

**THE IMMUNOGENICITY OF IRRADIATED  
*THEILERIA PARVA* (KATETE) SPOROZOITES  
IN THE IMMUNISATION OF CATTLE  
AGAINST MALIGNANT THEILERIOSIS**

**By**

**Stephen Mutoloki**

**A dissertation submitted to the University of Zambia in partial fulfillment of  
the requirements of the degree of Master of Veterinary Medicine in**

**Veterinary Parasitology.**

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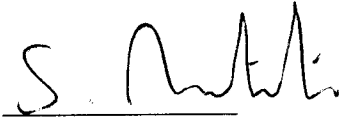
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**Declaration**

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A handwritten signature in black ink, appearing to read 'S. Mutoloki', written over a horizontal line.

Stephen Mutoloki

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Date

## **Dedication**

To my wife Emma, for giving me the strength and support when I needed it most.

To my daughter Joy, for the inconvenience she suffered all that time that I was away from home.

To my mother Meriah and in loving memory of my late dad Samuel H. Mutoloki  
... May his soul rest in peace.

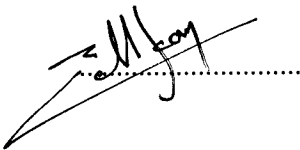
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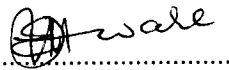
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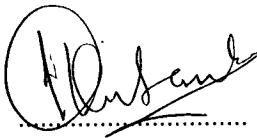
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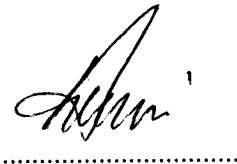
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### Abstract

The effect of irradiation on the Katete stock of *Theileria parva* sporozoites either directly (stabilate form) or indirectly (in their tick vectors) was compared to the 'infection and treatment' method of immunisation of cattle against malignant theileriosis. Fifteen steers were randomly divided into five groups using a computerised random number generator. The first group comprising three steers was immunised with *T. parva* sporozoites harvested from *Rhipicephalus appendiculatus* ticks and then irradiated at 8.4 krad using the <sup>60</sup>Cobalt source. In the second group, four steers were immunised with *T. parva* sporozoites irradiated at 20 krad in their *R. appendiculatus* tick vectors. The third and fourth groups consisting of three steers each were immunised with non-irradiated *T. parva* sporozoites. In addition, the fourth group was also treated with tetracycline (infection and treatment). The fifth group (control) with two steers was introduced during the challenge of immunised animals and was treated with lethal doses of *T. parva* stabilates only.

The steers in all experimental groups, except the control, resisted lethal challenge with homologous *T. parva* stabilates. Mild and moderate reactions were observed in the group of steers immunised with stabilates produced from *T. parva* sporozoites irradiated in their tick vectors (group two) and non-irradiated stabilates only (group three), respectively. Steers in the control group died from East Coast fever.

The best results were obtained from the groups of steers immunised with directly irradiated *T. parva* sporozoites and non-irradiated stabilates with concurrent tetracycline therapy. There was no statistically significant difference

( $P>0.05$ ) between the infection and treatment method of immunisation and that of directly irradiated stabilates.

Estimates of costs showed that farmers would save between \$0.39 (calf) and \$2.39 (adult) per animal if they used irradiated stabilate instead of tetracycline in the immunisation.

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# CHAPTER ONE

## INTRODUCTION

### 1.1 General introduction

Malignant theileriosis refers to East Coast fever (ECF) and its related syndromes of Corridor and January disease. It is a disease of cattle characterised by a febrile reaction, swelling of superficial lymph nodes, dyspnoea, anorexia and often leading to death. Manifestation of the disease varies from hyperacute to mild or inapparent infections.

Malignant theileriosis is one of the most important tick-borne diseases of cattle in Africa responsible for enormous direct economic losses in Eastern, Central and Southern Africa (Mukhebi, 1992). In 1989, it was estimated that 25 million cattle in Eastern, Central and Southern Africa were at risk of getting infected with theileriosis (ILRAD, 1990). In the same year, regional losses due to theileriosis in eleven countries (Burundi, Kenya, Malawi, Mozambique, Rwanda, Sudan, Tanzania, Uganda, Zaire, Zambia and Zimbabwe) were estimated at US\$ 168 million (Mukhebi *et al.*, 1992).

Wherever the disease occurs, vigorous control measures which include vector control, cattle movement control, chemotherapy and immunisation have to be undertaken for the beef and dairy industries to be sustained.

The average cost of dipping one animal per year using a hand pump was estimated at US\$ 11.63 in 1995 (Minjauw, 1996) while the cost of treating one case of ECF weighing 400 kg with buparvaquone was estimated at about US\$ 50 (two injections given intramuscularly at a dosage of 2.5 mg/kg, 48 h apart). The cost of immunising one animal with the infection and treatment method varies depending on

the place, country and time (Mukhebi *et al.*, 1990). The estimated cost of immunising one animal varies from US\$ 2.51 to US\$ 20.00 for its life time (Elyn, personal communication, 1997; Irvin, 1984; Kiltz, 1984; Mukhebi *et al.*, 1990 and Radley, 1981). The bulk (60% - 70% of the total cost of the exercise) of the difference between these estimates is mainly due to the transport operational costs. In Zambia, Elyn (personal communication, 1997) estimated the cost to be equal to US\$ 11 on average. This cost could be further reduced if the transport operational costs were minimised. These estimates show that, where an enzootically stable situation for other tick-borne diseases exists, it is cheaper to immunise cattle against theileriosis than any other means of control. These expensive control measures, which have often been applied in combinations, have often and largely been the responsibility of Governments in respective countries. In Zambia, with the inception of the Agricultural Sector Investment Program (ASIP), this responsibility has been shifted from the Government to the cattle owners (small scale farmers), who own about 82% of the total cattle population (Mwenya *et al.*, 1994). Most of the small scale farmers consider the cost of acaricides, chemotherapeutic drugs or even immunisation by infection and treatment to control theileriosis too high (Chilonda, personal communication, 1997).

For the infection and treatment method, the cost of the chemotherapeutic drugs (tetracycline) accounts for the next single highest cost of the whole exercise after the transport operational costs (Elyn, personal communication, 1997 and Mukhebi *et al.*, 1990). This is especially true in the first year of immunisation when both calves and adult cattle have to be immunised. Transport operational costs are administrative costs which are dependant on the method of delivery of field immunisation. Immunisation campaigns are likely to cost more than an on-going process because costs incurred on

transport are high in the former, irrespective of the number of animals immunised. The more the number of cattle immunised, the lower the cost (Elyn, personal communication, 1997). However, the method of delivery of immunisation is ultimately the responsibility of the person immunising and it is in his interest to keep the costs as low as possible.

For the vaccine producer, the use of tetracycline in the infection and treatment immunisation remains a hitch in as far as the cost of the exercise is concerned. It is for this reason that it was decided to revisit irradiation of *Theileria parva* sporozoites in this study, as an alternative to the use of chemotherapeutic drugs in the immunisation against theileriosis.

Although irradiation of sporozoites either directly or in their arthropod hosts has been tested by many researchers in the past and is currently not in use, most of their results, especially on the irradiation of *T. parva* sporozoites in their tick hosts at dosages of 20 to 30 krads, had indicated a strong possibility of reducing the pathogenicity and virulence of the parasites to levels which could be used for immunisation (Mugera *et al.*, 1973 and Munyua *et al.*, 1973).

Further, while irradiation of *T. parva* sporozoites has been investigated extensively in Kenya, it has never been tried in Zambia and specifically on the Katete stock. It may thus be possible that the Katete stock of the parasite would respond differently to irradiation from those studied in Kenya.

Finally, the cost of irradiating one dose of *T. parva* stabilate either directly or in their tick hosts is estimated to be less than US\$ 1. Therefore, the success of this study could be very important in the price reduction of the malignant theileriosis vaccination exercise.

## 1.2 Objectives of study

- 1) To compare the immunogenicity of irradiated Katete strain of *T. parva* sporozoites with non-irradiated ones with tetracycline (infection and treatment immunisation).
- 2) To assess the suitability of using directly (stabilate form) irradiated *T. parva* sporozoites in the immunisation of cattle against malignant theileriosis.
- 3) To assess the suitability of using indirectly (in their tick vectors) irradiated *T. parva* sporozoites in the immunisation of cattle against malignant theileriosis.

## CHAPTER TWO

### LITERATURE REVIEW

#### 2.1 Historical background and aetiology of theileriosis

It is believed that malignant theileriosis originated from east Africa during the 19th century when cattle were introduced into areas where the parasite (piroplasms), wild buffalo hosts, and their tick vectors were living in a stable enzootic relationship (Sonenshine, 1993). The disease spread to the rest of eastern, central and southern Africa as a result of subsequent importation of cattle from east Africa which were meant to replace the losses of cattle following Rinderpest outbreaks in 1896, as well as due to losses caused by the Anglo-Boer war of 1899-1902 (Norval *et al.*, 1992).

Malignant theileriosis was initially prevalent along the east African coast before spreading deeply in-land, hence the name “East Coast fever” which was used to emphasise the absence of the disease in the south African coast at that time. In Zimbabwe (Southern Rhodesia), the name “January disease” was adopted to denote an atypical form of the same disease when it was identified on seven farms on the eastern border of Southern Rhodesia (Lawrence, 1937). “January disease” was used because of the seasonality of the disease. “Corridor disease” was the name given to the disease when it caused high mortality in cattle in the corridor between two game reserves in southern Zululand (South Africa) in April, 1953. Here, the disease was characterised by a more rapid course, absence of many characteristic lesions of ECF, rare piroplasms in the blood and inconsistent cross-immunity (Lawrence, 1935).

In Zambia, theileriosis was first reported at Fife in 1922 in the Nakonde area of Northern Province (Akafekwa, 1976). Thereafter, the disease was diagnosed in Mbala, in the Northern Province and Chipata (Eastern Province), in the years 1946

and 1947 respectively. In 1977/78, the disease was detected in Monze, in the Southern Province (Chizyuka and Mangani, 1985). Today, the disease is found in all except Luapula, Western and North-western provinces of Zambia.

Malignant theileriosis is caused by various sub-species of a protozoan parasite *Theileria parva*. The disease is caused by a syndrome involving ECF and Corridor disease in Zambia. The causative agent of ECF is *T. parva* (Theiler, 1904). Corridor and January diseases are caused by *T.p. lawrencei* (Neitz, 1955) and *T.p. bovis* (Neitz, 1957) respectively.

Another species of *Theileria* parasites known to exist in Zambia is *T. mutans* (Theiler, 1906). Until recently, this species has been considered to be non pathogenic.

Malignant theileriosis is transmitted transtadially by the nymphal and adult stages of the three host tick *Rhipicephalus appendiculatus*. The larva or nymph of this tick gets infected when it feeds on an infected mammalian host. The parasites undergo development in the tick before they can infect the next host during the tick's next feeding. African buffaloes (*Syncerus caffer*) are well known natural hosts of *T. p. lawrencei* although various other wild animals are suspected reservoir hosts for these parasites. In contrast, *T.p. parva* is mainly associated with cattle to cattle transmission. It is interesting to note that *Theileria* parasites transmitted from buffaloes to cattle tend to be very virulent strains which cause a very severe form of the disease called Corridor. Such *Theileria* strains are generally referred to as *T.p. lawrencei*. However, when the parasites are repeatedly passaged through cattle, by cattle to cattle transmission, they tend to become transformed, and cause ECF (Maritim *et al.*, 1992 and Sonenshine, 1993).



The parasites of *T.p. parva*, *T.p. lawrencei* and *T.p. bovis* are morphologically and serologically indistinguishable and have the same tick vectors thus evidence to combine them is conclusive. Therefore, the trinomial system of classification for *T. parva* has been dropped (Anonymous, 1989).

## 2.2 Life cycle of *Theileria parva*

The life cycle of *T. parva* has been reviewed in detail by Irvin and Cunningham (1981) and Norval *et al.* (1992). The main biological vector of *Theileria* parasites is *R. appendiculatus* Neumann, 1901, commonly known as the 'Brown ear ticks' of cattle although other *Rhipicephalus* species like *R. zambeziensis* have been shown to be important vectors as well (Lessard *et al.*, 1990).

The ticks are infected as larvae or nymphs when they feed on the *Theileria*-infected erythrocytes from carrier or sick cattle. *Rhipicephalus appendiculatus* nymphs require a quantum of ingestion of about  $1 \times 10^7$  piroplasms to establish infection (Purnell *et al.*, 1974). The infected erythrocytes are lysed in the intestinal lumen of the tick to release the piroplasms which differentiate into macro- and microgametes, which then fuse to form zygotes. The zygotes then invade the intestinal epithelial cells where they develop into kinetes. During and after the moulting process of the tick, the kinetes migrate through the gut wall into the hemolymph to the E cells of the type III acini of the salivary glands where they become sporoblasts. The salivary glands only become available for infection by *T. parva* kinetes after re-development, following each moult (Fawcett *et al.*, 1981a, b and 1982).

In the salivary glands, only about 10 out of the about 700 type III acini cells are actually infected and it appears that most of the parasites die before completing the

life cycle (Purnell *et al.*, 1974). The salivary glands seem to have no chemo-attraction to the kinetes as is evident in *in-vitro* systems (Bell, 1980). The sporoblasts in the salivary glands undergo several nuclear divisions in the resting tick to become a multinucleate growing sporont syncytium.

The life cycle *T. parva* is summarised in the schematic diagram presented in Fig. 1.

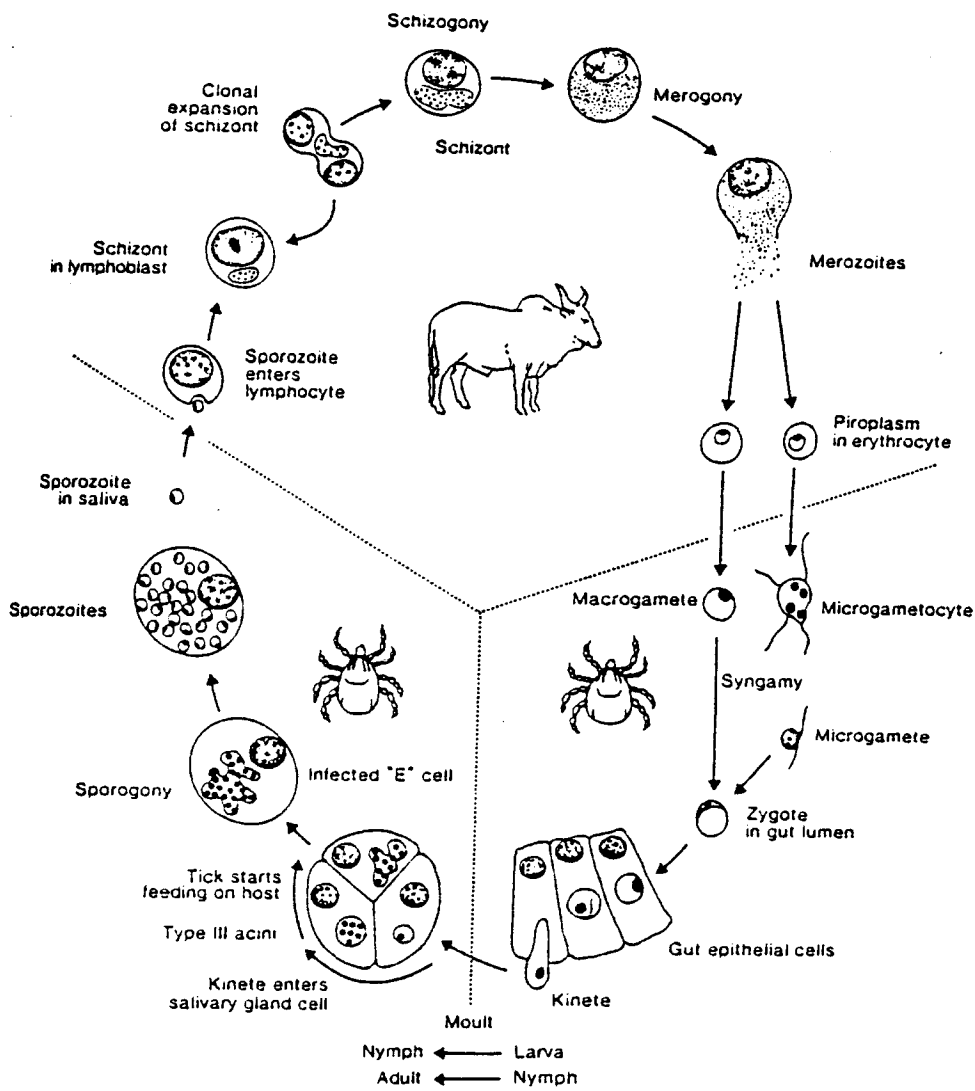


Fig. 1. Life cycle of *Theileria parva* (from Norval *et al.*, 1992)

When the tick feeds onto the next host, the sporoblasts undergo sporogony which results in the production and release of numerous mononuclear sporozoites. The tick feeds for at least 4 days before the sporozoites mature. Sporozoites are the infective stage and are injected into the next mammalian host through the tick's saliva when taking their blood meal.

In the bovine host the sporozoites invade lymphocytes by means of passive endocytosis. Destruction of the host cell membrane by the sporozoites enables them survive lysis by the host cell. Each parasite then associates itself with the Golgi apparatus and induces an increase in cytoplasm transforming the lymphocyte into a lymphoblastoid cell. The parasites are then transformed into multinucleate schizonts (Sonenshine, 1993). As the schizonts mature, the parasitised cells then begin a process of merogony, budding off merozoites from peripherally located nuclei around the central meroblast (Sonenshine, 1993). These merozoites lie free in the cytoplasm of the degenerating lymphoid cells and are liberated into the blood plasma as the host cells disintegrate. The merozoites then invade erythrocytes and in the process transform into piroplasms which are believed to function as gametocytes or their precursors (Norval *et al.*, 1992). It is at this stage that the parasite gets infective to the tick host.

### **2.3 Clinical signs and pathology of theileriosis**

Clinical signs vary according to the level of challenge, virulence of the pathogen and immune status of the host. The levels may be inapparent, mild to hyperacute, severe or fatal infections. The incubation period and severity of infection is directly dependent on the sporozoite quantum of infection, breed and individual

resistance of cattle, arthropod vector, parasite species and strain (Irvin and Cunningham, 1981). However, there is a minimum prepatent period of five days which is not quantum dependant (Irvin and Cunningham, 1981). Swelling of the lymph nodes draining the site of tick attachment occurs followed by appearance of macroschizonts 7 to 10 days post infection. The animal then becomes pyretic with temperatures between 39.5 °C to 42 °C. Lymph node swellings then become pronounced and generalised. Later, anorexia develops and the animal loses condition rapidly. Lacrimation and nasal discharge may also occur. Diarrhoea and respiratory distress are not uncommon just before the death of the animal. The ultimate cause of death is the uncontrolled multiplication of infected lymphoblasts replacing normal lymphoid tissue and the infiltration into various tissues, for example the interstitial tissues of the lungs, kidneys, liver and gastro-intestinal lining (Irvin and Cunningham, 1981).

The most common post-mortem findings include pulmonary oedema, hyperaemia and emphysema, enlarged liver and spleen (in chronic cases the spleen is not usually enlarged). The trachea and bronchi may be filled with frothy exudate. The heart may be dilated with extensive haemorrhages and infarcts. There may also be ulcers lining the abomasum. The kidneys may show pinpoint haemorrhages and tiger-stripings of the caecum may be observed (Irvin and Cunningham, 1981).

## **2.4 Diagnosis of theileriosis**

Several methods have been developed for the diagnosis of malignant theileriosis. The Indirect Fluorescent Antibody test (IFAT) has been useful in the detection of antibodies of cattle that have been infected with the disease. This

technique has also been modified to detect piroplasms and schizonts of *Theileria* in blood smears and tissue sections (Morrison *et al.*, 1981). However, IFAT is cumbersome to carry out and depends on subjective observations by the operator. Cross-reactions with *T. annulata* and *T. taurotragi* occur thus IFAT is mostly used in serological surveys rather than for diagnostic purposes.

Indirect Haemmagglutination (IHA) test, Complement Fixation test (CFT) and Capillary Agglutination (CA) have also been used in the past. However, these tests lack specificity and allow for cross-reaction with antibodies of other parasites.

In Zambia, the most widely used diagnostic technique involves the demonstration of schizonts and piroplasms in Giemsa stained lymph node biopsy and blood smears respectively. This method is easy to execute and the results are obtained quickly.

## **2.5 Control of theileriosis**

Control of theileriosis can be categorised into four main groups: vector control, cattle movement control, chemotherapy and immunisation. These methods have been reviewed in detail by Norval *et al.* (1992). Vector control refers to the use of acaricide application in the control of ticks. Different types of acaricides and their applications have been discussed comprehensively in FAO (1984). The initial high cost of constructing dip tanks and spray races, coupled with the high cost of acaricides and the subsequent development of resistance by ticks to acaricides are however, a major limitation.

Other methods of vector control with limited practical feasibility in Africa include habitat modification (Hornby, 1935; Yeoman, 1967; Norval, 1977a, b; c and

1983), tick killing plants e.g. *Melinis minutiflora* and *Stylosanthes* (Thompson *et al.*, 1978; Sutherst *et al.*, 1982 and Norval *et al.*, 1983), host resistance to ticks, tick vaccines, predators, parasites, pathogens and the use of pheromones in combination with acaricides.

Quarantine and livestock movement restrictions have also been used but without much success in some cases due to the difficulty in implementation. These methods are unpopular among farmers. They are further complicated by the presence of other animals besides cattle which serve as vectors of ticks (eg. *Hippotragus niger*) and hosts for *Theileria* parasites (eg. *Syncerus caffer*) (Norval and Perry, 1990).

Chemotherapy in theileriosis control is widely used. Drugs such as tetracycline, buparvaquone and halofuginone are effective against *T. parva*. Tetracycline slows down the multiplication of parasites when administered early during the infection. Buparvaquone is effective against both the schizont and piroplasm stages while halofuginone is effective against the schizont stage only. The major limitation in the use of chemotherapeutic control of *T. parva* infection is basically the high cost of the drugs.

Immunisation of cattle against theileriosis (infection and treatment) has become one of the most popular control methods in the field. It has been observed that animals which survive *T. parva* infection become immune to homologous challenge (Burridge *et al.*, 1972 and Dolan *et al.*, 1980).

Immunisation of cattle against theileriosis is done by injecting the animal with *T. parva* sporozoites subcutaneously behind the ear and subsequently treating it with long acting tetracycline, a technique approved by the Food and Agricultural Organisation (FAO) of the United Nations. Infection and treatment immunisation

against *T. parva* is done in a number of countries such as Burundi, Kenya, Malawi, Rwanda, Tanzania, Zambia and Zimbabwe (Dolan, 1988). This method uses live sporozoite vaccines.

## 2.6 Immune response of cattle to theileriosis

The response of cattle to *T. parva* infections has been described in detail in the ILRAD annual report (1989). An animal undergoing immunisation or challenge against *T. parva* generates cytotoxic cells that are restricted in activity to autologous parasitised cells (Emery *et al.*, 1981a and Eugui and Emery, 1981). This activity is confined within the peripheral blood mononuclear cells (PBM) (Emery *et al.*, 1981b). The nature of this genetically restricted cytotoxic T-lymphocyte (CTL) responses has been identified as CD2+CD4-CD8+ (Goddeeris *et al.*, 1986). The restricting elements in *T. parva* CTL responses have been found to be type 1 Major Histocompatibility Complex (MHC) molecules (Morrison *et al.*, 1987).

As the immunology of *T. parva* infection became more clearly understood, it became apparent that the immunological response of cattle to *T. parva* infections was cellular mediated (Emery, 1981). This revelation led to the isolation of the *T. parva* sporozoite antigen associated with the stimulation of immunity in cattle. This antigen, known as the p67 because of its molecular weight of 67 kDa, is present on the surface of the sporozoites and has become a potential candidate in the production of a novel vaccine against malignant theileriosis (Norval *et al.*, 1992).

Cattle that are naturally or artificially infected with *T. parva* and recover become solidly protected against homologous challenge for at least three and half years (Burridge *et al.*, 1972). Humoral immunity is not considered to play a major role



in the protection of cattle against the disease although other studies indicate that it should not be totally ignored (Musoke *et al.*, 1982 and 1984; Dolan *et al.*, 1985).

## 2.7 Background of immunisation against theileriosis

The history of immunisation against theileriosis has been reviewed in detail by Norval *et al.* (1992). Gray and Robertson (1902) started the work on immunisation when they discovered that a single injection of blood from a recovered animal did not confer immunity to susceptible animals. Koch (1903a and b) developed a vaccination regime against *T. parva* involving the use of defibrinated blood obtained from recovered animals. The results of this regime were not satisfactory.

Sergent *et al.* (1924) demonstrated that *T. annulata* could be transmitted to susceptible cattle with infected blood. This was because *T. annulata* piroplasms replicate in the blood of the host, making it possible for them to develop in the recipient bovine. Based on this principle, a method of vaccination using blood from mildly infected animals was adopted in Algeria, Israel and USSR. It was later discontinued because of high mortalities and the risk of transmitting other disease agents with the blood.

Experimental immunisation against *T. mutans* using infected blood has also been reported (Uilenberg *et al.*, 1977 and Young, 1985). *Theileria mutans* piroplasms also replicate in the blood.

Although blood from a *T. parva* infected animal may contain antibodies, its transfer to susceptible animals fails to protect them because their piroplasms are dormant (Muhammed *et al.*, 1975).

Theiler (1911a and b) and later Spreull (1914) then attempted to develop a vaccine using various preparations of cells of spleen and lymph nodes. The cells were suspended in peptone water and inoculated into susceptible animals. The results they obtained were promising, offering between 49.5 to 62.1% protection. However, this method of immunisation was later discontinued because:-

- a) the proportion of animals that died because of the exercise was too high.
- b) the number of cattle protected was comparatively too low.
- c) the immunisation procedure was too difficult and
- d) the need to use immunisation material immediately after harvesting from sick animals was considered too inconvenient.

An *in-vitro* system for propagating a *Theileria* infected schizont lymphoblastoid cell line was later established. In this system, schizonts and host cells divide synchronously to produce immortalised cell lines. Similar systems were also developed for *T. annulata*, *T. taurotragi* and *T. hirchi* (Malmquist *et al.*, 1970; Stagg *et al.*, 1976 and Tsur and Pipano, 1966). This development increased the prospects of immunisation using this method.

*Theileria annulata* infected cells grown *in-vitro* can be used to infect and immunise cattle (Pipano, 1981). After a certain period of cell line passages, the parasites seem to lose their ability to produce merozoites (and hence virulence is lost) when infected into cattle. This change in immunogenicity led to the development of cell culture vaccine for *T. annulata*. However, the mechanism behind the reduction in virulence of *T. annulata* parasites after several passages in cell culture is not well understood. Unlike *T. parva*, *T. annulata* infections are not quantum dependent.

The use of schizont infected lymphocytes for immunisation against *T. parva* was tried but was not feasible (Brown, 1987). Dolan *et al.* (1984) demonstrated that this failure was associated with the positive histocompatibility barrier in cattle which received unmatched cells infected with donated *T. parva* schizonts which were bovine lymphocyte antigen (BOLA) related.

Spreull (1914) showed that unfed *R. appendiculatus* ticks contained *T. parva* sporozoites. These unfed ticks were however too pathogenic for use in immunisation. This approach was re-investigated years later by others like Brocklesby *et al.* (1965); Jarrett *et al.* (1966) and Pirie *et al.* (1970) but without much improved results. Other methods were also investigated for immunisation purposes. These included the *in-vitro* cultivation of *T. parva* infected lymphoid cells. This method was followed by exhaustive testing of the dose and passage attenuation. Inactivation of parasites by irradiation either directly or in their mammalian or arthropod hosts was also done (described further in section 2.9).

Other studies included the use of anti-theilerial drugs such as parvaquone and halofuginone in the ‘infection and treatment’ immunisation. Currently, one technique is predominant. This is the ‘infection and treatment’ method in which a therapeutic dose (tetracycline) is used to slow down the multiplication of the parasites (Spooner, 1990).

Infection and treatment technique (Radley *et al.*, 1975) involves active infection of recipient cattle with cryopreserved suspensions of *T. parva* sporozoites grown in *R. appendiculatus* ticks while simultaneously treating the animal with long acting tetracycline to partially protect the animal. The result is a mild or inapparent disease reaction and hence the development of an immune status.

Recently, a *T. parva* antigen molecule specific to sporoblast and sporozoite stages of the parasite has been reported. This molecule has a relative molecular weight of 67 kiloDaltons and appears to be invariant between strains. Field trials have been done with this sporozoite p67 antigen and has been shown to protect about 70% of immunised cattle to homologous challenge (Musoke *et al.*, 1992).

## **2.8 Future prospects in the immunisation against theileriosis**

Given the limitations associated with each of the control methods described above, the most promising seems to be the immunisation technique. Subsequently, the most appropriate vaccine against *T. parva* infections will depend on the improvement of the preparation of the appropriate immunogenic antigen. Such a preparation should be able to effectively stimulate the host cell mediated immune response which should eventually protect the immunised animal.

With the identification of the p67 antigen as a vaccine candidate for the immunisation against theileriosis, a solution to this disease may be in sight (Norval *et al.*, 1992).

Meanwhile, irradiation of *T. parva* in the production of a vaccine against theileriosis requires further investigation in order to increase the understanding of the effect of ionising radiation on *Zambian strains of T. parva*.

## **2.9 Irradiation of parasites in vaccine production**

Irradiation of parasites is known to inactivate them without altering their antigenic structure (ILRAD, 1990). This effect, caused by the heat or excitation of ionisation radiation, causes free hydroxyl radical formation (Little, 1968). Depending

on the dose involved, the hydroxyl radical formation results in a range of effects from temporary impairment of cellular functions to cell death. Not all cells however, are equally susceptible, and certain phases of cell development, for example, those involved with DNA synthesis and mitosis are most affected. This principle has been applied to attenuate parasites so that they can be used as vaccines.

Clyde *et al.* (1975) successfully immunised human volunteers with irradiated *Plasmodium falciparum* sporozoites. Irradiation of *Babesia bigemina* is also known to slow down development of the parasites but induces the host immune reaction (Gil *et al.*, 1986 and Shah-Fisher and Say, 1989). Wright *et al.* (1982) found that irradiation of *B. bovis* at 350 Gy rendered it avirulent but sufficiently immunogenic to protect the immunised animals against clinical symptoms and subsequent challenge against babesiosis. However, the problems associated with these *babesia* vaccines were the short shelf life and the possibilities of incidental transmission of other pathogens such as bovine leukosis, since these vaccines were produced from whole blood (Taylor, 1989).

In the USA, irradiation was applied in the production of a vaccine against *Anaplasma marginale* of ovine origin (Shah-Fisher and Say, 1989). Ristic (1968) and Ristic and Carson (1977) also subjected a virulent form of *A. marginale* (Florida isolate) to irradiation combined with passage attenuation through deer and sheep. This resulted in low virulence of the parasites which were then used for preimmunisation of young cattle and is so far the most effective method available.

### **2.9.1 Irradiation of *Theileria* parasites in vaccine production**

The difficulty in obtaining the best titration of *T. parva* sporozoites for use in immunisation (Cunningham *et al.*, 1974), pioneered the possibility of sublethally modifying the *T. parva* parasites using irradiation. This was done either in their tick vectors, their free-living infectious sporozoite stage or in their recipient bovine host stage.

#### **2.9.1.1 Irradiation of *Theileria parva* parasites in their bovine hosts**

Irvin *et al.* (1975) used parasitised lymphoid bovine cells to study the effect of irradiation on *T. parva*. Irradiation was found to affect the host cells and the parasites differently. It inhibited host cell division while the macroshizont nuclear particles increased in number. This method could therefore not be used as a possibility for vaccine production against theileriosis.

Other studies aimed at exploiting the possibility of attenuating *T. parva* in laboratory animals were also done (Irvin *et al.*, 1972a and b; 1973, 1974; 1975 and 1976). Infection of laboratory animals, for example mice (Irvin *et al.*, 1972b) with *T. parva* was unsuccessful because of the failure by the parasites to adapt themselves in the host cells.

#### **2.9.1.2 Irradiation of *Theileria parva* parasites in their tick vectors**

Purnell *et al.* (1972a and 1973a) investigated the effects of  $^{60}\text{Co}$ -irradiation on the tick *R. appendiculatus*. Their main objective was to find the doses of irradiation tolerated by various stages of the tick vector. Irradiation of adult ticks with doses up to 16 krad was found to have had no effect on their subsequent ability to attach to hosts.

From these experiments, it appeared that the best time to irradiate the parasites without interfering with the feeding of the ticks would be during the stage of unfed adults.

Morphological studies on the development of *T. parva* in salivary glands of feeding adult irradiated ticks at various doses were also done (Purnell *et al.*, 1972b). These studies revealed that doses as low as 0.5 krad appeared to reduce the number of parasites developing in salivary glands of the infected ticks, though the parasites were still observed in ticks irradiated at 32 krad. Doses in excess of 4 krad appeared to alter the morphological development of the parasites. For ticks receiving higher doses, the parasites appeared to agglomerate suggesting that the viability had been greatly affected. Purnell *et al.* (1974) further carried out three experiments in which infected ticks at different stages of their life cycle were irradiated with different doses within the range they could tolerate. Engorged nymphs were irradiated with doses of 1, 2 and 4 krad. Those irradiated with 4 krad failed to moult. The adult ticks resulting from nymphs irradiated with doses of 1 and 2 krad were fed on rabbits and then their salivary glands examined for parasites. The parasites which developed were seen to be normal morphologically. The same applied to adult ticks resulting from irradiated moulting ticks with doses up to 16 krads.

In a preliminary experiment in which parallel batches of irradiated (at doses 4-32 krad) adult ticks were fed on cattle and rabbits, it was shown that the results obtained by Purnell *et al.* (1972b) were a partial interpretation of the effects of irradiation. Irradiation slowed down the development of parasite masses rather than reducing their number (Purnell *et al.*, 1974).

In the subsequent experiment, infected unfed adult ticks of *R. appendiculatus* were irradiated with doses of 5 to 60 krad. They were then allowed to feed on rabbits for 5 to 7 days. All ticks in each treatment group were 'harvested' on the same day. The ticks obtained were either dissected to remove salivary glands or ground into stabilate. The results of this experiment showed that increasing the dose of irradiation resulted in decreased number of morphologically normal parasites. On the other hand, increasing irradiation resulted into milder reactions with few exceptions. For example, all animals except one, which received doses irradiated above 20 krad had no reactions. In one exceptional case, parasites irradiated at a dose of 45 krad, when inoculated into an animal produced a long prepatent period and the animal died of typical ECF. These results discouraged the workers from further optimism in using this method for vaccine production.

Table 1 below is a summary of the effect of irradiating sporozoites at different doses in their tick vectors.



Table 1. Summary of the effect of irradiation of *Theileria parva* sporozoites in their *Rhipicephalus appendiculatus* tick vectors on cattle (Compiled from Purnell *et al.* (1972a and b, 1973a and b); Purnell *et al.* (1974) and Mugeru *et al.* (1973)).

Dose	Average no. of cattle used	Immunisation		Challenge	
		died (av)	survived (av)	died (av)	survived (av)
0	5	4	1	0	1
10	8	7	1	0	1
15	5	2	3	1	2
20	4	0	4	0	4
30	14	0	14	0	14
25-40	2	0	2	2	0
45	2	1	1	1	0
50	5	0	5	3	2
50	1	0	1	1	0
60	1	0	1	0	1
70	4	0	4	4	0

### **2.9.1.3 Direct irradiation of *Theileria parva* sporozoites**

Cunningham *et al.* (1973a and b) investigated the effects of irradiation on infective particles obtained from vector ticks by the methods of Purnell and Joyner (1968). Three experiments were carried out. In one of them, infective particles in foetal calf serum were irradiated with doses ranging from 4 to 32 krads. Then groups of five animals were inoculated with this material. Increasing irradiation was seen to reduce the severity of the disease. In this case, the critical dose appeared to be 10 krad, at which only one animal had an ECF reaction and three were immune to subsequent challenge.

Tables 2 is a summary of the irradiation of sporozoites in relation to the corresponding reaction of immunised cattle.

Table 2. Summary of the effect of direct irradiation of *Theileria parva* sporozoites on cattle (Compiled from Cunningham *et al.* (1973 a and b)).

Dose (krads)	Average no. of cattle used	Immunisation		Challenge	
		died (av)	survived (av)	died (av)	survived (av)
0	6	5	1	0	1
4	5	3	2	1	1
4.3	2	1	1	0	1
7	5	1	4	1	3
8.6	2	0	2	0	2
10	5	1	4	1	3
13	5	0	5	3	2
16	5	0	5	5	0
17.2	2	0	2	1	1
32	5	0	5	5	0
34.4	2	0	2	1	1
68.8	2	0	2	2	0
137.6	2	0	2	2	0

## CHAPTER THREE

### MATERIALS AND METHODS

#### 3.1 *Rhipicephalus appendiculatus* ticks

*Rhipicephalus appendiculatus* (Neumann) ticks were used in the study.

##### 3.1.1 Source of ticks

Ten engorged adult female *R. appendiculatus* ticks were obtained from the National Institute for Science Research (formerly NCSR) Animal Production and Research Unit in Chilanga. They were originally collected from cattle at Hamutete in Mazabuka. In the laboratory, they were in their eighth generation of rearing.

##### 3.1.2 Laboratory maintenance of ticks

Laboratory maintenance of *R. appendiculatus* ticks was done using the methods of Bailey (1960) and Irvin and Brocklesby (1970), with minor modifications. The ticks were kept in a dark incubator at 25 °C, in a desiccator containing a saturated solution of potassium chloride to maintain a relative humidity of about 80-85% (Solomon, 1951).

The engorged ticks laid eggs which hatched within four to six weeks of incubation. The resultant larvae hardened in one week and were then fed on rabbits. Cloth earbags were stuck to the base of each ear of the rabbits using Tuffstuff contact adhesive, reinforced with Elastoplast adhesive bandage (Healoplast, Zambia) bound around it. The distal ends of the bags, which were also open, were sealed using a piece of adhesive bandage. Then both earbags of each rabbit were bound together using another piece of the adhesive bandage to prevent drooping of the ears. No collars were used. After twenty-four hours, the tube which had contained the larvae, cotton wool,

dead and unattached larvae as well as debris were removed from the earbags on the rabbits.

Engorged larvae were collected twice daily, in the mornings and evenings. All developmental stages were kept at 25 °C and 80-85% relative humidity. Moulting from larvae to nymphs took an average of 10 to 14 days.

### 3.2 Experimental animals

#### 3.2.1 Cattle

Nineteen healthy *Bos Taurus* cattle were purchased from Enviro Flor farm, east of Lusaka. The farm had no history of overt theileriosis. All the cattle purchased tested sero-negative for *Theileria parva* antibodies on IFAT. The average age of the animals was one year. The cattle were all treated with imidocarb dipropionate at a dosage of 3 mg/kg, followed by an injection of tetracycline at a dosage of 20 mg/kg. This was necessary because the farm from which the animals originated had a history of anaplasmosis. The cattle were also dewormed with albendazole at a dosage of 0.75 ml/kg and ivermectin at a dosage of 0.2 mg/kg. Dipping against ticks was done once weekly with amitraz at a dilution of 1 ml per 2 litres of water.

Cattle were housed in stables at the quarantine area of the School of Veterinary Medicine, UNZA, Lusaka. They were fed on hay (approx. 2.5% of bodyweight per animal per day); dairy meal (approx. 10 kg per animal per day), mineral licks without urea (*ad lib*), and maize bran mixed with di-calcium phosphate and common salt (approx. 2 kg per animal per day). Water was provided *ad lib*.

### 3.2.2 Rabbits

Fifteen New Zealand White and Californian crossbred rabbits were purchased from the Central Veterinary Research Institute (CVRI) in Lusaka. The rabbits had an average age of three months and had not been used in any experiment previously.

The rabbits were housed in metal cages in the small laboratory animal facility at the School of Veterinary Medicine. Each rabbit occupied a separate cage. The rabbits were fed on rabbit pellets and dairy meal (approx. 140 g per rabbit per day). Drinking water was provided *ad lib*.

## 3.3 Isolation of *Theileria parva* from infected cattle using *Rhipicephalus appendiculatus* ticks

### 3.3.1 *Theileria parva*

*Theileria parva* (Katete stock) was used in this study.

#### 3.3.1.1 Source of cryopreserved *Theileria parva*

The source of *T. parva* was the Belgian funded Assistance to the Veterinary Services of Zambia project (ASVEZA) in Chipata, Eastern province. The stock was isolated from cattle in Katete district of the same province by the Belgian Animal Disease Control Project (BADCP) in 1985. The *T. parva* stocks were stored in liquid nitrogen and were only revived once in cattle in 1996.

#### 3.3.1.2 Resuscitation of cryopreserved *Theileria parva* stored in liquid nitrogen

Preparation of the *T. parva* stablate for infecting cattle was done as follows: -

Three vials of the 1/10 (1 tick per ml) dilution were retrieved from the liquid nitrogen storage container. They were thawed immediately in a bowl containing water at 40 °C by the method described in FAO (1984).

### 3.3.2 Infection of steers with *Theileria parva* stabilate

After resuscitation, the *T. parva* stabilates were pooled together. Three steers were then injected, each with 2 ml of the *T. parva* stabilate, subcutaneously behind the right parotid lymph node on day zero.

The following day (day one), the ears of the steers were washed twice (in morning and afternoon) with acetone in order to remove the wax deposits and dirt.

On days two and three, the ears of the steers were washed with soap, once in the mornings and afternoons, so as to wash away the dirt and traces of acaricide and acetone. On days four to seven, they were washed twice each day with plain water to thoroughly clean them. An injection of cortisol was also given to improve tick attachment as reported by Norval *et al.* (1992).

Rectal temperature of each steer was recorded twice daily, once in the morning (08 00 h) and afternoon (16 00 h). Lymph node biopsy and blood smears were made from each steer starting from the time lymph nodes got enlarged.

### 3.3.3 Application of *Rhipicephalus appendiculatus* nymphs on infected steers

When the parasitaemia of each steer reached about one percent, nymphs (four weeks post-moulting) were applied onto each ear of the steers. This was done as described in 3.1.2 above. The two hind legs of each steer were loosely tied together to prevent the steers from scratching off the earbags.

Collection of engorged nymphs was done twice daily, once in the mornings and afternoons. These nymphs were first washed in running tap water to remove contaminants, blotted on filter paper to dry and then kept in an incubator at 25 °C with 80% relative humidity during moulting. Moulting into adults took on average three weeks. The adult ticks were removed from the moulting tubes and put into clean ones to reduce further contamination from dead ticks and exoskeletons.

### 3.3.4 Pre-feeding of adult *Rhipicephalus appendiculatus* ticks on rabbits to mature *T. parva* sporozoites

Adult *R. appendiculatus* ticks five weeks post-moulting were pre-fed on rabbits using the method described by FAO (1984). The ticks which attached in the first 24 h were left to feed for a further four days to allow sporozoites to mature before harvesting them. Harvesting was done by plucking the semi-engorged ticks off the ears of the rabbits and putting them into 10 ml test tubes. Approximately 100 ticks were put into each test tube. A total of thirty tubes were collected in all.

### 3.3.5 Determination of *Theileria parva* infection rates in ticks

The infection rate of *T. parva* in the salivary glands of *R. appendiculatus* ticks were estimated using the method developed by Blewett and Branagan (1973) and later modified by Walker *et al.* (1979). Fifty pairs of male and female ticks were embedded on candle wax. A small amount of dissecting buffer was then poured on the embedded ticks. The dorsum of each tick was dissected and removed using a fine scapel blade (No. 11) under a stereoscopic microscope. The intestines were removed using a pair of forceps. Salivary glands were teased out, put on a glass slide and then fixed in absolute methanol for at least 10 minutes. They were then stained in methyl green pyronin stain for 30 minutes. After that, they were washed in absolute methanol for 2 minutes followed by dehydration in absolute methanol again for an additional 5 minutes. Clearing of the slides was done in xylol for 5 minutes followed by mounting with depex mixed with xylol. Examination of the samples was done under a light microscope at X100 or X200 magnification.

The acini were considered infected when exhibiting blue-stained particles and cytomeres while the nuclei were blue. The normal acinar cell contents appeared pinkish-red.



### 3.3.6 Irradiation of *Rhipicephalus appendiculatus* ticks

Six hundred infected but unfed adult *R. appendiculatus* ticks were irradiated using a  $^{60}\text{Co}$  Source (Multipurpose Gamma Irradiation Facility, National Institute for Science Research, Lusaka, Zambia). Four batches of 150 adult ticks were put into small plastic containers measuring 0.5 cm (radius) and 2 cm (height). The top of the tubes were closed using cotton wool plugs to prevent the ticks from escaping. 20 krads (0.2 kGy) was chosen as an optimal dose to be used based on past experiments (Mugera, *et al.*, 1973; Purnell *et al.*, 1974).

The radiation facility which was used is a Multipurpose Gamma Irradiation Facility (MPGIF) built with the help of the International Atomic Energy Agency in 1986. It is housed in a concrete cell with a system of eight product turntables arranged circularly around the source, on which the products are exposed to the radiation field for a specified period. It is controlled by a remote control panel.

The MPGIF is used primarily for treatment of medical and health products, foodstuffs, agricultural products and other miscellaneous products as required by the clients. For foodstuffs and agricultural products, FAO/WHO have cleared irradiation treatment for foods as a method of preservation as long as a dose of 10 kGy (1,000 krads) is not exceeded (NCSR, 1989).

Before irradiating the ticks, the dosage to be used was calibrated by exposing three Fricke docimeters to 20 krads of irradiation for about 30 minutes. This was necessary to ensure that the correct radiation was administered to the ticks. These docimeters were then read using a UV-visible spectrophotometer (Shimadzu UV240). The average exposure of the docimeters to the irradiation was 20 krads.

During the actual irradiation of the ticks, three Fricke docimeters were again put in-between the tick samples in order to monitor the dose of irradiation.

### 3.3.7 Stabilate production

Stabilate production was done according to the method of FAO (1984), with minor modifications. The harvested ticks were washed in running tap water, then

dipped in methanol and rinsed in distilled water in order to clean the ticks as well as to kill the bacteria on their surface.

Mortar and pestle were used to homogenise the ticks in Eagle's Minimum Essential Medium (MEM) using sterile commercial sea sand at a rate of ten ticks per 0.5 ml of MEM. The supernatant was removed and put into a centrifuge tube and spun at 450 rpm in order to remove the coarse debris. The supernatant was removed and the volume adjusted to 0.5 ml with MEM. The MEM suspension was always kept on ice.

An equal volume of MEM with 15% glycerol was then added drop-wise to bring the total volume to 1 ml (or a stabilate with a total concentration of 10 ticks per ml) while stirring. Glycerol is necessary for cryopreservation of *T. parva* sporozoites (Cunningham *et al.*, 1973c). Dispensation was done using a 20 ml syringe. About twenty vials of stabilate at this concentration (10 ticks per ml) were then dispensed and stored in an Ultra deep freezer at  $-82^{\circ}\text{C}$ . Each vial contained 2 ml of stabilate. The rest of the stabilate was diluted to 1 tick per ml of medium by adding MEM with 7.5% glycerol. This was also dispensed into 2 ml vials and stored in the Ultra deep freezer.

### **3.4 Viability of *Theileria parva* stabilate**

To test the viability of the *T. parva* stabilate, one steer was infected with 2 ml of the 1/1 (10 ticks per ml) dilution of the new stabilate. This was done using the method described in section 3.6.

## **3.5 Immunisation trials against theileriosis**

### **3.5.1 Selection of animals**

Fifteen steers were randomly divided into five groups using a computerised random number generator. Four of these groups were used to test the response of cattle to directly irradiated (three steers), indirectly irradiated (four steers), non-irradiated (three steers) and non-irradiated *T. parva* stabilate with concurrent tetracycline treatment (three steers). The details are given below. A total of thirteen steers were used for immunisation while a further two steers (fifth group) were

reserved to act as a control during the challenge of immunised animals against lethal doses of *T. parva* stabilates (section 3.6).

### 3.5.2 Preparation of *Theileria parva* stabilates for immunisation

Eight vials, each containing 2 ml of *T. parva* stabilates were transported in liquid nitrogen from the University of Zambia (UNZA) School of Veterinary Medicine to the National Institute for Science Research where the  $^{60}\text{Co}$  source was located. This was done to facilitate irradiation of part of the stabilate.

Thawing of the stabilate was done according to the method of Musisi *et al* (1996). Six vials each containing 2 ml of 10 ticks/ml of *T. parva* stabilate were retrieved from the liquid nitrogen container, wiped dry and immediately immersed into a bowl containing water at 40 °C. Upon thawing, the contents of all the 6 vials were pooled into a common bottle with a screw cap. The stabilate was then divided into two bottles, one containing 8 ml (to serve as non irradiated inoculum) of stabilate and the other, 4 ml (for direct irradiation) to equal the number of animals to receive each treatment of stabilate.

All vials containing stabilates were kept on ice at all times after thawing. The 2 vials each containing 2 ml *T. parva* stabilate made from irradiated *R. appendiculatus* ticks were also thawed, pooled and kept on ice.

The final distribution of the different treatments of stabilates was as follows:

- a) 4 ml irradiated directly (DIS).
- b) 8 ml not irradiated (NIS).
- c) 4 ml produced from irradiated ticks (ITS).

### **3.5.3 Irradiation of *Theileria parva* stabilates**

Irradiation of the *T. parva* stabilate was done at the NCSR using a  $^{60}\text{Co}$  source. Four ml of 1 ticks/ml *T. parva* stabilates made from non-irradiated *R. appendiculatus* ticks were exposed to 8.4 krad for about 30 minutes of irradiation while being kept on ice. The choice of 8.4 krad dosage was selected after reviewing previous experiments (Cunningham, *et al.*, 1973a and b).

### **3.5.4 Immunisation of cattle with directly irradiated *Theileria parva* stabilates**

The first three steers were inoculated with directly irradiated *T. parva* stabilate (suspension of sporozoites directly exposed to irradiation). Each steer received 1 ml of the 1 tick/ ml stabilate administered subcutaneously behind the right parotid lymph node. The whole process from thawing of the stabilates to administration into the animals took two hours.

### **3.5.5 Immunisation of cattle with indirectly irradiated *Theileria parva* sporozoites (in their arthropod hosts)**

The second group of four steers were inoculated with *T. parva* stabilate made from irradiated *R. appendiculatus* ticks as described in 3.3.6 above.

### **3.5.6 Immunisation of cattle with non-irradiated *Theileria parva* stabilates**

The third group of three animals were inoculated with non-irradiated stabilate only as described in 3.5.4. above.

### 3.5.7 Immunisation of cattle with non-irradiated *Theileria parva* stabilate administered with concurrent tetracycline treatment

The last group of three steers was treated with non-irradiated stabilate administered simultaneously with long acting tetracycline as described in 3.5.4. above. Tetracycline was given intramuscularly at a dosage of 20 mg per kg body weight.

### 3.5.8 Summary of *Theileria parva* stabilate treatments of cattle in immunisation trials against theileriosis

The table below is a summary of the *T. parva* stabilate treatments of cattle in the immunisation trials and challenges (experimental design).

Table 3. Summary of *Theileria parva* stabilate treatments of cattle in the immunisation trial and challenges against theileriosis.

Treatment of stabilate	Abbreviation	Number of animals	
		Immunised	Challenged
Directly irradiated	DIS	3	3
Irradiated ticks	ITS	4	4
Non-irradiated	NIS	3	3
Non-irradiated tetracycline treated	NIT	3	3
Positive control	Control		2

### 3.6 Experimental challenge of cattle

Twenty-nine days post immunisation, all cattle in the four treatment groups (section 3.5) were challenged with a double (2 ml of 10 ticks/ml) dose of *T. parva* stabilate. This stabilate was non irradiated and at this dosage was expected to be virulent and lethal. Two control steers were also included to check the lethality of the

stabilate. One ml of stabilate of 1/1 concentration (10 ticks/ml) was administered subcutaneously behind each parotid lymph node of every animal. Each animal received a total of 2 ml of stabilate.

### **3.7 Monitoring of the immunisation and experimental challenge of cattle**

#### **3.7.1 Temperature**

Rectal temperatures of all steers was taken twice daily, once in the mornings and afternoons. Cattle with temperatures above 39.5 °C were considered to have fever.

#### **3.7.2 Packed cell volume (PCV)**

Fresh blood was collected from each steer daily in heparinised vacutainer tubes. The PCV was read as follows: blood from each steer was drawn into a capillary tube. The capillary tubes were then sealed on one end with plastaseal. The capillary tubes were then centrifuged in a micro-hematocrit centrifuge at 10,000 rpm for 5 minutes. A hematocrit tube reader was then used to read the PCV which is just the percentage of cells in the blood.

#### **3.7.3 Parasitaemia**

Blood smears were made from each steer from the seventh day post-infection. Lymph node biopsy smears were made from each steer starting from when the lymph nodes got swollen. Staining was done using Giemsa (Cica, Japan). The slides were first fixed in 100% Methanol for 2 minutes, air-dried, and then stained in 10% Giemsa for 30-45 minutes. After staining, the slides were washed in running tap water and then air-dried. Examination was done at X1000 magnification under a light microscope.

The following formulae were used to calculate the schizont and piroplasm

indices: The schizont index (%) =

$$\frac{\text{The number of infected lymphocytes in 10 microscopical fields} \times 100}{\text{The total number of lymphocytes in the 10 fields.}}$$

Piroplasm index (%) =

$$\frac{\text{The number of infected erythrocytes in 10 microscopical fields} \times 100}{\text{The total number of erythrocytes in the 10 fields.}}$$

The ten fields were chosen randomly.

#### **3.7.4 Indirect Fluorescent Antibody test (IFAT)**

The indirect fluorescent antibody test was done according to the method of Goddeeris *et al.* (1982). *Theileria parva* schizont antigen was used. This test was done on the four groups of cattle used to study the response of different treatments of stabilates. This was necessary because the production of antibodies gives an idea of response of the animals to the stabilate, although the main mechanism of immunity of cattle to *T. parva* infections is not humorally mediated.

##### **3.7.4.1 Test serum**

The immune response of experimental animals was monitored using IFAT once every week. This was done by collecting about 5 ml of blood from all experimental animals in plain vacutainer tubes using a jugular vein puncture technique. The blood was then allowed to clot at room temperature (37 °C) for about 2 h. The blood clot was then removed and the tubes centrifuged at 450 rpm for five minutes, before removing the serum into vials.

#### 3.7.4.2 Control serum

*Positive control serum was initially prepared from one of the steers which had recovered from an experimental infection with *T. parva*.*

Negative control serum was prepared from a steer obtained from Kane farm, a commercial farm known to be free of ECF in Lusaka.

#### 3.7.4.3 Conjugate

The conjugate was rabbit antiovine globulins conjugated with fluorescent isothiocyanate (Nordic Immunological laboratories). The conjugate was used at a dilution of 1:32 in phosphate buffered saline (PBS). This dilution did not give any loss in titre of the positive serum. At the same time, no reaction was observed using the negative serum. For the batch of conjugate used, fluorescence below the dilution was 1:80 was considered positive.

#### 3.7.4.4 Schizont antigen preparation

Teflon-coated slides were left in methanol for 1 hour to remove any grease.

Lyophilised (freeze dried) infected cells were then re-hydrated in distilled water to their original volume (before lyophilisation). The cell suspension was then diluted with PBS + 0.2% BSA to a concentration giving approximately 50 cells per microscopic field.

Clumped cells were broken down by passing the suspension at least thrice through a syringe with a 26 G needle.

While shaking the suspension, 10 µl of the suspension was dispensed on each well of the teflon-coated slides. The slides were then left to dry at 37 °C for 1 hour, wrapped in tissue paper and stored at -20 °C.



### **3.7.4.5 Indirect Fluorescent Antibody test procedure**

Positive and negative reference sera as well as test serum were diluted to 1/80 in PBS.

Teflon-coated slides were retrieved from the deep freezer and then dried at 37 °C while wrapped in tissue paper to prevent condensation of water on the slides. They were then fixed in cold acetone for 10 minutes.

A humid chamber was prepared by soaking blotting paper with water. Diluted serum samples including reference sera were then dispensed onto the slides. The slides were then incubated for 30 minutes in the humid chamber at 37 °C and thereafter washed in PBS.

The conjugate was then applied onto the samples and incubated for a further 30 min. The slides were then washed in PBS. Mounting was done with 50% glycerol and examination carried out under a fluorescence microscope (Olympus, BH 2).

## **3.8 Statistical analysis**

Regression analysis with corresponding F-test was used to analyse the temperature and packed cell volume (PCV) data. All analyses were done with the help of the Glim statistical software.

## CHAPTER FOUR

### RESULTS

#### 4.1 Tick colony

The ten engorged adult female *Rhipicephalus appendiculatus* ticks which were obtained from the National Institute for Science Research in Chilanga to establish a colony yielded an average of 1500 larvae per batch of eggs laid per tick. From each instar (both larvae and nymphs), 50% of the ticks attached and fed efficiently to reach engorgement. Ultimately, only 19% of the ticks that hatched reached the adult stage and were available for stabilate production.

##### 4.1.1 Infection rate of *Theileria parva* in *Rhipicephalus appendiculatus* ticks

A total of one hundred pairs of *R. appendiculatus* ticks were dissected. The infection rate, calculated as a percentage of infected ticks over the total number of ticks dissected and examined, was 56.5%. The mean number of infected acini per infected tick was calculated to be equal to 7.5%. More female ticks were infected than males by a ratio of 14 to 1.

#### 4.2 Temperature

The daily average temperatures of steers infected with non-irradiated *Theileria parva* stabilate (NIS), steers infected with lethal doses of *T. parva* sporozoites for stabilate production (SP), and those used in the test for the viability of the stabilate (VS) are shown in figure 2. This figure shows that the daily average temperatures for the three groups of steers had a very similar pattern. Statistically, there was no

significant difference between the groups ( $p>0.05$ ). In all cases (NIS, SP, VS), the steers developed fever between day 14 and 16.

The fever lasted an average of six days except for the steer used in the test for the viability of the stabilate. This animal was treated with Butalex® on day 18 after schizonts had been demonstrated in the lymph node biopsy smears and thus no more daily average temperatures were recorded thereafter.

Comparison of the daily average temperatures between the NIS group and the non-irradiated tetracycline treated (NIST) group (fig. 3) showed a significant difference ( $p>0.05$ ) with the latter having no remarkable increase throughout the initial infection period. The NIST group was simulated to a negative or immunisation control.

There was a significant difference ( $p>0.05$ ) between the daily average temperatures of the NIS group and the directly irradiated stabilate (DIS) group (fig. 4); and the steers infected with stabilate produced from irradiated ticks or irradiated 'tick' stabilate (ITS) group (fig. 5). However, there was no significant difference ( $p>0.05$ ) in the mean daily temperatures between DIS and NIST (fig. 6) and ITS and NIST (fig. 7). Similarly, no significant difference was recorded in the mean daily temperature between DIS and ITS groups (fig. 8). Figure 9 is a summary of the mean daily temperature of all the groups (DIS, ITS, NIS and NIST).

Following challenge with lethal doses of homologous stock, none of the immunised groups DIS (fig. 10); ITS (fig. 11); NIS (fig. 12) and NIST (fig. 13) showed any significant raise in temperature. However, the control group of steers developed fever eight days post infection which lasted a mean period of 11 days. These animals died soon after the temperature returned to normal on day 19. Fig. 14 is

a summary of the daily mean temperature of all groups of experimental steers following challenge with lethal homologous stock.

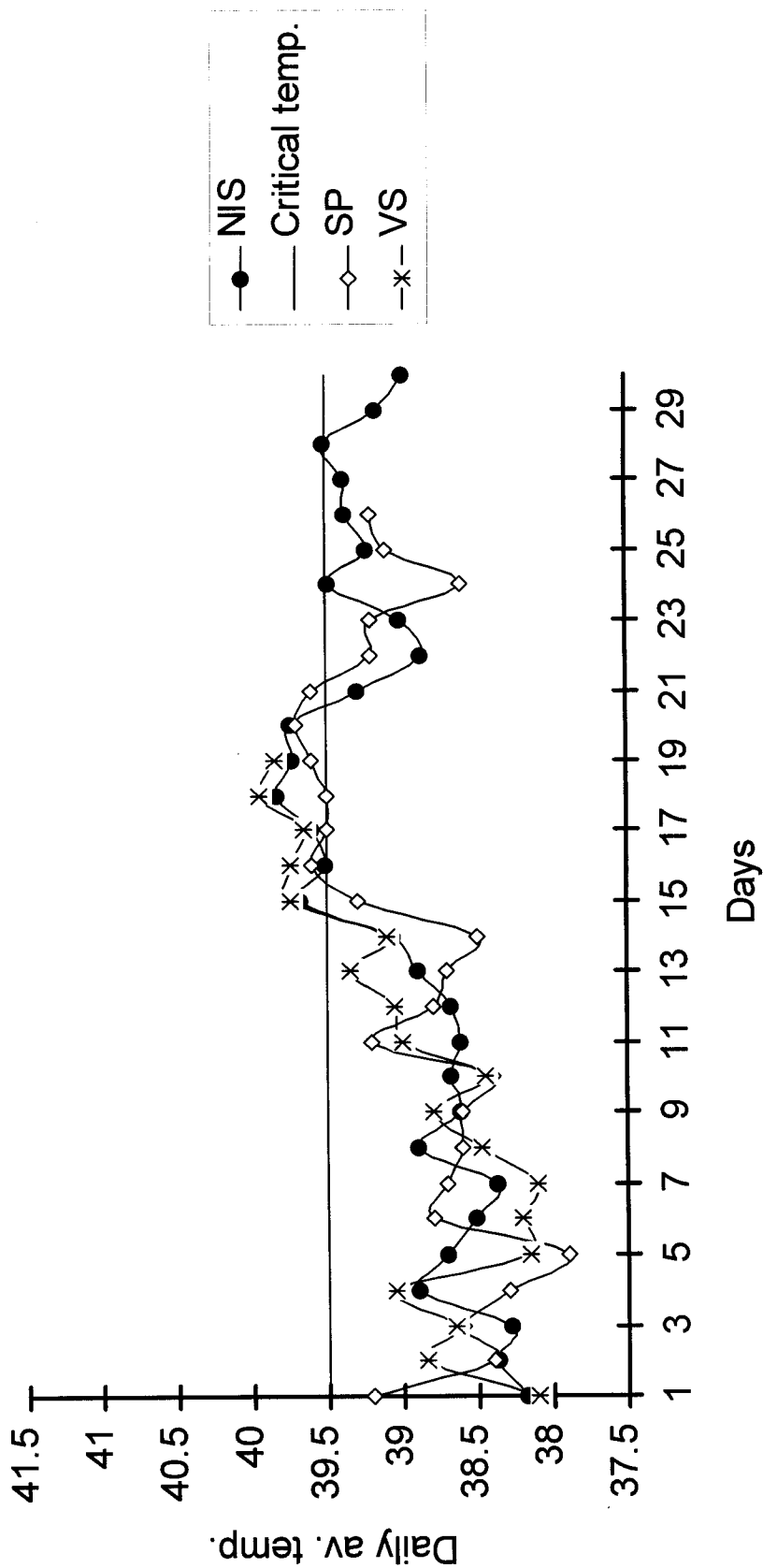


Fig. 2. Daily average temperatures of steers infected with non-irradiated *Theileria parva* stabilate (NIS), steers used for stabilate production (SP), and to test the viability of the stabilate (VS).

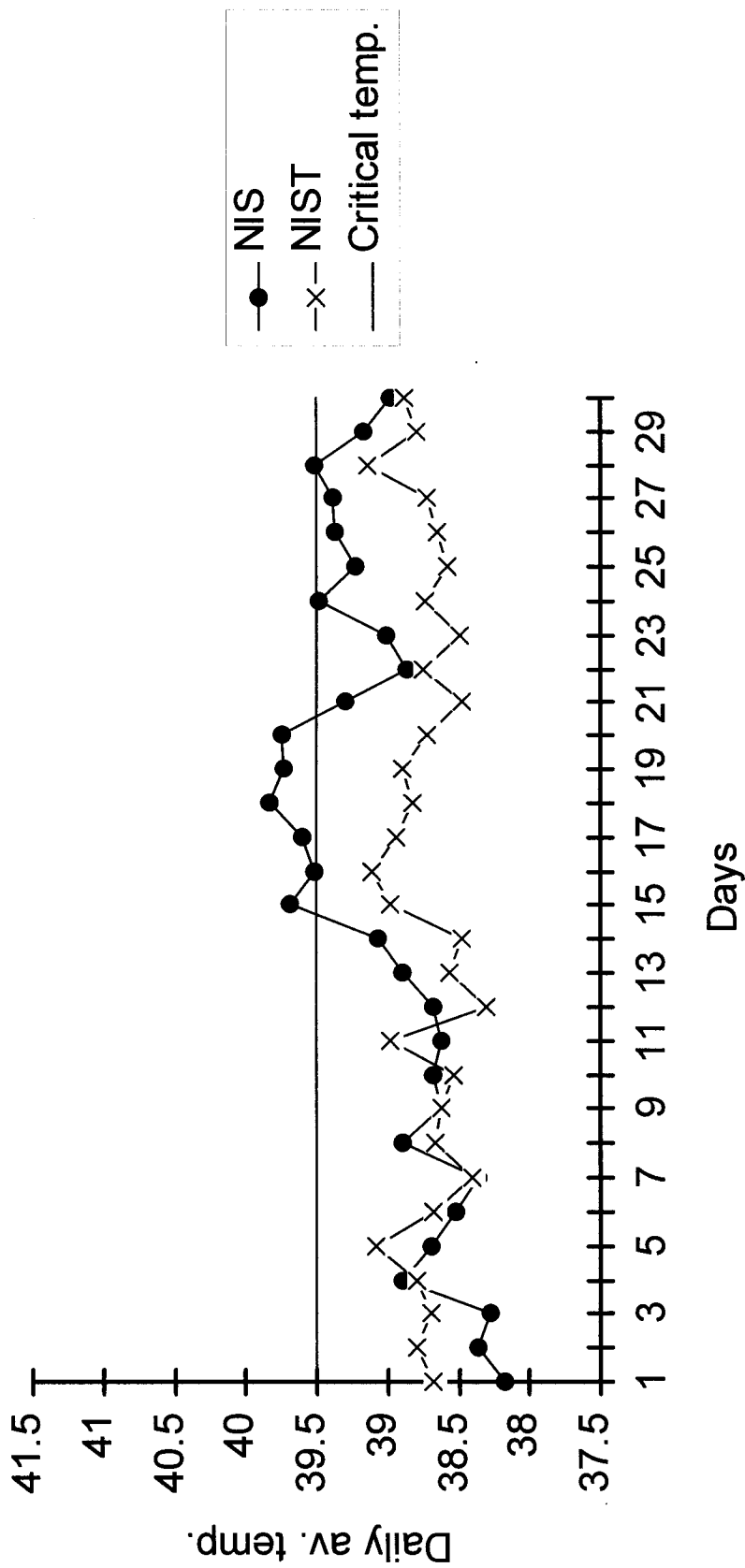


Fig. 3. Daily average temperatures of steers infected with non-irradiated *Theileria parva* stablate (NIS) and those infected with non-irradiated *T. parva* stablate with concurrent tetracycline treatment (NIST).

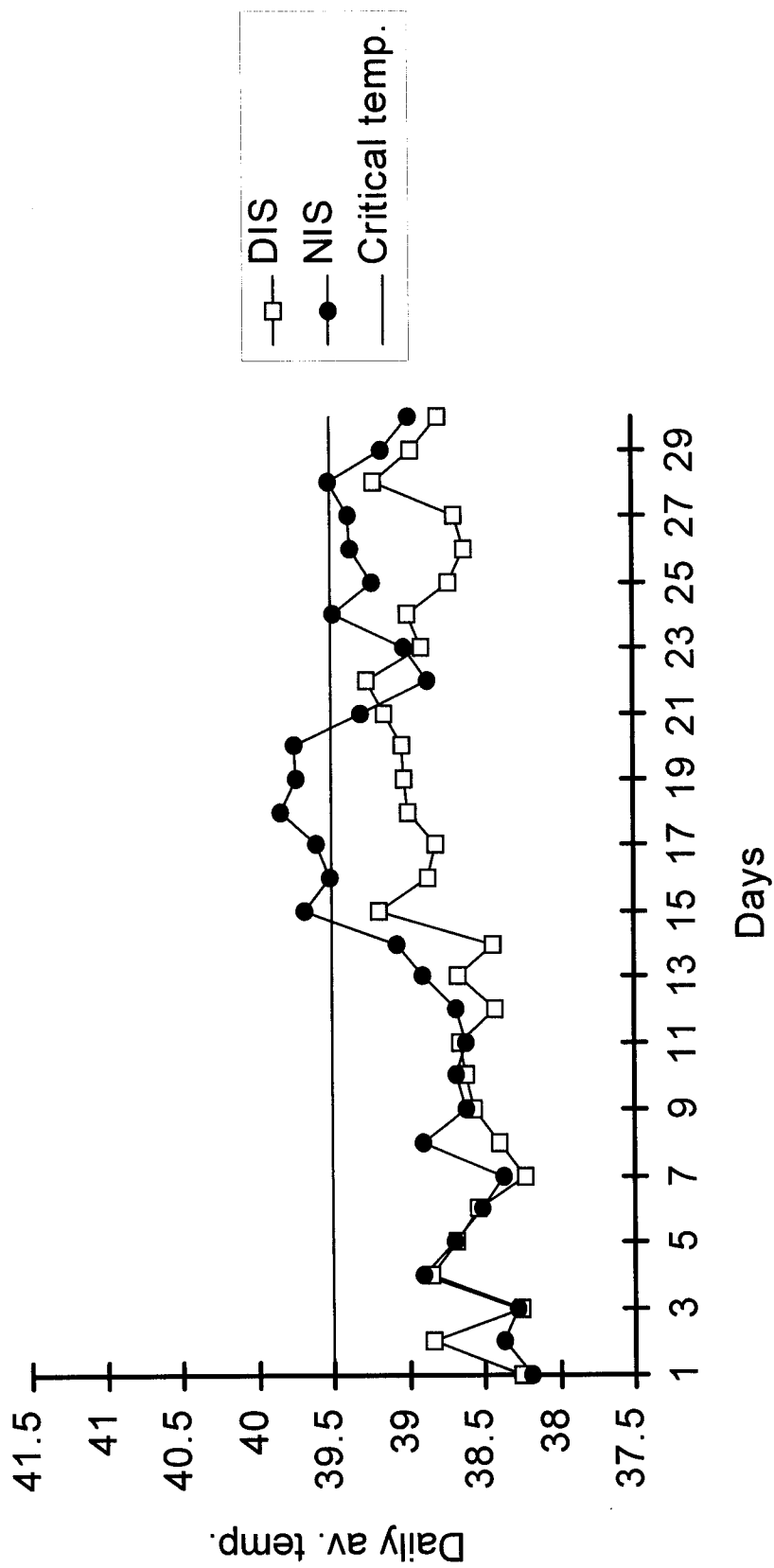


Fig. 4. Daily average temperatures of steers infected with directly irradiated *Theileria parva* stabilate (DIS) and non-irradiated stabilate (NIS).

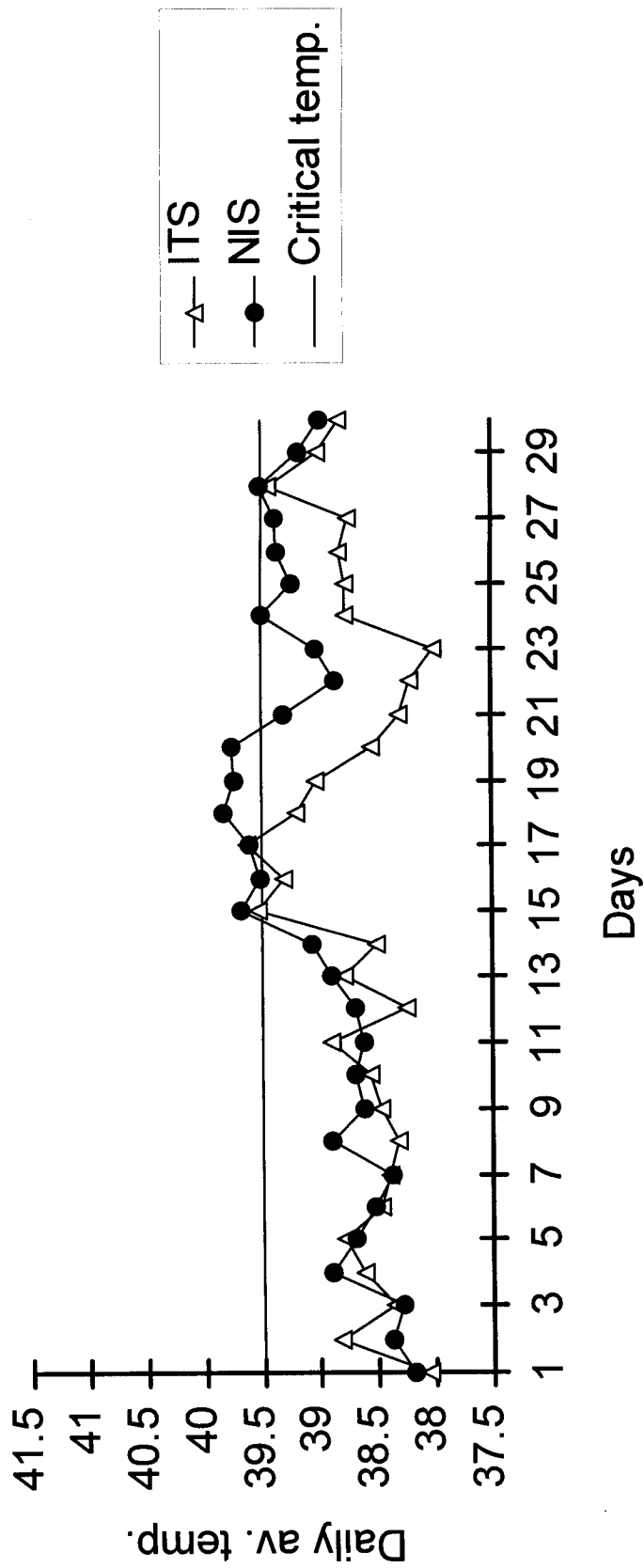


Fig. 5. Comparison of daily average temperatures between steers infected with non-irradiated *Theileria parva* stabilate (NIS) with those infected with stabilate produced from irradiated *Rhipicephalus appendiculatus* ticks (ITS).



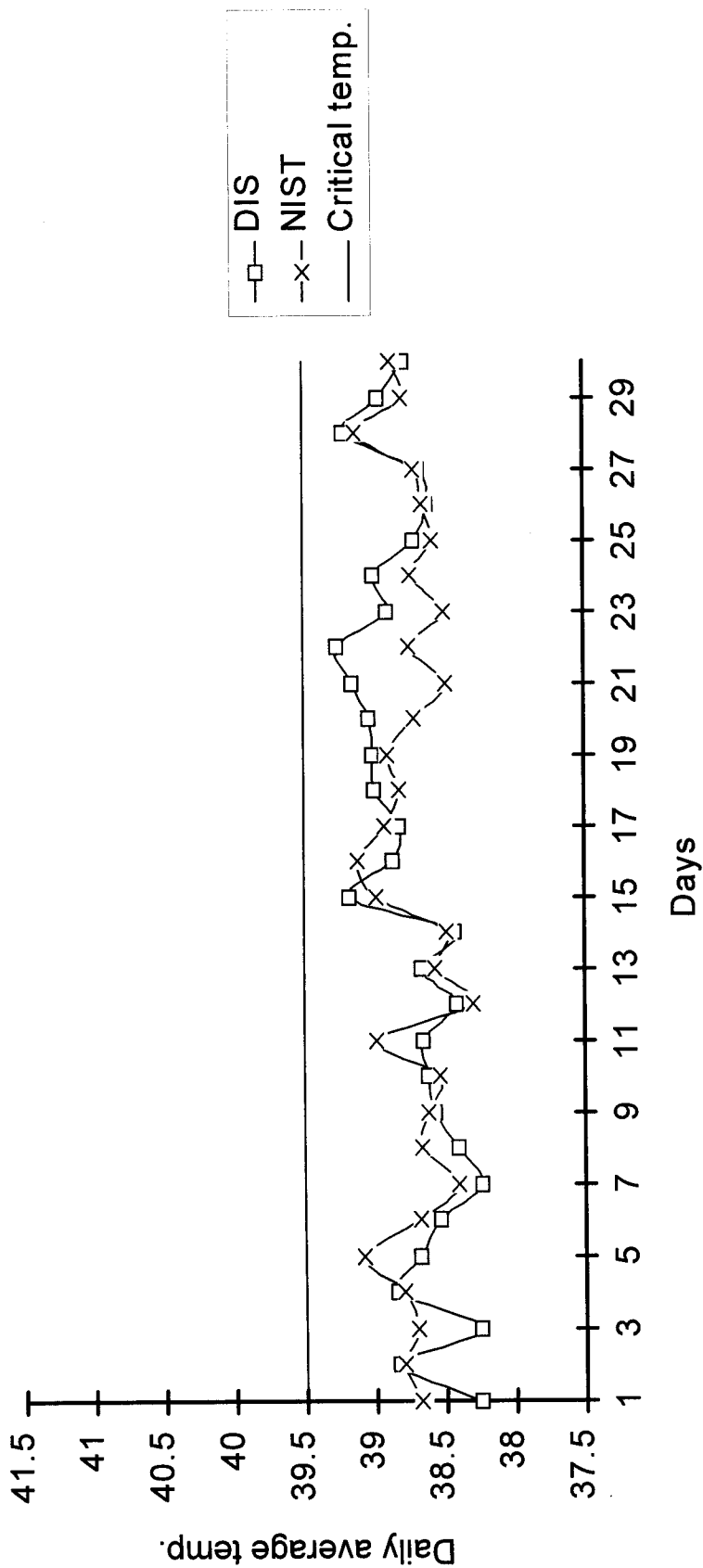


Fig. 6. Daily average temperature of steers immunised with directly irradiated *Theileria parva* stabilates (DIS) and non-irradiated stabilates with tetracycline treatment (NIST).

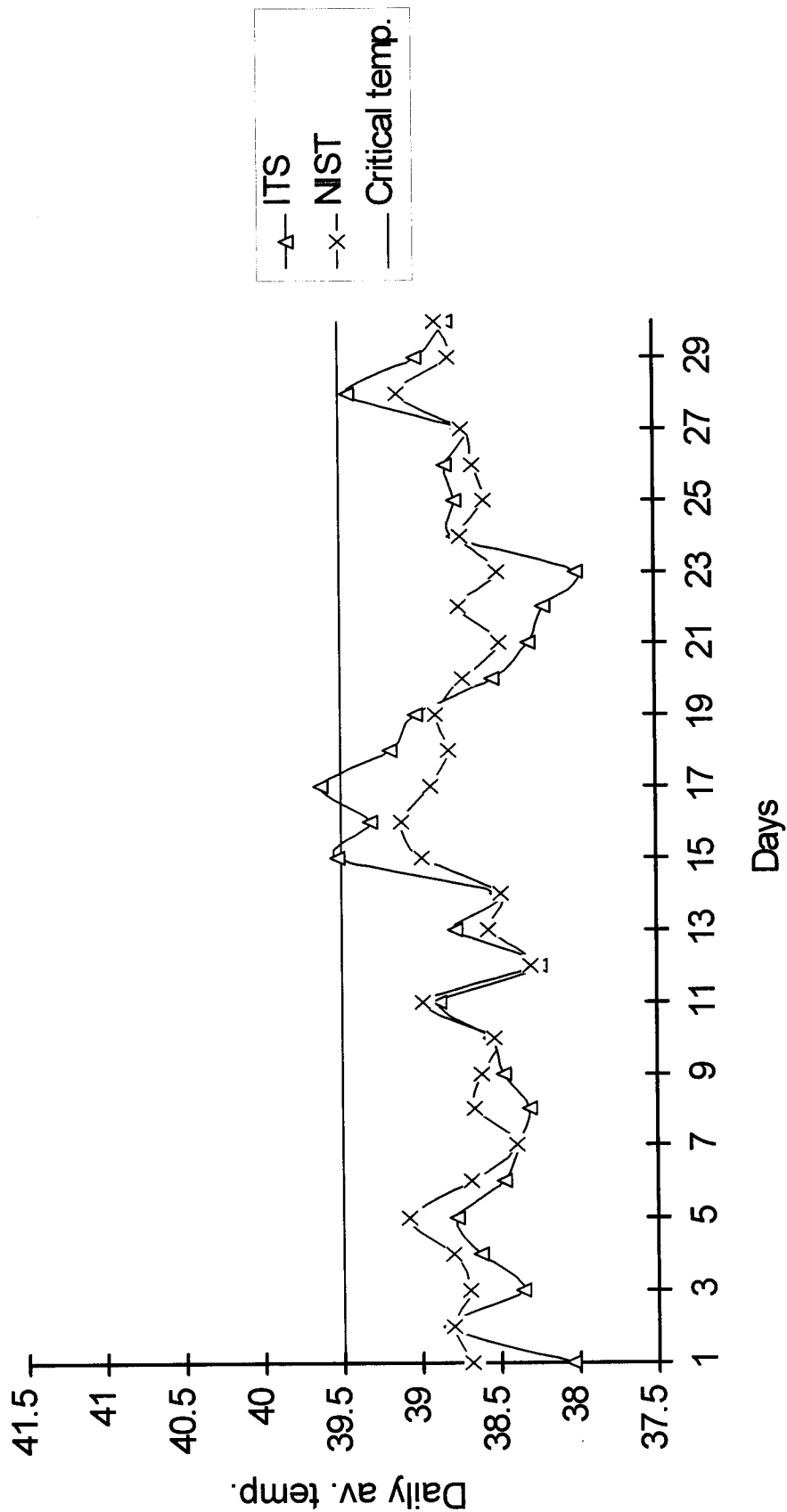


Fig. 7. Daily average temperatures of steers immunised with *Theileria parva* stabilate produced from irradiated *Rhipicephalus appendiculatus* ticks (ITS) and non-irradiated stabilates with tetracycline treatment (NIST).

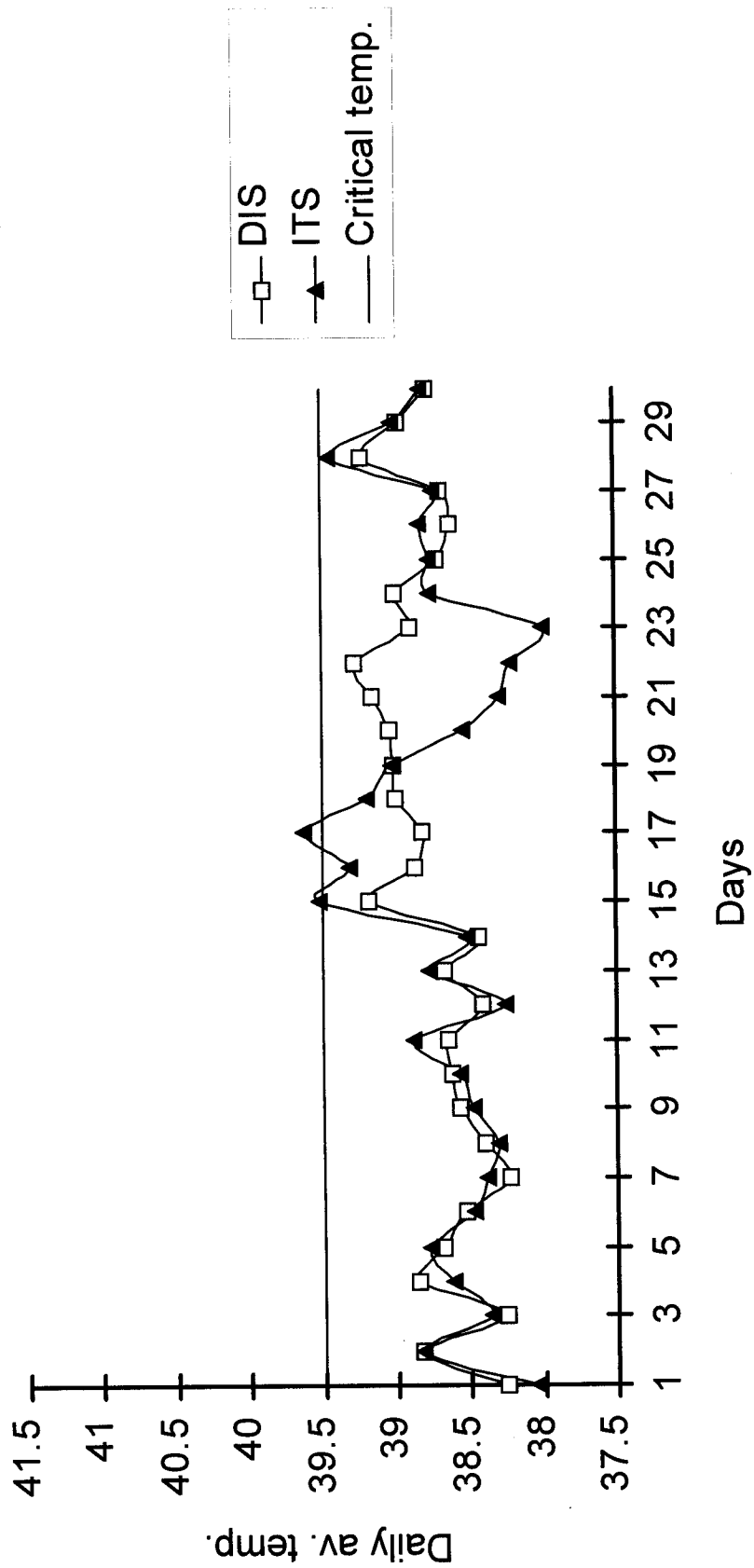


Fig. 8. Daily average temperatures of steers infected with directly irradiated *Theileria parva* stabilate (DIS) and those infected with stabilate produced from irradiated *Rhipicephalus appendiculatus* ticks (ITS).

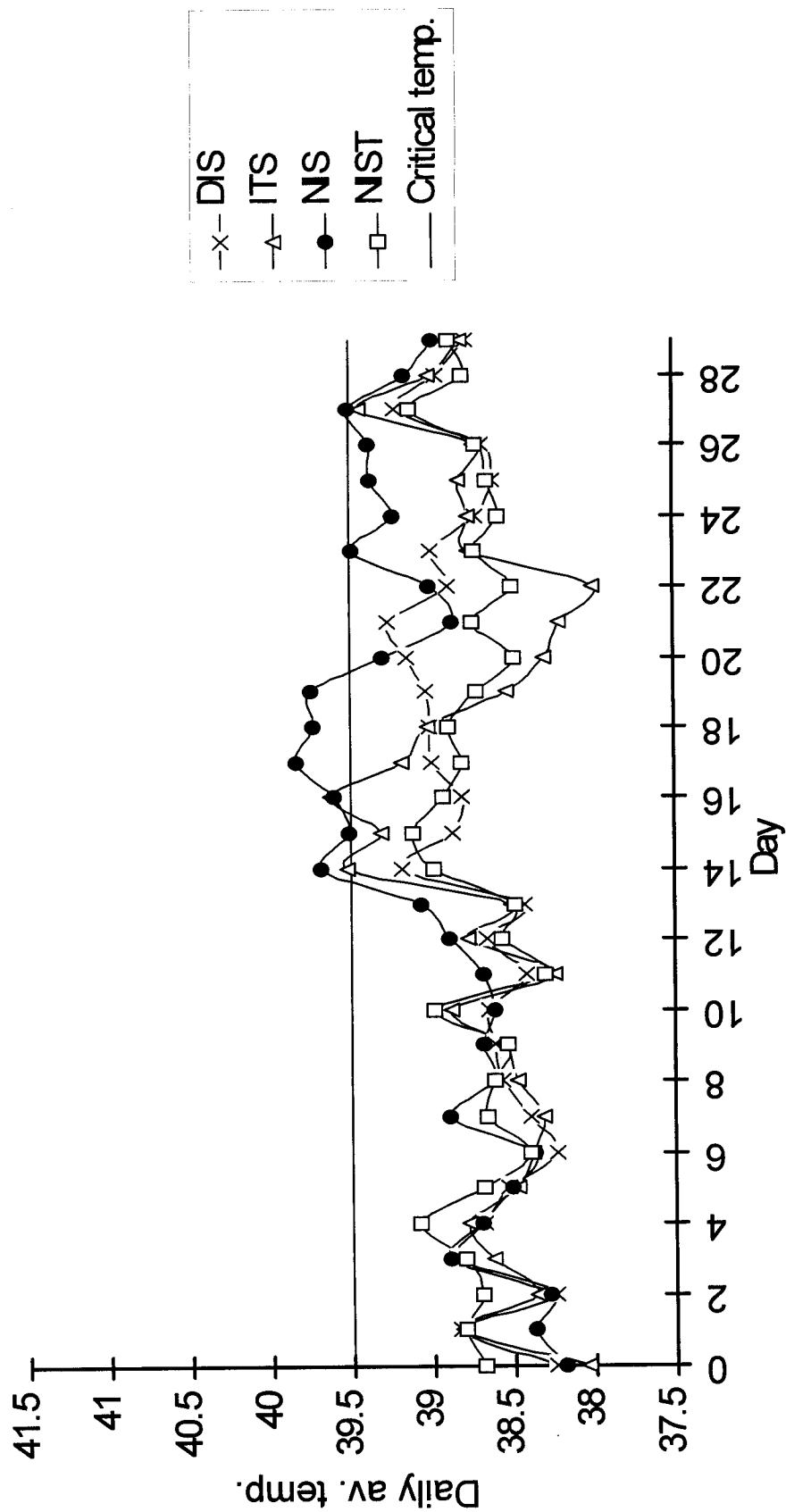


Fig. 9. Summary of daily average temperatures of steers infected with directly irradiated *Theileria parva* stabilate (DIS), irradiated *Rhipicephalus appendiculatus* ticks stabilate (ITS), non-irradiated stabilate (NIS) and non-irradiated stabilate with tetracycline treatment (NST).

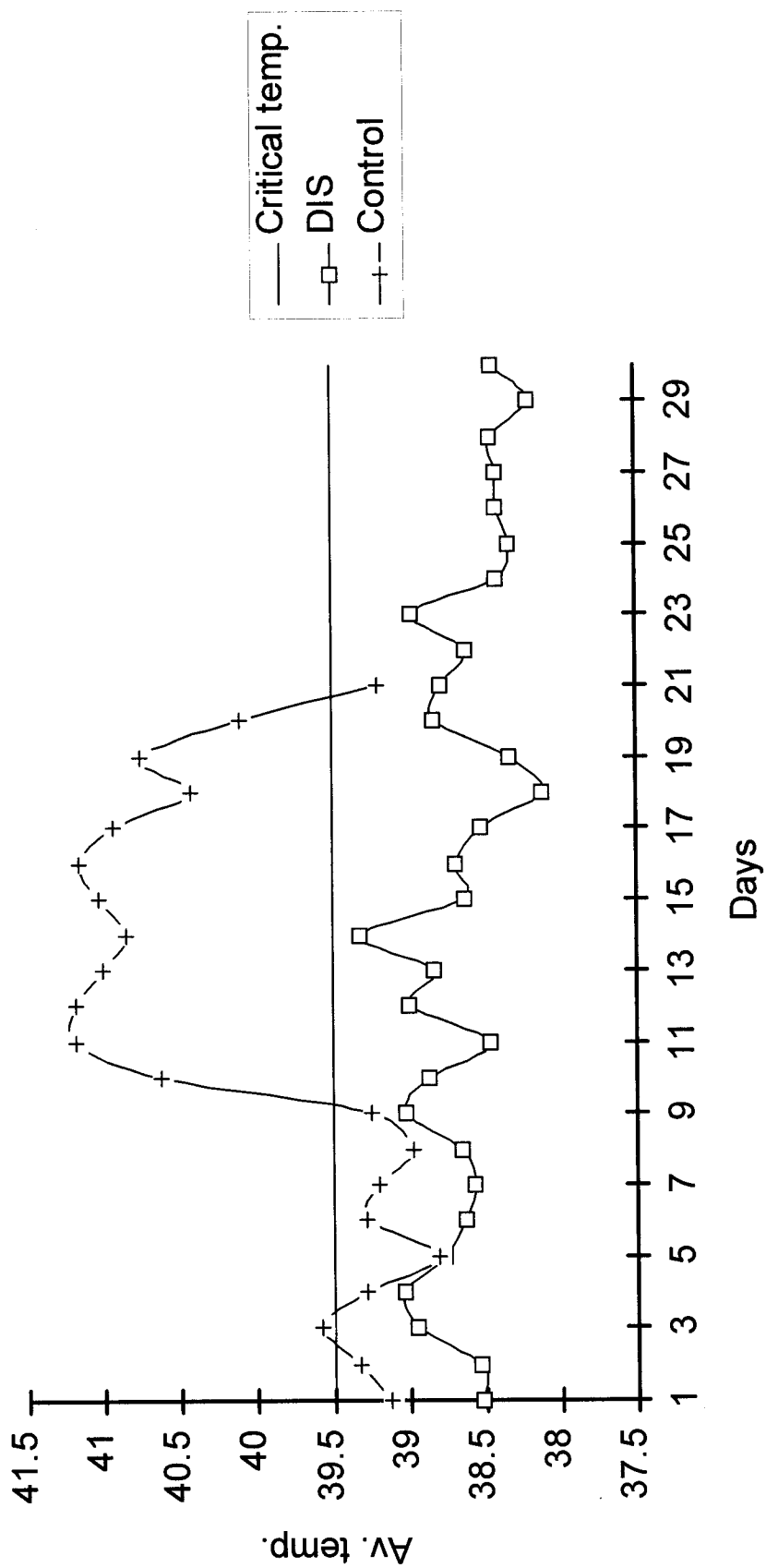


Fig. 10. Daily average temperatures of steers immunised with directly irradiated *Theileria parva* sporozoites (DIS) following challenge with double dose of non-irradiated homologous stock.

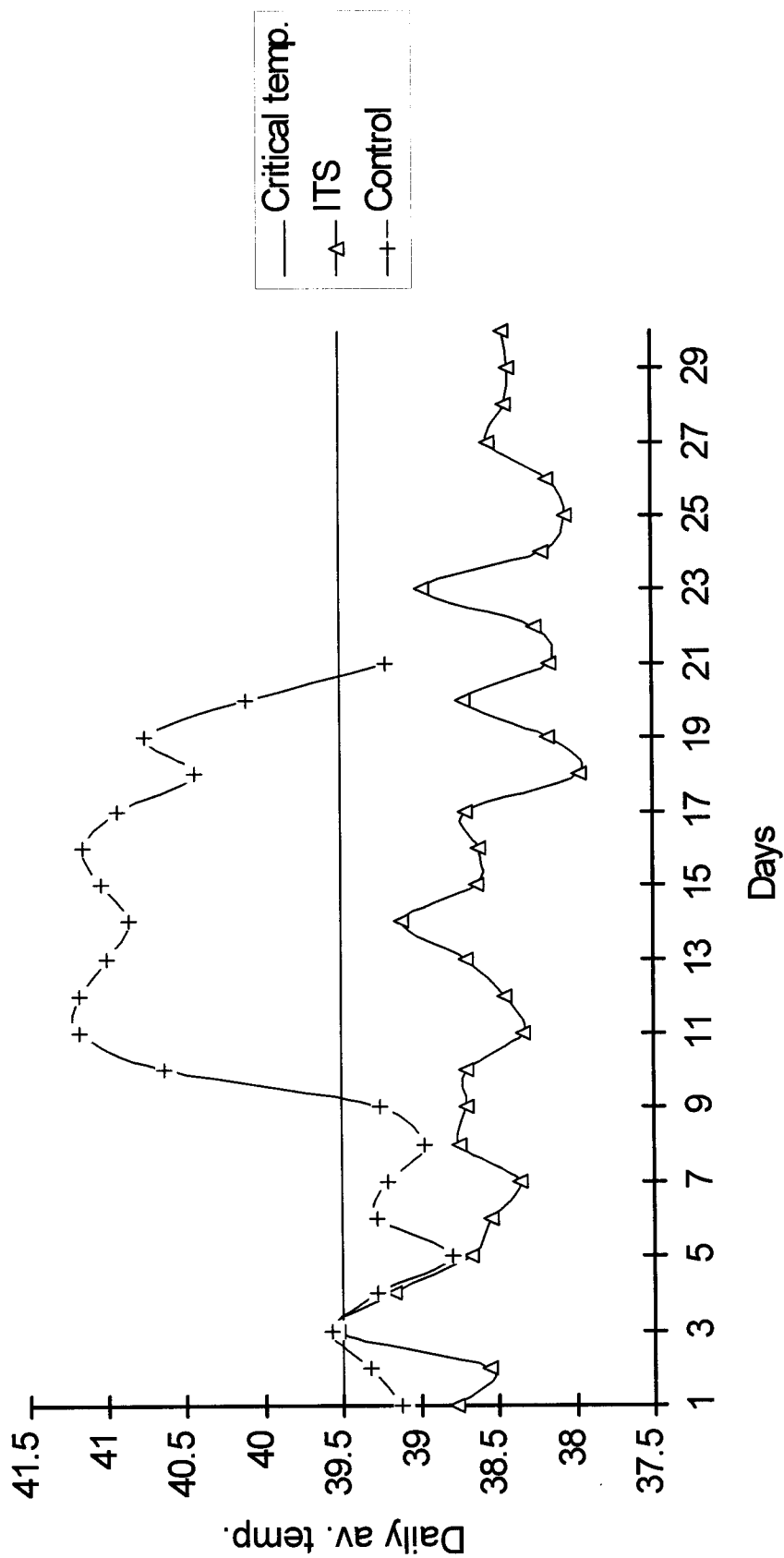


Fig. 11. Daily average temperatures of steers immunised with *Theileria parva* stabilate produced from irradiated *Rhipicephalus appendiculatus* ticks (ITS) following challenge with double dose of non-irradiated homologous stock.

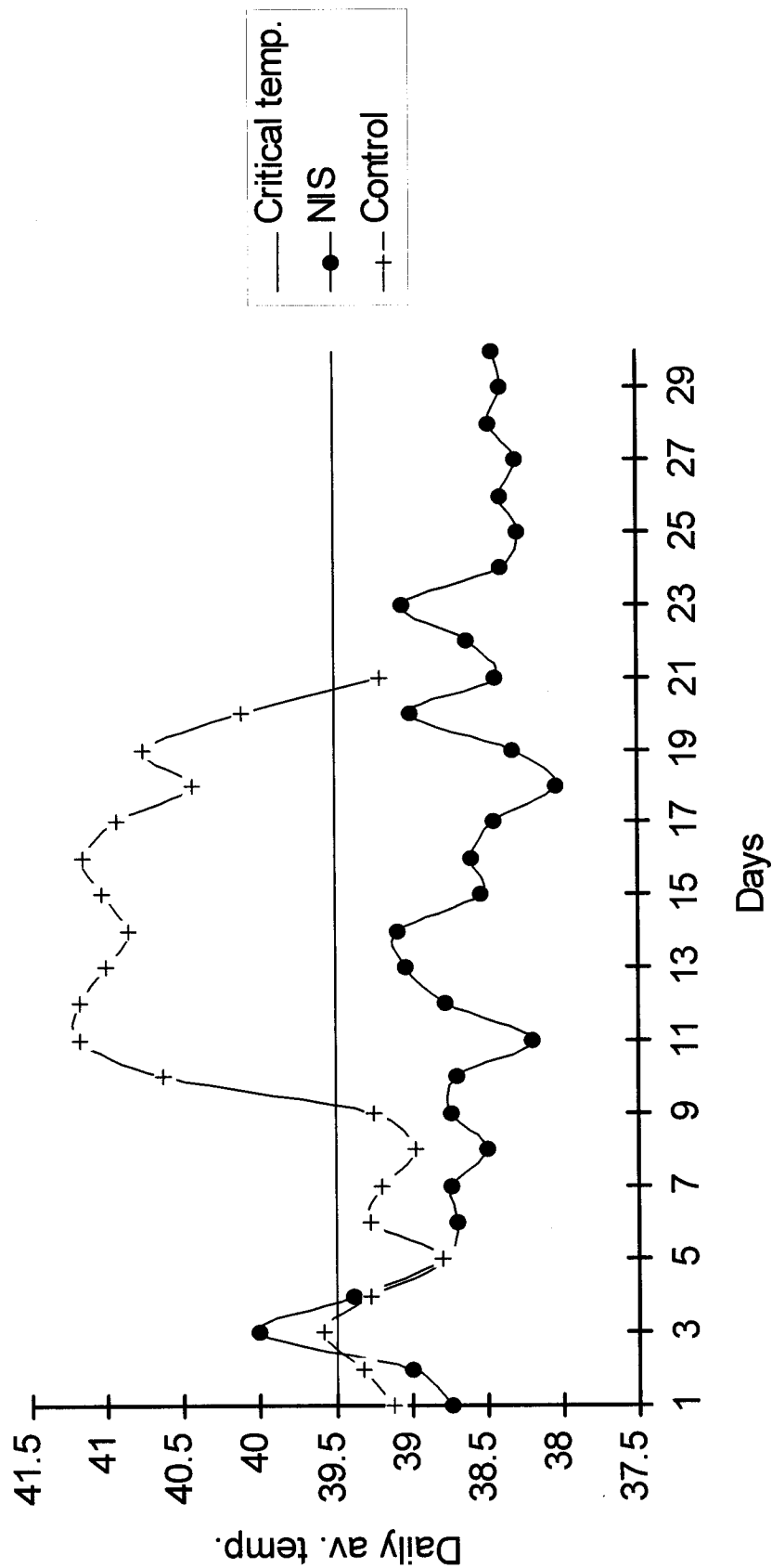


Fig. 12. Daily average temperatures of steers immunised with non-irradiated *Theileria parva* sporozoites (NIS) after challenge with double dose of non-irradiated homologous stock.

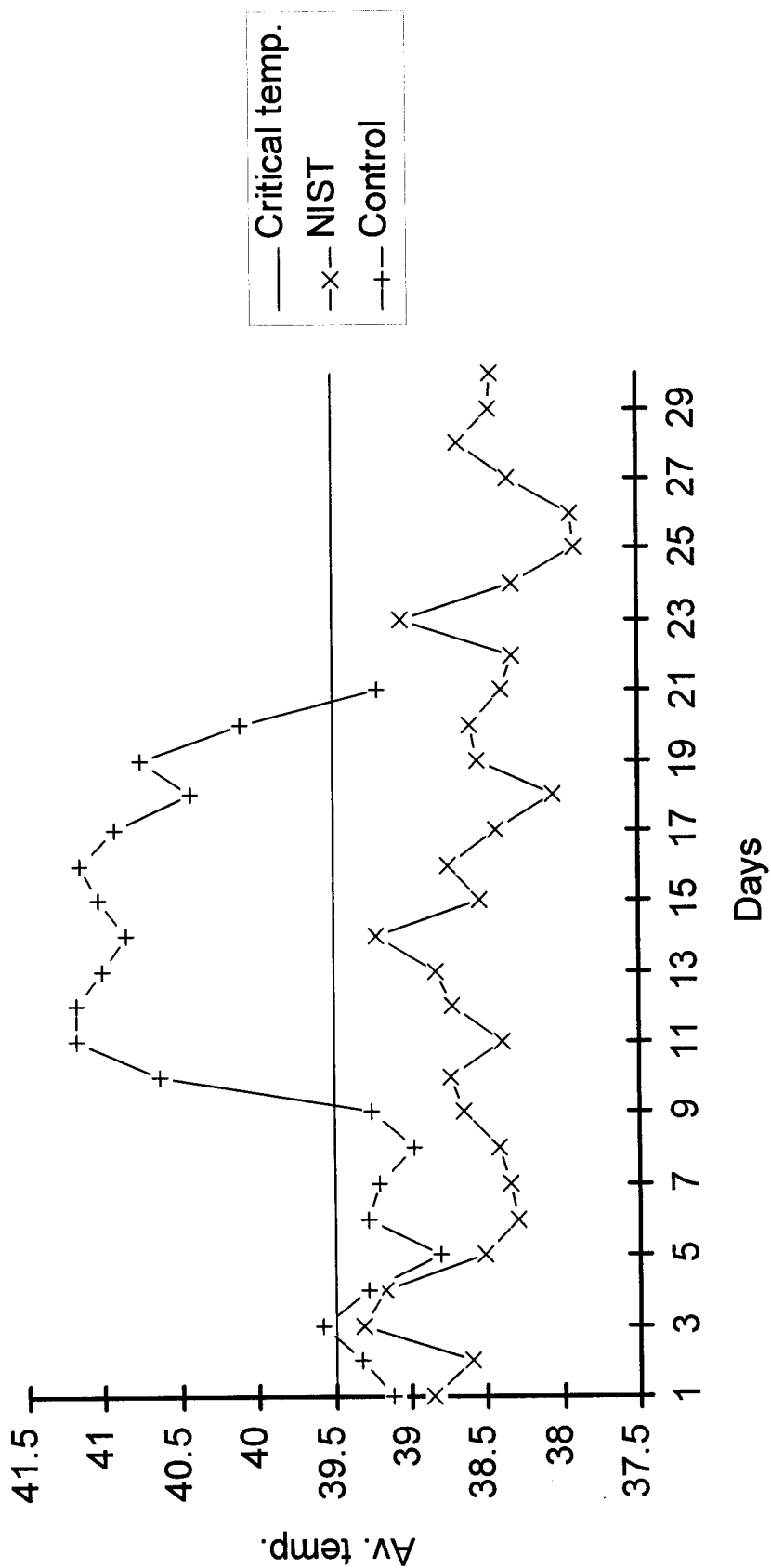


Fig. 13. Daily average temperatures of steers immunised with non-irradiated *Theileria parva* sporozoites with concurrent tetracycline therapy (NIST) following challenge with double dose of non-irradiated homologous stock.



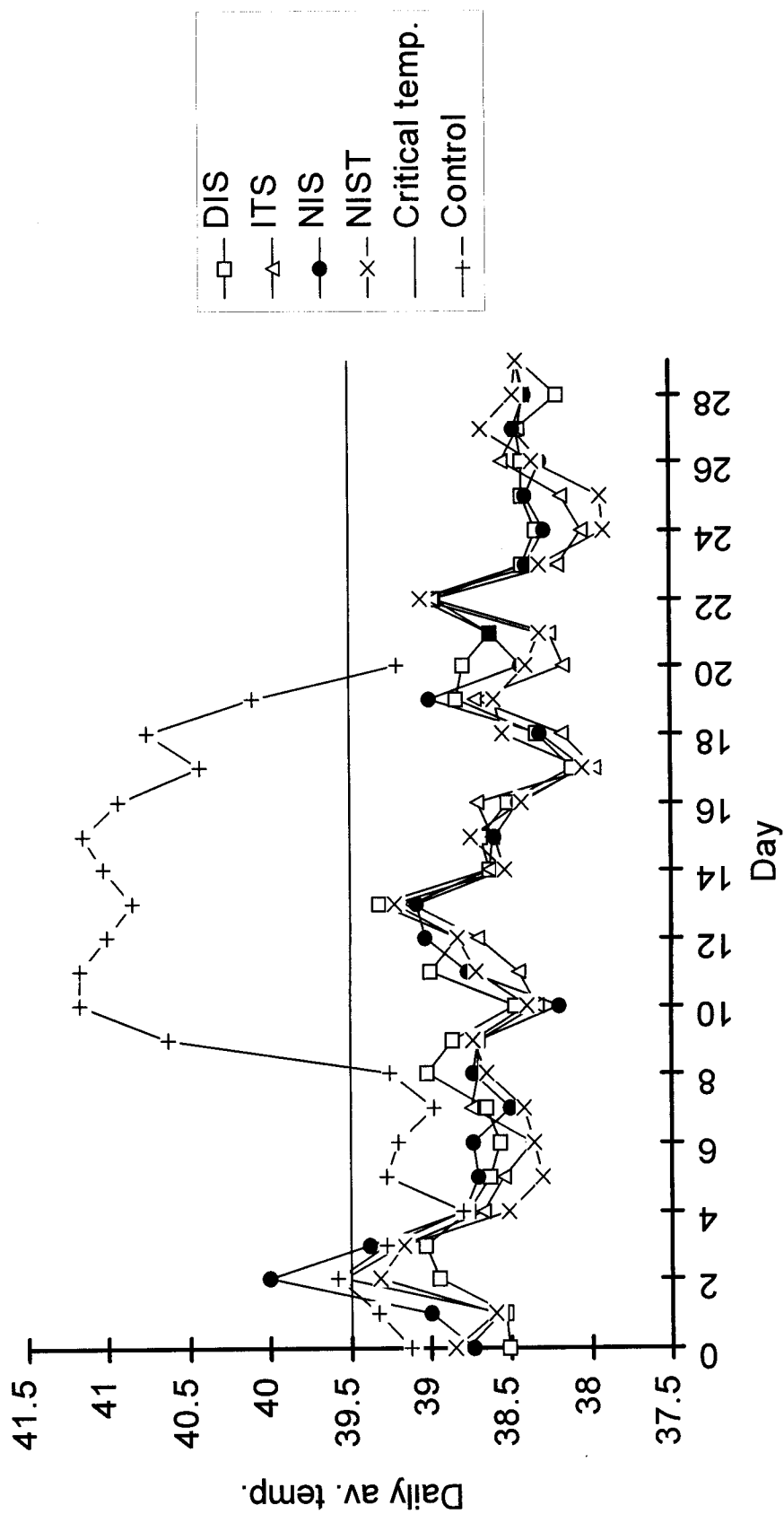


Fig 14. Summary of the daily average temperatures of steers infected with directly irradiated *Theileria parva* stabilate (DIS), irradiated *Rhipicephalus appendiculatus* ticks stabilate (ITS), non-irradiated stabilate (NIS) and non-irradiated stabilate with tetracycline (NIST), following challenge with double doses of non-irradiated stabilate.

### 4.3 Packed cell volume (PCV)

The changes in the mean daily packed cell volumes (PCV) for steers immunised with non-irradiated *T. parva* stabilates (NIS), those used in stabilate production (SP) and viability test (VS) are shown in fig. 15. The daily average PCV of NIS and SP groups of steers showed a gradual drop from about 27% to about 23%. The mean daily PCV for the single VS steer remained fairly constant at about 27% until the animal was treated 18 days post infection. The PCV of other treatment groups, the directly irradiated stabilate DIS (fig. 16), ITS (Fig. 17), showed a similar pattern with the PCV dropping gradually from about 25% to 22%. The mean daily PCV of the NIST (fig. 18) group dropped slightly by about 1% only. Fig. 19 is a summary of PCV changes of all the treatment groups following initial infection.

Following challenge with lethal homologous stock of *T. parva* stabilates, the PCV of all groups of steers, DIS (fig. 20); ITS (fig. 21); NIS (fig. 22); and NIST (fig. 23) remained low but fairly constant or slightly increasing by at least 1% in the following three weeks. The summary of all challenged groups is shown in fig. 24. The PCV of the control group (Fig. 20 to 24) dropped sharply from about 31% to about 21% in about three weeks. This showed that the more severe the reaction was, the faster the PCV dropped.

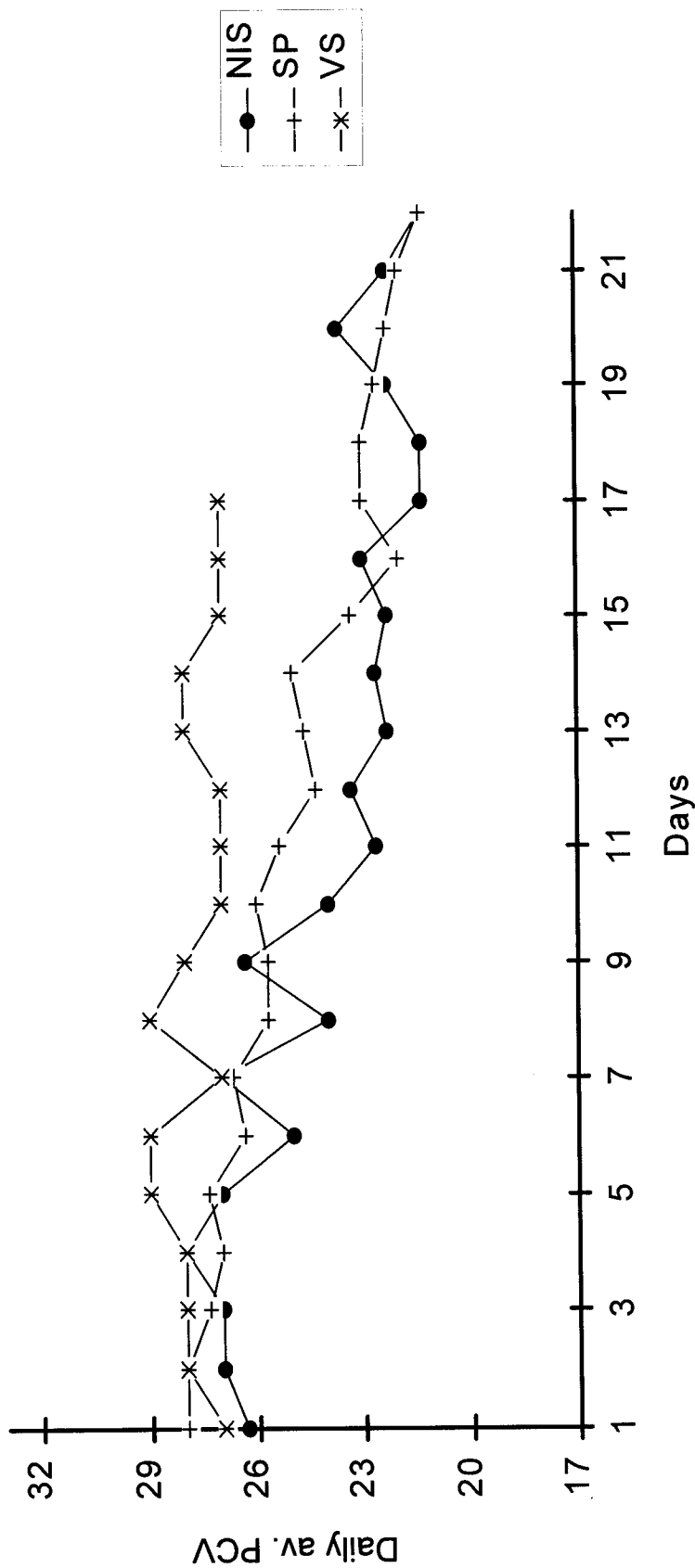


Fig. 15. Mean daily PCV of steers administered with a lethal dose of *Theileria parva* (SP) in comparison with those infected with a non-irradiated *T. parva* stabilate (NIS).

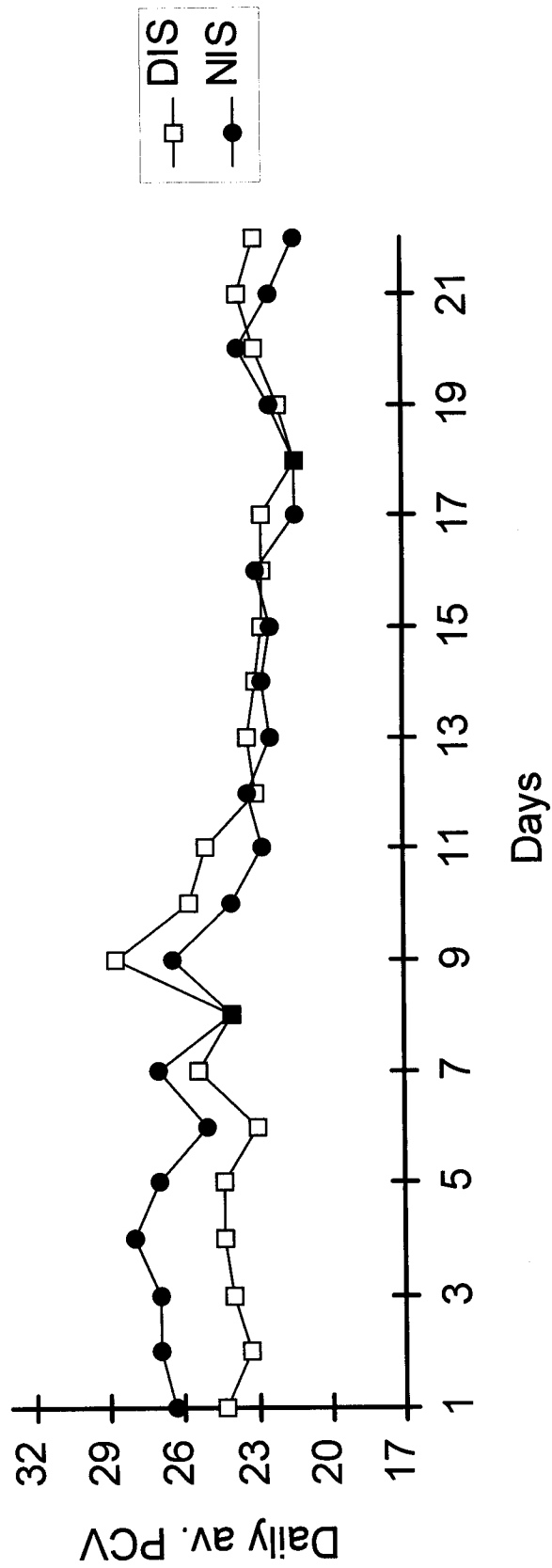


Fig. 16. Mean daily PCV of the steers immunised with directly irradiated *Theileria parva* stabilate (DIS) and steers infected with non irradiated stabilate (NIS).

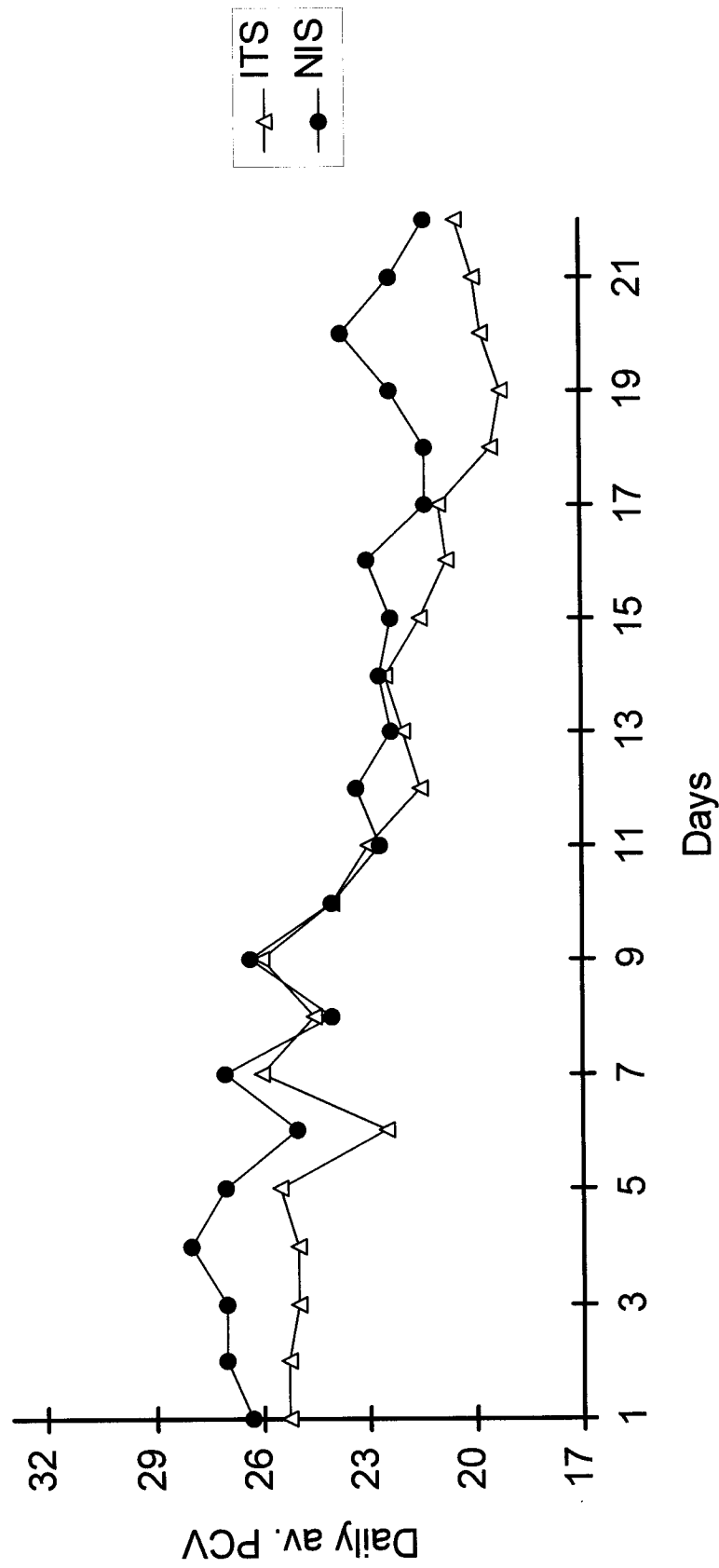


Fig. 17. Mean daily PCV of the steers immunised with *Theileria parva* stabilate produced from irradiated *Rhipicephalus appendiculatus* ticks (ITS) and steers infected with non irradiated stabilate (NIS).

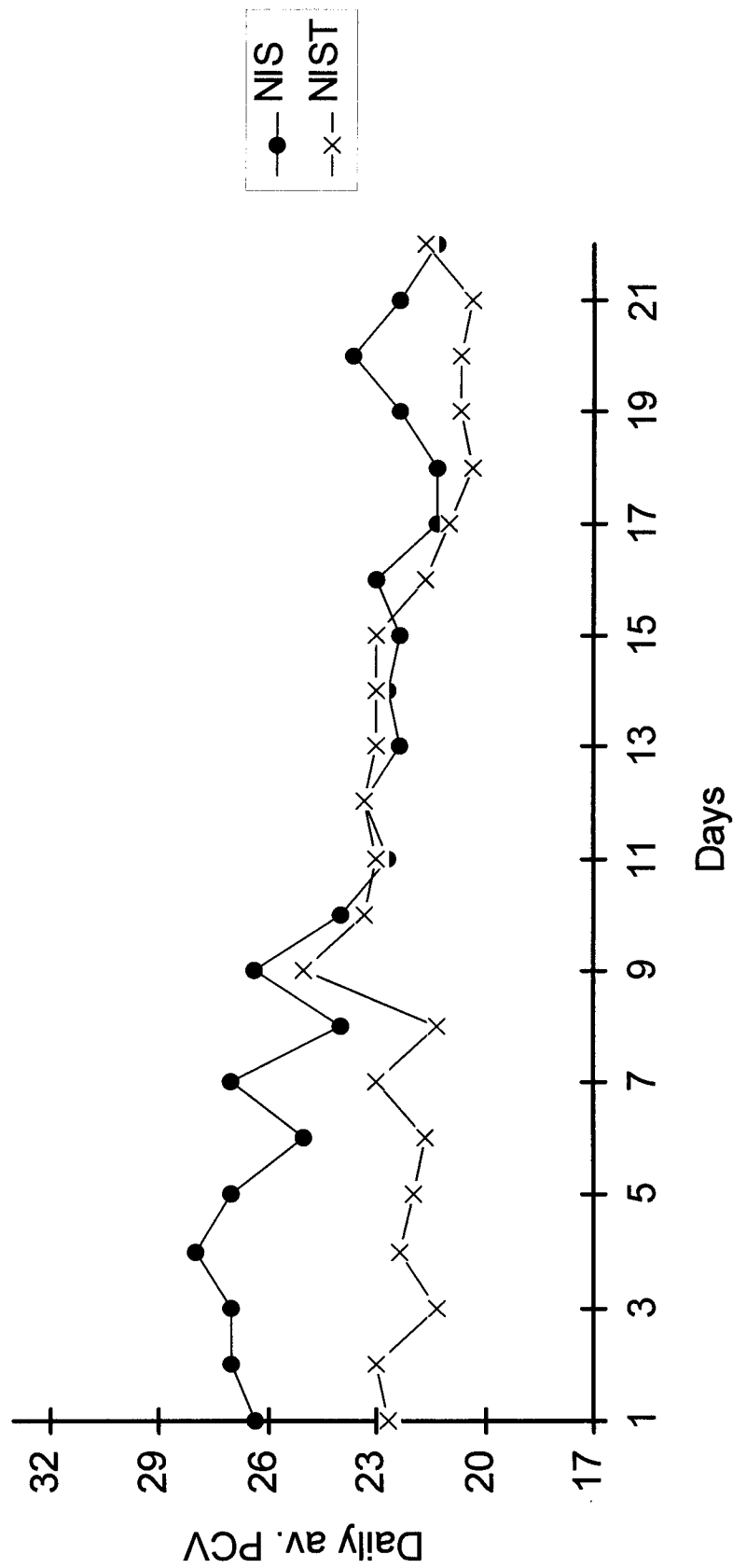


Fig. 18. Mean daily PCV of the steers immunised with non irradiated *Theileria parva* stabilate (NIS) and those infected with non irradiated stabilate but with concurrent tetracycline (NIST).

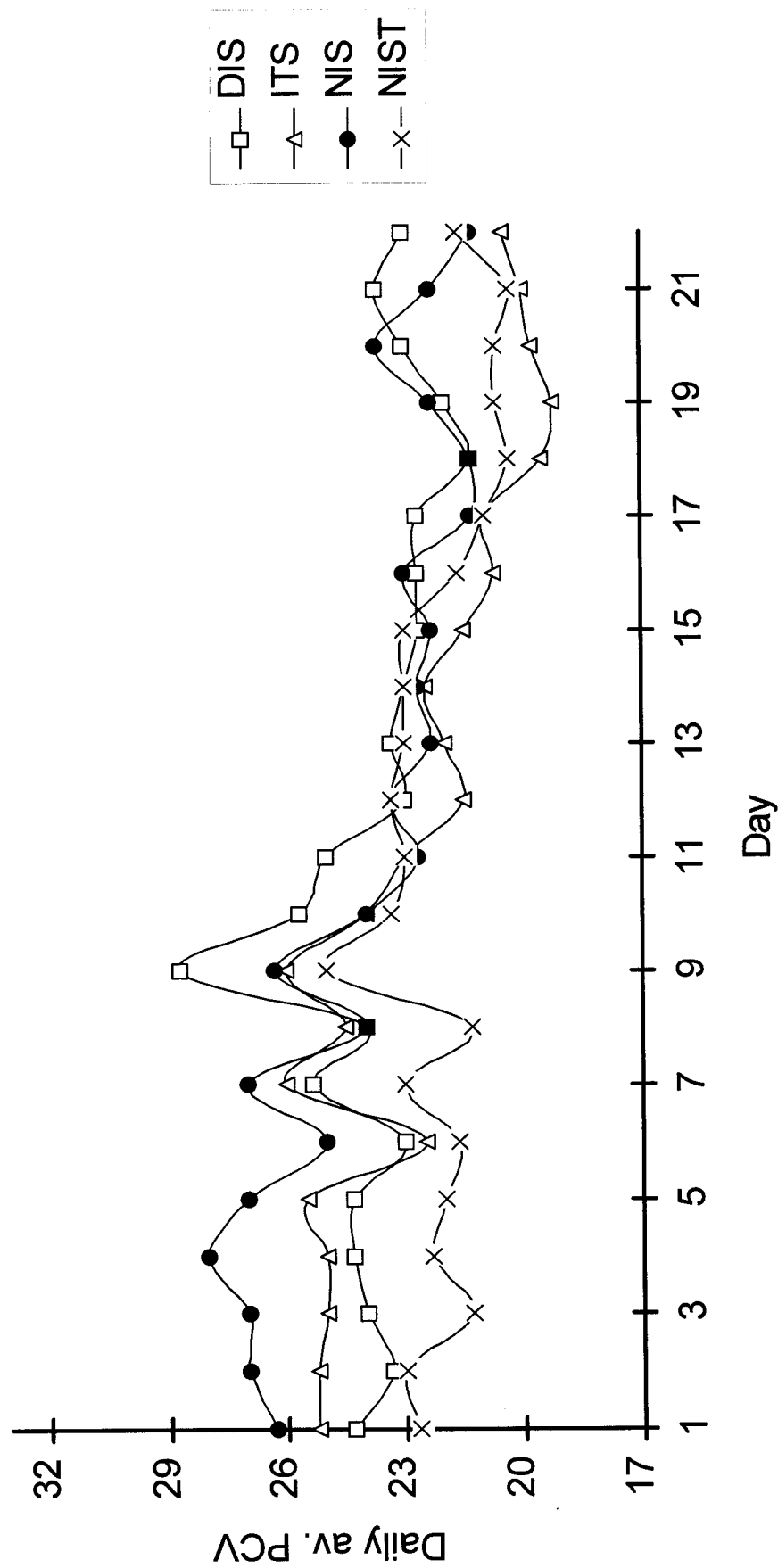


Fig. 19. Summary of the mean daily PCV of steers immunised with directly irradiated *Theileria parva* stabilate (DIS); irradiated *Rhipicephalus appendiculatus* ticks stabilate (ITS); non-irradiated stabilate (NIS); and non-irradiated stabilate with tetracycline treatment (NIST).

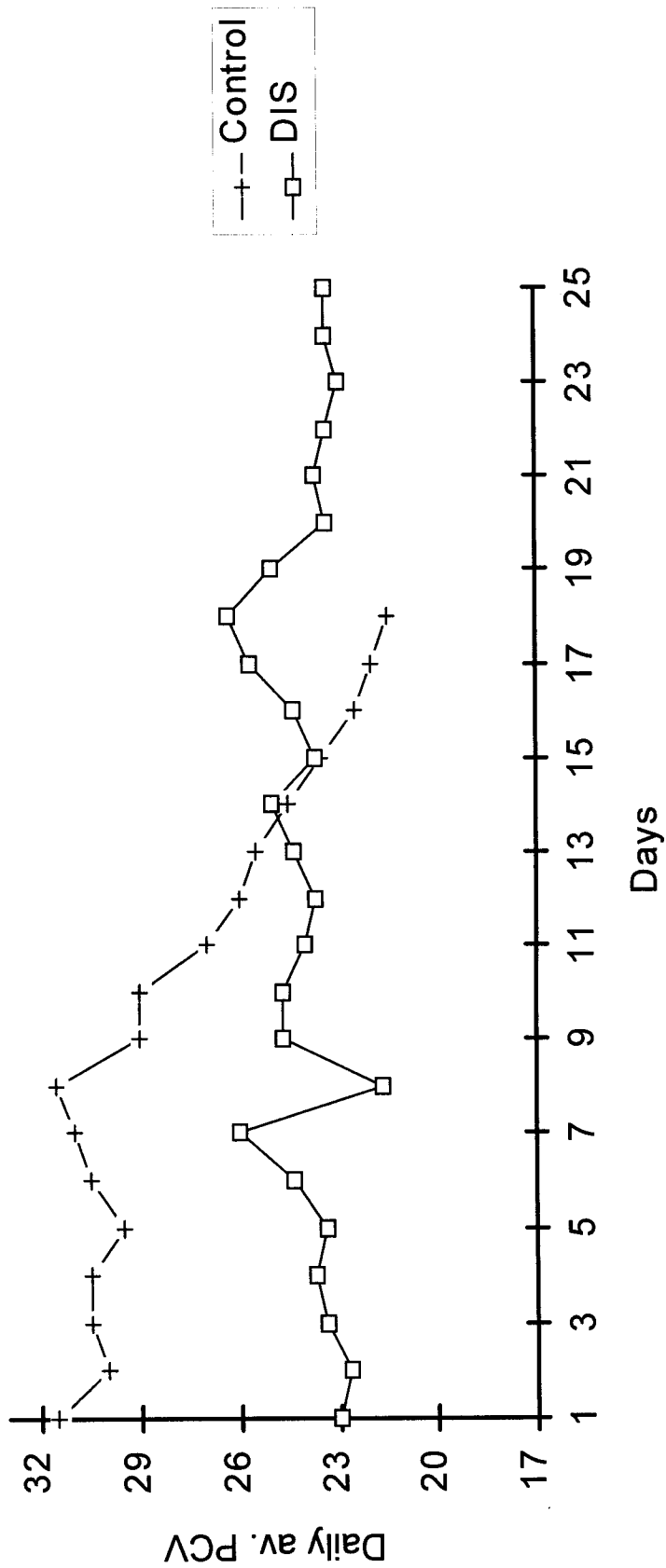


Fig. 20. Mean daily PCV of steers immunised with directly irradiated *Theileria parva* stabilate following challenge with a lethal homologous stock.



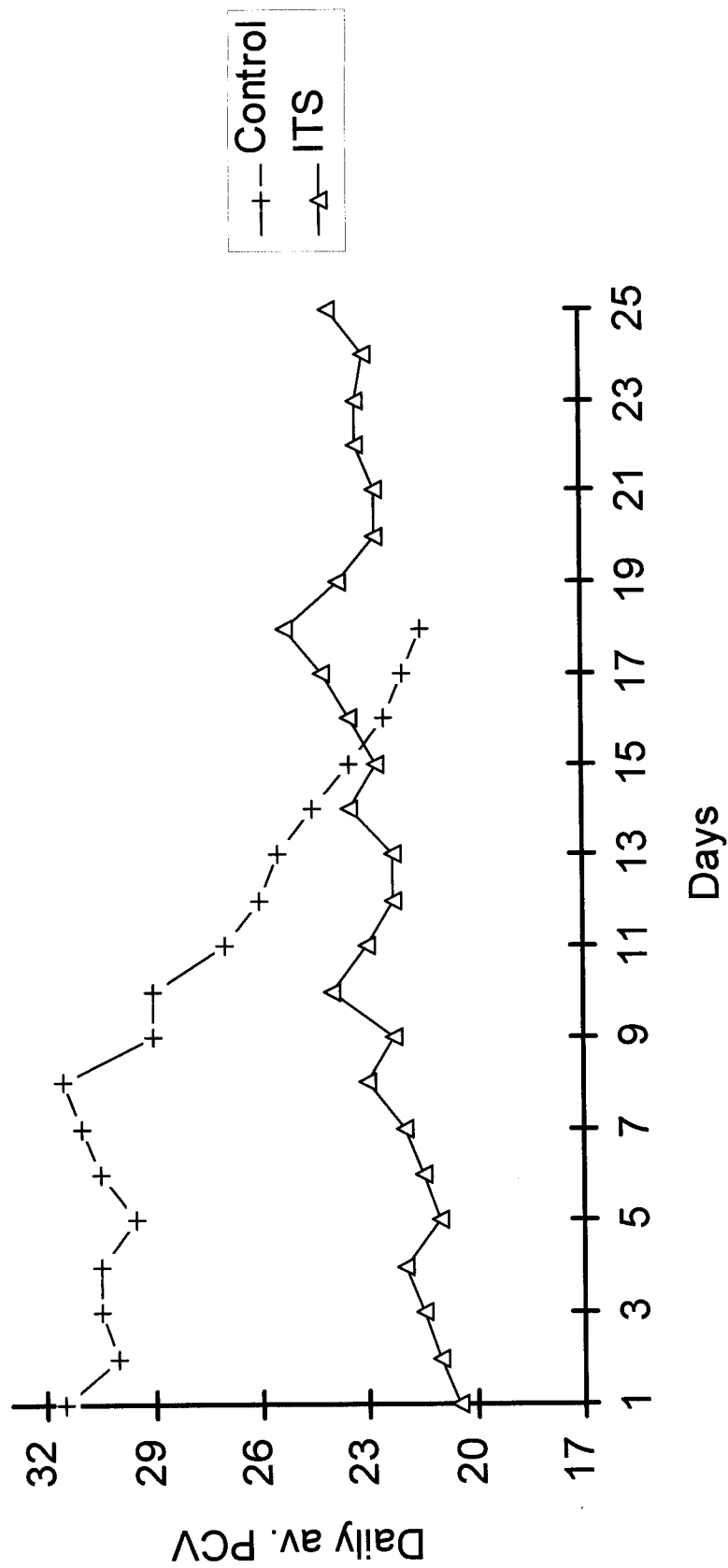


Fig. 21. Mean daily PCV of steers immunised with *Theileria parva* stabilate produced from irradiated *Rhipicephalus appendiculatus* ticks (ITS), following challenge with a lethal homologous stock.

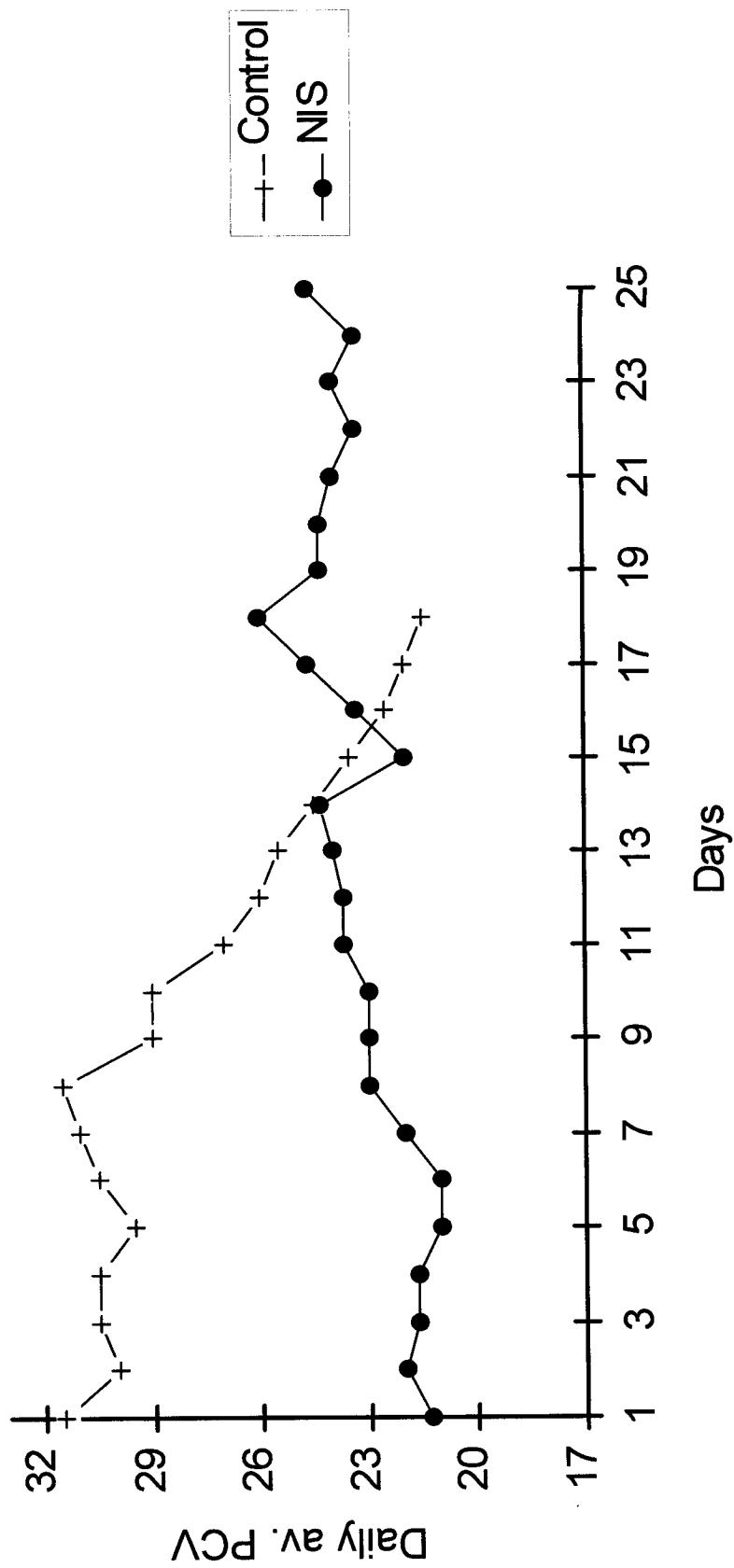


Fig. 22. Mean daily PCV of steers immunised with non-irradiated *Theileria parva* stabilate (NIS) following challenge with a lethal homologous stock.

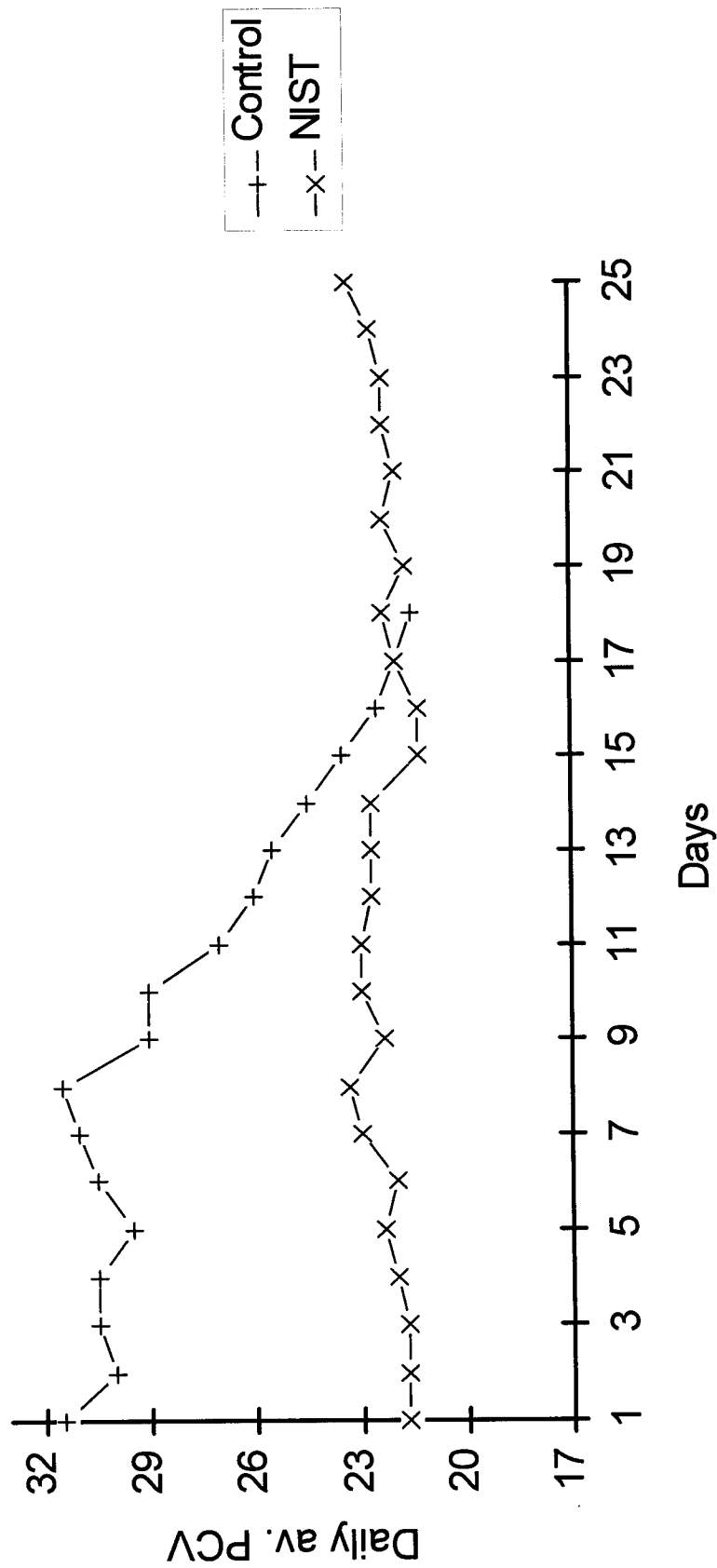


Fig. 23. Mean daily PCV of steers immunised with non-irradiated *Theileria parva* stabilate with concurrent tetracycline treatment (NIST), after challenge with a lethal homologous stock.

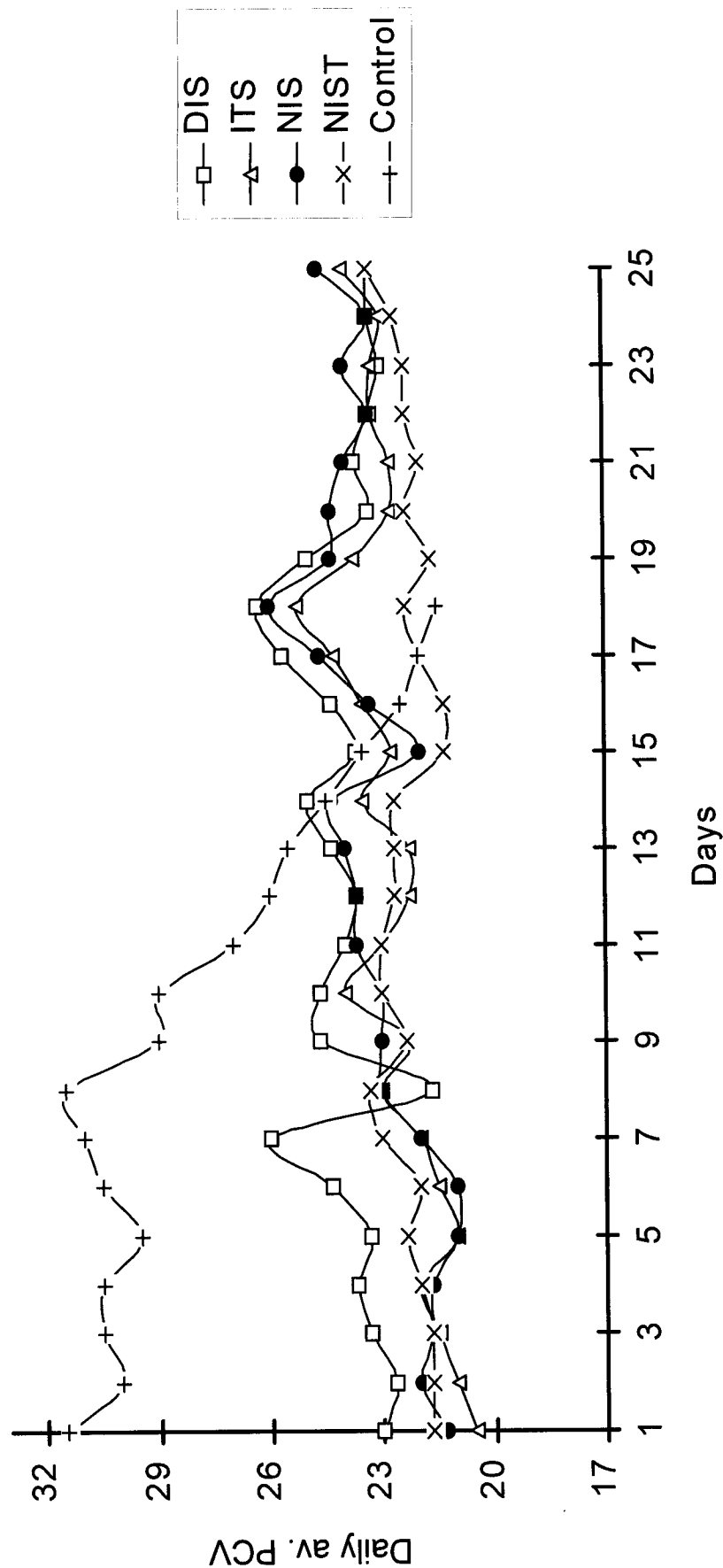


Fig. 24. Summary of the mean daily PCV of steers immunised with directly irradiated *Theileria parva* stabilates (DIS); irradiated *Rhipicephalus appendiculatus* ticks stabilate (ITS); non-irradiated stabilate (NIS); non-irradiated stabilate with tetracycline treatment (NIST)

## **4.4 Serology**

The mean period to seroconversion, measured by the Indirect Fluorescent Antibody Test (IFAT), for three groups of steers infected with directly irradiated stabilates (DIS); irradiated 'tick' stabilate (ITS) and non-irradiated stabilate with tetracycline treatment (NIST) was three weeks post-inoculation of the initial challenge. The group which was given non-irradiated stabilate (NIS) seroconverted only seven days after challenge with lethal dose of homologous stock.

## **4.5 Parasitaemia**

### **4.5.1 Schizonts**

The median period to schizonts detection following initial immunisation for the groups of steers used in the stabilate production (SP) and viability of stabilate test (VS) was ten days. Maximum schizont indices (MSI) of 30.3% and 13% were observed in lymph node biopsy smears of the SP and VS groups respectively.

No schizonts were detected in the lymph node biopsy smears of cattle in the groups DIS and NIST.

For the groups immunised with irradiated 'tick' stabilate (ITS) and non-irradiated stabilate (NIS), the median period to schizont detection following initial immunisation was 14 days. Maximum schizont indices of 7.4% and 9.7% were observed in these groups respectively.

Following challenge with double doses of homologous stock, no schizonts were seen in the lymph node biopsy smears of all the immunised groups.

The mean period to the detection of schizonts in lymph node biopsy smears of the control group was eight days post inoculation. Maximum schizont index of 50% was observed in this group following inoculation of double doses of stabilate.

#### **4.5.2 Piroplasms**

The median period to piroplasm detection in blood smears of steers was shortest in the secondary challenge control group (10 days), followed by VS (12 days); SP (13 days) and then NIS and ITS (19 days) post immunisation (table 4). Maximum piroplasm indices of 38%, 28.3%, 0.7%, 3.7% and 5.2%, respectively, were observed in the blood smears.

No piroplasms were seen in the blood smears prepared from the steers in the NIST as well as in the DIS group following initial immunisation and challenge.

Table 4. Summary of parameters used to measure the response of cattle to various treatments of *Theileria parva* stabilates.

Experiment	No. of cattle used	1st fever (median)	Day fever (median)	Days fever (median)	1st schizonts (median)	Day schizonts (median)	Max. MSI (%)	1st Piroplasm (median)	day Piroplasm (median)	Max. piros (%)	Reaction score	Days to Death (median)
SP	3	11		7	10		30.3	13		28.3	++++	21
VS	1	15		1*	10		13	12		0.7	++	0
DIS	3	0		0	0		0	0		0	+	0
ITS	3	14		2	14		7.4	19		5.2	++	0
NIS	3	14		6	14		9.7	19		3.7	+++	0
NIST	4	0		0	0		0	0		0	+	0
Control	2	8		11	8		50	10		38	++++	19

Key: MSI - schizont index; SP - steers infected with *Theileria parva* for the purpose of stabilate production; VS - Steer used to test the viability of the stabilate produced in this trial; DIS - steers infected with directly irradiated *T. parva* stabilate; ITS - steers infected with stabilate produced

from irradiated ticks; NIS - steers infected with non irradiated stablate; NIST - steers infected with non irradiated stablate and were also treated with tetracycline. \* Single steer treated with buparvaquone on day 18 (i.e. the course of experimental theileriosis was terminated prematurely)

Reaction score:-

- + = no reaction (No parasites are detected; no clinical signs are apparent)
- ++ = mild reaction (no fever or fever less than four days; the animal is otherwise clinically normal and recovers)
- +++ = moderate reaction (schizonts are detected; fever between 4 and 9 days; animal shows mild transient clinical signs and recovers)
- ++++ = severe reaction (schizonts are detected, fever over 9 days; obvious signs of ECF and animal may recover or die)



#### 4.7 Clinical Signs

Based on the severity of the steers' response to the infection, the reactions were classified as none, mild, moderate and severe (table 4). According to this classification, the animals which reacted most severely were those used in the stabilate production as well as the controls. Out of the immunised groups, the ones immunised with non-irradiated stabilate reacted moderately while those immunised with stabilate produced from irradiated *R. appendiculatus* ticks reacted mildly. There was no reaction from the group immunised with directly irradiated stabilate (DIS) as well as the group immunised with non-irradiated stabilate with concurrent tetracycline treatment (NIST).

## CHAPTER FIVE

### DISCUSSION

The results of this study showed that irradiation of the Katete strain of *Theileria parva* sporozoites either directly or indirectly (in their tick vectors) at doses of 8.4 krads (0.084 kGy) and 20 krads (0.2 kGy) respectively retained sufficient immunogenic properties to elicit solid immunity against homologous challenge in immunised cattle. *T. parva* sporozoites directly irradiated in stablate form had their virulence reduced to levels that could be used for immunisation. This method was found to be suitable for preparing an attenuated 'vaccine' against malignant theileriosis and could be used as a substitute to tetracycline treatment in the exercise. However, at 20 krads, the indirectly irradiated *T. parva* sporozoites were still virulent enough to cause a mild disease when used for immunisation without concurrent tetracycline treatment.

While infection and treatment is currently one of the most popular methods of malignant theileriosis control, immunisation of cattle using irradiated *T. parva* sporozoites has not been employed in Zambia or elsewhere. This has partly been due to the difficulty in defining the safety margin of irradiated *T. parva* parasites for 'vaccine' production (Cunningham *et al.*, 1973a and b, and Purnell *et al.*, 1974). Secondly, the effect of irradiation on the *T. parva* sporozoites has not been well understood.

The results obtained in this study confirm previous findings by Cunningham *et al.* (1973a and b), ILRAD (1990), Mugeru *et al.* (1973) and Purnell *et al.* (1972a, b and 1974) that irradiation of *T. parva* sporozoites either directly or in their arthropod vectors reduces the virulence of the parasites. All steers immunised with different

treatments of stabilates (directly irradiated (DIS); irradiated ticks (ITS); non irradiated (NIS); non-irradiated stabilates with concurrent tetracycline treatment (NIST) survived the initial immunisation and became solidly immune to subsequent homologous challenge with *T. parva*.

The aim of any immunisation protocol is to elicit a protective immune response in the vaccinated animal. A vaccine for use in animals or in man, will be assessed by its efficacy and safety. These are the two main characteristics of importance for the manufacturer as well as for the vaccinated individual. The safety issue is of major importance for attenuated vaccine preparations, especially since attenuated vaccines have proven to be of high efficacy for a number of diseases compared to inactivated or killed preparations of the same antigen.

One important tool for assessment of vaccine efficacy is to monitor the response in the vaccinated animal. In this study it was shown that the daily average temperature of steers in the NIS, SP and VS exhibited a pattern typical of theileriosis. This was also true for the secondary challenge control group (fig. 24). However, the vaccinated animals showed no signs of clinical infection and it was these findings that were interpreted as being typical of a subclinical theileriosis. This was based on the observation that the packed cell volume (PCV) of all groups dropped by between 1 and 3% in all the treatment groups during immunisation. This shows that *T. parva* had a negative effect on the PCV of cattle. This is in line with the findings of Mbassa *et al.* (1994) and Shatry *et al.* (1981) that some species of *T. parva* cause mild anaemia. However, after challenge, the PCV remained at a low value. Further, some schizonts and piroplasms were respectively demonstrated in the lymph node biopsy and blood smears of the ITS group of animals. This indicated a mild disease in this group.

Despite the differences between groups concerning schizont/piroplasm counts, no statistically significant difference ( $P>0.05$ ) in the daily average temperatures between ITS, DIS and NIST were found.

The infectivity of the stabilate that was used for immunisation was confirmed by the observation that schizonts and piroplasms were respectively demonstrated in the lymph node biopsy and blood smears of the single steer used for testing the viability of the *T. parva* stabilate. This steer was treated with buparvaquone on day 18 post-infection. The early demonstration of schizonts (10th day post-infection) followed by piroplasms (12th day post-infection) in the lymph node biopsy and blood smears respectively, as well as the appearance of fever, was confirmatory of the viability of the stabilate.

The results obtained from the ITS group confirmed previous findings by Mugeru *et al.* (1973) and Munyua *et al.* (1973) that irradiation of *T. parva* sporozoites in their tick vectors at 20 krads reduces the virulence of *T. parva* sporozoites. Mugeru *et al.* (1973) and Munyua *et al.* (1973) irradiated *T. parva* parasites at doses of 20 krads and 30 krads in their *Rhipicephalus appendiculatus* tick vectors and used these ticks to successfully immunise experimental cattle against theileriosis. The immunised cattle were able to withstand a ten infected tick challenge.

In addition to the findings of Mugeru *et al.* (1973) and Munyua *et al.* (1973), this study has further shown that *T. parva* parasites irradiated at 20 krads in their tick vectors remained sufficiently virulent to induce a mild disease and to raise a protective immune response. In the case of Mugeru *et al.* (1973) and Munyua *et al.* (1973), immunisation using irradiated *T. parva* infected *R. appendiculatus* ticks was successful because the effect of irradiation on the parasites was further regulated by

the slow inoculation of the parasites into the host animals over several days (Purnell *et al.*, 1973a; Young, 1977 and Young *et al.*, 1975). This low grade inoculation of parasites over a long period of time gave the cattle time to mount an effective immune response without any clinical symptoms being observed. This is especially so because severity of *T. parva* infections is dose dependent (Cunningham *et al.*, 1970 and Irvin and Cunningham, 1981).

This study has also shown that the steers belonging to the NIS group recovered after going through a moderate theileriosis reaction. The dosage used in this group was half that of the control group.

There are, however, additional factors that should be considered when evaluating the safety of the stabilate vaccine preparations, including the concentration of parasites subjected to various doses of irradiation and the viability or virulence of the *Theileria* species.

In previous studies where low and high concentrations of parasites were subjected to different doses of irradiation, it had been shown that the dosage of irradiation required to reduce the virulence of *T. parva* parasites increased directly with an increased concentration of sporozoites (Cunningham *et al.*, 1973a and b). Some degree of attenuation or inactivation of parasites was reached in both cases but the low safety margin within which this could be achieved deterred these workers from doing further work using this method.

With regard to virulence differences between strains, different *T. parva* stocks have been observed to have varying biological characteristics and respond differently to stabilate treatments. *Theileria parva* (Boleni) has low pathogenicity and has cross-protection properties against *T. parva* (Muguga) (Hove *et al.*, 1995), although the

latter is very virulent and pathogenic (Brocklesby, 1962 and Brocklesby *et al.*, 1961 ). When subjected to various doses of irradiation, *T. parva* (Muguga) expressed some degree of attenuation although with unpredictable results in certain instances (Cunningham *et al.*, 1973 a and b).

In Zimbabwe, Irvin *et al.*, (1989) studied some of the biological characteristics on the Boleni stock of *T. parva*. The stock appeared to induce protection against a wide spectrum of *Theileria* stocks. These characteristics were later used to produce a vaccine against *T. parva* (Boleni) which was used without concomitant tetracycline chemotherapy (Kanhai *et al.*, 1997). Like the Muguga cocktail, *T. parva* (Katete) is also virulent and is not suitable for immunisation without concomitant tetracycline therapy. Although dilutions as low as 0.03 ticks per ml have been used successfully for immunisation under experimental conditions (Berkvens, personal communication), this was not done under field conditions because of the possibility of losing some immunogenic properties, thus the threat of reducing the number of cattle protected by the vaccine. However, subjecting concentrated stabilates of *T. parva* (Katete) to irradiation seems to reduce their virulence to levels that can be used for immunisation without concomitant tetracycline treatment. What can be concluded from these observations, is that titrating stabilates has to be standardized for each *T. parva* strain. Results obtained for one strain can not be extrapolated to others. This also makes any comparison on the safety issue between studies difficult, and should at least be performed cautiously.

In the present study, the best immunisation results came from groups infected with directly irradiated sporozoites (DIS) as well as the non-irradiated sporozoites with concurrent tetracycline treatment (NIST). The results for these two groups were

similar in all respects. The groups encountered no febrile reaction, seroconverted within the first three weeks and withstood lethal homologous challenge. No schizonts nor piroplasms were detected in the lymph node biopsy and blood smears. This shows that using directly irradiated *T. parva* (Katete) sporozoites at a dosage of 8.4 krad to immunise cattle is as good as the infection and treatment method using tetracycline.

The challenge in the stabilate production is to adjust the dose used to attenuate the parasite so that sufficient viability or potency is retained. Sufficient in this sense meaning the ability to induce a protective immune response in the vaccinated animal. The mechanisms involved are not well understood, but in the case of stabilate “vaccination”, sporozoites are introduced by one single injection thereby creating a clonal division of lymphocyte-infected with schizonts. This clonal division is important for widespread dissemination of infected host cells throughout the body of the host (DeMartini and Moulton, 1973), which again will result in a strong immune response. The potency of a *T. parva* vaccine produced by irradiation depends on many factors such as the infection rate of *T. parva* sporozoites in ticks and/or the concentration of parasites in the stabilate (dilution); the dosage of irradiation; and the virulence and pathogenicity of the strain of parasites.

The dosage of irradiation that was used in this study was chosen on the basis of a compilation of the average responses of cattle per dosage of irradiation used by Cunningham *et al.* (1973a and b), irrespective of the number of infectious particles (concentration). The best response was found to have been at about 8 krad ( this was the dosage at which most animals did not succumb to immunisation and at the same time, resisted the challenge). The dosage of irradiation used as well as the difference

in the stock of parasites (Katete stock) could be attributed to the success of attenuating *T. parva* parasites.

The cost of the infection and treatment immunisation varies depending on the place, country, time, quantities and cost components involved (Mukhebi *et al.*, 1990). Generally, in the first year of immunisation the cost of tetracycline is very high because of the weight of cattle that have to be immunised. This results from the need to immunise both adult cattle as well as calves at the start of the program. However, these costs reduce in the subsequent years as only calves whose average weight is low have to be immunised. Radley (1981) estimated the cost of immunising one animal with *T. parva* vaccine to be equal to US\$ 2.51 while Irvin (1984) estimated that in Malawi it would cost between US\$ 4.00 and US\$ 5.00. In both these cases, the cost of tetracycline was estimated to range between 97.5% and 99.6% of the total cost per single immunisation. In Burundi, Kiltz (1984) estimated the cost of immunising one animal to be equal to US\$ 20.00. Mukhebi *et al.* (1990) identified, quantified and cost the major components in the infection and treatment exercise based on a hypothetical ECF immunisation in Kenya. They estimated the cost of the infection and treatment exercise to range between US \$ 2.37 and 2.97 (Mukhebi *et al.*, 1990), with about 60% of the cost being due to transport costs. The cost of tetracycline was estimated to be less than 1%.

In Zambia, the cost of infection and treatment vaccination for an animal weighing 50 kg was estimated at US\$ 10.87 (Elyn, personal communication, 1997). This cost was calculated on the basis of the production of 40,000 doses and immunising 6,000 animals annually. Like Mukhebi *et al.* (1990), the cost of the



exercise included capital and vaccine delivery costs (approximately 66%). Stabilate production accounted for 27% and tetracycline (Terramycin® LA) 7%.

In real terms, vaccinating a calf weighing about 50kg with the infection and treatment method requires 5 ml of terramycin® LA (1ml/10Kg body weight) while an adult cow weighing 400kg requires about 40ml. The cost of terramycin® LA per ml is \$0.08. Thus the cost of terramycin® LA required to immunise a calf and cow respectively would be \$0.40 and \$2.40.

In contrast, the cost of irradiating *T. parva* sporozoites directly or through their tick vectors varies inversely with volume and time. The larger the volume of stabilate to be irradiated, the cheaper it becomes.

In Zambia, there is a multipurpose gamma irradiation source owned by the NCSR. This is the irradiation source which was used in this study. Use of this facility which is currently underutilized would be cheaper than purchasing one, if it is assumed that all stabilates required to immunise cattle for one year are irradiated at the same time.

A partial budget analysis, according to the method of Putt *et al.* (1987) was used to estimate the cost of irradiation. Based on the assumption that 100,000 doses of stabilate are irradiated once every year and that a corresponding number of animals are immunised, the cost of irradiating one dose of stabilate was estimated to be \$0.012 (own calculations, see appendix 5). Therefore, for every calf (50 kg) immunised, a farmer would save about \$0.39, and \$2.39 per adult animal weighing 400 kg. Thus if 100,000 calves and adults are immunised using irradiated stabilate, the farmers would save between \$38,800 and \$239,800 per annum. The benefits would be much higher if

heavier calves or adult animals are immunised as this requires more usage of terramycin®.

## CHAPTER SIX

### CONCLUSION

The following conclusions can be drawn from this study:-

a) Irradiated *Theileria parva* (Katete) sporozoites both directly and indirectly (in their tick vectors) are immunogenic enough to induce immunity in immunised cattle against homologous challenge.

b) Attenuation of *T. parva* (Katete) parasites by direct irradiation of the stabilates at 8.4 krad is suitable for vaccine production. This method could be used as a substitute for the use of tetracycline in the infection and treatment exercise if the attenuation was found to be consistent.

c) Indirect irradiation of *T. parva* sporozoites (diluted to 0.1 ticks per ml with an infection rate of 56.5%) at 20 krad reduces the virulence of the resulting stabilate. However, this stabilate is virulent enough to cause disease in immunised animals if not used with concurrent tetracycline therapy.

#### Future work

a) To compare *T. parva* strains in Zambia in terms of virulence, pathogenicity and cross-immunity to ascertain which ones have the broadest cross-protection properties.

b) To define the infection rates and dilutions of *T. parva* stabilates that will be attenuated at particular doses i.e. to define the safety margins of irradiated *T. parva* stabilates for use in immunisation.

- c) To define the dosage at which irradiation of *T. parva* sporozoites in their tick vectors can reduce the virulence to levels that can be used for immunisation of cattle.
- d) To do a comprehensive study on the cost per unit of irradiation in Zambia.

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## APPENDICES

### Appendix 1

Table 1.1 Duration of developmental stages of *Rhipicephalus appendiculatus* ticks maintained in the laboratory.

Developmental stage	Developmental period (range in days)
Adult pre-oviposition	5-11
Pre-eclosion (incubation)	24-35
Larval pre-feeding*	3-6
Larval pre-attachment	1
Larval pre-moulting	10-13
Nymphal pre-feeding*	5-8
Nymphal pre-attachment	1
Nymphal pre-moulting	11-16
Adult pre-feeding*	5
Adult pre-attachment	1-4

\* Immatures fed on rabbits

♥ Intermediate stage of ticks observed to be active when stimulated

- Adult stage fed on rabbits in order to mature *T. parva* sporozoites

## Appendix 2

Table 2.1 Useful parameters on the infection rate of *Theileria parva* in *Rhipicephalus appendiculatus* ticks used in this study.

Item	Estimate
Infected male <i>R. appendiculatus</i> ticks	7
Infected female <i>R. appendiculatus</i>	106
Male <i>R. appendiculatus</i> mean infected acini/ infected tick	6.5
Female <i>R. appendiculatus</i> mean infected acini/ infected tick	8.5
Total number of ticks dissected	200
Ratio of infected male to female ticks	1:14
Infection rate (Infected ticks/total ticks examined) (%)	56.

Appendix 3

Table 3.1 Serological profiles of cattle following immunisation with different treatments of *Theileria parva* stabilates. The indirect fluorescent antibody test was used.

Stabilate treatment	Tag No.	Week									
		0	1	2	3	4*	5	6	7	8	9
DIS	115	-	+/-	+/-	+/-	+/-	+	+	+	+	+
DIS	178	-	+/-	+/-	+/-	+/-	+	+	+	+	+
DIS	212	-	+/-	+	+/-	+	+	+	+	++	++
ITS	166	-	+/-	+	+/-	+	++	++	++	++	++
ITS	182	-	+/-	+	+	+	++	+	+	+	+
ITS	305	-	-	+/-	++	++	++	++	++	++	+
ITS	117	-	+/-	+/-	+/-	+	++	+	+	+	+
NIS	119	-	+/-	+	+/-	+/-	+	+	+	+	+
NIS	104	-	-	+/-	+/-	+/-	+/-	+	+	+	+
NIS	185	-	+/-	+	+	+	++	+	+	+	++
NIT	113	-	+/-	+	+/-	+/-	++	+	+	+	+
NIT	121	-	+/-	+	++	+	+	+	+	+	++

Stabilate treatment	Tag No.	Week										
		0	1	2	3	4*	5	6	7	8	9	
NIT	160	-	-	+/-	+/-	+/-	+	+	+	+	++	
Control	431	-	-	-	+							
Control	432	-	-	-	-							

**Key**

- ... No fluorescence seen
- +/- ... Doubtful
- + ... Positive
- ++ ... Very positive
- \* ... Secondary challenge of steers with homologous stock of *Theileria parva*.
- DIS ... Directly irradiated *Theileria parva* stabilate
- ITS ... Stabilate produced from irradiated *Rhipicephalus appendiculatus* ticks.
- NIS ... Non-irradiated *Theileria parva* stabilate
- NIT ... Non-irradiated stabilate with concurrent tetracycline therapy

The base line titre was found to be 1/80. Titres 1/80 and below were considered positive.



## APPENDIX 4

**Partial analysis of the cost of irradiating 100,000 doses of *T. parva* stabilates using the multipurpose 60 Cobalt source viz-a-viz the cost of tetracycline in the infection and treatment immunisation** (Method according to Putt *et al.*, 1987)

In partial analysis, the costs and benefits of the situation *with* the change is compared to that *without* the change and are itemised under the following headings:

<u>Costs</u>	<u>Benefits</u>
Extra costs incurred	Costs saved
Revenue foregone	Revenue gained

The extra costs of using irradiation to reduce the virulence of *T. parva* sporozoites in the immunisation against theileriosis were compared with the costs of using tetracycline (terramycin®) on an annual basis.

### Assumptions

- a) The multipurpose gamma irradiation source used to irradiate the *T. parva* sporozoites is hired from NCSR for one day only. During other days, it is used for other purposes.
- b) One hundred thousand doses of stabilates are irradiated and used to immunise a corresponding number of animals for that year.
- c) The life span of the irradiator is five years.

**Capital costs:**

Cost of irradiator \$450,000.00

Life span of irradiator (if used daily) 5 years

$$\text{Therefore, Depreciation} = \frac{\text{RC} - \text{SV}}{n}$$

Where:

SV = Salvage Value (assumed to be equal to 0)

RC = Replacement cost

n = Life span of irradiator.

$$\begin{aligned} \text{Depreciation} &= \frac{\$450,000}{5} = \$90,000. \\ &= \frac{\$90,000}{365} = \$246.56 \end{aligned}$$

$$\text{Total depreciation per dose of stabilate} = \frac{\$246.56}{100,000} = \$0.002$$

**Other costs**

$$(\text{Labour, docimetry etc}) = \$0.01$$

Total extra cost per dose of stabilate

Capital costs \$0.002

Other costs + \$0.01

\$0.012

Benefits of irradiation	Cost of irradiation
extra benefits	extra costs
0	0.012
costs saved	benefits lost
Tetracycline	0
Calf (50 kg) \$0.39	
Adult (400 kg) \$2.39	

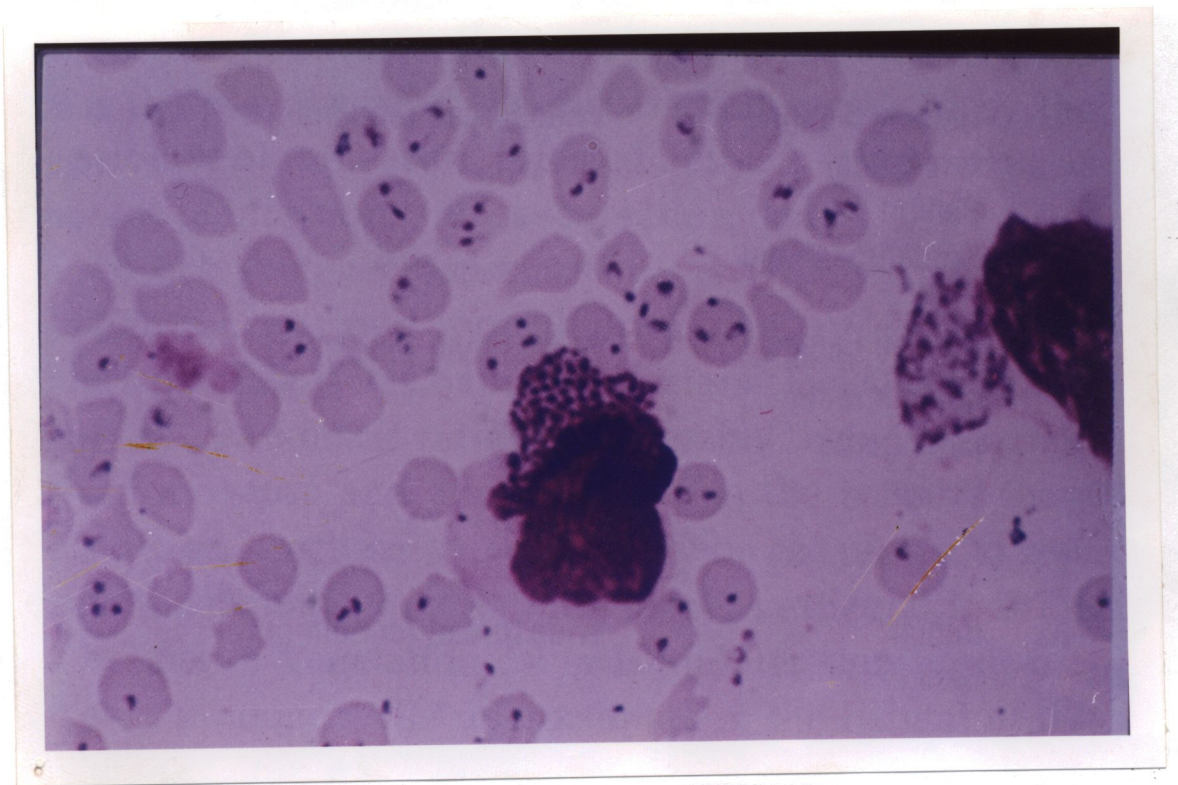
The total cost saved by irradiating *Theileria parva* stabilates instead of using tetracycline (infection and treatment) is estimated to be between \$0.39 and \$2.39 per animal.

## APPENDIX 5

## MISCELLANEOUS PHOTOGRAPHS

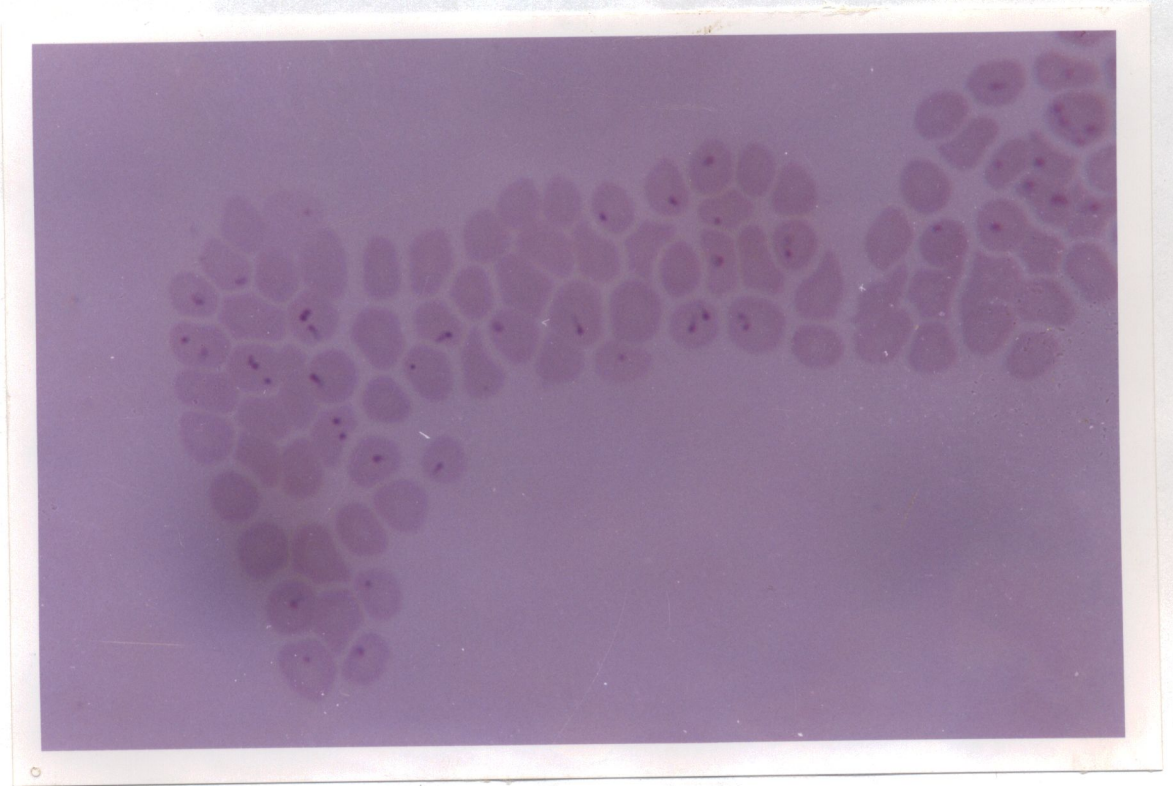


5.1 Rabbit with cloth earbags containing *Rhipicephalus appendiculatus* ticks



5.2 Lymph node biopsy smear showing schizonts of *Theileria parva* in a lymphocyte.





5.3 Blood smear showing piroplasms of *Theileria parva*.



5.4 Multipurpose gamma irradiator (60 Cobalt Source).

