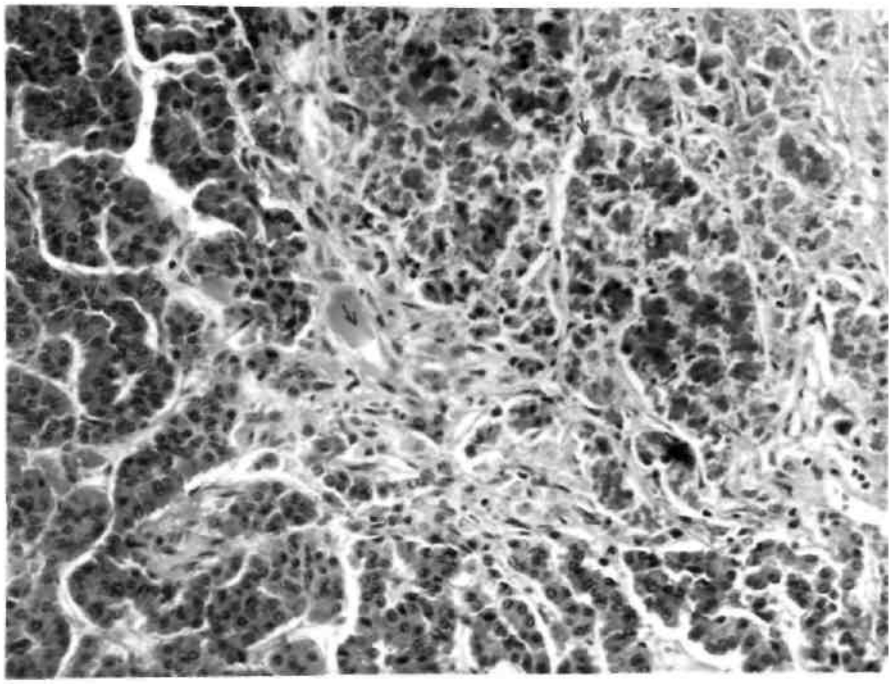


A. Pituitary of a control ewe showing normal parenchymal cells H&E x 400

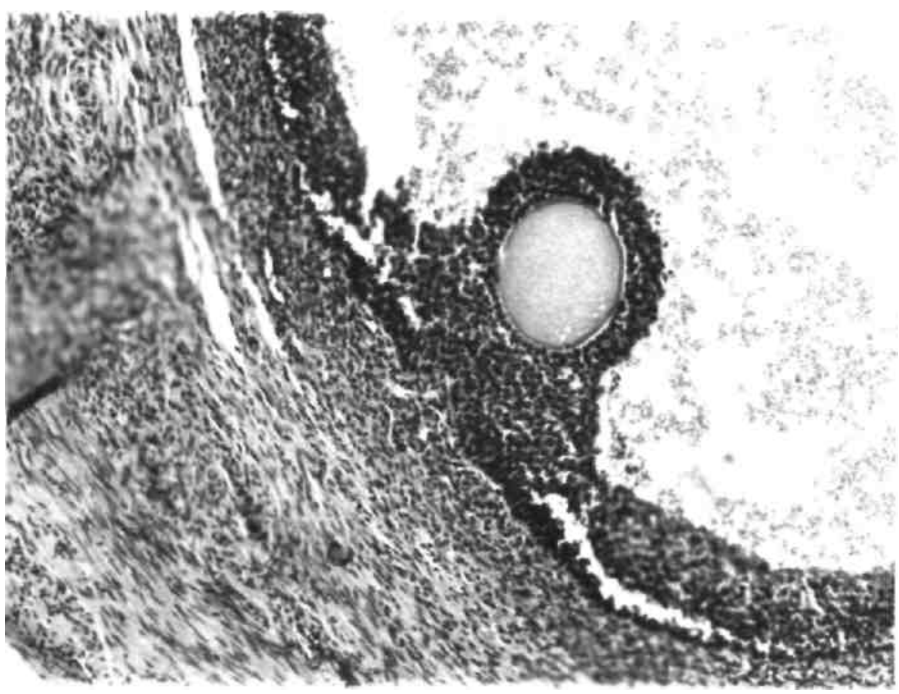


B. Pituitary of an infected ewe showing mononuclear (mostly lymphocytes and macrophages) cellular infiltration into the capsule and the parenchyma (arrowed) H & E x 200.

LEGENDS TO PLATE 3

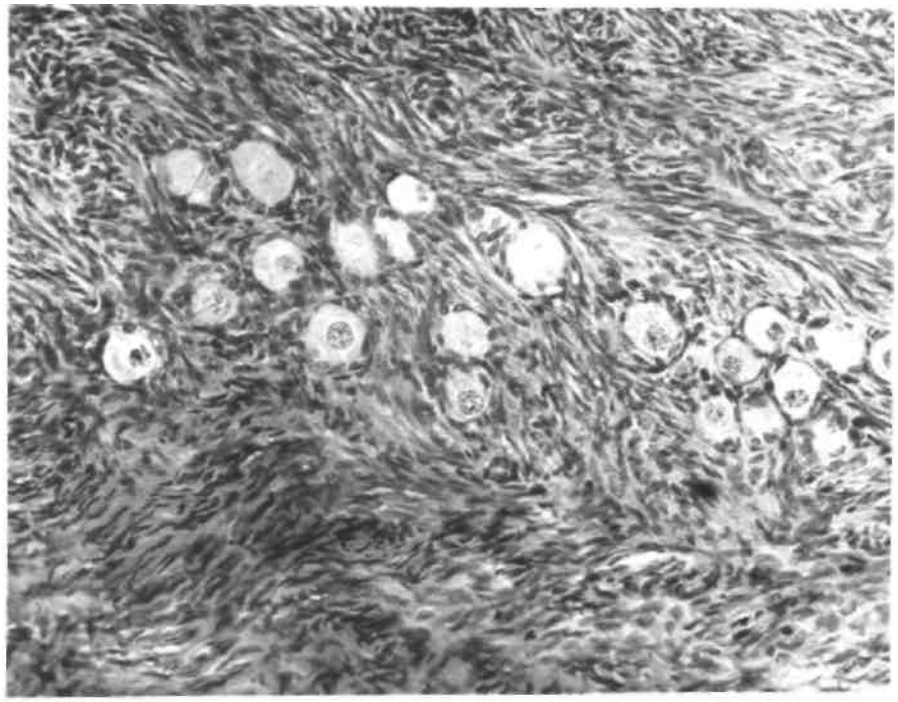


A. Pituitary of an infected ewe showing necrosis and fibrosis of acidophilic and basophilic cell groups (arrowed) H & E x 400

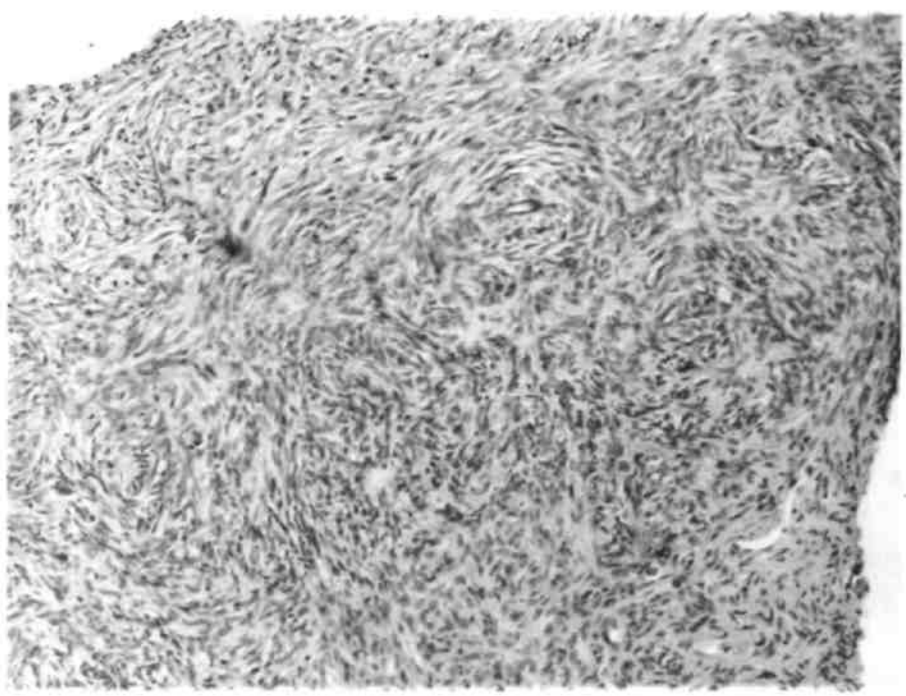


B. Ovary of a control ewe showing a Graafian follicle embedded in parenchymal epithelial cells and dense irregular connective tissue H & E x 400.

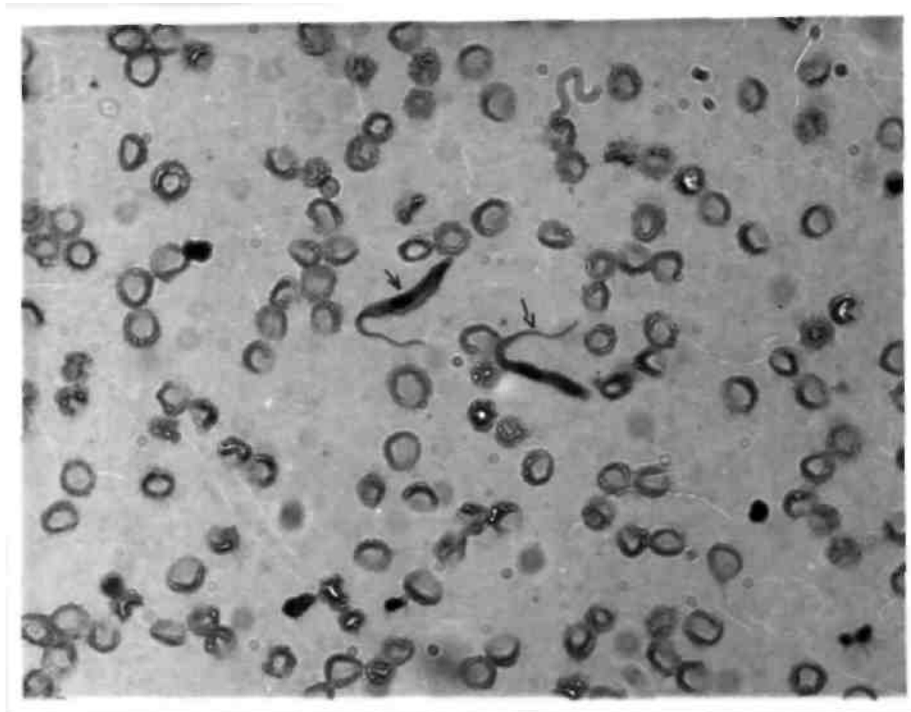
LEGENDS TO PLATE 4



A. Ovary of an infected ewe showing fibrosis of the ovarian stroma with few primary, secondary and atretic follicles H&E x 400.



B. Ovary of an infected ewe showing extensive fibrosis of the ovarian stroma, with no evidence of follicular formation H&E x 400.



A. A thin blood film showing low parasi-taemia of _T_. _vivax_ (arrowed) in the jugular blood smear of an infected ewe H & E 1000.

CHAPTER 5

DISCUSSION

The clinical observations made in. this study, such as undulating parasitaemia which was accompanied by intermittent hyperthermia, are in agreement with previous observations in *T*. *vivax*-infected sheep and goats (Anosa and Isoun, 197U. 1980b; Arunsi et ai.,1985; Maikaje *et al*., 1986) and in *T*. *vivax*-infected cattle (Ogwu *et al*,, 1984; Uzoigwe, 1984). The marked drop in PCV and Hb values observed in this study are indications of anaemia (Saror, 1975). These are in agreement with results obtained from *T*. *vivax*-infected sheep- and goats (Clarkson, 1968; Anosa and Isoun, 1976), and in *T*. *Congolense*-infected sheep and goats (Mackenzie and Cruikshank, 1973; Luckins *et al*., 1986).

The ranges of lengths of oestrous cycle (16 - 21 days), the duration of oestrus (24 - 32 hours) and the intensity of oestrus (l4 - 18 mounts per oestrus) observed in control ewes in this work agree with those reported by Igono *et al*. (1982), Osinowo (1982) and Oyedipe *et al*., (1986b) for normal Yankasa sheep. The progesterone profiles were normal and remained within the range of 4 - 6 ng/ml during the luteal phase of the cycle for the control ewes throughout the experiment and the infected ewes before infection. This is similar to those reported by other workers for goata (0’ hara and Gombe, 1986a; Liewelyn *et al*., 1986) and higher than those reported for sheep (Quirke *et al*., 1979; Oyedipe *et al*., 1986a).

The absence of clinical oestrus observed in this study following *T. vivax* infection is in contrast to the observation of clinical oestrus in some of the *T. congolense*-infected goats (Llewelyn *et al*., 1986). The progesterone concentrations decreased within the range of 1 - 2 ng/ml for 2 ewes in the first grouping of infected animals during the luteal phase of the oestrous cycle. This is about a 33% decrease when compared to the value obtained during the luteal phase of the control (uninfected) ewes. Progesterone concentration was basal for about 40 days in the second grouping of 2 of the infected ewes, but later rose again possibly as a result of recovery from *T. vivax* infection. Two ewes in the third grouping of infected animals, however, became completely acyclic, exhibited prolonged basal progesterone concentrations throughout the duration of the experiment.

These observations show that *T. vivax* infection has adverse effects on the oestrous activities of mature Yankasa ewes. However, the machanisms by which the organism adversely affects reproduction are poorly understood. Several causes have been suggested, including pituitary hormonal defects in man (Apted 1970), in goats (Griffin and Allonby, 1979), and in sheep (ikede and Losos, 1972b). Depressed plasma progesterone levels were associated with degenerative lesions of the hypothalamus, pituitary glands and the ovaries of the infected ewes. These observations agree with those of other investigators who observed reduction of overall sizes of the organs and extensive tissue or organ damage, effecting the central nervous system, endocrine and reproductive organs in T. vivax-infected sheep and goats (Losos and Ikede, 1972; Isoun and Anosa, 197ij.b; Anosa arid Isoun 1980a), in T. brucei-infected sheep and goats (Losos and Ikede, 1972; Ikede and Losos, 1972b), in T. congolense-infected sheep and goats (Losos and Ikede, 1972; O′ hard and Gombe, 1986b; Luckins et al.,1986) and in bulls infected with T. vivax and T. congolense (Isoun et al,, 1975; Luckins et al,, 1986, Sekoni, 1987). On the contrary, Griffin (1978) ovsorved that pituitary lesions did not occur in T. congolense infections of sheep and goats, a disease in which the main pathogenic effects are associated with the anaemia. Other suggested causes of trypanosome-induced reproductive dysfunction are fever (Ikede and Losos, 1975; Kaaya et al., 1977; Griffin and Allonby, 1979c) and stress (ikede, 1979). Hyperthermia and nutritional stress conditions arising from the infections may also influence the production of gonadotropins (Llewelyn et al., 1986).

Degenerative changes observed in the reproductive organs of ruminants (Wloff and Monty, 1974; Wolf et al., 1977), had been attributed to prolonged hyperthermia (Sekoni, 1987). In a similar investigation, a significant decrease in progesterone concentrations and pituitary gonadotropins had been reported in cows subected to heat-stress (Madam andJohnson, 1973; Miller and Alliston, 1973; Rosenberg et al., 1977)

In the present study, hyperthermia was commonly recorded in infected ewes, with time (temperatures > 38.8°C occuring in about 82 per cent of days post-infection. It is therefore quite possible that fever contributed to the degeneration of the reproductive system. The difference lies in the sources of hyperthermia; one was as a result of a pathogen (T. vivax)while the other was due to an increase in environmental temperature.

However, Konthy, (I984) has pointed out that more studies are needed to clarify the pathogenesis of infertility due to heat stress.

Undulating fever and hyperthermia could also be responsible for the clinical anoestrus observed in T. vivax infected ewes, by causing the animals to "switch-off. This is a mechanism by which the ewes become irresponsive to various challenges made by the ram. The hypothalamic neurones provide an important link between the central nervous system and the pituitary gland in the integration of behavioural responses (Wood 1971). It is thus not improbable that hypothalamus being a thermoregulatory centre is responsible for the switch-off, coupled with its proximity to and influence upon the pituitary gland.

The pituitary effect is the most likely cause of reproductive dysfunction considering the gonadotropin requirements in the stimulatinn and maintenance of gonadal activities in animals (Sehoni, 1987).

Reproduction is under the control of neuro-endocrine glands (Arthur, 1977). Diseases that have pathological effects on the endocrine glands (especially the anterior pituitary gland and the ovaries) are likely to interfere with the endocrine control of reproduction. Hence the abnormal progesterone profiles and various types of anoestrus observed in this study for T. vivex-infected ewes.

This study has therefore complemented earlier reports on the interaction between trypanosomiasis and reproduction because of the information on ovarian activity provided by the clinical heat detection, plasma progesterone estimations and reproductive pathology of mature Yankasa ewes. There are number findings from this investigation which could have important implications on our understanding of the pathophysiology of T. vivax infection in sheep and their adverse effects on reproduction. Some of these are:

1. Infection with T. vivax causes distruption of normal cyclical overian activity.
2. The occurrence of irregular oestrous activities following trypanosome infection suggests some distruption of the normal patterns of pituitary gonadotropin release or a lack of ovarian responsiveness to such stimuli.

3. The pattern of responses following infection corresponds with the pathological findings in the reproductive organs. This suggests that one of the possible mechanisms by which trypanosomes alter oestrous activities in infected animals is by inducing severe damage to their hypothalamo-hypophysial-gonadal axis, resulting in depressed ovarian activities.

Further studies can be carried out to find the effect of T. vivax on fertilization, implantation and pregnancy. It will also be worthwhile to study the effect of T. vivax infection on the reproductive performance of other Nigerian breeds of sheep such as the West African Dwarf ewes (WAD) and their crosses. Based on this investigation, it is evident that T. vivax is of economic importance causing infertility in sheep. Therefore any attempt by the Government to improve livestock production must take into consideration the effect of trypanosomiasis in sheep particularly in tests infested areas.

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APPENDIX la

CLINICAL PARAMETERS OF UNIFECTED CONTROL) YANKASA EWES (N = 5)**

Pre/Post Infection (Days)	Rectal Temp. (°C)	P.C.V. (%)	Hb (g/dl)	Body Weight (kg)
-32	38.8±0.9	25±1 .4	9.9±0.6	26.7±2.6
-26	38.6±0.3	25.6±2	9±0,8	26,8±1 .6
-17	38.2±0.4	26.6±1.6	9.6±1.6	26.9±2.4
-9	38.3±0.2	25.3±2.1	9±1.5	27.4±2.8
-3	38.6±0.2	26.8±2.3	9.2±0.8	
0	38+0.3	*	*	27.5±0.5
2	38.2±0.4	*	*	
4	38.7±0.2	25.6±2.3	8.8±0.4	
6	38.4±0.3	23.6±1.9	9.6±1	27.6±2.3
8	38±0.3	23.8±2.7	8.4±0.6	
11	38±0.4	26.2±1.9	*	
15	37.6±0.1	22.8±1 .6	8.4±0.6	
18	38.4±0.3	25±0.7	10.9±3.6	
20	38.4±0.3	27.4±1.3	9.6±1	27.9±0.4
22	38.3±0.2	26±2.9	*	
25	38.1±0.6	24.6±1.6	8.6±0.6	
27	38.2±0.2	24±3.3	*	
29	38.2±0.2	23.8 ±1 .5	*	
32	38.9±0.3	22±2.2	*	
34	38.7±0.2	23.8±1 .5	*	
36	38.8±0,3	23.4±1.5	8.3±0.6	28.5±0.3
39	38.5±0.2	23±2	8.7±1	
41	38.4±0.2	22.8±3.7	8.9±1.4	
43	38.4±0.1	26.2±1.9	9.4±0.9	28.7±0.1
46	38.3±0.1	25.8±3	9.6±1	
48	38.4±0.2	*	*	
49	38.4±0.3	*	*	
50	38.4±0.4	*	*	

* Not analysed
** Mean ± SD.

APPENDIX Ib

CLINICAL PARAMETERS OF T.VIVAX INFECTED YANKASA EWES (N = 6)**

Pre/Post Infection (Days)	Rectal Temp. (°C)	Parasitaemia (HCT)	P.C.V. (%)	Hb (g/dl)	Body Weight (kg)
-32	38.5±1	0	29±1.4	10.9±1	24.9±4
-26	38.4±0.5	0	28.7±1.6	10.8±0.8	26.7±3.2
-17	38.2±0.4	0	31±1.9	10.9±1.1	27.8±2.7
-9	38.3±0.6	0	28.3±2.3	9.6±1.1	28.8±3.9
-3	38.7±0.5	0	29±2.9	10.3±0.9	
0	38.5±0.9	0	*	*	29±2
2	40±1.4	3±1.2	28.6±2	9±1	
4	39.9±1.2		26.3±2	10.5±1.5	
6	39.2±0.6	3.2±1	24.6±2.8	10.9±2.5	25.3±4
8	39.8±1.0	4±0	23.6±3.6	8.1±1.2	
11	39.1±0.9	2.9±1.2	23.6±4.1	*	
15	38.9±1.1	2.9±1.2	20.6±3.9	7.7±1.7	
18	39.4±1	2.4±1.3	20.3±3.4	7.2±1.2	
20	39.5±0.6	2.7±1.4	21.3±2.9	7.4±0.9	24.1±3.8
22	39.3±0.5	3±1.4	21.4±3.3	*	
25	39.4±1.1	3.1±1.2	19.9±4.2	6.3±1.2	
27	39.9±0.8	2.4±1.1	18.9±3.4	*	
29	39.6±0.9	2.7±1.6	19.4±3.4	*	24.1±2
32	39.8±0.8		16.4±4.3	*	
34	39.7±0.8	3.3±1.1	19.2±3.3	*	
36	38.1±0.7	2.6±1.4	17±3.7	6.5±1.2	
39	39.3±1.1	3.1±1.5	16.4±4	6±1.6	24.1±3.1
41	39.3±0.6	3±0.8	17.7±3.4	6.8±1.4	
43	39±0.3	3.7±0.8	17.7±3.5	6.4±1.4	
46	39.6±0.3	2.9±1.2	17.9±3.6	6.5±1.6	23.8±2.7
48	39.2±0.6	3.7±0.8	15±3.4	6±1.4	
49	39.1±0.7	2.5±0.9	15.3±3.2	*	
50	39±0.2	1.3±1.5	12.3±2.1	*	

* Not analysed
** Mean ±SD.

APPENDIX IIa. PROGESTERONE CONCENTRATIONS OF FIVE CONTROL EWES

DAYS	ng/ml
-32	0.10±0.06
-31	0.12±0.07
-29	0.11±0.03
-28	0.11±0.06
-26	0.49±0.25
-25	1.10±0.04
-23	2.65±0.13
-19	4.11±0.7
-13	0.24±0.15
-11	0.16±0.07
-9	0.47±0.26
-7	0.59±0.35
-5	2.71±1.42
-3	4.16±1.30
0	4.01±0.62
2	3.27±1.17
4	2.14±0.33
6	0.45±0.14
8	0.31±0.04
11	0.51±0.14
13	2.95±0.78
15	3.85±0.97
18	4.25±0.61
20	3.83±0.49
22	2.26±0.1
25	0.28±0.18
27	0.23±0.06
29	1.42±1.33
32	3.71±0.95
34	5.71±0.08
36	4.68±0.11
39	3.40±0.52
41	1.72±0.86
43	0.36±0.3
46	0.63±0.04

APPENDIX IIb PROGESTERONE CONCENTRATIONS OF *T.VIVAX*-INFECTED EWES (ng/ml)

DAYS	344 ng/ml	379 ng/ml
-32	0.1	0.1
-31	0.1	0
-29	0	0
-28	0.2	0.1
-26	0.2	0.6
-25	0.1	1.1
-23	0.2	2.3
-19	1.4	4.7
-13	3.9	0.1
-11	3.8	0.1
-9	0.2	0.7
-7	0.1	2.8
-5	1.2	3.7
-3	3.4	5.6
0	9.9	4.2
2	3.8	0.1
4	3.6	*
6	4.7	0.1
8	0.3	0
11	0.1	0.1
13	0.2	*
15	0.1	*
18	0.1	0.1
20	0.2	*
22	0.2	*
25	0.2	0.2
27	0.3	0.1
29	0.2	0.3
32	0.9	0
34	0.2	0.3
36	8.6	0.1
39	0.5	0
41	1.5	0.9
43	2.4	2.3
46	2.8	2.3

APPENDIX IIb - CONT'D.

DAYS	340 ng/ml	236 ng/ml	355 ng/ml	3207 ng/ml
-32	0.2	0.1	0	0.1
-31	0.1	0.1	0	0.0
-29	0.1	0.2	0	0
-28	0.2	0.2	0.2	0.1
-26	0.2	0.2	1.6	0.1
-25	0.7	0.4	5.4	0.2
-23	1.3	1.8	0.4	0
-19	3.2	3.4	0.1	4.8
-13	4.8	5.3	0.2	0.5
-11	0.2	0.3	1.1	0.1
-9	0.1	0.2	0.2	0.4
-5	2.8	1.1	2.4	1.3
-3	4.8	3.4	4.3	2.9
0	6.3	5.3	4.8	5
2	5.4	6.1	3.5	4.9
4	5.2	5.3	0.2	4
6	2.9	5.2	0	0.3
8	0.4	2.7	0.6	0.1
13	0.3	0.4	2.4	0.9
14	0.3	0.4	3.9	2.9
15	0.2	0.3	4.7	3.8
18	8.2	0.3	5.1	4.3
20	0.3	0.3	3.9	6.1
22	0.3	0.3	0.3	4.6
24	0.9	0.4	*	0.4
25	0.4	0.3	1.6	0.5
27	2.1	1.1	1.4	2.3
29	0.3	0.4	*	1.6
32	0.2	0.3	1.7	2
34	0.6	0.6	*	2.4
36	0.3	0.3	2.2	2.4
39	0.3	0.3	0.1	0.3
41	0.2	0.3	0.1	0.2
43	0.6	0.6	0.2	0.3
46	0.2	0.3	0.1	0.2

* Not analysed.