Toxicological assessment of a tsetse fly repellent (Patent No.Ke 00185, 2004) on the health of exposed animals.

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

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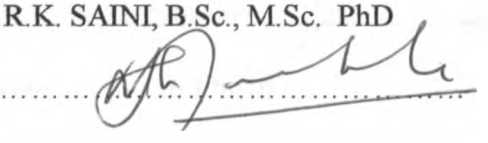

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DEDICATION

This work is dedicated to my loving husband Henry, and our children Mishael, Christa and Earl.

ABSTRACT

Tsetse and trypanosomosis have been extensively studied and an array of vector and disease control measures have been developed and applied with varying degrees of success. Current trypanosomosis control strategies rely on use of trypanocidal drugs, breeding of trypanotolerant cattle and tsetse control or eradication. Since none of these control options has proved to be a fully viable solution to the control of trypanosomosis, search for more tsetse fly and trypanosomosis control measures continues.

A synthetic tsetse fly repellent (Patent No. Ke 00185, 2004; Saini and Hassanali), was identified in 1999 at the International Center for Insect Physiology and Ecology (ICIPE). The repellent is based on the potent phenolic analogue of a mild natural repellent of Savannah tsetse fly species. This single- component synthetic compound was discovered from a structure-activity study of the phenolic constituents of body odors of tsetse bovid hosts and their aged urine. Preliminary field trials undertaken among the pastoralist community in Nguruman Kenya, showed that the repellent caused more than 80% reduction in tsetse challenge and reduced the feeding efficiency of the tsetse fly on cattle by over 90%. This research technology needs to be packaged, ready for release and transfer to livestock farmers in tsetse fly infested areas.

No chemical agent is entirely safe and likewise no chemical agent should be considered as being entirely harmful. This concept is based on the premise that any chemical can be permitted to come into contact with a biologic mechanism without producing an effect on that mechanism provided the concentration of the chemical is below the toxic level. This study was therefore undertaken to generate data on acute toxicity of the repellent in

laboratory mice and effects of prolonged exposure to vapours of the repellent in goats as a prelude to its potential utilization for tsetse fly control in livestock. Data obtained from the acute toxicity studies in mice may be extrapolated to reflect the toxicity of the repellent to domestic animals and humans. In another experiment, rabbits were used to determine the ocular and dermal irritant effects of the repellent. Haematological and biochemical effects of the repellent were determined in goats exposed to low -2mg/hr and high doses -4mg/hr of the vapours of the repellent through repellent collars for nine months. After the nine months of exposure to the repellent, two goats from the low dose, high dose and the control group each were sacrificed and the chronic pathologic effects of the repellent determined using standard histopathology techniques.

The 24 hours intraperitoneal (i.p) median lethal dose (LD_{50}) of the repellent in mice was found to be 40.2mg/kg body weight. The clinical signs of acute toxicity were decreased locomotor activity, an initial increase followed by a decrease in respiratory rate and an increased depth in respiration, terminal convulsions, coma and death. Death was probably due to depression and paralysis of the respiratory center in the brain. The repellent caused mild dermal irritation characterized by edema and erythema. There was moderate eye irritation affecting the cornea, iris and conjunctiva. Plasma levels of gamma glutamyl transpeptidase (GGT), alkaline phosphatase (ALP) and aspartate aminotransferase (AST) were monitored monthly for nine months. There were no significant differences between the treated and the control animals in the plasma levels of the enzymes over the experimental period ($p > 0.05$). For hematological parameters, the monthly means in all the groups for the RBC count were within the normal range

for the species. There were also no significant differences between the means of the three groups ($p>0.05$). There was variation in the mean monthly values of the granulocyte and lymphocyte count that was not associated with the treatment since the trend was similar in both the treated and the control groups. The monthly group means for the WBC count were above the reference values for the species in all the groups including the pre-treatment values. There were no significant differences in the monthly means of the WBC count between the treated and the control group ($p>0.05$). Similarly, there was no significant difference in mean body weight between the treated and the control groups of the goats ($p> 0.05$). Histological sections prepared from the lungs, liver, kidney and heart tissues showed normal cellular and organ structure except for mild lymphocyte infiltration in the lung tissue in all the groups.

According to the standard scheme for classifying relative toxicities, the LD_{50} in mice indicates that the synthetic repellent is highly toxic, manifesting central nervous system (CNS) effects. Ingestion of the repellent should, therefore, be avoided. The repellent is a mild skin irritant and causes eye irritation. However, the vapours of the repellent caused no effects on haematological and biochemical parameters and general health of exposed goats over the experimental period. Although the reactions in mice, rabbits and goats may not necessarily be the same as in humans, from the results, it is recommended that the tsetse fly repellent should be handled properly to avoid skin and eye contacts of the animal and the handlers. Clear information on the toxicity should be availed to the animal handlers. Any accidental contact would be treated symptomatically.

CHAPTER ONE
INTRODUCTION

Trypanosomosis is one of the major disease constraints to livestock production in sub-saharan Africa. It is a disease of humans and animals, caused by haemoparasites of the genus Trypanosoma. In Africa, the important trypanosome species affecting domestic ruminants are transmitted by tsetse flies (Leak, 1996). Trypanosomosis is prevalent in Africa between latitudes 15^0N and 21^0S representing a surface area of approximately 10 million-km^2. (Ford and Katondo, 1973; Leak, 1996). Thirty-seven sub-Saharan countries are affected by the disease, with an estimated 160 million cattle and 260 million sheep and goats being at risk of trypanosomosis (Leak, 1996).

Trypanosomosis is more important in the sub-humid than in the wetter parts of the semi-arid zones of Africa (Leak, 1998). The incidence and severity of the disease in different regions are dependent upon local conditions. In some areas virtually no economical livestock development is achievable due to the disease. Some livestock breeds such as Ndama are, however, known to be tolerant to the disease. Trypanosoma-susceptible livestock are reared in endemic areas through the support of curative and prophylactic trypanocides as the main disease control option (Ford and Katondo, 1973).

Budd (1999), estimated the total losses to agricultural production alone due to trypanosomosis in Africa to be $4.5 billion per year. In addition to direct losses due to the disease and the financial requirements for control operations, animal trypanosomosis also indirectly affects human health through protein deficiency caused by the shortage of meat and milk. The disease also causes a decrease in livestock productivity since it

prevents the introduction of improved breeds and causes overstocking in tsetse free areas (Ford and Katondo, 1973).

The impact of trypanosomosis on African agriculture is most obviously felt at herd level as reduced milk and live animal offtake and reduced work efficiency of oxen used for cultivation (Swallow, 2000; Erkelens *et al.*, 2000). In susceptible cattle breeds, the disease reduces calving by upto 20% and causes deaths of 20% of young stock. Meat and milk offtake is reduced by at least 50% (Swallow, 2000). For example trypanosomosis reduces by 33% the availability and efficiency of draught animals used for preparing land for crop production in Ethiopia (FAO, 1998). In mixed farming systems where trypanosomosis is severe, it constrains the number of oxen that farmers own and reduces the average area planted per household by as much as 50% (Swallow, 1997).

Current control options of trypanosomosis are based on three principal strategies viz.; Use of trypanocidal drugs, rearing of trypanotolerant cattle, and tsetse fly control or eradication. Each of these strategies has advantages and disadvantages, but generally none has proven to be fully satisfactory as viable and sustainable solutions to trypanosomosis control. Presently, the use of trypanocidal drugs is the primary approach to the control of animal trypanosomes throughout most of Africa (Stevenson *et al.*, 2000). However, cost, availability and growing drug resistance limit its adoption as a sustainable method to prevent or treat the disease. Trypanotolerant cattle are only found in certain areas of West and Central Africa and at the Kenyan coast and, although they retain a

certain level of productivity under tsetse challenge conditions, they are less productive in terms of meat and milk.

Tsetse fly vector control methods relying on large-scale bush clearing and aerial spraying have largely been discontinued due to environmental concerns. The use of baits both olfactory and visual (traps and insect impregnated targets) are currently the most common methods of tsetse control. Stationary odor-baited traps or pesticide-treated targets can be placed throughout a geographic area (based on tsetse distribution and human/livestock movements) to increase tsetse mortality and reduce prevalence of trypanosomosis. Traps and targets appear to be the most efficacious in reducing prevalence, but benefits are a pure public good; there are no private benefits in the form of individual animals protected nor are there benefits from reducing other biting fly and/or tick diseases. Parallel difficulties in initiating and sustaining the necessary collective action at the community level have greatly limited their impact.

Recent surveys conducted by the International Livestock Research Institute and its partners have shown a high proportion of resistant trypanosomes in areas with high trypanosomosis risk and trypanocidal drug use (McDermott & Coleman, 2001).Pyrethroids, insecticidal pour-ons and sprays applied to individual animals protect the individual animal from trypanosomosis and from other biting fly/tick diseases, and also have a relatively large impact on tsetse mortality and thus on overall prevalence of the disease. The disadvantages include, the relatively high costs of pour-ons needed to treat animals, and potential free-riding behavior due to the positive externalities in use.

To date, most community-based traps/targets projects still operational are those supported by external agents (governments, donors, Non-Governmental Organizations) with varying degrees of community participation, while the distribution of pour-ons has been supported by projects or paid for by individual rich farmers.

Recent research at the International Center for Insect Physiology and Ecology (ICIPE) offers a promising alternative technology for tsetse control that would expand the options for trypanosomosis control, and reduce dependence on trypanocides and the environmentally hazardous acaricides. The technology is based on the potent phenolic analogue of a mild natural repellent of Savannah tsetse species, *G.pallidipes* and *G. morsitans morsitans* (Patent No. Ke 00185, 2004; Saini and Hassanali 2004).

The active ingredient of the repellent has been derived by molecular optimisation studies to act as an olfactory antagonist of a key kairomone which flies use to locate their host for feeding. The repellent significantly reduces tsetse challenge (>80%) and feeding efficiency on cattle (>90%) (Saini and Hassanali 2004). The repellent technology could be used in conjunction with other tsetse and trypanosomosis control tactics. The new technology, if adopted may offer a cost effective tsetse fly bio-control strategy. However, before the technique is adopted for use, there is need to generate adequate data to support safe use of the product. Since every substance that comes into contact with the body has some potential for affecting the body at least at the site of contact this study was therefore undertaken to generate toxicological data of the repellent in exposed animals. The results of the study provides safety information on the

repellent for use in the control of tsetse flies. Toxicological study is a standard requirement for the registration of the product for use in animal husbandry.

Hypothesis: The synthetic tsetse fly repellent is safe for use in livestock.

The broad objective of the study was therefore to determine the toxic effects of the synthetic repellent.

The specific objectives of this project were: -

i. To evaluate the adverse effects of the repellent by carrying out acute toxicity studies including determination of signs of acute toxicity, median lethal dose, dermal and ocular irritation tests in laboratory animals.

ii. To determine the chronic toxic effects of exposure of the repellent on hematological and biochemical parameters in goats.

iii. To determine the effects of the repellent on weight gain in exposed goats.

iv. To investigate histopathological effects of the repellent in goats after nine months of continuous exposure.

CHAPTER TWO
REVIEW OF LITERATURE

2.1 The aetiology of trypanosomosis

Trypanosomosis is a vector borne protozoal disease caused by trypanosomes. Trypanosomes are flagellate protozoa belonging to the Class *zoomastigophorea* and Order *kinetoplastine*. Characteristically they possess a kinetoplast, a body of deeply staining nucleoprotein (DNA) situated near the base of the flagellum.

All trypanosomes capable of completing the life cycle in Glossina posses a kinetoplast. Some strains of *T. evansi* and *T. equinum* do not possess an obvious kinetoplast and are incapable of cyclical transmission in Glossina (Hoare, 1972).

The tsetse- transmitted trypanosomes of African mammals are elongate, usually slightly curved protozoan with a single nucleus. They are 10-35µm long and their organelle of locomotion is a single flagellum that originates near the posterior end of the body, extending forward but attached to the body of the trypanosome by a fold of the pellicle, the undulating membrane. Near the basal body of the flagellum is the dark-staining Kinetoplast (Jordan, 1986).

The diagnostic characters of the blood forms of the various species of mammalian trypanosoma are the size and shape of the body, the position of the nucleus and kinetoplast, and the length and form of the undulating membrane and flagellum as observed in stained preparations of the parasites (Jordan, 1986).

2.2 The Vectors of trypanosomes

Trypanosomes are transmitted from the infected to healthy animals by the *Glossina* species. Adult *Glossina* species are dull in appearance varying in colour from a light yellowish-brown to a dark blackish-brown. In some species the abdomen may have alternate darker and lighter bands. The smallest species is some 6-8 mm long and the largest 10-14 mm (Jordan, 1986). The tsetse can be classified in the order of *Diptera* (2 wings), family *Glossinidae* and within the genus *Glossina.* Within the genus, 23 species and 8 sub-species have been identified (Moloo, 1993).

Three subgenera can be differentiated using ecological characteristics. These subgenera are: -

- Morsitans group found in Savannahs of Africa
- Palpalis group found in riverine forest vegetation.
- Fusca group found in rain forests

The most important vectors of the Morsitans group that transmit disease to domestic ruminants are *G. morsitans* (South and East Africa), *G. pallidipes* (thicketed areas of savannahs*), G.m. submorsitans* (Savannahs of West Africa extending eastwards to Ethiopia), whilst *G. fuscipes* of the palpalis group is an important vector of human trypanosomes in West Africa, Southern Sudan and Uganda. The fusca group is less important for the transmission of trypanosomes to domestic livestock because the habitat of livestock and the habitat of the vectors hardly overlap, except for 2 species which occur outside rain forest areas: G. *brevipalpalis* and *G. longipennis* (Leak, 1996).

In Kenya, *G. pallidipes* has a wide geographic distribution where there are two major fly-belts. A western fly belt that includes Nguruman, Maasai Mara and Labwe Valley and an eastern fly-belt located in the coastal areas, Kibwezi and Meru (Van, 1982).

2.3 The ecology of tsetse flies

The trypanosomes are insect-borne and their epidemiology is determined by the ecology of their insect vectors. The genus *Glossina* occurs over some 11 million km^2 of Africa (Jordan, 1986). Its northern limit extends across the continent from Senegal in the West to Southern Somalia in the East. This limit is about 14^0 N but in Somalia it is only 4^0 N. The Northern limit corresponds closely to the Southern edges of the Sahara and Somali deserts. In the South West it varies between 10^0 and 20^0S, corresponding closely to the northern edges of the Kalahari and Namibian deserts, whereas in the Southeast it is generally at about 20^0S but extends as far as 29^0S along the East African Littoral (Bursell, 1960).

These limits are determined by climate, through its effect on vegetation. The flies can penetrate drier areas within the shelter of vegetation lining water sources. In Southeast Africa, where rainfall is high and there are no deserts, the limit of *Glossina* is related to seasonal low temperatures. This may affect the adult flies which are inactive below 16^0C and so lengthen the period spent in the soil by the pupa before development to the adult stage can be completed (Bursell, 1960).

2.4. The epidemiology of trypanosomosis

2.4.1. Interactions of vector and hosts

Habitats are composed of different mixtures of soil, water, air and vegetation, each with different thermal characteristics and often, different temperatures (Miller, 1994). Many arthropod vectors of parasites are sensitive to climate, which is generally expressed as temperature, humidity and rainfall (Hay *et al.*, 1996). The general distribution of tsetse flies is determined principally by climate and influenced by altitude, vegetation and the presence of suitable host animals. Each of these factors may directly affect the birth, death or migration rates of the vector and thus the population size (Hay *et al.*, 1996). For example, the most favorable temperature for *Glossina* is between 21 and 25°C for the adult stage while too high (> 35^0C) or too low temperatures (<14^0C) hinder puparia from completing their development (Glasgow, 1963). Generally, the limit of tsetse distribution is closely correlated to the tropical Savannah climate; it follows the 508 mm annual isohyets (Leak, 1998). Vegetation is important in providing shade and maintaining a suitable microclimate for the vector as well as a habitat for their vertebrate host (Leak, 1996). Vegetation types vary with climate and altitude. Vegetation profiles for tsetse habitats are directly related to the ecological zones where tsetse species are present and can roughly be described as woodlands, thicket woodlands, thicket or Savannah (Leak, 1998).

2.4.2. Transmission of trypanosomosis

In Africa, certain species of trypanosoma are transmitted from one vertebrate host to another by the *Glossina spp*. The normal vertebrate reservoir for the T*rypanosoma spp* are the wild large mammals (buffalo, warthog) of Africa, which, unless stressed,

generally do not suffer any deleterious effects from trypanosome infection. Some of these animals can harbour trypanosome infections for long periods of time, and act as reservoir hosts from which tsetse flies themselves become reservoirs of the parasite, as trypanosomes undergo continuing cycles of development within the fly (Jordan, 1986).

Once forms of trypanosomes infective to the vertebrate host have developed, the tsetse fly remains infective for the remainder of its life (Blood *et al*, 1989). Kleine (1909) noted that the pathogenic African trypanosomes undergo a definite cycle of development in the tsetse vector. He also noted that the circulation of trypanosomes between wild animals and tsetse fly is of no practical significance until man or his domestic animals, which are usually susceptible to infection by the trypanosomes, intrude into the cycle and become hosts of the parasite. Cyclical transmission is the commonest and the more important mode of trypanosome transmission than mechanical transmission that has been attributed to biting flies transmission of *T. evansi* in equines and camels (Jordan, 1986).

In cyclical transmission, a tsetse fly ingests trypanosomes in a blood meal from a mammal. The trypanosomes undergo a cycle of development in the insect. In the insect gut, only trypanomastigote forms occur and these transform into epimastigote forms in the mouthparts or salivary glands prior to their further transformation into metacyclic forms which are infective to the mammalian host. This cycle of development varies in duration according to the species of trypanosome, possibly the species of the tsetse fly, and temperature. *T. vivax* in *G. palpalis palpalis* takes some 12-13 days at 22 ^{0}C and 5

days at 29^{0}C (Desowitz and Fairbairn, 1955), *T. congolense* takes 23 days at 28^{0}C and 12 days at 30^{0}C (Fairbain and Culwick, 1950).

2.5 Pathogenesis of trypanosomosis

Trypanosomosis in all species is a progressive and usually fatal disease. The trypanosomes exert their effect by multiplying rapidly in the bloodstream, causing disseminated intravascular coagulation and then entering and blocking capillaries causing ischaemia and anaemia (Blood *et al*, 1989). *T. brucei* has the added capability of passing out of capillaries into the tissues and causing injury to all organs. It also passes through the placenta and into foetus in pregnant animals causing abortion and premature births (Ogwu *et al.*, 1986). A cerebral form of trypanosomosis occurs with mixed infections (Masake *et al.*, 1984), or as a relapse after an apparently successful treatment (Moulton, 1986 and Masake *et al.*, 1984). The latter is thought to be due to the protection of the trypanosomes against the chemotherapeutic agent by the blood-brain barrier (Moulton, 1986).

2.6 Control of trypanosomosis

Tsetse flies are widely distributed across tropical Africa, and are the primary vectors of trypanosomosis. Tsetse and trypanosomosis have been extensively studied and an array of vector and disease control measures have been developed and applied with varying degrees of success. Current control methods are based on three principal strategies namely:- use of trypanocidal drugs, breeding of trypanotorelant cattle and tsetse control or eradication. Each of these strategies has advantages and disadvantages, but generally

none has proven to be fully satisfactory as viable sustainable solutions to control of trypanosomosis.

2.6.1. Chemotherapy and chemophylaxis

This is a strategy aimed at reducing losses in animal husbandry due to trypanosomosis, or in making animal husbandry at all possible in regions where trypanosomosis is endemic (Blandl, 1988). The use of trypanocides is the most widespread strategy against trypanosomosis in most of Africa (Leach and Roberts, 1981; Stevenson *et al.*, 2000). However, only a small group of chemotherapeutic and chemoprophylactic compounds are currently in use (more than 50 years old) and new compounds are unlikely to become available in the near future (Peregrine, 1994).

Animals are given trypanocides for treatment or prevention. The drugs kill off the trypanosomes in the animal or interfere with their development.

Two different situations and objectives exist (Blandl, 1988).

I. Sporadic and exclusively curative use of trypanocide. This is the case in most regions of Africa where an attempt is made to reduce the losses with the use of trypanocides without being able to avoid the losses. It amounts to an average of one treatment per year for each head of cattle.

II. Regular prophylactic treatment with the objective of totally avoiding losses due to trypanosomosis. This is used in large- scale cattle enterprises and up to 6 treatments per animal per year are necessary. A precondition for regular blood tests of all conspicuously sick animals at 4 weeks intervals makes it only feasible

on well-run ranches or feedlot. This regime is impossible in small scale livestock enterprise, semi-nomadic or nomadic farming systems.

Trypanocides: Various trypanocides have been used in the treatment and prevention of trypanosomosis:-

1. Acid naphthylamines (Suramin^{R}) – This drug has gained much acceptance in treatment of camels infected with *T. evansi* and horses infected with *T. brucei*, *T. evansi* and *T. equiperdium*. Suramin is a competitive inhibitor of l-glycerol 3-phosphate oxidase. The drug is highly protein bound and this provides its prophylactic properties through its slow release and therefore prolonged action.

2. Ethidium (Homidium bromide)

Ethidium has been used extensively as a curative agent against *T.vivax* and *T. congolense* infections in animals since 1948 and parasites have developed resistance against the drug (Ford *et al.*, 1953). Various formulations exist in the market.

1. Samorin (Metamidium, isometamidium)

This is a hybrid product synthesized from berenil^{R} and ethidium in the prescence of sodium acetate. Samorin (isometamidium M and B 418 OA) is recommended for the treatment of trypanosomosis at a dose of 0.5mg/kg body weight for prophylaxis. The drug is remarkably useful in areas where drug resistance to antrycide or berenil has developed (Williamson, 1971). Samorin is active for between 2-4 months as a trypanocide and is used adequately for prophylaxis (Leach and Roberts,1981).

2. Antrycide, Antrycide dimethylsulphate and Antrycide prosalt.

The chloride form of Antrycide is readily soluble in water. It has been used as a trypanoprophylactic agent because of its slow release from the site of injection. Antrycide dimethylsulphate has been used against *T. simiae* in pigs and *T.evansi* in camels. Antrycide prosalt has prophylactic and curative values against *T. simiae*. It acts by inactivating cytoplasmic ribosomes and by inhibiting synthesis of RNA from exogenous purines.

3. Aromatic dimidines (Pentamidium and Berenil^{R})

Berenil is largely eliminated from kidneys within 24 hours of injection and considered primarily for curative purposes (Leach and Roberts, 1981). Opinions as to its duration of prophylaxis differ considerably (Cunningham *et al.*, 1964) and vary between 3-4 days (Gitatha and Maudlin, 1968) and 2-3 weeks (Cunningham *et al.*, 1964).

The widespread use of these trypanocides has led to the development of resistant strains of trypanosomes in many locations and cross-resistance has also been observed. This has been attributed partially to the close chemical relationship of several trypanocides (prothidium, homidium, dimidium, and isometamidium all derived from pyrithidium). Pyrithidiumbromide, homidium, and quinpyramin salts can no longer be used due to resistance (Leach and Roberts, 1981; Geerts and Holmes, 1998).

Whiteside (1960) developed the concept of the "sanitive pair of drugs" from his analysis of occuring cross-resistance by use of two substances between which no cross-resistance had been observed. At the time of Whitesides' analysis, two such pairings: Homidium and Berenil as well as Samorin and Berenil were found to be effective.

Current medical treatment relies on the use of the Berenil and Samorin pair (Blandl, 1988). However, resistance for these substances and cross-resistance has also been

reported (Whiteside, 1963; Jones-Davies, 1967a, b; 1968; Graber, 1968; Haase *et al.*, 1981; Williamson, 1970; Lewis and Thompson, 1974; Kupper and Wolters, 1983).

The profitability of the use of trypanocides is determined by the cost of the drugs and effective treatment and by the additional revenues due to the reduction of or prevention of productivity losses and mortality of animals. The cost varies greatly from location to location depending on the frequency of treatment and cost per treatment. Jahnke (1974) combined various frequencies of treatments with various densities of stock and establishes ranges for the cost of using trypanocides. According to his figures then, to totally avoid losses due to trypanosomosis, the cost for the use of trypanocides would amount to 50% of the family income of pastrolists in Uganda, if the cost of drug and application is taken into account.

2.6.2. Trypanotolerance

This is based on the observation that if different breeds of cattle are kept in areas with trypanosomosis challenge, they will show symptoms of disease and impairments of performance in varying degrees (Brandl, 1988). In particular, the *Bos taurus* cattle of West Africa, whose origin is traced back to the hamitic longhorn cattle and to shorthorn (Brachyerous), (Epstein, 1971), are reputedly less susceptible than the B*os indicus* (Zebu) introduced much later to Africa and the European *Bos taurus* breeds. The low susceptibility is defined as trypanotelerance. N'Dama, Baoule, Ghana Shorthorn, Somba and Muturu belong to the trypanotolerant breeds. On the other hand, the Zebus and European breeds of cattle are considered highly susceptible to trypanosomosis (Brandl, 1988).

The first observations on trypanotelerance go back to the beginning of the twentieth century (Pierre, 1906). In the mean time the trypanotolerance of the *Bos taurus* cattle of West Africa is considered assured (Stewart, 1951; Chandler, 1952, 1958, Desowitz, 1959, Roberts and Gray, 1973; Toure *et al*, 1978; Murray *et al.*, 1979 b, 1981, 1984; Sarrol *et al.*, 1981). A series of more recent surveys shows that differences in trypanosensitivity exist, not only between breeds (e.g. N'Dama compared to Zebu) but also between individuals of one and the same breed (Brandl, 1988). This means that trypanotolerant, as well as susceptible N'Damas, can be found, just as distinct tolerance was proved in Zebus (Roelants *et al.*, 1983). However, majority of works on breed comparisons show a higher percentage of trypanotolerant animals in the *Bos taurus* breeds of West Africa (Roelants *et al.*, 1983, Murray *et al.*, 1984). Whereas the immunity has a genetic basis (Murray *et al.*, 1979 a), the intensity of the tsetse challenge has a strong influence on the degree of tolerance (Brandl, 1988). However, productivity drops rapidly with tsetse intensity of infection and N'Damas also show common symptoms combined with a very bad general condition and high mortality rates (Godfrey *et al.*, 1960; Stephen, 1966; Roberts and Gray, 1973; Toure *et al.*, 1978; Murray *et al.*, 1979 a; Roelants *et al.*, 1983). Should the animal recover spontaneously or after treatment with a trypanocide, some consequential damages, such as long-term infertility, often remain (Ige and Amodu, 1975). Further, tolerance breaks down easily under stress such as malnutrition, pregnancy, lactation, disease and migration (Murray *et al.*, 1982).

Stephen (1966) had preferred Zebus to trypanotorerant N'Damas owing to perceived low productivity but comparison studies by Trail (1985), using productivity index based on

weight of calves at 1 year, plus the meat equivalent of the milk produced per cow per year, showed that the productivity of the trypanotolerant *Bos taurus* cattle (compared to the zebu stock) is by no means so much lower, than is frequently assumed.

Blandl (1988) notes that the proportion of trypanotolerant breeds in the total cattle population of the countries affected by trypanosomosis is at 5% (8/147 million); this is relatively low, even if the percentage to West African coastal countries is very high, in some cases lying at 100% (ILCA/FAO/UNEP, 1979). This stock size limits the spread of the trypanotolerant cattle. Shaw and Hoste (1987), in a study in which 19 West African countries were included, concluded that in these countries a maximum of 50,000 heifers are available annually for the extension of keeping trypanotorelant cattle or for their export. However, according to this study, no more than 10,000-14,000 heifers are available annually for the new build up of trypanotolerant stocks.

Trypanotolerant breeds represent a valuable potential for regions with tryponosomosis and in particular in regions in which tsetse control is either not possible or practical, their husbandry is a promising strategy whose potential requires further examination (Blandl, 1988).

Studies in Kenya in which the Orma Boran and the Galana were compared under natural tsetse challenge (Njogu *et al.*, 1985; Mwangi *et al.*, 1998) and following experimental infection, showed that the Orma Boran is trypanotolerant. These studies indicated that the Orma Boran, because of their superior body weights, could be used as part of integrated trypanosomosis control program in trypanosomosis endemic areas in East Africa. The

impact and sustainability of trypanotolerance in the pastoral communities has not been given much attention (Maichomo *et al.*, 1999).

2.6.3 Vaccines

One important biological feature of the pathogenic trypanosomes is their ability to vary the structure of their external surface coating of variable surface glycoprotein (VSG). This process known as antigenic variation, results in the ability of the parasite to evade the immune response of the mammalian host most effectively (Vickerman, 1978; Cross, 1978). The number of variant antigenic types (VATs) of a single strain of trypanosomes is controlled genetically by complex gene switching processes that result in a vast repertoire. There are usually several species, subspecies or types, and strains circulating in any given area, all of which have distinct antigenic repertoires. Hence, cattle do not normally develop immunity to trypanosomosis and can undergo repeated infections throughout their lifetimes.

In spite of an extraordinatry research effort directed at this problem over the last 30 years, notably by the International Livestock Research Institute, (ILRI), the mechanisms of antigenic variation appear to have been effective in thwarting attempts to immunise cattle artificially and the prospect of a vaccine against trypanosomosis in any species is no closer. The one area where there has been some progress has been in the development of an anti-disease vaccine, in which efforts are directed towards preventing the pathogenic effects of the parasite rather than infection itself (Authie *et al.*, 2001).

2.6.4. Insecticide impregnated traps.

Traps are applied in order to acquire information on a population of flies, or with the aim of effectively decimating the maximum numbers of flies through continually capturing a

certain proportion of them and thereby decreasing losses due to trypanosomosis. Reduction of fly density can thus be enhanced if the traps are impregnated with a suitable insecticide. Studies have shown that odours from urine of cattle can increase by several times the number of *G. pallidipes* Austen and *G. morsitans morsitans* Westwood caught in traps or attracted to insecticide treated targets (Chorley, 1948; Owaga, 1984, 1985; Dransfield *et al.*, 1986; Vale *et al.*, 1986). Much of the efficacy of the urine is due to phenols (Hassanali *et al.*, 1986; Bursell *et al.*, 1988). This method has been used on large scale since 1982, when it was used for the first time in the Northern Ivory Coast for the control of riverine tsetse flies (Kupper, 1983).

The traps are set up at a distance of 300m from each other in the control regions gallery forests every year at the beginning of the dry season. At the beginning of the rainy season the traps have to be removed to avoid losses through flooding. The traps are checked once a week. Maintenance involves repair of the nets, replacement and clearing of overgrown vegetation to secure a wide visibility of the traps for the flies. The effect of the traps on the fly population is supervised through regular entomological surveys. Unlike the sterile insect technique and insecticide application by helicopter it is impossible to eradicate tsetse with the trap method. It is employed in order to keep the fly population at a low level which can indeed be as much as 98% below the population size before application (Kupper, 1983).

In Lambwe Valley Kenya, a tsetse suppression trial using baited insecticide impregnated targets was initiated by KETRI in 1988 and achieved a 95% reduction in fly numbers

within the first 12 months. The trial was extended over a wider area with 99% suppression (Bourn, 1998). In Galana ranch, use of impregnated targets begun in December 1987 and by August 1988 no tsetse fly were being caught in the traps and only 6 cases of trypanosomosis were recorded in 2000 cattle over a period of three months (Bourn, 1998). Fly reinvasion however later occurred after termination of the trial.

A study of the trap method in Northern Ivory Coast (Mbengue region) showed that the control cost per unit area decreased with the increasing size of the region. This is attributed to the fact that the capacity of the management unit is then more balanced and therefore more economically utilized with the increasing size of the control region. The study also showed that the cost reduction per unit area declines with increase in control area. The expansion of the control region from 1750 km sq. to 10,000 km sq. resulted in decrease of the cost per hectare by 54%. This is based on the fact that the management unit share of the overheads in the total cost drop as the control area increases; while the proportion of measure in the control region increases. Kupper (1983) demonstrated that trap spacing could be increased to 600m with negligible effects to the technical efficiency of the method.

2.6.5. Aerial spraying of insecticides

Tsetse control with aid of helicopters, takes place through a single spraying of vegetation with a residual insecticide. The insecticide must have residual effect for at least six weeks to kill the flies present at spraying time and those that hatch in the following weeks. The aim of these measures is total eradication of the tsetse fly in the control region. The insecticides mostly used in the past were endosulfan and dieldrin. The method has been

tried in Kenya (Le Roux and Platt, 1968) where good residual deposits of dieldrin were achieved in dense thicket habitats of *G. pallidipes.* In Lambwe Valley, aerial experimental application of dieldrin was initiated in 1968 with little success due to budgetary constraints and also reinvasion. Other methods that had been attempted were ground application of insecticide (dieldrin and cypermethrin) and bush clearing primarily within the park (Bourn, 1998). The environmental impacts of aerial spraying is a major concern that limits the utility of this method.

2.6.6. Sterile insect technology (SIT)

The SIT was successfully used for the control of screwworm fly, *Cochliomyia hominivorax,* from South East US and melon flies, *Dacus cucurbitae* in Rota, an island in the Marianas Islands of the Pacific Ocean. When used after an insecticide treatment, SIT is an effective method of tsetse control (Dame, 1970).

The SIT was first employed on large scale against riverine tsetse species in 1981-1984 in Sideradougou region of Burkina Faso (Blandl, 1988). The SIT is based on the release of male tsetse flies bred and sterilized in the laboratory, which then compete in the control region with the wild fertile males for the females to be fertilized. The reproduction potential of fly population is thereby so strongly impaired that an eradication of flies is possible in the region concerned.

In order to achieve the desired effect on the wild fly population, a ratio of 7 sterile males to 1 fertile male has to be reached in the control region (Dame and Schmidt, 1970). The number of sterile males required for this would exceed the capacity even of very large fly

production units for only a moderate fly density in the control region. Hence, the wild fly population has to be reduced prior to the release of the sterile males by putting up insecticide-impregnated screens or traps in the control region for a few months to reduce the initial population by up to 95%. The utilization of SIT in the tsetse control is made possible due to particular biological characteristics of this insect, the reproduction rate of the carniviparous fly at one larva every 10 days per reproductive female, is extra ordinarily low for an insect.

The female mates only once or rarely a few times, in the first days after hatching. The sperm cells transmitted by the males are stored in a special organ, spermathecae. Hence insemination by a sterile male leads to a female remaining sterile for the rest of its life. There are two ways of sterilizing insects; by gamma radiation or radio-sterilization that utilizes a cobalt-60 source. In *G. morsitans*, the male pupae are exposed to 6,000-12,000r. The adult male arising from the pupae exposed to cobalt-60 are released into infested areas to exceed and compete numerically with the natural male tsetse population. Chemosterilization of adult males is also practised by application of alkylating agents such as apholate and metepa (Blandl, 1988). Sterilizing is possible by physical (gamma rays) or chemical (Azaridin derivatives) methods, without their vitality being too strongly impaired. SIT has been practised in Zimbabwe and Tanzania.

2.6.7 Use of pour - ons and repellents.

The earliest attempts at controlling tsetse using insecticide-treated cattle were disappointing, probably because the DDT formulations used did not persist (Whiteside, 1962; Burnett, 1954). In the late 1960s a wide range of insecticides were tested on cattle in Zimbabwe, but none killed the flies or knocked them down for more than a week after

the cattle were treated, confirming earlier indications that insecticide-treatment of cattle was unlikely to be cost effective. In the mid 1980s however, when deltamethrin was shown to be effective on targets, it was also tested on cattle and found to be effective for 2-4 weeks (Thompson, 1985).

Following this, a large-scale trial of the technique was initiated in eastern Zimbabwe (Thompson and Wilson, 1992a,b). Thirteen cattle inspection centres were selected for the trial. Cattle were dipped at fortnightly intervals with a 0.00375% formulation of Deltamethrin s.c. Between 80 and 90% of the 22,000 cattle in the area (2,500km2) were treated at 14 day intervals and the monthly incidence of trypanosomosis declined from 257 in June 1986 to 35 in August 1987.

Munsibwe *et al.* (1999), in a preliminary study on the effectiveness of cyfluthrin treated-cattle to control tsetse in Eastern Zambia, demonstrated that the incidence of trypanosomosis declined from 9.7% to 0.9% and the animals had higher than maximum monthly average PCV recorded during the period preceding the application of the pour on. There was also a decline in sales of trypanocidal drug Diminazine aceturate by 71% in the area of study.

Insect repellents have been for long produced for human use to reduce disease threat and to provide personal protection from insect borne diseases such as malaria, sleeping sickness meningitis and West Nile virus. Various formulations have been produced (Melnik, 1988) to meet the requirements of long lasting efficacy under extreme

environmental conditions, low initial cost and water resistance. Since they are for human use, they should also be non-greasy, sweet tasting, easy to apply and pleasing to the olfactory senses. The common insect repellent agent is N,N-diethyl-m-toluamide (DEET) in amounts ranging from 7% to 33% by weight (wt%). Other insect repellents, such as citronella (an extract of lemon grass) which is safe to use in up to 10% concentrations have also been used.

Early studies by Vale (1974a) showed that chemicals present in human odour reduce the numbers of tsetse attracted to a host and also the proportion that subsequently feed. Such chemicals could provide a useful tool in the integrated management of trypanosomosis. In areas where cattle are protected from trypanosomosis by use of prophylactic drugs, disease challenge might reduce by treating cattle with repellents.

In a study of the olfactory responses of tsetse flies, *Glossina species* to phenols and urine in the field, Vale, *et al.* (1988) observed that for *G. pallidipes* and *G. morsitans morsitans*, 4-methylphenol and 3-*n*-propylphenol were attractants whereas 2-methoxyphenol was repellent. Recent studies by Torr *et al.* (1996) showed that low doses (10mg/h) of acetophenone, 2-methoxyphenol, pentanoic and hexanoic acid (all known repellents to the tsetse fly) reduced the catch of baited traps by 45-85%, confirming previous findings by Vale (1980) and Vale *et al.* (1988). The results also showed that 2-methoxyphenol was the most potent repellent of those tested, reducing trap catches by 85% and its repellent effect was not enhanced by adding either pentanoic acid or acetophenone. However, the author cautions against making deductions regarding the behavior of the tsetse based solely on their responses to traps as opposed to hosts. The

repellents however did not demonstrate effect on landing, hence the data suggests that baiting an ox with these chemicals would only halve the number that is attracted to the ox, and at best reduce the proportion that subsequently feed by 25%. The net reduction in biting rate would then be 60%. The repellents would be useful in situations with low fly densities and, or low infection rates (Torr, *et al.*, 1996).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Animals used

3.1.1 Mice

One hundred and thirty (130) Swiss white mice comprising of 65 male and 65 female, aged three months from the same litter were purchased from the International Livestock Research Institute (ILRI). They were kept in cages and fed on commercial feed (mice diet cubes) and provided with clean water *ad libitum* .The males and females were housed separately. The mice were given four weeks to acclimatize.

3.1.2 Rabbits

Fourteen (14) New Zealand White rabbits aged between three to six months were purchased from the Ngong Veterinary farm. They were housed in individual cages and fed on commercial rabbit pellets and provided with water *ad libitum*. They were allowed one month to acclimatize.

3.1.3 Goats

Forty-five (45) small East African goats were purchased from Kitengela in Kajiado District and transported to Veterinary Investigation Laboratories, Kabete. They were between one year and two years of age and of both sexes. The goats were tagged and given numbers for identification. The animals were initially housed together and fed on hay (Rhodes grass) and given drinking water *ad libitum*. They were also fed on wheat bran as a concentrate and provided with Red Rockies® as the salt lick. They were dewormed using Levamisole Hydrochrolide Bp 15mg and Oxyclozanide BP (Vet) 30mg

(Nilzan plus® Coopers K) and injected with multivitamins. They were allowed one month to acclimatise. Before the administration of the repellent, all the goats were weighed.

3.1.4 Synthetic tsetse fly repellent.

The synthetic repellent was manufactured by ICIPE (Patent No. Ke 00185, 2004; Saini and Hassanali). The repellent is a colourless liquid, highly volatile and insoluble in water with a specific gravity of 1.090 -1.100 at 20 °C. The concentration of the formulation used was 99mg/ml.

3.2 Determination of the intraperitoneal LD_{50} of the repellent in mice

3.2.1 Preliminary toxicity testing

Ten mice (five female and five male) were randomly selected from the cages. They were weighed and body weights recorded. Trial and error was used to approximate the range of toxic dose of the repellent. Two mice were injected intraperitoneal with 0.1ml of the repellent whose concentration was 99% using disposable sterile 1 ml syringes. This represented a dosage of 259-mg/kg bwt. The mice, however, went into prostration, coma and died immediately and this necessitated dilution. Corn oil was used as the diluent, since the repellent is insoluble in water. A dilution of 1: 10 was done to make a 10% solution with a concentration of 9.9 mg/ml. A further two mice were randomly picked and a dosage of 100mg/kgbwt administered. Both mice went into prostration, coma and finally died within 45 minutes. Dosages of 10-70mg/kg body weight were then administered and animals observed. A further dilution of 1:100 was made, resulting

in a 1% solution used for the lower dosages of 10-20-mg/kg body weight. Sixteen mice were then selected, injected and observed for 24 hours (Appendix 1).

3.2.2 Experiment 1: Determination of intraperitoneal LD_{50} in mice of the tsetse repellent

The Reed and Muench (1938) method of LD_{50} determination was used in this experiment Ninety mice were randomly divided into nine groups of ten mice each, comprising of five female and five males. The mice were weighed individually and marked. The nine groups were then randomly allocated to various doses (30-65-mg/kg bwt) of the repellent (Appendix 2).

The repellent was administered via intraperitoneal route using sterile 1ml disposable syringes. The mice were observed for the development and the sequence of signs of acute toxicity and the numbers that survived or died were recorded (Appendix 2).

3.3 Experiment 2:Evaluation of dermal irritation of the tsetse repellent in rabbits.

The method described by Draize (1959) was used. Six rabbits were used in this experiment. The area over the back of each animal extending from the base of the neck to the hindquarters was shaved using a hair removal cream (Veet®). Prior to application of the cream, the hair was clipped using scissors. After five minutes the hair was gently wiped off using gauze bandage and the area thoroughly cleaned using plenty of clean tap water. The area was then dried by gently mopping with a cotton wool. The rabbits were then returned to their cages for 24 hours before application of the repellent. Prior to test application of the repellent, each rabbit received four parallel epidermal abrasions with a sterile needle at one test site while the skin at the opposite site remained intact. To each

site, 0.5g of the repellent was applied by introduction under a double gauze layer to an area of skin approximately 1 square inch. The patches were backed with plastic, covered with a non-reactive tape and the entire test site wrapped with a binder. Two rabbits served as the control and received no repellent but everything else was similar. The rabbits were then returned to their cages.

After 24 hours exposure, the binders were removed. The test sites were rinsed with tap water to remove any remaining test material. At 24 and 72 hours after test application, the test sites were examined for dermal reactions in accordance with the Federal Hazardous Substances Act (FHSA) - as recommended by Draize scoring criteria (Appendix 3). The Primary Irritation Index (P.I.I) of the test material was calculated following test completion.

3.4 Experiment 3: Evaluation of ocular irritation of the tsetse repellent in rabbits.

A procedure commonly employed for evaluating the irritant capacity of liquids and solids was used (Draize 1944). Six white albino rabbits (New Zealand white breed) were weighed individually and their sexes noted. The eyes were examined visually and their health status established. 0.1ml of the repellent was instilled into the lower conjuctival sac of the right eye, in each of the rabbits using sterile 1ml pipettes. The eyelids were held together for one second and then released. The eye was not washed and the left eye served as the control. Ocular reactions were observed at 24, 36, and 72 hours and at 4, 7 and 21 days after application. The status of the cornea, iris and conjunctiva was rated

according to the grades for ocular lesions provided for in the EPA Health Effects Test Guidelines OPPTS 870.2400 Acute Eye Irritation (Appendix 5)

3.5 Experiment 4: Evaluation of the effects of the tsetse repellent on the haematological, biochemical and pathological changes in goats.

Forty five goats were divided into three experimental units of fifteen goats each. The three experimental units were then randomly allocated to three experimental groups: (i)a high treatment level of the repellent (4mg/hr) (ii) a low treatment level of the repellent (2mg/hr) and (iii) a control group. During the study the groups were housed separately but similar feeding and management regimes were applied to the three units. All the goats were weighed and haematological and biochemical parameters were evaluated for each animal before and during the experiment.

3.5.1 Administration of the repellent.

The repellent was administered in specially made dispensers tied around the neck of the goats (Plate 1). The dispensers allowed diffusion of the repellent at a rate of 4mg/hr. The animals in the high treatment group, each received one dispenser, hence a release rate of 4mg/hr while animals in the low treatment group received one dispenser each with the repellent diluted 1:1 with liquid paraffin, hence a release rate of 2mg/hr. The control group were given only the dispensers with liquid paraffin. The quantity in the dispensers lasted for thirty days after which replenishment was done. Release rate of repellent was estimated as per body weight of the goats.

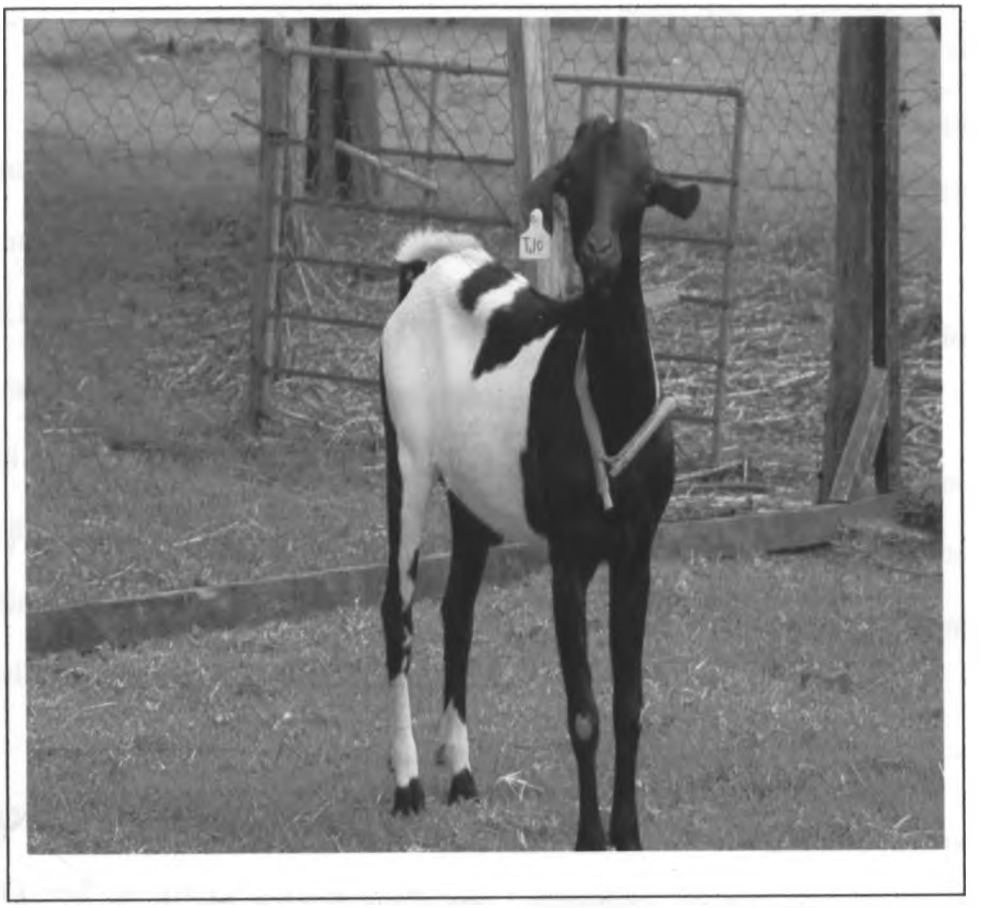

Plate 1 **An experimental goat with a repellent collar around the neck. The tygon tube tied to the collar contains the tsetse fly repellent and allowed diffusion of the chemical.**

3.5.2 Blood for haematological and biochemical analyses.

Blood samples were collected monthly from the jugular vein, using 4.5m vacutainers with disodium ethylene diaminetetracetic acid (EDTA) as the anticoagulant for haematological analysis. Plain 4.5ml vacutainers were used for the collection of blood for biochemical analysis.

3.5.2.1 Determination of the total red blood cell counts (RBC), the total white blood cell counts (WBC) and the differential leucocyte counts.

A full automatic hematology cell counter (Melet schloesing laboratories France) was used for the cell counts. A card specifically for goats (MS 4 Vet pack) was inserted in the machine. After mixing of the blood, a sample was fed to the machine and an output of the cell counts and the differential cell counts was given.

3.5.2.2 Determination of alkaline phosphatase (ALP), gamma -glutamyl-transferase (GGT) and aspartate aminotransferase / glutamate oxaloacetate transaminase (AST/GOT)

Diagnostic kits Alkaline phosphatase fluitest® ALP, Kinetic test optimized, Gamma - GT,fluitest® GGT, Kinetic test acc. SZASZ '74 and Biozyme GOT ASAT, GOT / ASAT mono, UV-METHOD (Biocon ®) were used for kinetic determination of the activity of the enzymes in serum.

Procedure

The spectrophotometer (Visual Ref: 99 875 Version D 05/2001) Biomeriux was used in all the biochemical determinations. The procedure outlined in the kits was used in the

mixing of the reagents and substrates. The spectrophotometer was adjusted to zero absorbance using distilled water and the calibration co-efficient of 952; temperature of $+30^0$ C and wavelength was adjusted depending on the enzyme being read as follows: Hg 405 nm (for ALP), Hg334 nm for (GGT) and Hg 340nm (for AST). After mixing the prescribed serum and reagent in a 1cm light cuvette, the spectrophotometer made automatic readings and recorded at the output.

3.6 Determination of body weight in goats

The goats were weighed monthly and the body weight recorded.

3.7 Post-mortem examination.

At the end of the study two animals from each group were randomly selected and sacrificed. Gross examination of the organs was done. Specimens obtained from the lungs, liver, kidneys and heart tissues were fixed in 10% buffered formalin, embedded in paraffin wax, sectioned at 4 microns and stained with hematoxylin and eosin for microscopic examination.

3.8 Statistical analysis of the data.

A calculation method was used to analyse data on LD_{50} determination (Reed and Muench, 1938) and dermal irritation (Draize, 1944). Analysis of the haematological, biochemical and body weight data were compared using repeated measures analysis of variance, SAS (SAS, 2003).

CHAPTER FOUR
RESULTS

4.1 Intraperitoneal median lethal dose (LD_{50}) of the synthetic tsetse fly repellent

Intraperitoneal administration of the tsetse fly repellent at dosages ranging from 30-65 mg/kg body weight caused death of mice within 24 hours. The details of percent mortality at each dosage level are given in Table 1. From the table, the LD_{50} lies between 40 and 45-mg/kg bwt. The actual calculated 24 hour intraperitoneal LD_{50} for the repellent in this experiment was determined to be 40.2-mg/kg body weight.

Table 1. Percent mortality in mice after intraperitoneal injection with the synthetic repellent within 24 hours post administration

Dose (mg/kg bwt)	Log. Dose	Died	Survived	Accumulated			Percent mortality
				Died	Survived	Total	
30	1.4771	0	10	0	25	25	0
35	1.544	8	2	8	15	23	34.8
40	1.6020	4	6	12	13	25	48
45	1.6532	7	3	19	7	26	73.1
50	1.6989	9	1	28	4	32	87.5
55	1.7403	9	1	37	3	40	92.5
60	1.7781	8	2	45	2	47	95.7
65	1.8129	10	0	55	0	55	100

4.2 Acute toxic effects of the repellent in mice

During the preliminary determination of LD_{50}, mice receiving dosages of 10-40 mg/kg bwt survived and only exhibited decreased locomotor activity and exploratory behavior. The observed signs of acute toxicity for the mice receiving 35-65 mg/kg bwt of the repellent were an initial decreased locomotor activity and prostration. The mice then exhibited decreased respiratory rate with increase in the respiratory depth. Mixed convulsions were noticed in the unconscious mice and this preceded coma and death at varying times. Some mice given lower dosages showed, decreased locomotor activity and then recovered. A varying degree of convulsions and decreased respiratory rate accompanied by increased respiratory depth were noted. At 24 hours, the surviving mice exhibited rough hair coats, and photophobia. However, feeding and drinking of water was not affected. Decreased locomotor activity and decreased exploratory behavior was noted. The control group exhibited normal activity in all aspects.

4.3 Dermal irritation with the tsetse fly repellent in rabbits

The skin reactions for the intact and abraded skin 24 hours and 72 hours post-application are shown in Table 2. At 24 hours post-application of the repellent, 67% of the rabbits treated with the test material (n =6) exhibited varying degrees of erythema, from very slight erythema to eschar formation. In the rabbit that exhibited eschar formation, the skin was dark and had hardened patches with elevated edges that were hyperaemic and oedematous. At 72 hours, 83% of the experimental rabbits exhibited erythma. Sixteen percent of the experimental animals and the control group did not show any changes on the skin. At 24 hours post application of the repellent, 50% of the rabbits exhibited very slight to slight oedema and by 72 hours, only 16% had slight oedema. The primary

irritation index (PII) was then calculated, based on the sum of the scored reactions divided by 24 (two scoring intervals multiplied by two test parameters multiplied by six rabbits). A primary Irritation Index of 2.4 was obtained and according to the method the repellent causes mild irritation to the skin in rabbits (Appendix 4).

Table 2 Skin reactions in rabbits after application of the synthetic repellent

Rabbit No.	Reaction	24 hours		72 hours	
		Intact	Abraded	Intact	Abraded
10	Erythema	3	3	4	4
	Edema	2	2	1	1
11	Erythema	0	0	0	0
	Edema	0	0	0	0
12	Erythema	4	4	4	4
	Edema	2	2	2	2
13	Erythema	1	1	1	1
	Edema	0	0	0	0
14	Erythema	2	2	2	2
	Edema	1	1	0	0
15	Erythema	0	0	0	0
	Edema	0	0	0	0

(n= 6)

KEY: **Erythema** 1- slight erythema; 2- well defined erythema; 3- moderate to severe erythema; 4- beet red erythema with injuries
Edema 1- slight edema with barely perceptible raised edges; 2 slight edema.

4.4 Ocular irritation with the tsetse fly repellent in rabbits

The effects on the eyes and associated mucous membranes are presented in Table 3 and the scores in Appendix 5 At 24 hours, all the test eyes in the six rabbits had the eyelids swollen with 67% of the rabbits having partial eversion of the eyelids. The chemosis, however, had receded at 48 hours to only slight swelling and at 21 days post-application of the repellent, there was no swelling. At 24 hours the cornea in the test eyes of all the rabbits had easily discernible translucent areas with the iris slightly obscured. This persisted for three days post-application of the repellent in 33% of the rabbits and four days in 67% of the rabbits. At 24 hours, the iris of the test eyes in all the rabbits showed marked congestion but was still reacting to light shone to the eye. This persisted in 83% of the rabbits to the 7^{th} day but at 21 days, the iris was normal. In 16% of the rabbit at day 4 and 7 had the iris giving slow reaction to light shone in the eye. There was diffuse hyperemia of the conjunctiva in the test eyes in all the rabbits at 24 hour post-application of the repellent. This persisted for two days in 83% of the rabbits and for 7 days in 16% of the rabbits.

Table 3 (0.5 ml) **Eye reactions in rabbits after application of the synthetic repellent**

Rabbit no	24 hrs				48 hrs				72 hrs				4 days				7 days				21 days			
	C	I	R	H	C	I	R	H	C	I	R	H	C	I	R	H	C	I	R	H	C	I	R	H
1	2	1	2	2	2	1	2	1	2	1	2	1	2	1	1	1	2	1	1	1	0	0	0	0
2	2	1	2	2	2	1	2	2	2	1	2	2	2	2	2	1	3	2	2	1	0	0	0	0
3	2	1	2	2	2	1	2	1	2	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0
4	2	1	2	1	2	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0
5	2	1	2	1	2	1	1	1	2	1	1	1	2	1	1	1	1	1	1	1	0	0	0	0
6	2	1	2	2	2	1	2	1	2	1	1	1	2	1	1	1	1	1	1	1	0	0	0	0

(n=6)

KEY: C =Cornea: 1 for diffuse opacity; 2 for easily discernible translucent area with details of iris clearly visible.
I = Iris: 1 for congestion, swelling and hyperemia; 2 for no reaction to light
R = Conjunctivae redness: 1 for some blood vessels hyperemic; 2 for diffuse hyperemia
H = Chemosis: 1 for swelling of the eyelids; 2 for obvious swelling with partial eversion of eyelids

4.5 Effects of the repellent on hematological parameters in goats

In Fig. 1a, the serum mean monthly levels of WBC were in the range of 15.0± 0.5 to 20.2± 1.0, 14.6± 0.5 to 18.3±1.2 and 14.4±0.6 to17.4± 1.0 for goats exposed to high, low and control groups respectively. In Fig. 1b, the mean monthly levels of RBC (M/mm^3) were in the range of 10.5± 0.4 to 12.9± 0.6, 8.8± 0.8 to 13.0±0.4 and 11.4±0.3 to14.1± 0.6 for goats exposed to high, low and control groups respectively. In Fig. 1c, the mean monthly levels of lymphocyte cell count (%) were in the range of 40.7± 2.5 to 68.8± 2.8, 38.7± 1.4 to 71.0±2.3 and 38.0±2.3 to 69.4± 2.6 for goats exposed to high, low and control groups respectively. In Fig. 1d the mean monthly levels of granulocytes cell count (%) were in the range of 31.1± 3.0 to 52.5± 2.6, 23.7±2.2 to 54.8±2.2 and 24.3±1.6 to55.2± 2.2 for goats exposed to high, low and control groups respectively.

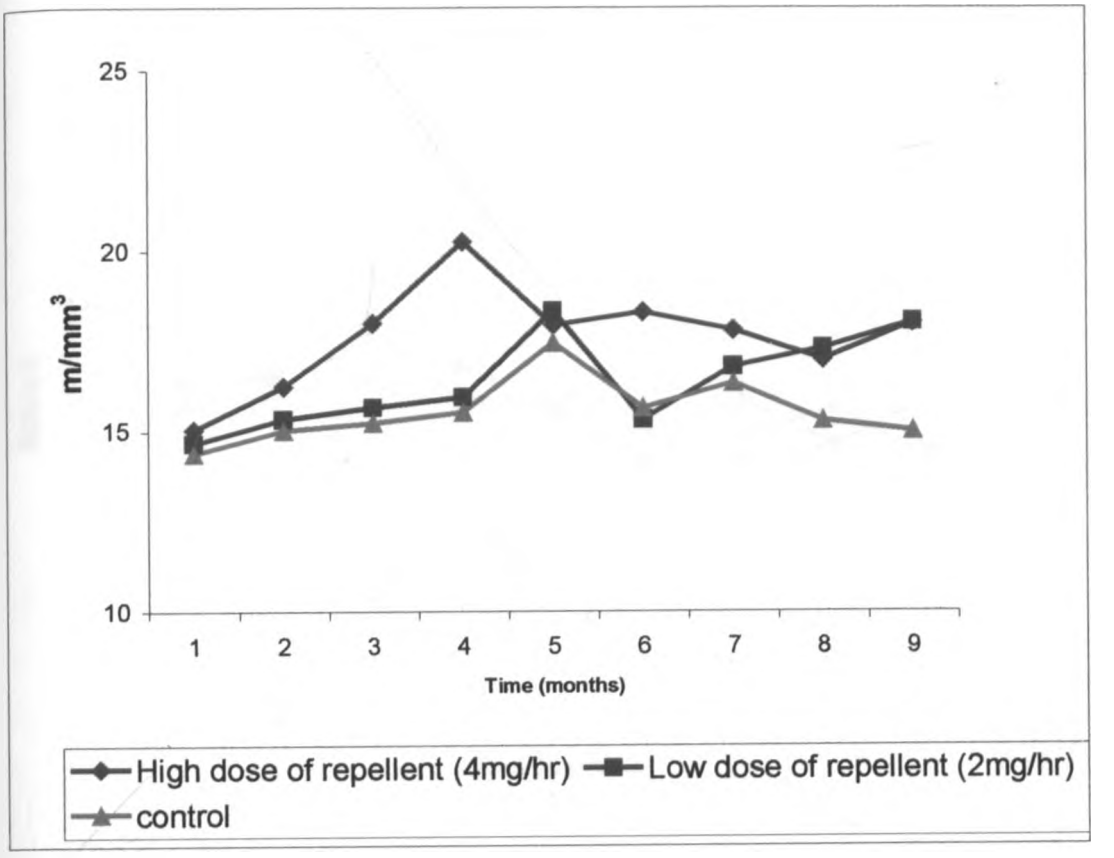

Figure 1(a) Mean monthly value of White blood cells in goats after exposure to synthetic repellent (ref value 4 – 13 x $10^3/mm^3$) (n= 12)

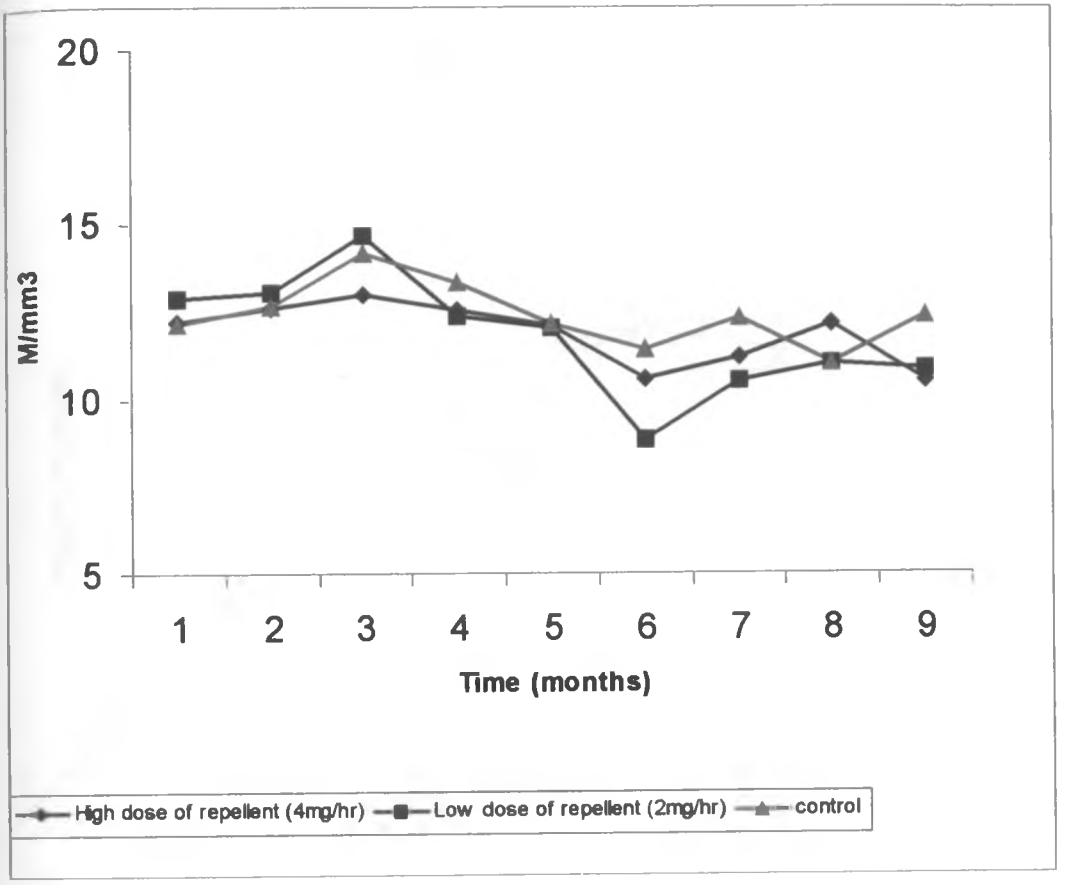

Figure 1 (b) **Mean monthly value of Red blood cells in goats after exposure to synthetic repellent (ref value 8-18 x 10^6/mm^3) (n= 12)**

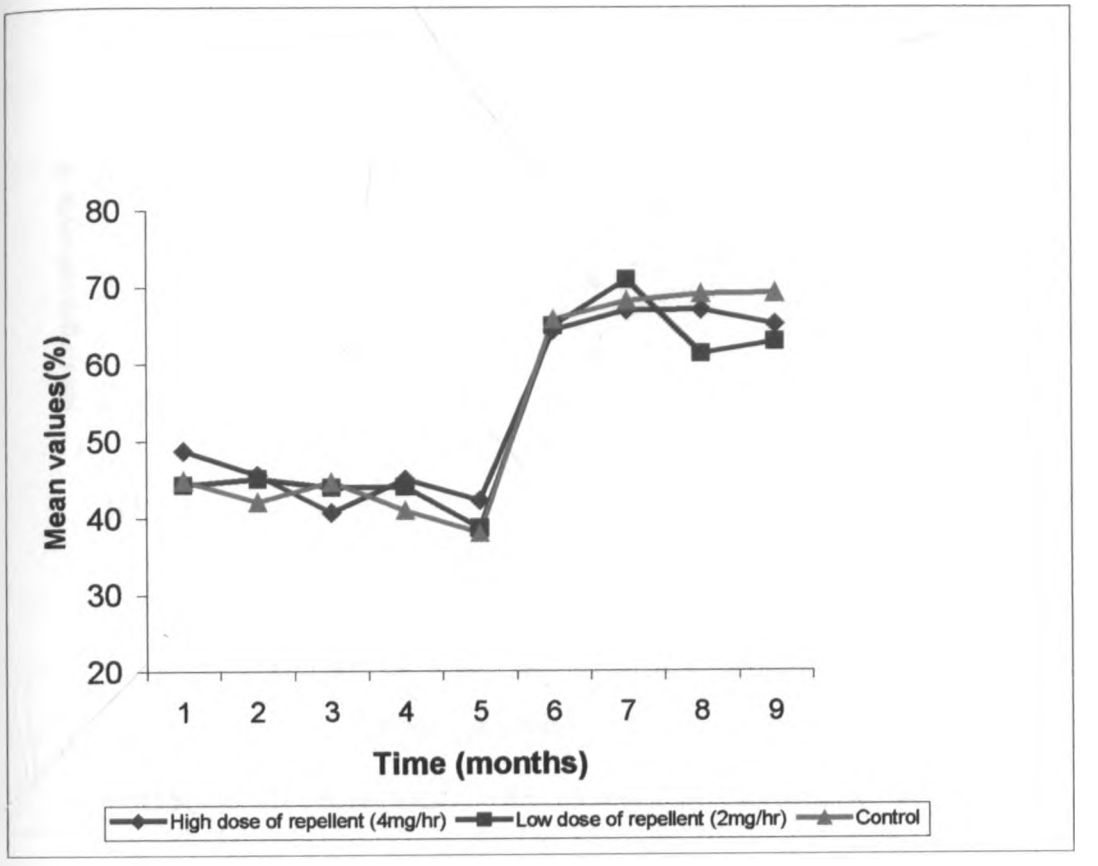

Figure 1(c) Mean monthly differential counts of blood lymphocytes in goats after exposure to synthetic repellent (ref value 50 –70 %) (n= 12)

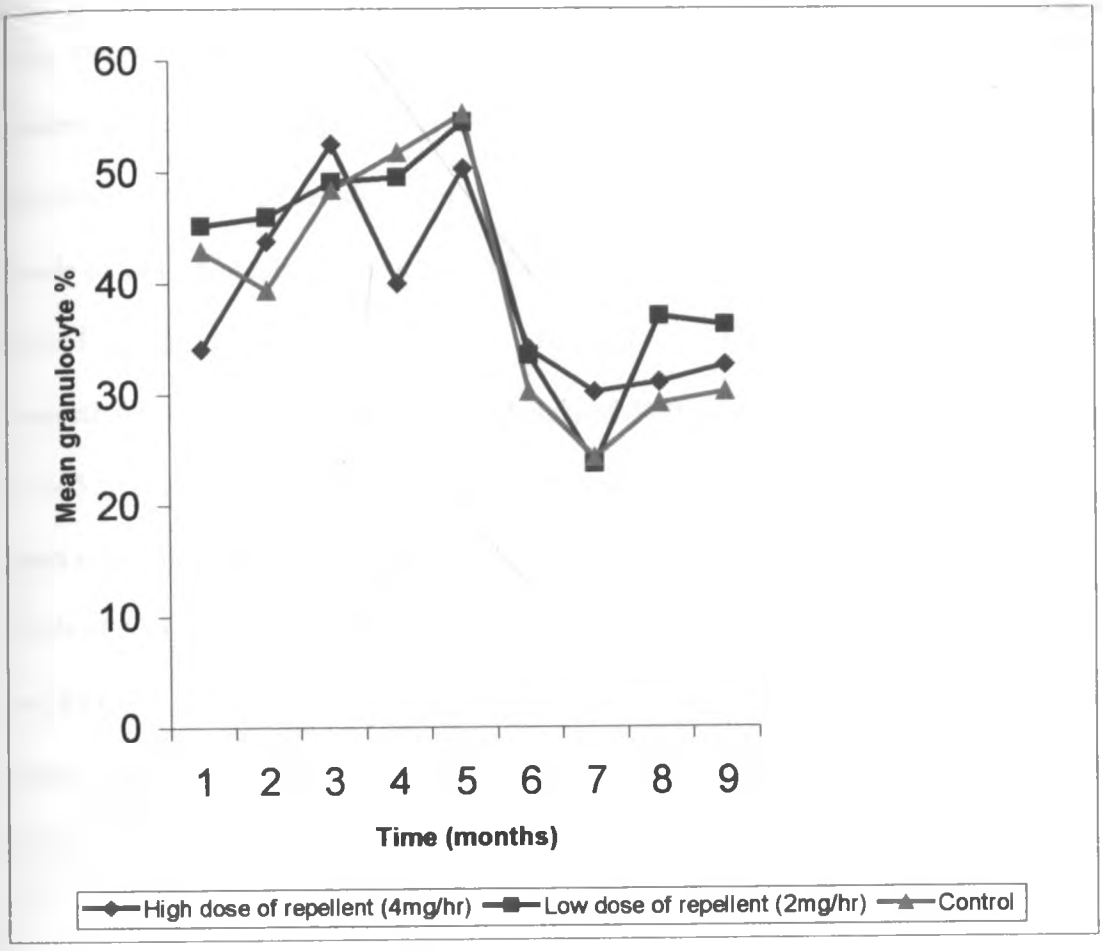

Figure 1(d) Mean monthly differential counts of blood granulocytes in goats after exposure to synthetic repellent (ref value 30 –48 %). (n= 12)

4.6 Effects of the synthetic repellent on biochemical parameters in goats

The mean monthly levels of hepatic enzymes, aspartate aminotransferase (AST), gamma glutamyl transpeptidase (GGT) and alkaline phosphatase (AK) obtained over the observation period are shown in Figures 2(a), (b), and (c). The serum mean monthly levels of GGT were in the range of 27.6± 3.6 to 51.1± 5.6, 31.7± 1.5 to 56.8±4.7 and 28.3±1.2 to54.1± 9.7 (U/l) for goats exposed to high, low and control groups respectively. The serum mean monthly levels of ALP were in the range of 209.7± 53.0 to 645.1± 81.5, 204.1± 27.1 to 573.9±65.5 and 298.1±47.0 to707.0± 121.1(U/l) for goats exposed to high, low and control groups respectively. The serum mean monthly levels of AST were in the range of 85.5± 10.5 to 134.7± 32.2, 93.7± 15.5 to 126.0±10.6 and 85.6±11.0 to116.6± 15.6 (U/L) for goats exposed to high, low and control groups respectively. Appendix 6, 7 and 8 display the mean monthly levels of AST, GGT and ALP.

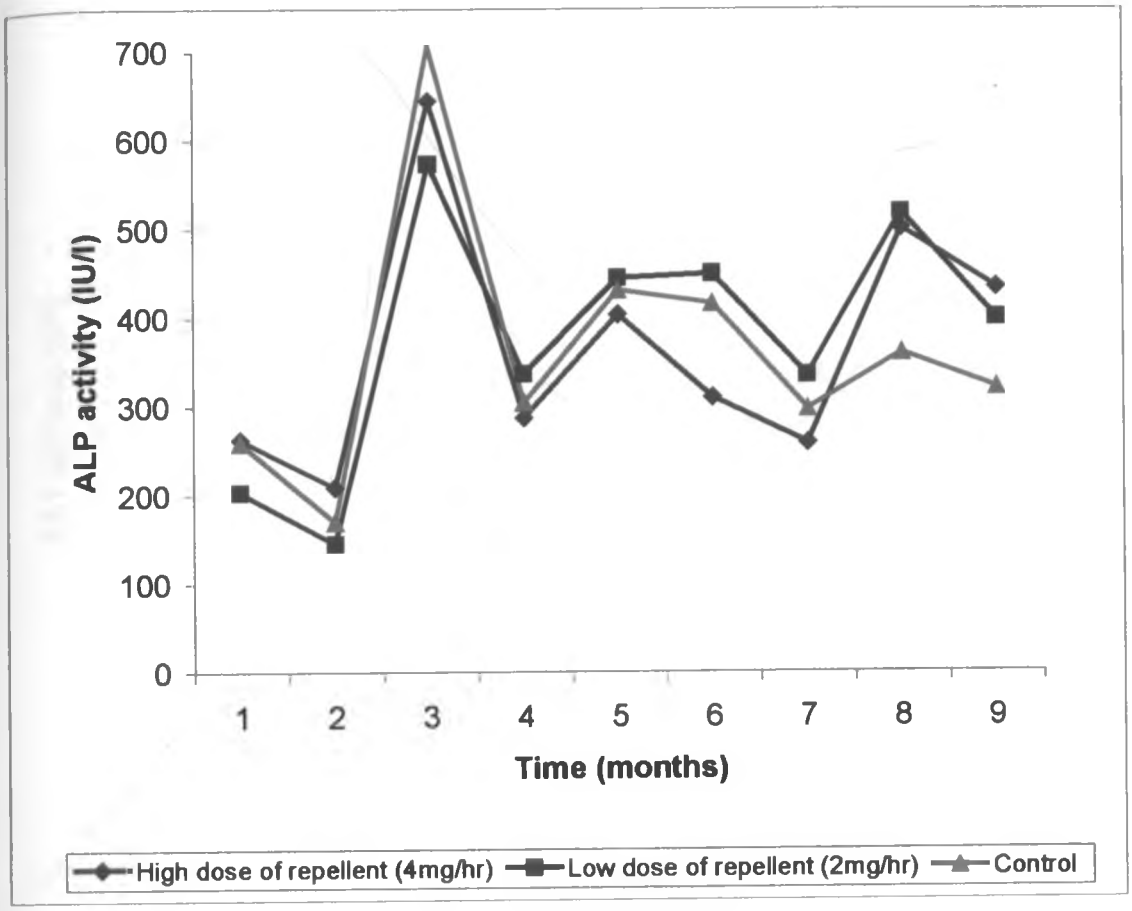

Figure 2 (a) Monthly mean levels of Alkaline phosphatase (ALP) in goats after exposure to synthetic repellent (ref value 93-387 U/I) (n=12)

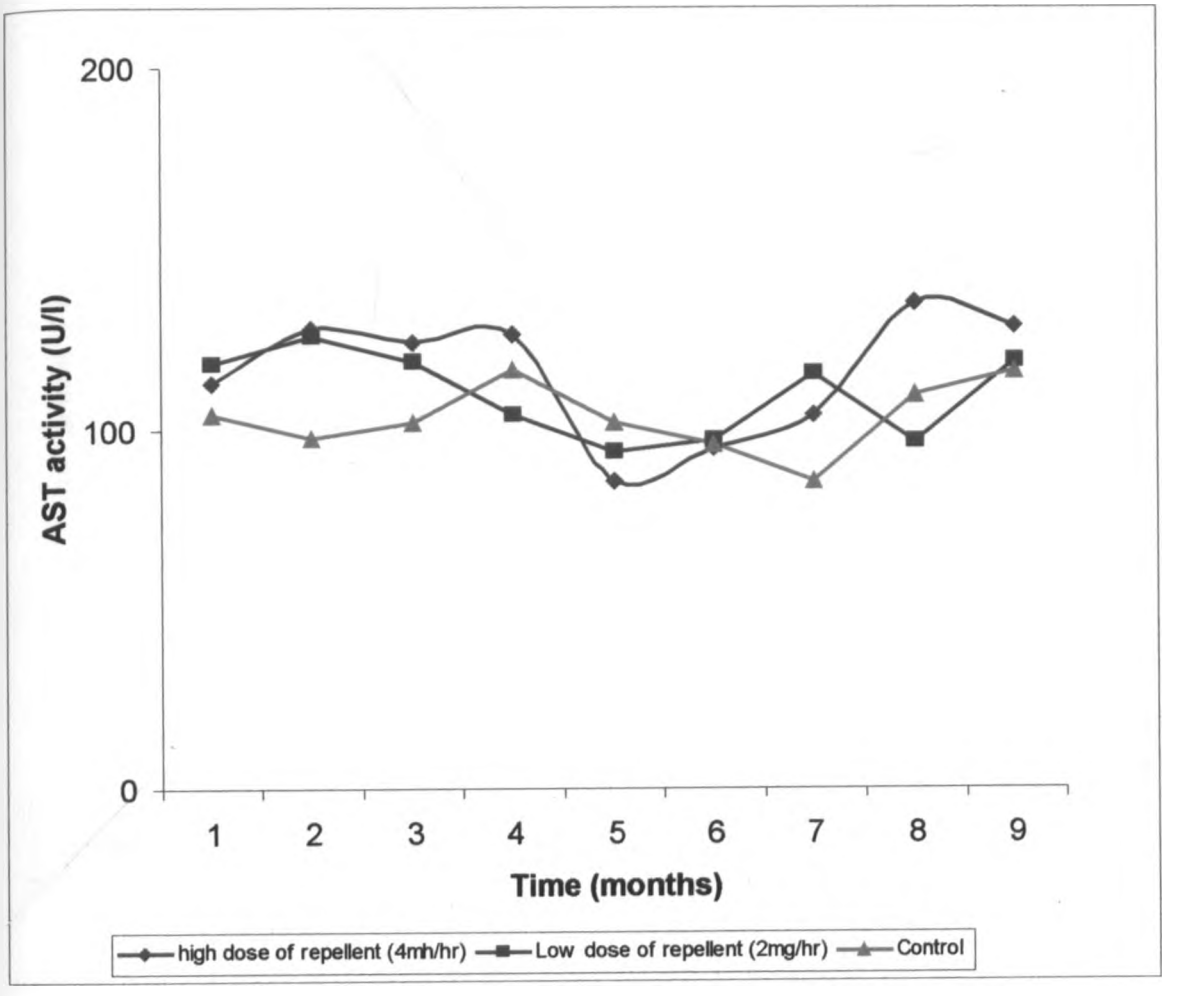

Figure 2 (b) Mean monthly levels of Aspartate aminotransferase (AST) after exposure to the synthetic repellent (ref. Value 167-513 U/l) (n = 12)

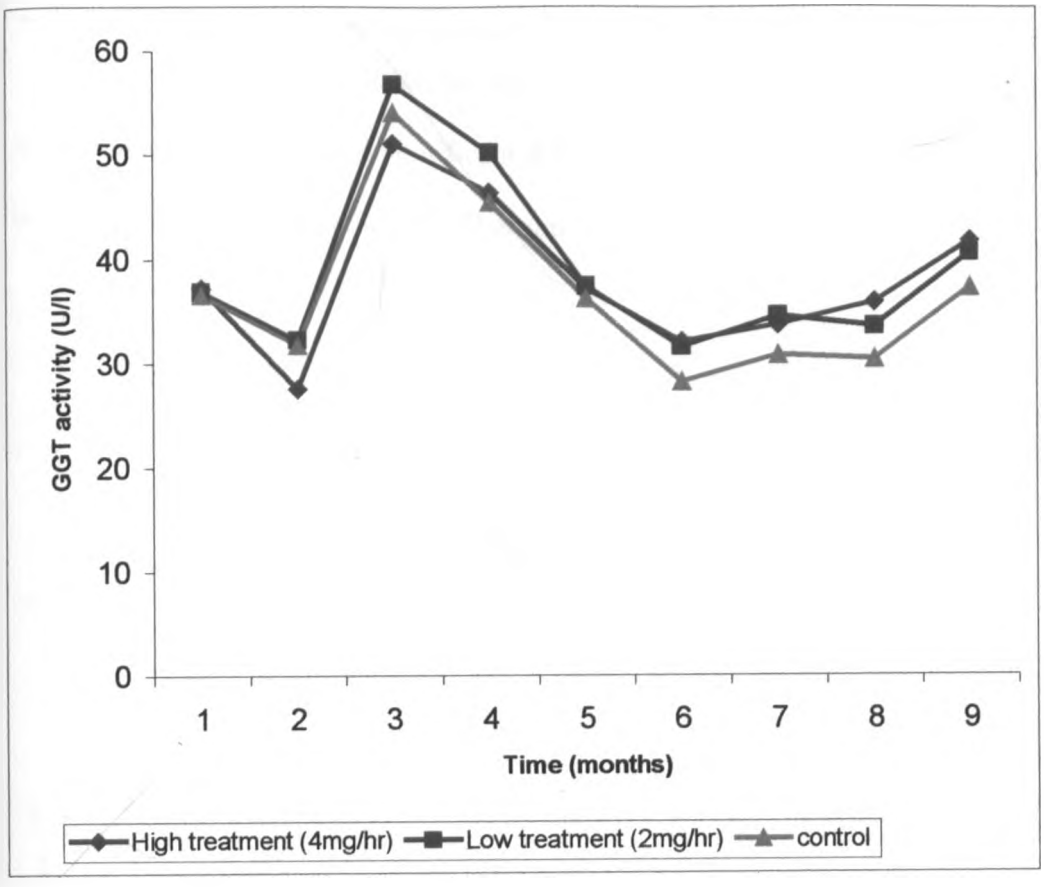

Figure 2 (c) Mean monthly levels of Gamma glutamyl transpeptidase (GGT) in goats after exposure to synthetic repellent (ref. Value 20 –56 U/I) (n= 12)

4.7 Effects of the repellent on body weight in exposed goats

The changes in body weights are illustrated in Figure 3. There was a weight gain in all the groups and there was no significant difference in the weight gain between the groups exposed to low, high and the control group.

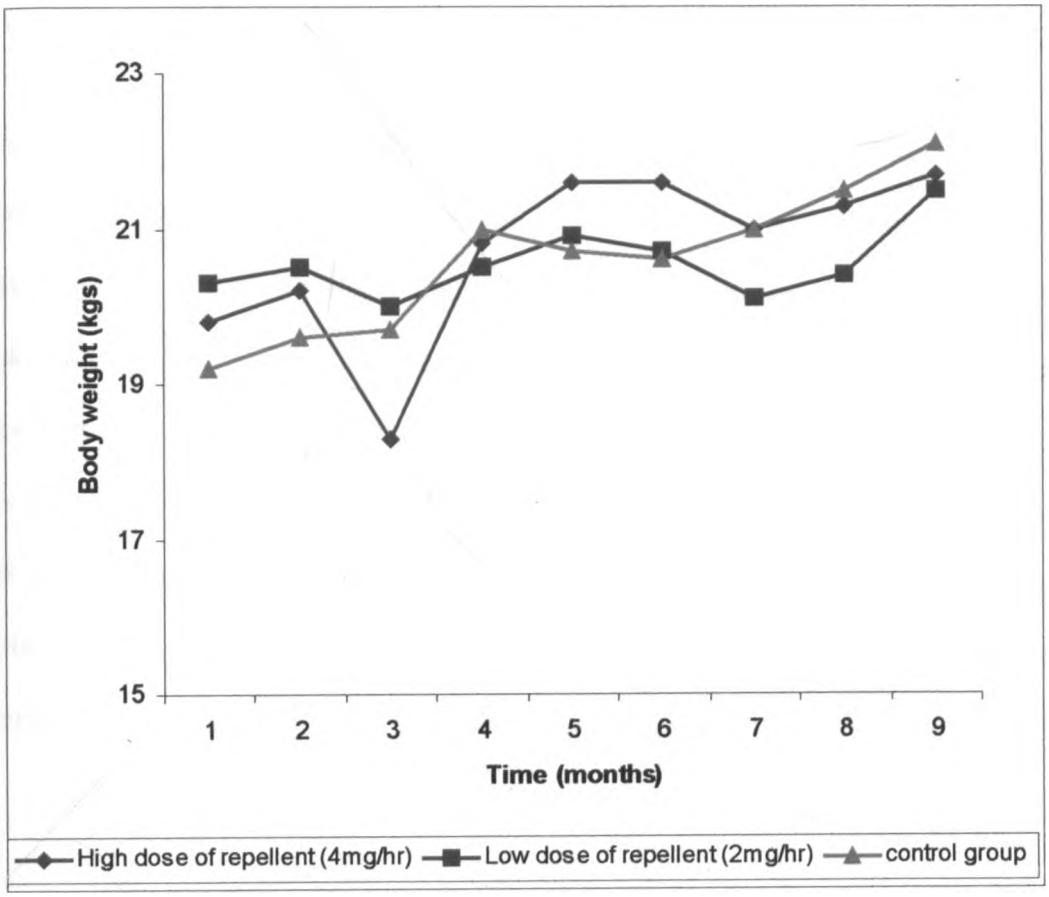

Figure 3 Mean monthly body weights (kg) in goats after exposure to the synthetic repellent

4.8 Post-mortem changes

At post-mortem examination, the liver, kidneys, heart, trachea, bronchi and lung tissue were normal. There were also no significant histological changes in the liver, kidney and heart tissues. Histology of the sections (Plate 2 and 3) showed areas of alveolar wall thickening and alveolar collapse interspersed with emphysema. The alveolar wall thickening was attributed to mononuclear cell infiltration (mainly lymphocytes). The same cells were also seen as aggregates around blood vessels and air passages. This was observed in all the animals presented for postmortem examination. In the myocardium, 83% of the animals had varying numbers of sarcocysts and small foci of lymphocytic infiltration. In the kidney, 33 % of the sections had small foci of lymphocytic infiltration otherwise the other sections had no significant changes.

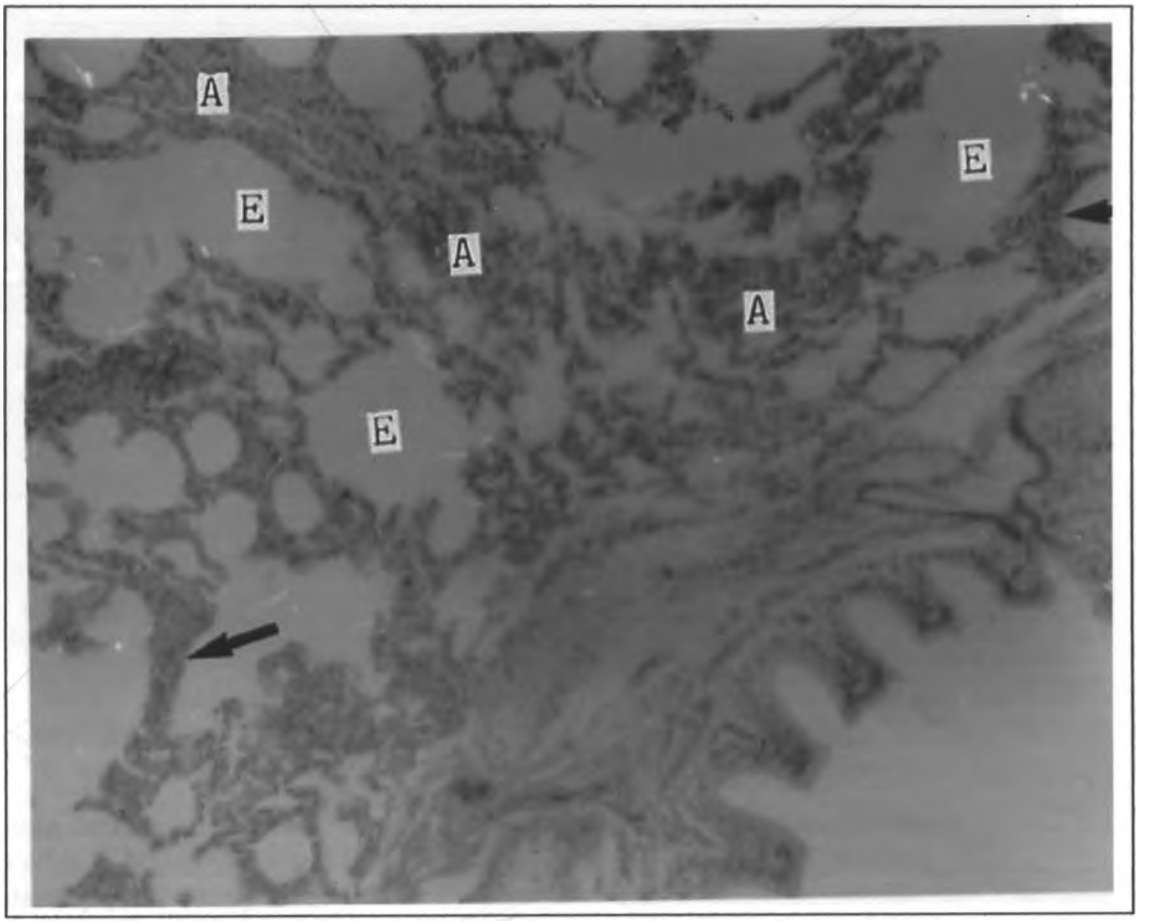

Plate 2L **Lung section from a goat exposed to high dose of repellent vapors. Note alveolar wall thickening (arrows), alveolar collapse (A) and emphysema (E). (H/E x100)**

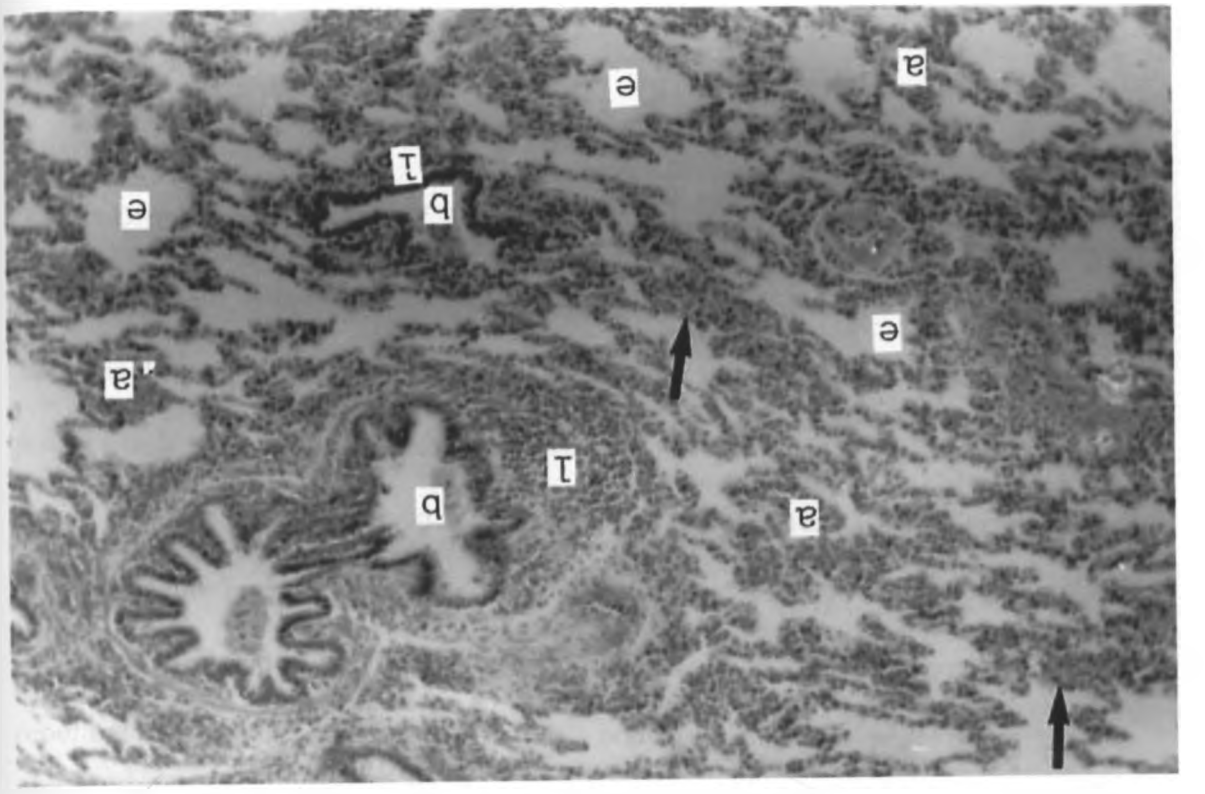

Plate 3 Lung section from a control goat. Note alveolar wall thickening (arrows), alveolar collapse (a), emphysema (e) and lymphocytic aggregations (l) around bronchioles (b). (H/E x100)

CHAPTER FIVE

DISCUSSION AND CONLUSIONS

5.1 Discussion

Several methods, either calculation or graphical, have been used for LD_{50} determination. These include the graphic method of Litchfield and Wilcoxon (1949), the logarithmic probit graph paper method of Miller and Tainter (1944), the range finding procedure of Weil (1972) and the calculation method of Reed and Muench (1938). The Reed and Muench method was used in the present study since it is simple and easy to perform. Using this method, the 24 hours intraperitoneal LD_{50} of the synthetic phenollic tsetse fly repellent in mice was found to be 40.2 mg/ kg body weight. Hence, under the conditions of the present study, the repellent according to the classification given by Loomis (1978), can be classified as highly toxic after intraperitoneal administration (1- 50mg /kg body weight).

Reports on the toxicity of specific phenol compounds in farm animals and their acute toxicities are scanty possibly due to their limited use as pesticides. The toxicity of the specific phenols depends on the substituent group, for example nitro-, chloro-, methoxyphenol. Pentachlorophenol, dinitro-orthophenols, dinitro-orthocresols (DNOC) and dinitropenol (DNP), have been used as wood preservatives (hence animals may have access to freshly treated materials used in fencing and construction of stables). DNP and DNOC are usually toxic at doses of 25 – 50 mg/kg body weight in all species with much smaller doses producing toxicity when environmental temperatures are high. (Blood *et al*, 1989). Amstutz (1980), in a study using calves, found the acute toxic dose for

pentachrolophenol to be 14 g/ 100kg body weight and the chronic toxic dose to be 3.5-5g when given orally daily for 7 days The signs of acute poisoning were marked dyspnoea, weakness, depression, asphyxial tremors, collapse and death. The chronically affected calves had weight loss, lethargy, and mild respiratory distress. Inflammation of the abomasums also occurred due to the effect of the chemical on the abomasal lining. Dodman *et al* (1976) reported similar signs in a case of subcutaneous poisoning by this compound in a cat.

The toxicity of the phenol compounds may also be dependent on the placement of the substituent from the hydroxyl group. However, in a study to compare the toxicity of cresol isomers using precision-cut rat liver slices, Thompson *et al* (1994) found p-cresol to be 5-10 times more toxic isomer at equimolar concentrations to o- or m-isomer. The toxicity of 4-methylphenol is possibly dependent on the formation of a reactive quinone methide intermediate which covalently binds to cellular macromolecules, hence eliciting cytotoxicity (Thompson, *et al.*, 1995; Thompson, *et al.*, 1996). Kitagawa (2001), in a study to investigate the effects of o-, m- and p-cresols on the bioenergetic system using isolated rat liver mitochondria, reported that the cresols inhibited liver mitochondrial respiration and induced or accentuated the swelling of the mitochondria. This suggested that the liver mitochondria might be one of the targets for the hepatotoxic actions of cresols. In reproductive and developmental toxicity studies of methyl phenol isomers (o-, m-, and p-cresol) carried out in rabbits, dosages of 5 and 100mg/ kg body weight, produced hypoactivity, audible respiration and ocular discharge with no embryotoxicity or fetotoxicity (Tyl, 1988a). Rats on daily oral dosages of 175mg/ kg body weight

produced similar signs (Tyl, 1988b). Hence, though the species of the animals and the group of phenols were different, signs of muscle tremors and respiratory distress, leading to coma and death due to respiratory failure were the most common observations.

In the present study, signs of acute toxicity of the tsetse fly repellent in mice were decreased locomotor activity, an initial increase followed by a decrease in rate of respiration and an increased depth in respiration, terminal convulsions, coma and death. Death was probably due to respiratory depression. The signs of acute toxicity observed were consistent with some of the signs of poisoning reported for various phenols in various species which include restlessness, deep and rapid respiration, ataxia, tremors, fever and collapse (Blood *et al,* 1989; Tyl, 1988a; Tyl, 1988b; Amstutz (1980).

The effects of the synthetic tsetse repellent on the skin in rabbits were erythma and oedema formation of varying degrees. In primary irritation index, a score of 2.4 represents mild irritation to the skin. However, according to Draize (1959) classification, the repellent used in the present study is not a primary skin irritant (score < 5). Phenols are absorbed through the skin causing the oedema and hyperaemia. In the case of cat poisoning via skin contact as reported by Dodman *et al,* (1976), the concentration of the phenol in blood was 6.8 mg/100ml at the time of death. At necropsy, there was extensive subcutaneous oedema of the face and right hind limb.

In the assessment and evaluation of the toxic characteristics of a substance, determination of the irritant and or corrosive effects on the eyes of mammals is an important initial step. The information derived from the test indicates the existence of possible hazards likely to arise from exposure of the eyes and associated mucous

membranes. In the present study, the effects of the repellent on the eyes were varying degrees of corneal opacity, hyperaemia of the conjunctiva and oedema of the eyelids. According to this method when four or more of the animals show a positive reaction the test is considered to be positive. Phenols are caustic in nature and this could have resulted in the irritation in the eyes. The effects on the eyes were reversible and were absent at day 21 of the experiment.

Many toxins induce hepatic injury. The susceptibility of the liver to toxic insult is in part a consequence of its location between the digestive tract and the rest of the body and the central role it plays in the biotransformation and disposition of xenobiotics (Miyai, 1991; Synder, 1979). Extrahepatic metabolism of toxins by mixed function oxidases may affect the target organ and potential hepatotoxicity of a given xenobiotics (Gram *et al.*, 1986). In hepatocellular or in cholestatic forms of liver injury, whereby the cell is damaged and the membranes disrupted, the enzymes are released into blood, hence increased serum enzyme activity is often the first change noticed in liver disease or injury.

Initial AST serum values and those seen in the course of the experiment showed great inter-individual variation. During the experiment, the lowest AST plasma concentration in an individual goat was 42 U/l and the highest was 408 U/l while the monthly group mean AST values ranged from 85.5U/l to 128.2 U/L. Kaneko *et al* (1997) reported that the serum concentration of AST was 167-513 U/L for goats. Although the serum concentration of AST found in this study was generally lower than the normal range for the species, there was no significant difference between the mean monthly values of the

treated and the control group ($p>0.05$). The low level of the serum AST in this study could therefore not be attributed to the treatment effect. While aspartate aminotransferase (AST) is present in most tissues and organs at varying concentrations, it is useful in evaluating hepatocellular and muscular injury because of its high activity in these tissues. Two isoenzymes of AST are present in the liver; one is mitochondrial and the other is from the cytoplasm. AST is generally elevated in acute hepatic injury and may be normal or slightly elevated in chronic disease (Cornelius, 1989).

High concentrations of alkaline phosphatase (AP) are found in the intestines, kidney, bone and liver. The activity of the AP is highest on the absorptive or secretory surfaces of cells (Kaplan, 1972). There were great inter individual differences in the serum AP values in the present study. The monthly AP group means (172 to 707 U/L), were higher than the normal range of (93- 387 U/L, (Kaneko, 1997). However, there was no significant difference between the mean monthly values of the treated and the control group ($p>0.05$). The elevations could then not be associated with the exposure to the tsetse repellent. Elevations of serum AP are observed in normal growing animals or in adult animals with increased osteoblastic activity. Serum AP is also elevated in both acute and chronic liver diseases, but marked elevations are indicative of cholestasis, with the highest plasma concentrations observed in animals with cholangitis, biliary cirrhosis, or extrahepatic bile-duct obstruction (Bud, 1997).

Gamma glutamyl transpeptidase (GGT) is a membrane bound enzyme that catalyses the transfer of gamma glutamyl groups from gamma glutamylpeptides to other peptides and

amino acids which are necessary for transfer of certain substances across the cell membrane. The enzyme is found in cells with high rates of secretion or absorption and especially the liver, kidney, pancreas and intestines (Bud, 1997). GGT activity is relatively high in the livers of cows, horses, sheep, and goats but lower in dogs and cats. Elevation of GGT serum levels is also observed in liver diseases (Bud, 1997). In the present study, the individual serum levels of GGT and the monthly group mean values were all within the normal range for the species of 20-56 U/L (Kaneko *et al* 1997). There was no significant difference between the means of the treated and the control groups ($p > 0.05$).

The number of circulating blood cells in animals varies with normal physiological states as well as with pathological conditions. Considerable variations that normally exist among a given population are attributed to sex, age, nutrition, ambient temperature, diurnal and sex cycles. Therefore, the normal values given for comparison are usually considered as guidelines rather than rigid criteria. In the present study, monthly means in all the groups for the RBC count were within the normal range for the species. There was also no significant difference between the means of the exposed and the control groups ($p>0.05$). There was variation across the months in the mean monthly values of the granulocytes and lymphocyte count that could not be associated with the treatment since the trend was similar in the treated and the control groups. There was no significant difference between the mean cell levels of the exposed and the control group ($p> 0.05$). The lymphocyte levels were initially lower than the normal levels (< 50 %) in the first five months but within the normal range towards the end of the study in all the groups.

The monthly group means for the WBC count were above the reference values for the species (4-13 x 10^9 /L) in all the groups, including the baseline data with values in the range of 14.4 to 20.27 x10^9 /L. There was however no significant difference in the monthly means between the treated and the control group.

The weight gains over the months were different with some months (months 2 and 3) goats experiencing weight loss. This was associated with the quality of the hay being used during this period. Analysis with SAS, using Repeated measures analysis of variance found the weights in the three treatment groups not significantly different at 5% significant level ($p > 0.05$).

The usual lesion in toxic hepatitis is centrilobular cloudy swelling when mild in degree and accompanied by extensive necrosis when severe in degree. Fibrosis develops where the necrosis is severe enough (Blood *et al*, 1989). In the kidney, toxins generally cause degenerative and inflammatory nephrosis of the renal tubules. There is necrosis and desquamation of the tubular epithelium, with hyaline casts in the dilated tubules (Blood *et al*, 1989). Dodman *et al* (1976) in a case of cat poisoning by a phenol reported at histopathology, congestion of the major organs especially the lungs that also had oedema fluid in the alveolar spaces. There were also focal hemorrhages in the adrenal cortices and protein casts in the renal tubules. There were no histological findings in this study that would support phenol toxicity. This correlates well with the observations of normal biochemical and hematological parameters in the study. In view of the absence of pathological changes in the exposed animals, it is reasonable to conclude that either the

repellent was not adequately absorbed through the respiratory mucous membranes or it is effectively non-toxic to goats.

5.2 Summary of Observations and Conclusions

The following general conclusions can be made based on the findings of the current study.

1. The median lethal dose of the synthetic tsetse fly repellent is 40.3 mg/kg body weight.
2. The clinical signs of acute poisoning are decreased locomotor activity, an initial increase followed by a decrease in respiratory rate and an increased depth in respiration, terminal convulsions, coma and death.
3. The synthetic tsetse fly repellent is an eye irritant causing conjunctivitis, corneal opacity and chemosis.
4. The repellent is a mild skin irritant causing transient oedema and erythma.
5. Hematological parameters (RBC, WBC, Granulocytes and lymphocytes) in goats exposed to low and high doses of the repellent vapours were not significantly different from the goats not exposed to the repellent vapours hence exposure to the repellent caused no adverse effects to the goats.
6. Biochemical parameters (AST, ALP and GGT) in goats exposed to low and high doses of the repellent vapours were not significantly different from the goats not exposed to the repellent vapours.
7. There were no pathological changes in the liver, heart and kidney in all goats. The interstitial pneumonia observed in the lungs was not associated with the exposure to the repellent since the control group was equally affected.

8. The weight gain in the goats exposed to low and high doses of the repellent vapours was not significantly different from that of the goats not exposed to the repellent vapours.

5.3 Recommendations and cautions

The synthetic repellent can be used to protect livestock but caution should be exercised to prevent contact with the mucous membranes. Contact with the skin should be minimal. When used correctly according to label instructions and with the appropriate precautions, repellents should present minimal risk to the environment. However, the small drips and spillages, which occur as part of normal agriculture practice, can result in a significant amount of environmental contamination. Due to practicalities and lack of awareness of the danger involved, it is possible that many users do not comply with these instructions. It is highly recommended that thorough training of the user should be undertaken.

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APPENDICES

Appendix 1 Preliminary determination of the 24 hour intraperitoneal LD_{50} in mice: Survival/deaths at 24 hours.

Serial No.	Sex	Body wt (g)	Dosage (mg/kg)	Vol (ml)	Conc. (mg/ml)	Results (24 hrs)
1	M	32.54	10	0.32	0.99	Survived
2	F	30.81	10	0.31	0.99	Survived
3	M	32.42	20	0.65	0.99	Survived
4	F	19.44	20	0.39	0.99	Survived
5	M	28.05	30	0.08	9.9	Survived
6	F	24.93	30	0.07	9.9	Survived
7	M	28.69	40	0.11	9.9	Survived
8	F	32.14	40	0.12	9.9	Died
9	M	23.60	50	0.11	9.9	Died
10	F	26.80	50	0.13	9.9	Survived
11	M	29.85	60	0.18	9.9	Died
12	F	28.93	60	0.17	9.9	Died
13	M	25.95	70	0.18	9.9	Died
14	F	23.60	70	0.16	9.9	Died
15	M	32.95	0(corn oil)	0.6	0.0	Survived
16	F	25.07	0(corn oil)	0.6	0.0	Survived

Appendix 2. Deaths/ survival of mice after 24 hours of IP injection with the synthetic repellent.

Dose	Number alive			Number dead		
mg/kg bwt	Females	Males	Total	Females	Males	Total
30	5	5	10	0	0	0
35	1	1	2	4	4	8
40	3	3	6	2	2	4
45	1	2	3	4	3	7
50	1	0	1	4	5	9
55	0	1	1	5	4	9
60	1	1	2	4	4	8
65	0	0	0	5	5	10
0 (control)	5	5	10	0	0	0

Appendix 3. Draize Scoring Criteria (Draize, 1959).

Erythema and Eschar formation (most severely affected area graded)	SCORE
No erythma	0
Slight, barely perceptible erythma	1
Well defined erythma	2
Moderate to severe erythma	3
Severe, beet red erythma with injuries in depth	4
Edema formation (most severely affected area graded)	SCORE
No edema	0
Slight, barely perceptible with raised edges	1
Slight oedema	2
Moderate oedema with the surface raised approximately one millimeter	3
Severe oedema with the area raised more than before and extending beyond the area of exposure	4

Appendix 4. Evaluation of primary irritation index.

INDEX	EVALUATION
0.0	No irritation
0.04 - 0.99	Irritation barely perceptible
1.00 - 1.99	Slight irritation
2.00 -2.99	Mild irritation
3.00 -5.99	Moderate irritation
6.00 -8.00	Severe irritation

Appendix 5 Grades for ocular lesions

Cornea	
Opacity: Degree of density (area most dense taken for reading). No ulceration or opacity	0
Scattered or diffuse areas of opacity (other than slight dulling of normal lustre), details of iris clearly visible	1
Easily discernible translucent area, details of iris slightly obscured	2
Nacrous area, no details or iris visible, size of pupil barely discernible	3
Opaque cornea, iris not discernible through the opacity	4
Iris	
Normal	0
Markedly deepened rugae, congestion, swelling moderate circumcorneal hyperaemia, or injection, any of these or combination of any thereof, iris still reacting to light (sluggish reaction is positive)	1
No reaction to light, haemorrhage, gross destruction (any or all of these)	2

Conjunctivae	
Redness (refers to palpebral and bulbar conjunctivae, excluding cornea and iris)	
Blood vessels normal	0
Some blood vessels definitely hyperaemic (injected)	1
Diffuse crimson colour, individual vessels not easily discernible	2
Diffuse beefy red	3
Chemosis (refers to lids and /or nictating membranes)	
No swelling	0
Any swelling above normal (includes nictitating membranes)	1
Obvious swelling with partial eversion of lids	2
Swelling with lids about half closed	3
Swelling with lids more than half closed	4

Appendix 6: Serum mean (sem) monthly levels of GGT of the exposed and control groups over the experimental period.

Months		0	1	2	3	4	5	6	7	8
Mean (±se) Serum GGT	1	37.2±2.6	27.6±3.6	51.1±5.6	46.5±2.8	37.3±1.8	32.15±1.5	33.77±1.0	35.9±2.2	41.7±1.5
U/L		n =14	n =14	n =14	n =13	n =10	n =10	n =10	n =10	n =10
	2	36.9±2.8	32.3±4.2	56.8±4.7	50.3±3.0	37.5±1.9	31.7±1.5	34.6±1.5	33.6±2.1	40.6±2.5
		n =14	n =14	n =13	n =13	n =10	n =10	n =10	n =10	n =10
	3	36.6±2.8	31.8±2.8	54.1±9.7	45.5±3.4	36.3±2.7	28.3±1.2	30.9±1.3	30.5±2.0	37.3±1.0
		n =14	n =14	n =13	n =13	n =10	n =10	n =10	n =10	n =10

Appendix 7: Serum mean (sem) monthly levels of ALP of the exposed and control groups over the experimental period.

Months		0	1	2	3	4	5	6	7	8
Mean	1	263.5±33.3	209.7±53.0	645±81.5	288±57.2	404.3±70.3	310.8±84.7	259.6±56.6	501.2±85.2	434.5±76.3
(±se)		n =14	n =14	n =14	n =13	n =10	n =10	n =10	n =10	n =10
serum	2	204.1±27.1	146±38.3	573.9±65.5	337.7±74.2	445.5±62.3	450.3±64.3	336±85.8	519.2±76.1	399.9±54.8
ALP		n =14	n =14	n =13	n =13	n =10	n =10	n =10	n =10	n =10
U/L	3	259.9±30.3	172.6±34.1	707±121.1	305.3±62.2	431.7±75.9	416.6±83.6	298.1±47.0	360.9±83.9	321.3±89.0
		n =14	n =14	n =13	n =13	n =10	n =10	n =10	n =10	n =10

Appendix 8: Serum mean (sem) monthly levels of AST of the exposed and control groups over the experimental period.

Months		0	1	2	3	4	5	6	7	8
Mean	1	113.0±4.8	128.0±8.5	124.3±10.6	126.1±9.6	85.5±10.5	95.0±8.6	104.2±10.9	134.7±32.2	128.2±18.2
(±se)		n =14	n =14	n =14	n =13	n =10	n =10	n =10	n =10	n =10
serum	2	118.0±16.9	126.0±10.6	118.6±9.6	104.3±10.9	93.7±15.5	97.2±15.9	115.5±15.5	96.5±8.6	118.6±8.9
AST		n =14	n =14	n =13	n =13	n =10	n =10	n =10	n =10	n =10
U/L	3	104.0±14.1	97.6±11.9	102.1±14.1	116.6±15.6	101.7±26.6	95.7±9.6	85.6±11.04	109.7±19.5	116.2±14.1
		n =14	n =14	n =13	n =13	n =10	n =10	n =10	n =10	n =10

Appendix 9: Serum mean (sem) monthly levels of White blood cell count (WBC) of the exposed and control groups over the experimental period.

Months		0	1	2	3	4	5	6	7	8
Mean (±se) serum WBC	1	15.0±0.5	16.2±0.6	18.0±1.2	20.2±1.0	17.9±1.1	18.3±1.6	17.8±1.1	17.0±0.9	18.0±0.8
(10^3/mm)		n =14	n =14	n =14	n =13	n =10	n =10	n =10	n =10	n =10
	2	14.6±0.5	15.3±0.4	15.6±1.0	15.9±1.4	18.3±1.2	15.3±1.7	16.8±0.9	17.3±1.1	18.0±1.1
		n =14	n =14	n =13	n =13	n =10	n =10	n =10	n =10	n =10
	3	14.4±0.6	15.0±0.7	15.2±1.7	15.5±1.0	17.4±1.0	15.6±1.8	16.3±0.9	15.2±0.7	15.0±0.8
		n =14	n =14	n =13	n =13	n =10	n =10	n =10	n =10	n =10

Appendix 10: Serum mean (sem) monthly levels of Red blood cell count (RBC) of the exposed and control groups over the experimental period.

Months		0	1	2	3	4	5	6	7	8
Mean (±se) serum RBC	1	12.2±0.3	12.6±0.3	12.9±0.6	12.5±0.51	12.3±0.3	12.4±0.5	12.3±0.4	12.1±0.4	10.5±0.4
M/mm^3		n =14	n =14	n =14	n =13	n =10	n =10	n =10	n =10	n =10
	2	12.9±0.3	13.0±0.4	14.6±0.6	12.3±0.4	12.8±0.5	12.1±0.4	12.4±0.4	12.0±0.4	8.8±0.8
		n =14	n =14	n =13	n =13	n =10	n =10	n =10	n =10	n =10
	3	12.1±0.4	12.6±0.3	14.1±0.6	13.3±0.3	12.6±0.5	12.3±0.6	12.5±0.5	12.1±0.4	11.4±0.3
		n =14	n =14	n =13	n =13	n =10	n =10	n =10	n =10	n =10

Appendix 11: Serum mean (sem) monthly levels of Lymphocyte cell count of the exposed and control groups over the experimental period.

Months		0	1	2	3	4	5	6	7	8
Mean (±se) serum	1	48.8±1.4	45.6±1.8	40.7±2.5	45.0±1.7	42.3±2.8	64.4±3.5	67.0±3.0	68.8±2.8	67.1±2.7
Lymphocytes (%)		n =14	n =14	n =14	n =13	n =10	n =10	n =10	n =10	n =10
	2	44.3±1.4	45.1±1.3	44.0±2.3	44.0±2.2	38.7±1.4	65.0±4.3	71.0±2.3	64.8±3.9	61.4±3.4
		n =14	n =14	n =13	n =13	n =10	n =10	n =10	n =10	n =10
	3	41.9±1.4	42.1±1.4	44.7±2.4	40.9±2.1	38.0±2.3	65.8±2.5	68.2±3.0	69.4±2.6	69.1±3.3
		n =14	n =14	n =13	n =13	n =10	n =10	n =10	n =10	n =10

Appendix 12:Serum mean (sem) monthly levels of Granulocyte cell count of the exposed and control groups over the experimental period.

Months			0	1	2	3	4	5	6	7	8
Mean	(±se)	serum	1	34.1±2.6	43.8±1.7	52.5±2.6	48.0±1.7	50.3±2.8	34.1±3.5	30.2±2.9	31.1±3.0	32.6±2.9
Granulocytes (%)				n =14	n =14	n =14	n =13	n =10	n =10	n =10	n =10	n =10
			2	45.2±2.7	46.0±1.6	49.1±2.2	49.5±2.0	54.8±1.4	33.5±3.9	23.7±2.2	37.0±2.9	36.2±1.9
				n =14	n =14	n =13	n =13	n =10	n =10	n =10	n =10	n =10
			3	42.9±2.3	39.4±1.8	48.4±2.2	51.7±2.1	55.2±2.2	30.2±2.1	24.3±1.6	29.2±3.4	30.2±2.3
				n =14	n =14	n =13	n =13	n =10	n =10	n =10	n =10	n =10