STUDIES ON THE CELLULAR AND HUMORAL IMMUNE RESPONSES OF THE TROPICAL BONT TICK *AMBLYOMMA VARIBGATUM* AND THE BROWN EAR TICK *RHIPICEPHALUS APPENDICULATUS* AGAINST PATHOGENS AND PARASITOIDS

A Thesis

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Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria

in fulfillment of the requirements for the degree of

Doctor of Philosophy in Veterinary Entomology

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CERTIFICATION

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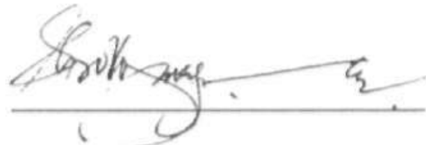

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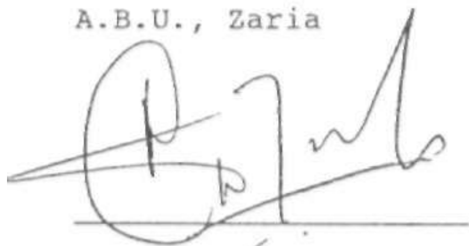

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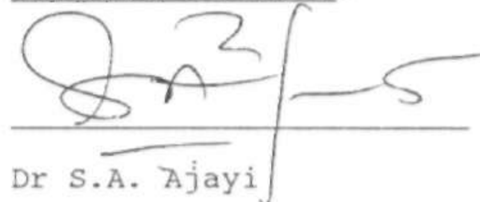

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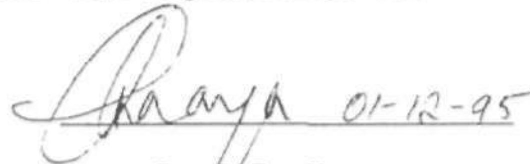

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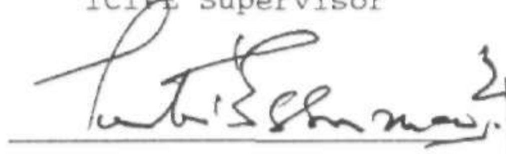

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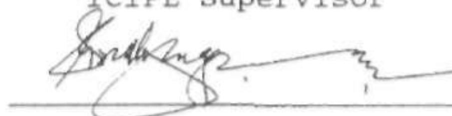

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The work of other investigators in the areas covered in this thesis is duly acknowledged and referred to appropriately. No part of this thesis has been submitted elsewhere for a degree or diploma.

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DEDICATION

To my parents, Niara and Tenimba Bengaly, whose wish has always been to see me reaching the highest level.

TABLE OF CONTENTS

Title	Page
Thesis approval	i
Declaration	iii
Acknowledgements	iv
Dedication	vi
Table of contents	vii
List of tables	xii
List of figures	xiii
Abstract	xviii

CHAPTER ONE

1.	Introduction	1
1.1	Objectives	6

CHAPTER TWO

2.	Literature review	8
2.1	The ticks	8
2.2	Classification	9
2.2.1	*Amblyomma variegatum*	10
2.2.2	*Rhipicephalus appendiculatus*	12
2.3	Life cycle	13
2.4	Economic, veterinary and medical importance	14
2.4.1	Rickettsial diseases	15

a.	Cowdriosis	15
b.	Boutonneuse fever	16
c.	Epidemic typhus	16
2.4.2.	Protozoal diseases (Theileriosis)	17
2.4.3.	Tick paralysis	18
2.4.4.	Viral diseases	19
1.	Arboviruses associated with *A. variegatum*	19
2.	Arboviruses associated with R. *appendiculatus*	21
2.5.	Immune mechanisms in arthropods	21
2.5.1.	Morphological types	22
2.5.2.	Cellular immune responses	26
2.5.2.1.	Phagocytosis	27
2.5.2.2.	Nodule formation	28
2.5.3.	Encapsulation	29
2.5.3.1.	Cellular encapsulation	29
2.5.3.2.	Humoral encapsulation	30
2.5.4.	Humoral immune response	31
2.5.4.1.	Antibacterial proteins	31
2.5.4.2.	Humoral lectins and lecti-like factors	34
2.6.	Insect immune responses to parasitoids	35
2.7.	Insect immune responses to fungal infections	37
2.8.	Tick control methods	39
2.8.1.	Chemical control	39
2.8.1.1.	Control of parasitic stages	39
2.8.1.2.	Treatment of free-living stages	40
2.8.2.	Non-chemical control methods	41

2.8.3. Use of tick-resistant cattle 41
2.8.4. Biological control 42
2.8.4.1. Predators 42
2.8.4.2. Pathogens 44
2.8.4.2.1. Bacteria 44
2.8.4.2.2. Fungi 45
2.8.4.3. Parasitic wasps 48

CHAPTER TRHEE

3. General materials and methods 52
3.1. Experimental animals and microrganisms 52
3.1.1 Ticks 52
3.1.2. Rabbits 52
3.1.3. Parasitoids 52
3.1.4. Bacteria 53
3.1.5. Fungi 53
3.2. Microscope 53
3.3. Haemocyte studies.................. 54
3.3.1. Morphology 54
3.3.2. Total and differential haemocyte counts 54
3.3.3. Cellular immune reaction 55

CHAPTER FOUR

4. Characterization and comparison of different morphological types of haemocytes in *Amblyomma variegatum* and *Rhipicephalus appendiculatus* as well as total and differential haemocytes counts

in bacteria, fungi and parasitoid infected and unfected ticks.

4.1	Materials and methods	58
4.1.1.	Characterisation of tick haemocytes	58
4.1.2.	Haemocyte count	60
4.1.2.1.	Uninfected ticks	60
4.1.2.1.1.	Total haemocyte count (THC)	60
4.1.2.1.2.	Differential haemocyte count (DHC)	61
4.1.2.2.	Infected ticks	61
4.1.2.2.1.	Bacterial infection	61
4.1.2.2.2.	Fungal infection	62
4.1.2.4.	Parasitization	63
4.2.	Results	65
4.2.1.	Characterisation	65
4.2.2.	Haemocyte count	68
4.2.2.1.	Uninfected ticks	68
4.2.2.2.	Infected ticks	69
4.3.	Discussion	71
4.3.1.	Characterisation	71
4.3.2.	Total haemocyte count	73

CHAPTER FIVE

5. Comparison of cellular and humoral immune reactions in different sexes and ages of ticks of both species, the level of pathogens that induce antibacterial activity and the type of humoral antibacterial factors produced.

in bacteria, fungi and parasitoid infected and unfected ticks.

4.1	Materials and methods	58
4.1.1.	Characterisation of tick haemocytes	58
4.1.2.	Haemocyte count	60
4.1.2.1.	Uninfected ticks	60
4.1.2.1.1.	Total haemocyte count (THC)	60
4.1.2.1.2.	Differential haemocyte count (DHC)	61
4.1.2.2.	Infected ticks	61
4.1.2.2.1.	Bacterial infection	61
4.1.2.2.2.	Fungal infection	62
4.1.2.4.	Parasitization	63
4.2.	Results	65
4.2.1.	Characterisation	65
4.2.2.	Haemocyte count	68
4.2.2.1.	Uninfected ticks	68
4.2.2.2.	Infected ticks	69
4.3.	Discussion	71
4.3.1.	Characterisation	71
4.3.2.	Total haemocyte count	73

CHAPTER FIVE

5. Comparison of cellular and humoral immune reactions in different sexes and ages of ticks of both species, the level of pathogens that induce antibacterial activity and the type of humoral antibacterial factors produced.

5.1. Materials and methods 95

5.1.1. Cellular immune reactions 95

5.1.2. Humoral immune reactions 97

5.2. Results 101

5.2.1. Cellular immune reaction 102

5.2.2. Humoral immune reactions 103

5.3. Discussion 105

5.3.1. Nodule formation 105

5.3.2. Encapsulation 106

5.3.3. Lysozyme activity 107

CHAPTER SIX

6. The immune status of ticks infected with the fungi *B. bassiana*, *M. anisopliae* and the hymenopteran tick parasitoid *I. hookeri*.

6.1. Materials and methods 122

6.1.1. Fungal infection 123

6.1.2. Parasitization 123

6.1.2.1. Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis 123

6.1.2.2. Development of the parasitoid 124

6.2. Results 125

6.2.1. Reaction to fungal infection 125

6.2.2. Parasitization 125

6.3. Discussion 128

CHAPTER VII

General discussion and conclusion

7.1. Discussion 143

7.2. Conclusion 148

7.3. Suggestion 149

REFERENCES 150

LIST OF TABLES

Table 4.1 Total haemocyte count haemolymph of *Amblyomma variegatum* and *Rhipicephalus appendiculatus* 77

Table 4.2 Effect of bacteria on total haemocytes of *Amblyomma variegatum* 78

Table 4.3 Percent differential haemocyte count on infected and uninfected ticks. Haemocytes of infected ticks counted at 24 hours post-infection 79

LIST OF FIGURES

Fig. 4.1 Total haemocyte number in nymphs of *Amblyomma variegatum* parasitized by *Ixodiphagus hookeri* 80

Fig. 4.2 Electron micrograph of a prohaemocyte with high nucleocytoplasmic ratio. Rough endoplasmic reticulum (rer) forms a narrow band varying in electron density around the nuclei. Filopodia (f), heterochromatin (hc), mitochondrion (m). (X 19500) 81

Fig. 4.3. Plasmatocyte (spindle shape) in the haemolymph of *A. variegatum*, X 1000. Modified Pappenheim stain 82

Fig. 4.4. Light micrograph of a spindle shaped plasmatocyte (centre) and Oenocytoid, (X 1500). Modified Pappenheim stain 83

Fig. 4.5. Electron micrograph of a spindle shaped plasmatocyte showing filopodial extension (f), mitochondria (m). Note rer around the nucleus and on the side. (X 12000) 84

Fig. 4.6. Electron micrograph of an oval shaped plasmatocytes showing filopodial extension (f), mitochondria (m). Note that the rer is well developped around the nucleus and on the side (rer), mitochondria (m), (X 19500) 85

Fig. 4.7. Electron micrograph of a round shaped plasmatocytes showing filopodial extension (f), mitochondria (m),

rough endoplasmic reticulum (rer), phagocytic particles, (X 19500) 86

Fig. 4.8 Granulocyte in *A. variegatum*. Modified Pappenheim stain (X 1500) 87

Fig. 4.9 Granulocyte (spindle shape) in *A. variegatum*, Modified Pappenheim stain. (X 1500) 88

Fig. 4.10 Electron micrograph of an oval granulocyte showing round and oval granules. Note that some granules are electron dense. Invaginations (i), filopodia (f), (X 15000) 89

Fig. 4.11 Electron micrograph of a round sperule cell showing several uniform granules. Rough endoplasmic reticulum (rer) around nuclei. Free ribosomes, Mitochondria, (X 12000) 90

Fig. 4.12 Sperule cell, round form. Modified Pappenheim method (X 1500) 91

Fig. 4.13 Oenocytoid. Pappenheim method. (X 1500) 92

Fig. 4.14 Electron micrograph of oenocytoid showing few granules. Nucleus (n) and mitochondria (m), (X 19500) 93

Fig. 4.15 *A. variegatum* infected with *Metarhizium anisopliae*, melanization 1 week post-infection, (X 1500) 94

Fig. 5.1 Lysozyme production in haemolymph of *Amblyomma variegatum* infected bacteria compared to haemolymph of non infected ticks and standard egg white lysozyme 109

Fig. 5.2 Lysozyme production in haemolymph of *Rhipicephalus appendculatus* infected bacteria compared to haemolymph of non infected ticks and standard egg white lysozyme 110

Fig. 5.3 Cell mass looking like granuloma in *A. variegatum infected with* E. coli 2 x $10^4\mu l$, (X 1500) 111

Fig. 5.4 Melanisation in *A. variegatum* infected with *Bacillus thuringiensis* 2 x $10^5\mu l$. (X 1500) 112

Fig. 5.5 Haemolymph of ticks collected at 24 hours post-infection. 1. Non infected *Amblyomma variegatum*, 2. *A. variegatum* infected with *Micrococcus luteus*, 3. Non infected *Rhipicephalus appendiculatus*, 4. *R. appendiculatus* infected with *M. luteus*, 5. *R. appendiculatus* infected with *E. coli* 113

Fig. 5.6 Tip of microcapillary tube covered with a withish capsule after being inserted in an engorged female of *R. appendiculatus*. (X 600) 114

Fig 5.7 Encapsulation 24 hours after implantation, debut of capsule formation. Deposition of melanized amorphous matrix on the surface of the araldite, haemocytes have loose contact with the surface of the implant, (X 15,000) 115

Fig 5.8a Encapsulation, 48 hours after implantation. Different types of haemocytes (plasmatocytes, and granulocytes) flatten around the melanized surface of the araldite, electron dense granules (G),

binucleated cells (arrow), mitochondria (m), (X 7,500) 116

Fig. 5.8b Encapsulation, 48 hours after implantation. Inner layer of the capsule composed of particles of haemocytes, (X 7,500) 117

Fig. 5.8c 1 Encapsulation, 48 hours after implantation. Haemocytes participating in the formation of capsule, binucleated plasmatocyte, mitochondria, structured granules, (X 15,000) 118

Fig. 5.8c 2 Encapsulation, 48 hours after implantation. Haemocytes participating in the formation of capsule, granulocyte with electron dense granules, (X 15,000) 119

Fig. 5.9a Encapsulation, 72 hours after implantation. Flatten haemocytes, gap junctions, (X 7,500) 120

Fig. 5.9b Encapsulation, 72 hours after implantation. Higher magnification of gap junctions, (X 42,000) 121

Fig. 6.1 Beginning of a Nodule formation in the haemolymph of *A. variegatum* infected with *M. anisopliae* (10^8), (X 1500) 132

Fig. 6.2 A female parasitoid laying eggs inside a nymph of *Amblyomma variegatum*. (X 187) 133

Fig. 6.3 Eggs of the tick parasitoid, 3 hours post-infestation, (X 1500) 134

Fig. 6.4 Immune reaction of the nymph of *A. variegatum* to the parasitoid, formation of a cell mass, (X 1500),

wet mount 135

Fig. 6.5 Embryo in cell mass at 3 hours post-infection, (X 600) 136

Fig. 6.6 First instar larvae, 3 days post-infestation, (X 240) 137

Fig. 6.7 Second instar larvae, 4 days post-infestation. Note see the trachea and the digestive system. (X 150) 138

Fig. 6.8 Third instar larvae of the tick parasitoid at 13 days post-infestation, (X 60) 139

Fig. 6.9 Parasitoid, inside the nymph, pupating (white) on one side and leaving their feces (dark) *on* the other side, (X 240) 140

Fig. 6.10 *The hole (arrow) through which adult parasitoids emerge from the body of the engorged nymph of A. variegatum*, (X 90) 141

Fig. 6.11 Haemolymph of nymphs of *A. variegatum* parasitized by *Ixodiphagus hookeri*. Haemolymph collected at 12 hours (1), 24 hours (2), 48 hours post-parasitization (3). Haemolymph from non infected nymphs and adult of *A. variegatum* (4 and 5) 142

ABSTRACT

Amblyomma variegatum and *Rhipicephalus appendiculatus* are pests of domestic and to a certain extend wild animals. While feeding they transmit important diseases, among which are cowdriosis and East coast fever. It is generally known that pathogens after being ingested with the blood by the tick, move through the gut and reach the salivary gland where they are injected in a host with the saliva of the tick during feeding. Hardly anything is known on tick immune system. The goal of this work was to study the immune system of both A. *variegatum* and *R. appendiculatus.*

Five types of haemocytes were found in both tick species. They were plasmatocytes, granulocytes, spherular cells, prohaemocytes and oenocytoids. There was no significant difference between total haemocyte count in adults and nymphs of A. *variegatum* and adults of *R. appendiculatus.* However total haemocyte count in infected ticks were significantly ($P<0.05$) higher than non-infected ones. Differential haemocyte count in non-infected showed a significant predominance of spherules and granular cells. The same trend was observed in ticks infected with bacteria.

Tick haemocytes reacted to the presence of foreign bodies like bacteria, fungi and parasitoids by forming nodules or granulomma. All the ticks injected with *Metarhizium anisopliae* and *Beauveria*

bassiana died within 5 to 10 days. None of the infected engorged females layed eggs. None of the engorged nymphs molted. Encapsulation was demonstrated using araldite as implant. Lysozyme activity was induced by injecting bacteria. *Bacillus thuringiensis* was observed to have induced more lysozyme activity than other bacteria.

The tick parasitoid *Ixodiphagus hookeri* laid eggs in all the stages of both *A. variegatum* and *R. appendiculatus* but completed its development in the nymph of *A. variegatum* despite the cellular and humoral reactions of the host. The parasitoid goes through 3 larval stages, pupates, molt and emerges generally through one hole after 4 to 6 weeks post-infestation, leaving a dried empty cuticle.

CHAPTER I

INTRODUCTION

Ticks are obligate blood suckers that parasitize all terrestrial vertebrates. The most vulnerable being mammals (Harwood and James, 1979). When feeding, ticks transmit a large number and a variety of infectious agents like *Babesia*, *Theileria*, *Anaplasma* and *Cowdria* to their host(s). Some of the pathogens are only slightly pathogenic to livestock but many cause disease in man (e.g. Lyme disease or Borreliosis caused by *Borrelia burgdorferi*). Others cause diseases of tremendous economic importance in livestock (Anonymous, 1991).

The Ixodidea order is composed of 3 families; Ixodidae (hard ticks), Argasidae (soft ticks) and Nuttalliellidae. The Ixodidae family is the most important because of the diseases they transmit and the amount of losses of animal proteins especially in underdeveloped countries.

Evidence shows that tick feeding alone, without the transmission of pathogens, can cause considerable harm to their hosts. Hunter and Hooker (1907) reported that as many as 200 pounds of blood may be withdrawn from a large host animal by ticks in a single season. Morel (1981) documented that a single tick can suck up to 2 ml of blood and cause a significant loss of productivity.

Sutherst et al. (1983) reported that the daily loss by a cow due to an engorged *Boophilus microplus* female can reach 1.52 g.

In addition to disturbances like loss of appetite, weight and productivity which they cause, ticks are responsible for the transmission of several diseases of economic importance to man and animals. Harwood and James (1979) mentioned 4 groups of diseases: (1) Dermatosis, inflammation, itching, swelling and ulceration at the site of the bite, or skin ulceration and lesions resulting from improper or partial removal of tick mouthparts; (2) Exsanguination, a serious condition in which a heavily infested animal develops anemia, (3) Otoascariasis, infestation of the auditory canal by ticks, with possible serious secondary infection. (4) Infections transmitted by ticks include Piroplamosis, Anaplasmosis, and Cowdriosis. The latter group is the most important economically because it is the first and second most important livestock disease in West and East Africa respectively (Neitz, 1968; Uilenberg, 1983). Cowdriosis is transmitted by *Amblyomma variegatum* the only tick species that covers the sub-saharan Africa (Walker and Olwage, 1987). The tick has been found on buffaloes, antelopes, elands, bushbucks, giraffes, canes, zebra, rhinocerous, and birds (Hoogstraal, 1956). The other important livestock disease is East Coast Fever. It is prevalent in the central and eastern regions of Africa where its vector *Rhipicephlus appendiculatus* is found. The causal agent is *Theileria parva*.

Several control methods have been used against ticks. Among them, the most widely used is chemical control which can be directed against the free-living stages or the parasitic stages on hosts. Acaricide treatment of vegetation has been done in specific sites (eg. along trails) in recreational areas in the USA and elsewhere to reduce the risk of tick attachment to people (Fraser et al., 1991). Treatment of hosts with acaricides to kill attached ticks is the most widely used method of tick control in livestock (Faser et al., 1991). Several chemical groups such as arsenic, organophosphates, carbamates, amidines and synthetic pyrethroids have been used to control tick and tick-borne diseases for years (Matthewson, 1984). These chemicals have led to the improvement of livestock production in Africa and other parts of the world (Garris et al., 1989; FAO, 1984). However the increasing cost of application (Sutherst et al., 1982) and the development of resistance by some ticks due to prolonged or incorrect use of acaricides have led to the development of alternative control methods. Pasture spelling (Wharton et al., 1969), habitat modification, use of chemically-baited pheromones (Norval and Rechav, 1979) have been tried. Attention is being paid to the development of other control approaches. These include the use of anti-tick grasses (Thompson et al., 1979) and legumes (Mwangi, 1991), parasitoids (Larousse et al. 1928; Mather et al., 1987; Mwangi, 1991), fungi (Mwangi, 1990; Kaaya, 1992) as well as resistant cattle (Latif, 1986). Immunological manipulation of the host's immune system to hinder successful feeding of the parasites

(the ticks) is also being tried with varying degrees of success. This involves the immunization of the host with tick antigen (Kemp et al., 1986; Opdebeeck et al., 1988; Willadsen, 1988; Mongi et al., 1986; Essuman, 1991).

Biological control of ticks using predators, pathogens and parasitoids have been tried but with little success. The cattle egret *Ardela ibis* was introduced in western Australia but was found to have little effect on ticks. The use of ants as biological agents of ticks was found impracticable and undesirable although they are predators of cattle ticks (Australian Government Publishing Service, 1975). The tick parasitoid, *Ixodiphagus hookeri*, after being released for 12 years in Maushion Islands, was found to reduce *Dermacentor variablis* population although not sufficient to eliminate the need for other control methods (Larousse, et al., 1928). This little success of the parasitoid may be due to the lack of knowledge of the biology, the relationship with the tick and the establishment of the parasitoid in the environment.

A lot of studies have been done on the resistance of cattle to ticks and also some immunomanipulation in order to render cattle resistant to ticks. A selection programme of animals designed to develop a breed resistant to cattle tick *Boophilus microplus* has been initiated in the sixties and seventies. Resistance to cattle tick has been increased by selection of animals which have the

fewest ticks resulting from artificial infestations of male animals by theoretical calculation from heritability estimates (Heweston, 1979). In Africa, reports indicate that the local breeds (Bos indicus) acquire a more effective natural resistance to tick infestation than the exotic ones (Latif, 1984; Norval, 1986).

A lot of studies have been done on the role played by the immune system in vertebrates and arthropods. Gupta in 1986 established some similarities between B and T lymphocytes in mammalians and granulocytes and plasmatocytes in arthropods. He mentioned their capacity of nonself recognition. There has been very few studies on tick haemocytes and the role they play in ticks defence system.

There have been a lot of studies on arthropod immune defence system and the recognition of foreigness (Gupta 1979, Ratcliffe 1982). Most of them have been found to use cellular and humoral defence to face foreign bodies. Studies on cellular defence have shown that insects blood cells ingest foreign bodies by means of phagocytosis and encapsulation. A lot is known about the immune responses of host animals to infection by pathogens, parasites, including tick and insects to foreign bodies, but hardly anything is known about the immune system of ticks and their response to pathogens and parasites. The ability of a tick to mount immune response (cellular and humoral) to invading pathogens in nature determines its ability to survive and reproduce. On the other hand,

if the immune response of a tick against a certain pathogen or a parasitoid is found to be inefficient, as it happens in insects with some pathogens eg. *Bacillus thuringiensis* producing 2 types of immune inhibitors which specially degrade Attacins and Cecropins in *Hyalophora cecropia* (Sidén et al. 1979), then this organism can be a good candidate for biological control of ticks. The objective of this study is, therefore, to study the cellular and humoral immune responses of the tropical bont tick *Amblyomma variegatum* and the brown ear tick *Rhipicephalus appendiculatus* to pathogens and parasitoid with the aim of sheding more light on the tick-pathogen and tick-parasitoid interactions. This will be achieved by the following objectives:

(1) to study, characterize and compare different morphological types of haemocytes in the above mentioned ticks; as well as total and differential haemocyte counts between bacteria, fungi and parasitoid infected and uninfected ticks;

(2) to study and compare cellular and humoral immune reactions in different sexes and ages of ticks of both species, the level of pathogens that induce antibacterial activity, the time taken for induction, the type of humoral antibacterial factors produced and to determine the ability of some factors released by insect pathogens eg. *Bacillus thuringiensis* and *Serratia marcescens* to inactivate tick immune proteins;

(3) to study the immune status of ticks infected with the fungi *Beauveria bassiana*, *Metarhizium anisopliae* and the hymenopteran tick parasitoid *Ixodiphagus hookeri*.

CHAPTER II

LITERATURE REVIEW

2.1 The ticks:

Ticks are obligatory bloodsucking arthropods (Gladney, 1978; Demaree, 1986; Arthur, 1962). They feed on man and animals (Sheals, 1973; Gladney, 1978; Arthur, 1962). While feeding they transmit diseases of economic importance among which are piroplasmosis, bovine anaplasmosis, theileriosis, heartwater (Gladney, 1978).

Although transmission of diseases is of economic importance, ticks have some other effects on animals, which are of major economic importance (Gladney, 1978; Arthur, 1962). All ticks must obtain blood from their host in order to survive. During feeding ticks can induce some injuries to the host especially when a large number of ticks engorge together. This may cause severe anemia, loss of production and even death of the animal (Gladney, 1978). Ticks can also induce dermatosis and other related reactions like inflammation, itching, swelling and ulceration at the biting site leading to the lesion on hides and skins providing a route for the invasion of pathogens which can be a cause of secondary infection (Harwood and James, 1978; Kettle, 1984; Yeoman and Walker, 1967).

2.2. **Classification**:

Ticks belong to the Phylum Arthropoda, class Arachnida (spiders, king crabs, scorpions and mites), and order Acarina (Ticks and mites) (Hoogstraal, 1973). Ticks constitute the superfamily Ixodoidea, which is divided into two families; Argasidae (soft ticks) and Ixodidae (hard ticks). The Ixodidae family is divided into 2 subfamilies Amblyomminae (comprising Haemaphisalis, Aponoma, Amblyomma and Dermacentor), and Rhipicephalinae (Rhipicephalus, Hyalomma and Boophilus) (Hoogstraal 1973; Harwood and James, 1978).

About 800 tick species in order Ixodoidea are classified into three families with the largest, the Ixodidae (hard ticks) having 650 species in 13 genera, the Argasidae (soft ticks) with 150 species in five genera, and the Nuttalliellidae with one genus and one species (Kettle, 1984). Among the 13 genera in the family Ixodidae, *Boophilus*, *Hyalomma*, *Rhipicephalus* and *Amblyomma* are the most important. There are 102 Amblyomma species in the world among which 10 are found in Africa. Most of them, if not all, parasitize livestock. They are reservoirs and vectors of *Cowdria ruminantium*, the rickettsial causative agent of Heartwater. Among the 5 described Boophilus species 4 are in Africa. Among them *Boophilus microplus* and *B. annulatus* are major vectors of *Babesia bigemina*, *B. bovis* and *Anaplasma marginale*. *Boophilus decoloratus* is an efficient vector of *B. bigemina* and *A. marginale* but not *B. bovis*.

Among the 55 Hyalomma species in Africa, 15 species of the subgenus Hyalomma are of veterinary and public health importance. They are involved in the transmission of *Theileria annulata, Babesia equi, B. caballi, A. marginale, Trypanosoma theileri* and some arboviruses. Seventy *Rhipicephalus* species have been described in Africa. The most important, *R. appendiculatus*, *R. sanguineus*, *R. e. evertsi*, are involved in the transmission of babesiosis, anaplasmosis, theileriosis and other diseases (Anonymous et al. 1991).

2.2.1. **Amblyomma variegatum**:

The most important Amblyomma species affecting domestic and wild animals is *Amblyomma variegatum*. In Africa it occurs south of the sahel regional transition zone (White 1983), right accross the continent from Senegal to South Africa. Outside the continent, *A. variegatum* has established itself in Madagascar, Yemen Arab Republic, the Indian ocean Island of Grand Comore, Reunion and Mauritius, Cap verde Island and Islands in the Eastern Carribean (Walker, 1987). *Amblyomma variegatum* is a parasite of domestic animals in Africa. It is found primarily on cattle, sheep and goat (Hoogstraal, 1956). The tick is also found on camels, horses and donkeys (Yeoman, 1968; Walker, 1974 and Pegram et al., 1981) as well as dogs, cats and pigs (Hoogstraal, 1956; Yeoman 1968 and Walker, 1974). Adult A. variegatum occur on a very wide range of mammals. They are most prevalent on medium and large animals

(Hoogstraal, 1956; Theiler, 1962; Hoogstraal and Aeschlimann, 1982). Colbo (1973) and Colbo and MacLeo (1976) found wild animals less affected than domestic ones. Medium and large mammals as well as birds have been listed by Hoogstraal (1956) to be hosts of nymphs while larvae were recorded predominantely from birds and small mammals (Hoogstraal, 1956).

Adults and nymphs occur commonly on the ventral surface of the host including the lower dewlap, brisket, abdomen, axillae and genitalia (Hoogstraal, 1956; Yeoman & Walker, 1967; Walker, 1974; MacLeod et al., 1977). Nymphs are also found on the heels (Walker, 1974), elbows, hocks and elsewhere on the legs where few adults are present (Yeoman and Walker, 1967). Larvae have been recorded from the head, including the ears of cattle (Wilson, 1948 cited by Hoogstraal, 1956; MacLeod et al., 1977), dewlap and abdomen (Kaiser et al., 1982).

Amblyomma variegatum is found from sea level to 2,590 m (Hoogstraal, 1956; Walker, 1974). It survives in areas where minimum rainfall is 400 - 750 mm p.a. (Elbl and Anastos, 1966; Walker, 1974; Paine, 1982) and maximum rainfall 2050 mm (eg. Kenya) to 2800 mm p.a. (eg. Tanzania) (Walker, 1974; Yeoman and Walker, 1967). *Amblyomma variegatum* is absent from drier, arid areas but can be found in semi-arid and humid areas (Walker, 1974) where it occupies a wide variety of different habitat types, mainly with tree and/or bush cover (Yeoman & Walker, 1967; Morel, 1980). It also occurs in cultivated steppe and grassland with a high grass

cover (Yeoman, 1968).

2.2.2. **Rhipicephalus appendiculatus**:

The brown ear tick *R. appendiculatus* is primarily a central and East African tick species ranging from the Transvaal in the south to the Equatorial province of the Sudan and to Ethiopia in the North (Elbl and Anastos, 1966). *Rhipicephalus appendiculatus* does not occur in West Africa (Hoogstraal, 1956). Its habitat ranges from sea level at the coast up to 2100 m inland but is absent from deserts and areas lacking shrub cover (Hoogstraal, 1956; Yeoman and Walker, 1967). It is commoner in areas of tall grass rather than short grass, and in areas of moderate or higher rainfall, which is often defined as more than 750 mm per annum (Arthur, 1966). In Tanzania, Yeoman and Walker (1967) found it rare in areas where the rainfall was less than 500 mm, and widely distributed in the 500-1000 mm rainfall zone, but not ubiquitous on cattle within that zone. In areas where the rainfall exceeds 1000 mm heavy infestation of *R. appendiculatus* could be expected wherever cattle were present in any number. The distribution of *R. appendiculatus* is the result of complex interaction between climate, vegetation and cattle (Kettle, 1984).

The brown ear tick is primarily a parasite of cattle but also occurs on sheep and goats and to a very limited extent on wild animals (Yeoman and Walker, 1967). Infestation of up to nearly 2000

of adults *R. appendiculatus* have been found on a single beast although usually they are considerably lower (Kettle, 1984).

The site most favoured by adult *R. appendiculatus* is inside the ear flap (which can support a maximum population of about 250 adults), but when there is heavier infestation they occur elsewhere on the head, spreading to the neck and body (Kettle, 1984). The brown ear tick is actually the most important vector of *Theileria parva*, the causative agent of East coast fever.

2.3. **Life cycle**:

Ticks pass through 4 stages (egg, larvae, nymph and adult). Fully engorged females, usually deposit their eggs on the ground, the number of eggs varying from 1000 to 18000 in some species (Harwood and James, 1979). Depending upon climatic conditions eggs hatch in two weeks to several months (Hoogstraal, 1956; Kettle, 1984). Six-legged (hexapod) larvae emerge, find hosts, engorge and moult in 14 days for *A. variegatum* and 10-49 days for *R. appendiculatus* (Hoogstraal, 1956). The emerged nymph has 8 legs, it is different from the adult by the absence of gonopore. The nymphs feed for four to eight days on the host, engorge, moult and become sexually mature adults (Kettle, 1984) in 19 days for *A. variegatum* and 10-61 days for *R. appendiculatus* (Hoogstraal, 1956; Petney et al., 1987).

Ticks use one to three hosts during feeding period. When the life cycle is completed on one animal the tick is called **ONE-HOST** tick e.g. *Boophilus annulatus*. When two hosts are involved, the tick is called **TWO-HOST** tick e.g. *R. evertsi*, *Hyalomma truncatum*. Larvae and nymphs of this type of ticks feed on the same animal. When a tick requires three diffferent hosts to complete its life cycle, it is called **THREE-HOST** tick, e.g *A. variegatum*, *R. appendiculatus*. In such a tick the larvae engorge on small animals, drop and molt, the nymphs find a host (small or medium size) engorge, drop and molt. The adults find a host (usually a large animal) feed and mate simultneously, drop to the ground where the females lay eggs (Harwood and James, 1979).

2.4. **Economic, veterinary and medical importance**:

Ticks are responsible for the loss of several animals throughout the world. In Australia the cost loss due to tick infestation in cattle in 1975 was estimated at 40 million dollars, of which one third was the cost of control and two thirds loss in production (Sutherst et al., 1979). Losses due to East coast fever in cattle industry in Africa was estimated to be US $168 million in 1989 (ILRAD, 1989). Heavy tick infestations damage hides and cause a loss in live-weight gain which has been estimated at 1 kg per beast per year for every 1,400 tick-years, or the equivalent of four female ticks dropping per day over a year (Sutherst et al., 1979).

Tick infestation can lead to secondary infections like abcesses and myasis due to *Cochlyomyia hominivorax* (Coquerel) in America and *C. macellaria* (Fabricius) in Africa (Harwood and James, 1979). Ticks are mechanically involved in the transmission of diseases like Cutaneous Streptotricosis which causative agent *Dermatophilis congolensis* is transmitted by *A. variegatum*. The tropical bont tick *A. variegatum* and other ixodid ticks are involved in the transmission of several other diseases. These are:

2.4.1. **Rickettsial diseases**:

1). **Cowdriosis**:

The most important disease transmitted by *A. variegatum* is Cowdriosis, very lethal to animals. Cowdriosis is ranked as the first or second most important livestock disease in Africa (Neits, 1968, Uilemberg, 1986). The causative agent is *Cowdria ruminantium*. The disease affects cattle (especially exotic breed), sheep and goat. In West Africa it is reported to cause 50% mortality in imported cattle breeds and small ruminants (Fao, 1984). The economic impact of heartwater has not been assessed because of difficulties in diagnosing it in live animals. The animal dies in a few hours without any clinical signs or may show paroxymal convulsions and dies within 36 to 48 hours (Van de Pypekamp and Prozesky, 1987). The disease is transmitted transtadially by nymphs and adults (Barré et al., 1987). Its maintenance is insured by wild animals, rodents and ground dwelling birds (Oberan and Bezuidenhout, 1987).

2). **Boutonneuse fever** (South African Tick Typhus, Kenyan Tick Typhus, Crimean Tick Typhus, Marseilles Fever, Indian Tick Typhus):

Caused by *Rickettsia conori*, the disease is found in Africa, European mediterranean regions, Israel, Turkey, Crimea and South east Asia. The disease affects mainly man, dogs and rodents. It is transmitted by *Rhipicephalus sanguineus*. Other tick vectors are in the genera *Haemaphysalis, Hyalomma, Amblyomma, Boophilus, Rhipicephalus, Ixodes* and *Dermacentor*. On the South African veld the chief vectors appear to be *A. hebraeum* and *R. appendiculatus* (Kettle, 1984). The normal route of transmission of *R. conori* from tick to man is through the bite of the tick but infection may also occur from contamination of eye and nasal mucosa from crushed ticks or tick faeces, particularly when dogs are being de-ticked (Rehacek, 1979).

3). **Epidemic typhus**:

Five strains of *Rickettsia prowazeki* have been recovered from *A. variegatum* and *H. rufipes* by inoculating ground-up ticks into guinea pigs. Larvae and nymphs of both species became infected when fed on rabbits and lambs harbouring *R. prowazekii*, but adults did not (Reiss-Gutfreund, 1956).

2.4.2. **Protozoal diseases**:

2.4.2.1. **Theileriosis**:

a. ***Theileria parva:***

Rhipicephalus appendiculatus is mainly involved in the transmission of *T. parva*, the causative agent of East coast fever (also known as Corridor disease and January disease). This is a highly pathogenic disease of domestic animals in eastern, central and southern Africa (Harwood and James, 1979). African buffalo can be infected but shows no clinical signs while cattle on the other hand cannot tolerate the infection. In endemically stable conditions, among indigenous animals, up to 50% of the calves exposed to *T. parva* die. In endemically unstable conditions, where there are livestock herds newly introduced to the parasite, 80 to 100% of animals of all age groups may die. It has been estimated that East Coast fever killed 1.1 millions head of cattle and caused US $168 million in losses in 1989 (ILRAD, 1991).

b. ***Theileria mutans:***

Theileria mutans is generally a non-pathogenic agent, associated with cattle (Harwood and James, 1979). It is transmitted by *A. variegatum* in East africa and *Haemaphysalis punctata* in Britain (Harwood and James 1979).

c. ***Theileria lawrencei:***

Found in wild african buffuloes (*Syncerus caffer*), *Theileria lawrencei* is transmitted by *R. appendiculatus*. The protozoa is highly pathogenic to cattle. Problems generally occur when these livestock are moved on land inhabited by wild buffaloes (Burridge, 1975).

2.4.3. **Tick paralysis**:

Tick paralysis is caused by several tick species around the world. In Africa, Karoo tick paralysis is caused by *Ixodes rubicundus*. The disease affects sheep, goats, cattle, and rarely dogs and jackals (Harwood and James, 1979). Paralysis of man is rare and regional in nature. It is attributed to *Rhipicephalus simus*, *Hyalomma tuncatus* and *I. rubicundus*. The most important paralysis causing tick is *I. holocyclus*. The tick is present in moist, densely vegetated areas of eastern coastal Australia. It was originally a parasite of native monotreme and marsupial fauna, but now parasitises introduced cattle, dogs, and cats, fequently causing paralysis in the first two (Kettle, 1984).

Ticks paralysis, or toxicosis, is caused by the introduction of toxin into the body of the host with the salivary secretion of the tick during feeding (Gladney, 1978). The toxin involved is not the same in different species. For example removal of *D. andersoni* usually leads to an immediate improvement whereas this does not necessarily occur in *I. holocyclus* and the animal (or man) may subsequently die (Stone et al., 1979). The severity of the paralysis depends on the susceptibility of the host (Gladney, 1978). Cattle paralysis was found to be caused by the feeding of *Dermacentor andersoni*. Sheep and rabbits were paralyzed subsequent to the feeding of *R. appendiculatus*, *R. evertsi evertsi* and *Ixodes rubicundus* (Morel, 1981; Latif, 1986 and Njau, 1985).

2.4.4. **Viruses**:

Aboviruses (arthropod born viruses) associated with ticks have been proved to be transmitted to vertebrates through tick salivary secretions. They are transmitted transovarially, transtadially and sexually (Plowright et al., 1974). Hoogstraal (1973) pointed out 68 viruses (about 21 known to affect man) identified from 60 Ixodid and 20 Argasid ticks.

a. Arboviruses associated with *A. variegatum*:

1.- ***Crimean congo Haemorragic fever virus*** (CCHF):

Observed for the first time in 1944-1945 among presumably nonimmune Russian troops assisting farmers in the war-devastated Crimea, the CCHF virus was mainly isolated form *Hyalomma m. marginatum* in southern USSR. In Africa it was isolated from ticks associated with livestock, especially *A. variegatum, B. decoloratus, H. m. rufipes, H. truncatum* and *H. impeltatum*. Human cases occur when ticks are crushed with fingers and tick bites (Harwood and James, 1979).

2.- ***Dugbe virus*** (DUG):

Has been isolated from *A. variegatum, H.m. rufipes, A. lepidum* and *B. decoloratus* in Nigeria from man and cattle. The virus causes moderately severe fever in latter (Harwood and James, 1979). Booth et al. (1991) demonstrated that the main sites of DUG virus replication were the epidermis, and haemocytes associated with

loose connective tissue. They also ascertained that the virus could not be detected in the salivary gland until the start of feeding.

3.- *Bhanja virus* (BHA):

Has been isolated from *A. variegatum, H. truncatum* and *B. decoloratus* in Nigeria and Cameroon (Vinograd et al., 1975). The virus affects domestic animals, man and birds.

4.- *Thogoto virus* (THO):

Associated with camel and cattle and rarely causes meningo-encephalitis in man. THO virus has been isolated from *H.a. anatolicum* in Egypt, *Rhipicephalus spp.* and *B. decoloratus* in Kenya and *H. truncatum* and *A. variegatum* in Nigeria (Harwood and James, 1979).

5.- *Lymphocytic choriomeningitis* **virus:**

Isolated from human blood in Ethiopia, the virus is transmitted by *A. variegatum* and *R. sanguineus* (Harwood and James, 1779).

6.- *Yellow fever virus*:

Has been isolated from *A. variegatum* collected in the Central African Republic and has been demonstrated to be transmitted transovarially to the progeny from infected females (Germain, 1984).

b. **Arboviruses associated with *Rhipicephalus appendiculatus*:**

1.- *Nairobi sheep disease virus* (NSD):

A severe virus disease of sheep and goats in which mortality may reach 90 per cent. The vector is the three-host tick

Rhipicephalus appendiculatus in which transovarial transmission occurs. Larvae can transmit the disease 100 days after hatching (Harwood and James, 1979). No virus or antibodies to NSD have been found in wild ruminants or rodents, and the virus appears to be restricted to sheep, goats and *R. appendiculatus*. Human infections with NSD occur rarely and the disease is mild (Woodruff, 1974).

2.- *Tick-borne encephalitis*: TBE virus is transmitted by *Rhipicephalus appendiculatus* and *Ixodes ricinus*. Labuda et al., (1993) used both species to demonstrate that efficent transmission of the virus can occur between cofeeding tick.

3.- *Thogoto virus*: The virus is transmitted by *R. appendiculatus*. Jones and Nuttal (1989) have shown that the virus can be transmitted from infected to unfected ticks when cofeeding. The virus can affect invertebrates and man.

2.5. Immune mechanisms in arthropods:

A considerable amount of studies have been done on arthropod immune mechanisms particularly on insects. Lackie (1988b) provided a list of insects subjected to insect immune studies. Some studies were done on arthropods immunity in general and very few reports focused on tick immune mechanisms. As mentioned by Gupta (1986), the most important measure of the competence of an immune system is its ability to distinguish between self and nonself. In arthropods this is believed to be accomplished by lectins and phenoloxydases (Kaaya, 1989).

2.5.1. **Morphological types of arthropod haemocytes**:

A lot of studies have been done on haemocytes of invertebrate groups. Among arthropods, the most extensively studied are insects, followed by crustaceans, arachnids and myriapods. Very few studies have been done on ticks.

Classification of haemocytes in insects and other arthropods has always been a subject of controversies and confusion. Attempts to improve classification methods have been on from Cuénot (1896), Hollande(1911), Wigglesworth (1959), Jones (1962) to Gupta (1979).

Several types of haemocytes have been identified in arthropods using light and electron microscopy. They are Prohematocytes (PR), Plasmatocytes (PL), Granulocytes (GR), Spherulocytes (SP), Adipohemocytes (AD), Coagulocytes (CO) or cystocyte, Oenocytoids (OE). Gupta (1979) summarized them as follow:

1. **Prohaemocytes**: Are small round, oval or elliptical cells with variable sizes (6-10 μm wide and 6-14 μm long). The plasma membrane is generally smooth, but may show vesiculation. The nucleus is large, centrally located, and almost filling the cell, nuclear size is variable (3.6-12 μm) in various insects. A thin or dense, homogeneous and basophilic layer of cytoplasm surrounds the nucleus, the nucleocytoplasmic ratio being 0.5-1.9 or more. The cytoplasm may contain granules, droplets, or vacuoles.

Prohaemocytes have been observed in most of the insect species but they are rare in the circulating blood. They are considered stem

cells for other haemocytes. In *Locusta migratoria* prohaemocytes were observed in the haemolymph after perturbations of the haemogram, in which they seemed to be released from haemopoitic organs (Hoffmann, 1969).

2. **Plasmatocytes (PL)**: Small to large polymorphic cells with variable size (3.3-5 µm wide and 3.3-4.0 µm long). The plasma membrane may have micropapillae, filopodia, or other irregular processes, as well as pinocytotic and vesicular invaginations. The nucleus may be round or elongated and generally centrally located. Plasmatocytes are abundant and may be confused with granulocytes and prohaemocytes. Their shape is not often regular with very few expansions. In some species, they are elongated. They form capsules around foreign bodies.

3. **Granulocytes (GR)**: Granulocytes are small to large cells, spherical to oval with variable sizes (10-45µm long and 4-32µm wide), rarely larger. The plasma membrane may or may not have micropapillae, filopodia, or other irregular processes. The nucleus may be relatively small (compared with that in the PL), round or elongated, and is generally centrally located. Nuclear size is variable (2-8µm long and 2-7µm wide). The cytoplasm is characteristically granular.

4. **Spherulocytes (SP)**: Spherules are ovoid or round cells with variable sizes (9-25µm long and 5-10µm wide) and usually larger than GRs. The plasma membrane may or may not have micropapillae, filopodia, or other irregular prcesses. The nucleus is generally small (5-9µm long and 2.56µm wide), central or eccentric, rich in

chromatin bodies, and generally obscured by the membrane bounded, electrondense, intracytoplasmic spherules that are characteristic of these cells. The number of spherules may vary from few to many, and the diameter from 1.5 to 5μm. The spherules contain granular, fine-textured, filaments, or floculent material.

In phase contrast microscopy (light microscopy), SPs appear irregular in shape, exhibiting an aspect of a "morula", which faciltates their recognition. The functions of these cells are unknown. They could play a role in the synthesis of mucopolysaccharides in *Bombix mori*.

5. **Oenocytoids (OE)**: Oenocytoids are small to large, thick, oval, spherical, or elongated cells with widely variable sizes (16-54μm or more) and shapes. The plasma membrane is generally without micropapillae, filopodia, or other irregular processes. The nucleus is generally small, round or elongate, and generally eccentrically located. Nuclear size may vary (3-15μm). Occasionally, two nuclei may be present. Oenocytoids are particularly fragile *in vitro* and lyse easily, ejecting materials into the haemolymph. They are non-phagocytic. OEs are readily recognized under a light microscope due to their large and regular shape. They are found in most of the insects but are absent in *Orthoptera* (*Locusta migratoria*). They never represent more than 1 to 2 % of all the haemocytes present in a normal insect.

6. **Adipohaemocytes (AD)**: Adipohaemocytes are small to large spherical or oval cells with variable sizes (7-45μm in diameter). The plasma membrane may or may not have micropapillae, filopodia,

or other irregular processes. The nucleus is relatively small (compared with that in PL and SP), round or slightly elongate, and centrally or eccentrically located. Nuclear size is variable (4-10μ in diameter). The nucleus may appear concave, biconcave, punctate or lobate. The cytoplasm contains characteristic small to very large refringent fat droplets (0.5-15μm in diameter) and vacuoles, which according to Arnold (1974), become filled with lipids under certain condition.

7. **Coagulocytes (CO)**: Are generally small to large cells (3-30μm long), spherical, hyaline, fragile and unstable cells, combining the features of GRs and OEs. The plasma membrane is generally without any micropapillae, filopodia, or other irregular processes. The role of COs in haemolymph is coagulation.

Studies on tick haemocytes have been going on since the beginning of the century until when Jones (1962) proposed the first nomenclature system. Using light microscope and using Jones system Dolp (1970) identified 3 types of haemocytes: prohaemocytes, plasmatocytes (early and advanced stages) and spherules cells (early and advanced stages). Brinton and Burgdorfer (1971) in their studies on *Dermacentor andersoni* used electronmicroscopy to recognized four types of cells, prohaemocytes, plasmatocytes, four sub-types of spherule cells (spherulocytes) and oenocytes (oenocytoid cells). By contrast, Balashov (1979) described 3 types: prohaemocytes, plasmatocytes and two types of granulocytes in *Hyalomma asiaticum*, his granulocyte type corresponding to the type

IV spherule cell of Brinton and Burgdorfer (1971) and type II granulocyte corresponding to type I spherule cell of Brinton and Burgdorfer (1971). Therefore, Bunnington and Obenchain (1986) concluded that there are at least 5 classes of haemocytes, including prohaemocytes, plasmatocytes, granulocytes, spherulocytes and oenocytoids.

2.5.2. **Cellular immune responses**:

Many functions have been ascribed to haemocytes, including the transport of lipids (Chino, 1985), proteins (Geiger et al., 1977) and tyrosine (Post, 1972; Vanderberg and Mills, 1974), coagulation of haemolymph and wound-healing, formation of connective tissue, secretion of humoral antibacterial factors and cellular immune responses (Lackie, 1988). The most important functions of these cells are phagocytosis, encapsulation and nodule formation (Gupta, 1986; Kaaya, 1989). These functions are generally performed by plasmatocytes and granulocytes (Kaaya, 1988). While phagocytosis and nodule formation act against particulate materials e.g. bacteria, the encapsulation reaction defends the insects against larger organism e.g. nematodes (Kaaya, 1989).

2.5.2.1. **Phagocytosis**:

This is a process by which the foreign body is engulfed. Phagocytosis is accomplished in three stages: (1) recognition of

the foreign body, (2) its ingestion, and (3) its final disposal, or clearance from the body (Ratcliffe and Rowley, 1979). Foreign bodies can be small biotic particles such as bacteria and yeast, and abiotic particles such as latex beads and colloidal carbon (Lackie, 1988). Careful quantitative work by Ratcliffe and Walters (1983) using various pathogenic and non-pathogenic bacteria in *Galleria* larvae, showed that low volume of bacteria (less than c. $10^3 \mu l^{-1}$) were removed by phagocytosis. Above this threshold level, nodule formation was a more efficient clearance mechanism. In higher arthropods, phagocytosis is generally performed by plasmatocytes (Gupta, 1986). In tsetse, bacteria injected into the haemocoel were phagocytized by plasmatocytes (Kaaya, 1989). Podboronov (1991) demonstrated phagocytic activities in ticks using *Ornithodoros papillipes, O. mubata, Alveonasus lahorensis* and *Hyalomma asiaticum*. He observed the phenomenon of phagocytosis 2 hours after the introduction of *Salmonella typhimurium* and *Staphylococci aureus*. He also noticed an increase in the number of prohaemocytes, plasmatocytes and spherule cells.

Apparently not all organisms are killed by phagocytosis. *Tubercule bacilli* have been reported to survive in haemocytes for prolonged periods (Cameron, 1934), while others e.g. *B. thuringiensis* in the armyworm, *Pseudaleutia unipuncta*, lyse the phagocytes and break free causing lethal septicaemia (Witting, 1965). Schmittner and McGhee (1970) reported that *Trypanosoma rangeli* in *Rhodnius prolixus* and *Crithidia spp.* in *Drosophila*

virilis use the haemocytes as sites of division and multiplication.

2.5.2.2. **Nodule formation**:

This operates when a large dose of particulate material invades or is injected into the haemocoel of insects and which cannot be cleared by phagocytosis alone (Kaaya, 1989). Haemocytes involved in this process are granulocytes and plasmatocytes. Granulocytes clump together, discharge granules and entrap the foreign particles (e.g. bacteria) in a clot-like gelatinous material. Within 5-6 min, melanization of the degenerating haemocytes and bacteria begins and compaction of the necrotic, melanized mass takes place. Plasmatocytes then start attaching to the melanized mass and flatten on its surface forming a multicellular sheath (Ratcliffe and Gagen, 1977; Kaaya, 1989). Like in the phagocyte, microorganisms entrapped in nodules may either be killed, remain alive for prolonged periods in insects or are released causing lethal septiceamia (Walters and Ratcliffe, 1983; Kaaya, 1989). Kaaya (1989) reported that nodules formed after injection of bacteria in adult *Glossina morsitans morsitans* were relatively small compared to those formed by lepidopterans. He attributed this to the low numbers of circulating haemocytes, characteristic of dipterans. The low numbers of circulating hamocytes has also been cited as the reason for the absence of nodule formation against *T. b. brucei* in tsetse (Kaaya, 1989).

2.5.3. **Encapsulation**:

2.5.3.1. **Cellular encapsulation**:

A mechanism that combats foreign bodies that are too large to be phagocytized. Encapsulation is accomplished in two stages: recognition of foreign body by granulocytes and capsule formation and melanization by granulocytes and plasmatocytes (Gupta, 1986). It has been reported to occur within 24 hours of parasitization and involves haemocytes in the formation of a multicellular sheath which encloses the foreign object (Ratcliffe and Rowley, 1979).

Once mature, capsules gradually become compacted and presumably remain in the haemocoel until the insect dies (Lackie, 1988). Not all organisms are killed. Sutherland et al. (1984) and Lafond et al. (1985) reported that some microfilariae have the ability to evade the host (*A. aegypti*) encapsulation. Sutherland et al. (1984) believed there are 3 possible explanations for the increased ability of the microfilariae *Brugia pahangi* to avoid the mosquito response following penetration of the midgut of Liverpool strain of *A. aegypti*: (1) during penetration of the midgut wall, the parasite acquires host materials and therefore are not recognized as foreign by the mosquito; (2) exposure to the midgut environment modifies or stimulates *B. pahangi* in such a way that they are able to inhibit the response in mosquitoes; (3) both of the above reactions might be occuring.

Eggenberger et al. (1990) reported that Epon was encapsulated by haematocytes in *Dermacentor variabilis* (Say). They observed that implant of Epon inserted in *Dermacentor variabilis* (Say) through incisions in the cuticle were encapsulated by haemocytes. By 3 hours post-implantation degranulation and disintegration of granulocytes had formed a matrix at the Epon surface. From 6 hours until encapsulation was completed (72 hours), plasmatocytes and granulocytes continued to respond to degranulation and formed multiple cell layers around the Epon implant.

2.5.3.2. **Humoral encapsulation**:

Humoral encapsulation occurs only in insects with few circulating haemocytes and is formed without apparent participation of haemocytes as a result of activation of haemolymph prophenoloxidases (Ratcliffe and Rowley, 1979). Humoral encapsulation has been reported only in Diptera and Hemiptera where it replaces cellular encapsulation reactions (Götz, 1986). In Culicidae, some limited heamocytes involvement in the formation of humoral capsules against invading parasites and fungi has been reported (Bronskill, 1962; Poinar and Leuteneger, 1971; Christensen et al. 1984). Poinar and Leuteneger (1971) observed that infection of mosquitoes with *Neoplectona carpocapsae* elicited formation of melanotic capsule, which were formed without direct participation of haemocytes. However, Iwama and Ashida (1986) showed immunochemically that the Oenocytoids of *Bombix* contain

prophenoloxidase. This view was supported by Drif and Brehelin (1983) who observed that mosquito Oenocytoids contain phenoloxidase activity and that they increased both in their relative percentage as well as in absolute numbers within 30 min of nematode penetration.

2.5.4. **Humoral immune responses**:

Insect humoral defence mechanism components comprise lysozyme and the inducible antibacterial proteins as well as lectins and the phenoloxidase system (Lackie, 1988).

2.5.4.1 **Antibacterial proteins**:

Synthesis of antibacterial proteins is induced by wounding and, to a greater extent, by injection of or infection by non pathogenic bacteria. At least 15 different inducible proteins have been identified from the hemolymph of *Hyalophora cecropia* pupae (Boman, 1986). Of these 15 emerge three main groups which comprise the Cecropins, attacins and lysozyme.

a. Cecropins:

Are strongly basic proteins with antibacterial activity against gram+ and gram- bacteria. The molecular weight is approximately 4000 daltons. Their existence in the haemolymph of immunized pupae of *Hyalophora cecropia* was demonstrated using SDS

polyacrylamide gel electrophoresis at pH 4 (Hultmark et al., 1980). Cecropins A and B were isolated one year later (Hultmark et al., 1980). Two years later *Cecropin D* and three minor forms believed to be precursors were found (Hultmark et al., 1982). Cecropins B and D were isolated from the chinese oath silk moth *Antheraea pernyi* (Qu et al., 1982).

Cecropinlike molecules were demonstrated *in* eight different lepidopteran species (Hoffman et al., 1981), in *Drosophila* (Flyg et al., 1987; Robertson and Postlethwait, 1986), in tsetse flies (Kaaya et al., 1987), and in darkling beetle (Spies et al., 1986).

b. **Attacins**:

Attacins are a group of larger proteins (Mw = 21 - 23,000 daltons). They were isolated as inducible antibacterial proteins P5 from an immunized pupae of *H. cecropia* and described as attacins (Hultmark et al., 1983). Six different components (A-F) were subsequently revealed and all of them cross-reacted with antisera prepared against P5 (identified as attacins). All the six components were shown to possess a common antigenic determinant, while another one was common only to attacins E and F, the two acidic forms (Boman, 1986). Five different attacinlike peptides were detected in *Manduca sexta* by two dimensional polyacrylamide gel electrophoresis. Attacins have also been found in two other moths besides *Cecropia*: *Antheraea pernyi* and *Manduca sexta* (Spies et al., 1986). A group of attacinlike proteins were also purified in *Sarcophaga peregrina* (Ando et al., 1987). Attacins act on the

outer membrane of bacteria (Engström et al., 1984), facilitating the action of cecropins and Lysozyme (Boman, 1986).

Molecules with similar characteristics to cecropins and attacins have been found in *Galleria melonella* (Hoffman et al., 1981), *Manduca sexta* (Spies et al. 1986), several species of Diptera (Okada and Natori, 1985; Keppi et al. 1986; Robertson and Postlethwait, 1986; Flyg et al. 1987; Ando et al., 1987; Kaaya et al., 1987) and in Coleoptera (Spies et al. 1986). The cecropins and attacins attack bacterial cell walls synergistically leaving the peptidoglycan layer to be digested by lysozyme (Boman and Hultmark, 1987).

c. Lysozyme:

Lysozyme is a bacteriolytic enzyme whose production in tsetse starts immediately after bacterial injection and reaches a peak 1-2 hr later (Kaaya et al. 1987). Its production is stimulated by live and dead bacteria (Kaaya and Dharji, 1988). Lysozyme which was the first antibacterial factor to be identified in insect heamolymph (Boman, 1986) was first purified from *Galleria melonella* (Powning and Davidson, 1976) and more recently two lysozymes from different ph optima have been purified from *Gryllus* (Schneider, 1985). It was also identified in the gut of several insects (Ribeiro et al., 1984), in haemocytes of *Spodoptera eridania* (Andersson and Cook, 1979) and *Locusta* (Zachary and Hoffmann, 1984) and in a haemocyte-like cell line (Landureau, 1976).

Cecropia lysozyme is composed of 120 amino acids with a molecular weight of 13.8 KD and shows great similarities with vertebrates' lysozymes of the chicken egg white type (Boman, 1986). Podboronov (1991) found the Molecular weight of Lysozyme in *Alveonasus lahorensis, Ornithodoros papillipes* and *Ornithodoros moubata* to be 13.8 KD while lysozyme in *Hyalomma asiaticum and Ixodes persulcatus* was 15 KD. The amino acid residues in a lysozyme molecule of *A. lahorensis* was 124, that of *O. papillipes* 125, *O. moubata* and *H. asiaticum* 127, *I. persulcatus* 130 and egg 129.

Lysozyme is bactericidal to only some gram+ bacteria e.g., *Bacillus megaterium* and *Micrococcus luteus* (Boman, 1986). The main function of lysozyme may not be to kill sensitive bacteria but to remove the murein sacculus which is left after the action of cecropins and attacins (Boman, 1986).

2.5.4.2. **Humoral lectins and lectin-like factors:**

Phagocytes of molluscs and arthropods detect and migrate towards invading micro-organims. This reaction and the subsequent phagocytosis event require specific receptors on the haemocyte surface to recognize different types of foreign molecules either produced by or located on the membrane of the micro-invader. Apart from different sugar determinants, the only type of binding molecule so far identified on the membrane of haemocytes (Amirante, 1976) reveals carbohydrate specificity and may be characterized as

a lectin (Renwrantz, 1986). Lectins (also referred to as haemoagglutinins) are widely distributed in the body fluids of invertebrates, including insects (Ey and Jenkin, 1982). Lectins have been detected on the surfaces of plasmatocytes and granular cells (Yeaton, 1980; Komano et al. 1983) but not on other non-phagocytic cells (Ratcliffe, 1986). Komano and Natori (1985) reported the involvement of the agglutinin of *Sarcophaga peregrina* larvae in the clearance and lysis of ^{51}Cr-labelled sheep erythrocytes from the haemocel.

Lectins present in the different region of the gut of *R. prolixus* and *G. austeni*, with agglutinating activity for *T. cruzi*; may control the development of such parasites outside the haemocel (Pereira et al., 1981; Ibrahim et al. 1984). Lectins present on the peritrophic membrane of larvae of the blow fly, *Calliphora erythrocephala*, may also regulate symbiotic and pathogenic microorganisms (Peters et al., 1983).

2.6. **Insect immune responses to parasitoids**:

Several defense mechanisms against parasitoids are known in insects. The most common being the formation of a capsule around the parasitoid eggs or larvae (Salt, 1970). This capsule, normally melanized, kills the parasitoid by asphyxia or by starvation (Fisher, 1971).

Some proteins called parasitism-induced proteins are produced in the host once parasitized. They could be playing some role in the host defense system but their function is still unknown. Some studies have demonstrated their production in most of the parasitized insects. For example, in the Lepidopteran-braconid host-parasite system of *Manduca sexta-Cotesia congregata*, a 33 KD parasitism-induced protein appears within 5 h of parasitization and larger molecular weight appear several days post-parasitization (Beckage et al., 1989).

According to Boulétreau (1986), the parasitoid can avoid these defense systems in two ways; it can be placed in tissues where an immunodepressive effect, activated perhaps by intermediate viral particles (Stoltz and Vinson, 1979; Edson et al. 1981) or by specific substances injected at the time of oviposition (Rizki and Rizki, 1984) or secreted by the eggs or larvae (Boulétreau and Quiot, 1972; Kitano and Nakatsuji, 1978).
Vinson and Stoltz (1986) have demonstrated the existence of a symbiotic relationship between some genera of endoparasitic Ichneumonids and endoparasitic Braconids and a virus which was identified as member of a new family of insect virus, the Polydnaviridae (Stoltz et al., 1984). It consist of segmented, double stranded, circular DNA genomes. It has only been isolated from the calyx or lateral oviducts of parasitoids (Stoltz and Vinson, 1979). The immunodepressive role of the virus was demonstrated by Edson et al. (1981).

2.7. <u>Immune responses of insects to fungal infection</u>:

Once the mycelia pass the insect cuticle, they bud into blastospores, while phagocytes concentrate around infection sites (Ferron, 1975). Vey (1967) described the cellular reaction of larvae of *Galleria mellonella* infected with *Aspergillus niger*; it is mainly plasmatocytes that gather tightly around the fungi. Hyphae are enclosed in a melanized sheath. After some days the pseudo-cell tissue takes a form of granuloma. This defense mechanism has been produced *in vitro* using invertebrate cell culture technics (Vey, 1967; Vey and Vago, 1971) which has revealed a phenomena of attractivity of haemocyte by mycelia. These haemocytic aggregates can grow big (diameter up to 1mm). The development of the disease may be stopped and the insect continues its development with the presence of granuloma, or the mycelia escape and multiply and the insect dies.

Vey and Vago (1971) distinguished the relative importance of these phenomena depending on the pathogenicity of the fungi; with moderately pathogenic fungi like *Aspergilus niger* v. Tiegh it looks like there is healing (Vey and Vago, 1969), the fungi remains isolated in its envelop for the rest of the insect's life; with highly pathogenic fungi like *Beauveria*, haemocytes may initially start gathering, even phagocytosis, may start but the process will be stopped later, most likely because of the toxins produced by the fungi. Death occurs because of a weak cellular reaction which

cannot prevent dispersion of blastospores in the haemocel and mainly because of toxins. Zacharuk (1971) observed that by the time the insect dies the ultrastructure of the cell in adipose tissue, malpighian tubules, digestive system, abdominal ganglion and muscles are all modified even if there is no mycelia around them (cytoplasms are vacuolated, *lysozomes increase in size*, mitochondria are degenerated). The role of toxins in the infection process was found important. Therefore some authors identified different toxins from cultures of fungi like *Beauveria, Metarhizium, Cordyceps* and *Entomophthorales*. Kodaira (1961) purified destruxins A and B from filtrates of *Metarhizium anisopliae*. Roberts (1969) decribed the symptoms they provoke after intrahaemocelic injection. Suzuki et al. (1970) isolated and described the structures of 3 other toxins, destruxins C and D and desmethyl-destruxin B. *Beauveria* was also shown to produced a toxic component called Beauverin (Hamill et al., 1969; Dorschner and Lardy, 1969) which is a cyclodepsiptide.

Post-mortem development of the fungi:

After the host dies the fungi continues its development as saprophyte, colonizing different tissues and growing through the cuticle and covering the entire cadavar. There is competition with bacterial flora, particularly abundant in certain insect species e.g. Oryctes (Ferron, 1975). In the majority of the cases insects that die of fungal infection do not putrefy, one may think the fungi produces antibiotic substances.

2.8. Tick Control methods:

2.8.1. Chemical control:

Tick control has been dependent on acaricides for decades. Chemical control of ticks has been directed at both free-living and parasitic stages but mainly at the parasitic stage.

2.8.1.1. Control of parasitic stages:

This method is widely used against livestock ticks. Several types of chemicals have been used, ranging from arsenicals to chlorinated hydrocarbons, Benzene hexachloride, Toxaphene, Organophosphorus compounds, carbamates and pyrethroids applied mainly dipping or hand spraying (Wellcome, 1976). Application of acaricides has given remarkable results as in the case of the eradication of *A. variegatum* in Puerto Rico (Garris et al., 1979) and the eradication of East coast fever in Zimbabwe (FAO, 1984). However, the misuse of acaricides and ability of ticks to develop resistance have favoured rapid development of resistance to acaricides throughout the world as in the case of *Boophilus decoloratus* against Arsenic, Benzene hexachloride and Toxaphene (Wharton and Roulston, 1970, Chema, 1984). In addition to the development of resistance, acaricide costs are exorbitant. The annual acaricide bill in Kenya is close to US$10 million (Young et al. 1988). Attempts have, therefore, been made to reduce the

dependency on acaricides by the use of integrated control method that increases the level of disease stability while reducing reliance on acaricides by natural exposure to diseases such as anaplasmosis and babesiosis when animals are young, and by artificial immunization if older animals (Dolan, 1988). This was suggested as a component of an Integrated Ticks and Tick-borne Management Programme by Kaaya (1992).

2.8.1.2. **Treatment of free living stages**:

Acaricide treatment of vegetation has been done on specific sites (eg. trails) in recreational areas in the USA and elsewhere to reduce the risk of ticks attacking people. Although not recommended for wider use because of environmental pollution, the method has shown some effectiveness, especially in fowl runs, pigeon lofts, pig sues and human dwellings (Anonymous et al., 1991).

2.8.2. **Non chemical control methods**:

2.8.2.1. **Pasture spelling or rotational grazing**:

Aims at starving the ticks by rotating animals from one pasture to another. Despite its technical efficiency, this method has shown some limitations. These include the cost of fencing and watering facilities, and also the fact that pasture if not grazed,

may deteriorate to the extent of being wasted (Australian Government Publishing Service, 1975).

2.8.2.2. **Burning of vegetation and cultivation**:

This includes burning of pastures or trees and cultivation of crops (Australian Government Publishing Service, 1975).

2.8.3. **Use of tick resistant cattle**:

A lot of studies on cattle resistance to ticks have been done. Various investigators have reviewed the acquired resistance in cattle and laboratory animals to feeding ticks (Willadsen, 1980; Wikel, 1982, 1984; Brown, 1985; Latif, 1986; Rechav, 1987; Latif et al., 1988; Dipeolu, 1990). Attempt to use tick resistant cattle as a control method for *B. microplus* ticks has been done in Australia and Asia with various successes (Australlian Government Publishing Service, 1975). The acquired resistance acquired in the decrease of engorgment period of feeding ticks, engorgment weight and reduction of normal eggs number and hatchability (Latif, 1984; Dipeolu and Harunah, 1984).

Some scientists have used tick antigens to render cattle resistant to tick infestation. Kemp et al. (1986) reported gut damage in *Boophilus microplus* fed on cattle vaccinated with extracts derived from whole adult ticks. Agbede and Kemp (1986)

demonstrated histopathologically that there was extensive gut damage in ticks fed on immunized cattle, enough to allow large substances like erythrocytes to pass across the tick midgut into the haemolymph (Kemp et al. 1986). Opdebeeck et al. (1988) reported that vaccines made from the midgut and syngaglion protected cattle against *B. microplus*, while a vaccine prepared from synganglion alone did not. Essuman et al. (in press) immunized Friesian cattle with semi-purified gut antigens of semi-engorged *R. appendiculatus* against all stages of the same species. They observed a significant decrease in the engorgement weight of females and an increase in the mortality in both sexes.

2.8.4. **Biological control**:

2.8.4.1. **Predators**:

a. **Birds:**

A group of predators of ticks has been listed by Kaaya (1992). Oxpeckers, *Buphagus* spp. black-billed magspies *pica pica*, the cattle egret *Ardeola ibis*, lizards, shrews, cotton rats, mice, hamsters and domestic chicken have all been reported to predate on various tick species (Cooksey and Davey, 1987). The red-billed Oxpecker *Buphagus erythrorhynchus* (Stanley) and the yellow-billed oxpecker *B. africanus* have been reported in Tanzania (Moreau, 1933), Kenya (van Someren, 1951), Zambia several other African countries (Attwell, 1966). The birds are associated with wild animals like eland, roan entelope, buffalo, rhinoceros and

hippopotamus (Attwell, 1966) as well as domestic animals cattle, pigs, sheep and donkeys (Moreau, 1933). They feed mainly on ticks and flies (van Someren, 1951) although they take advantage on the presence of sores and abrasions (Moreau, 1933). Moreau (1933) and van Someren (1951) examined stomach contents of red-billed oxpeckers in Tanzania and Kenya, respectively, they found that the birds feed mainly on ticks and flies. Oxpeckers could be potential biological control agents despite the fact that they failed to effectively control ticks in Australia. The cattle egret which was introduced in western Australia for tick control is present in most areas where cattle tick occur but has little effect on ticks and it is known to feed on insects disturbed from the grass by cattle (Australian Government Publishing Service, 1975).

b. **Ants**:

Ants have been found to be predators of several arthropod pests. The fire ant *Solenopsis invecta* (Buren) was reported to have controlled the lone star tick *Amblyomma americanum* (L) on the upland areas of Louisiana in USA (Burns and Melancon, 1977). Negm and Hensley (1967, 1969) also reported increased damage to sugar cane in Louisiana by the sugar-cane borer, *Diatraea saccharalis* (F) following treatment of sugar-cane fields with heptachlor for fire ant control. Ants play an important role in the predation of arthropods but their use as bological control agents appear to be impracticable and undesirable (Australian Government Publishing Service, 1975) especially in Africa where majority of the population have low income. Ants, especially fire ants, have been

proven to be polyphagous; they feed on anything even human being. They will end up becoming a nuisance to people instead of solving their problems.

2.8.4.2. **Pathogens**:

2.8.4.2.1. **Bacteria**

Bacillus thuringiensis, Salmonella enteritidis, Proteus mirabilis and *Serratia marcescens* have been reported as ticks pathogens, causing blackening, swelling and weakening of the cuticle (Hendry and Rechav, 1981). Five to ten percent of ticks in a laboratory colony of *Boophilus decoloratus* was reported to be affected by a blackening disease. The disease was due to *Proteus spp., Klebisiella pneumonie, Pseudomona sp., P. mirabilis.* Mwangi (1990) isolated *P. mirabilis, Pseudomonas sp. and S. marcescens* from engorged *R. appendiculatus*. How do these bacteria affect tick immune system? Could they be used as biological control agents?

2.8.4.2.2. **Fungi**:

a. **Use of fungi as pest control agents**:

The idea of using fungi in biological control was born more than 150 years after Agostino had elucidated for the first time the etiology of a contagious fungi in 1835 (Zimmermann, 1986). Metschnikoff (1879) and Krassilatschik (1888) were the first who mass-produced a fungus, *Metarhizium anisopliae*, for control of the wheat cockchafer *(Anisopla austriaca)*, and later on the sugarbeet

curculio (*Cleomus punctiventris)*. Many other attempts have been made to control insect pests with entomogenous fungi (Müller-Kögler, 1965) but due to varying and often disappointing results, the first enthusiasm on the possibilities and prospects of microbial control with fungi decreased. For the past 20 years there has been an increasing interest on fungal pathogens mainly because of the findings of insecticides' negative environmental impact (Zimmermann, 1986). Several fungi have been used for the control of various important arthropod pests of agriculture and forestry. Most of them are Deuteromycetes among which the two most prominent members are *Metarhizium anisopliae* and *Beauveria bassiana*. Howether, very little has been done to study the effects of these entomogenous fungi on haematophagous arthropod- pests and their biocontrol potentials.

i) **Beauveria bassiana**:

Beauveria bassiana is produced on a large scale for practical use in the USSR and the People Republic of China. The Russian preparation "BOVERIN" is recommended for the use against different insect pests, mainly *Leptinotarsa decomlineata* and *Cydia pommelia* (Ferron, 1981). Mostly it is mixed with reduced mixtures of chemical insecticides to increase the susceptibility of the target insect. In the People's Republic of China *B. bassiana* is, besides *Bacillus thuringiensis*, the most frequently used pathogen in microbial control. It is produced in several hundreds of production units or communes and is applied against the European corn borer *(Ostrinia nubilalis)*, pine caterpilars (*Dendrolinus spp*.) and

leafhoppers, *Nephotettit* spp. (Franz and Krieg, 1980; Hussey and Tinsley, 1981). Hsiao (1982) mentioned that *B. bassiana* killed 80% of L3-L4 pine caterpillars when applied either as dust at a rate of 191/ha containing 5×10^9 spores per gram or when sprayed at 1,2001/ha containing $0.1-0.2 \times 10^9$ per ml.

ii) **<u>Metarhizium anisopliae</u>**:

In Brazil, Metaquino (*Metarhizium anisopliae*) is used to control spittlebugs (*Mahanarva posticata*) on sugarcane (Ferron, 1981). More than 100,000 ha are treated annually, resulting in 40% of pest reduction (Roberts and Wraight, 1986). *Metarhizium* in conjunction with a bucalovirus has been used successfully for the control of coconut palm rhinoceros beetle (*Oryctes rhinoceros*) in Pacific Islands and in S.E. Asian countries (Zimmermann, 1986).

b. **<u>Infection of ticks by fungi</u>**:

There are records of ticks found to be infected with fungi. Samsinakova et al. (1974) in Czechoslovakia observed that *Ixodes ricinus*, *Dermacentor marginatus* and *D. reticulatus* in the field were infected with 17 fungi belonging to the groups *Deuteromycetes* and *Phycomycetes*. Mwangi (1990) isolated *Mucor* spp., *Fusarium* spp. and *Aspergillus* spp. from adult *R. appendiculatus*.

There are, however, very few records on the use of fungi as tick biological control agents. Mwangi (1990) recorded 75 and 35 percent mortality in engorged adult *R. appendiculatus* experimentally infected by immersion with a concentration of 10^9 spores/ml of *B. bassiana* and *M. anisopliae*, respectively after 2

weeks. The same mortalities, 73 and 35 percent respectively, were observed in unfed adults of *R. appendiculatus*. In contrary, Kaaya et al. (1995) observed 30% mortality in adults *R. appendiculatus* fed on rabbits. No significant reduction was observed in engorgement weight, egg mass or hatchability (Kaaya et al. 1995). In *A. variegatum*, *M. anisopliae* induced a mortality of 37% while *B. bassiana* induced no mortality. Both fungi induced significant reduction in engorgement weights, egg mass and egg hatchability. *Metarhizium anisopliae* reduced fecundity of *A. variegatum* by 94% while *B. bassiana* seems to have some effects on eggs; reduced egg hatchability to 0%. Eleven percent of female infected with *B. bassana* failed to lay eggs. Five percent of female *R. appendiculatus* infected with *B. bassiana* also failed to lay eggs.

In the field, Kaaya et al. (1995) reported that on zebu naturally infected with *R. appendiculatus*, *M. anisopliae* and *B. bassiana* induced 76-85% mortality. The fungi also reduced fecundity and egg hatchability by 85-99% and 94-100% respectively. On *R. appendiculatus* seeded in grass field *B. bassiana* and *M. anisopliae* induced 100, 76-95 and 36-64% mortality to larvae, nymphs and adutls respectively. When incubated in organophosphate acaricides both fungi retained normal growth, and morphological characteristics. The two fungi are therefore good potential biological control agents.

2.8.4.3. **Parasitic wasps**:

There are several parasitoids of insects reported in the literature, most of them belonging to the order Hymenoptera and Diptera. They predominate in eleven out of fourteen superfamilies in the sub-order Apocrita (Norris, 1991). The largest superfamily, Chalcidoidea, comprises twelve families among which the most important are Ichneumonidae, Braconidae, Encyrtidae and Aphelinidae (Claussen, 1972). Parasitoids are very small insects (mostly less than 3mm). They parasitize or hyperparasitize the eggs, larvae or adult stages of other arthropdos (Claussen, 1972; Richard and Davis, 1977; Naumann, 1991).

a. **Biology of *Ixodiphagus***:

Ixodiphagus hookeri belongs to the family Encyrtidae and the tribe Ixodiphagini. Parasitoids of this tribe are reported to attack the nymphal stage of their hosts. Their immatures feed on the blood imbibed by the tick from its mammallian host. Ticks of the genera Amblyomma, Dermacentor, Heamaphysalis, Hyalomma, Ixodes and Rhipicephalus are affected (Cooley and Kohls, 1934).

Ixodiphagus hookeri Howard was first described by Howard in 1908 from nymphs of *Rhipicephalus sanguineus* latreille collected in Texas, USA (Mather, 1933). The parasitoid has also been reported from South America in Brazil and Cuba, in Asia from China and Japan (Philip, 1954), the USSR (Nikolskaya, 1952), and India (Shastri, 1983). Reports from Europe include Britain (Noyes, 1977), France

(Du Buysson, 1912; Brumpt, 1930), Czekoslovakia (Davis, 1986). In Africa *I. hookeri* has been reported in Nigeria (Philis, 1931), South Africa (Cooley, 1934), Uganda (Steyn, 1955), Kenya (Philip, 1954; Mwangi, 1990) and Côte d'Ivoire (Graf, 1979).

The wasp has been found in Kenya parasitizing the nymphs of *A. variegatum* which had engorged on cattle (Mwangi, 1990). It had earlier been found emerging from the engorged *Rhipicephalus sanguineus* (Philip, 1954). Since 1990 a colony has been established at ICIPE and the wasp has been successfully reared in nymphs of *A. variegatum* and *A. sparsum* but not in any other stage of *Amblyomma* nor any other tick genera.

The general life cycle of the parasitoid varies from two to seven weeks under optimum temperature conditions (Claussen, 1972). Mating occurs soon after adults emergence, and the mating behavior varies according to species (Van Den Assem, 1985). Males of Ixodiphagini emerge first, and copulation takes place near the host (Davies, 1986). Mating of *I. hookeri* occurs immediately after emergence of adults and lasts for a few seconds (Cooley and Kohls, 1934). Immediately after mating the female starts searching for the host and oviposits (Cole, 1934). She pierces the host integument with the ovipositor and lays eggs for 2-20 seconds (Cooley, 1933) or more. The egg of *I. hookeri* has been described by dissecting and crushing the ovaries from adult females (Cooley, 1928). The number of eggs injected per oviposition has been estimated at 5-10

(Cooley, 1928). The duration of the larval development depends on the temperature. The eggs or young larvae remain latent in the unfed nymphs and development continues once the nymphs are fed (Cooley and Kohls, 1934). It is not known how the development of the parasitoid is delayed when the tick is not fed and why the tick immune system does not eliminate parasitoid eggs.

Adults of *I. hookeri* emerge through a hole which they chew in the integument of the host tick (Wood, 1911; Cooley and Kohls, 1934). The number of adults produced varies and depends upon the tick host attacked (Cooley, 1928; Smith and Cole, 1943). Longevity of adult *I. hookeri* is normally 1-2 days (Wood, 1911; Cooley, 1928). However, longevity was found to vary at different temperatures; with 28 days at 11°C, 6 days at 15°C and 2 days at 27°C (Smith and Cole, 1943).

b. ***Ixodiphagus* as biological control agent**:

Ixodiphagus hookeri despite its small size has shown some signs of potential as a biological control agent. The parasitoid was released in Montana and adjacent area in 1927-1932 to control *Dermacentor andersoni*. About 4,158,000 wasps were released but the parasite did not become established nor did it reduce the tick population (Cooley and Kohls, 1934). This parasite originating from France, was also released in Massachussetts and adjacent Islands in 1926 in an attempt to control *D. variabilis*. It became established and persisted for at least 12 years (Larousse et al, 1928). Nymphs of *D. variablis* became parasitized by the chalcids from a distance

of 100 yards. Larousse et al. (1928) stated that the parasitoid survived the winter after release. The tick population on Naushion island was significantly reduced the following season in contrast with high populations nearby although not sufficient to eliminate entirely the need for other control measures. Ninety one thousand adults parasitoids were released, in 1937-39 on Martha's Vineyard Island, Massachussetts, but no recoveries were made during the following years. Workers in the USSR have also studied the possibilities of using this wasp for controlling *Ixodes persulcatus*, but no data on releases has been reported (Jenkins, 19..). By 1987, the wasps released on Islands in 1926 were still persistant. They appeared to have reduced the prevalence of pathogen (*Borelia burgdorferi and Babesia microti*) infection in *Ixodes damnini* by as much as one third (Mather et al. 1987).

A better understanding of the biology, ecology and behavior of the wasp is needed.

CHAPTER III

GENERAL MATERIALS AND METHODS

3.1. Experimental animals and microorganisms:

3.1.1. Ticks:

Ticks used in the study have been reared and maintained by feeding on rabbits at the International Centre of Insect Physiology and Ecology (ICIPE) tick rearing unit according to the method described by Bailey (1960) and Irvin and Brocklesby (1970). Nymphs and adults of *Rhipicephalus appendiculatus* and *Amblyomma variegatum* were used during the experiment. They were kept in an incubator at 25°C and 85% relative humidity at a photoperiodicity of 16 out of 24 hours.

3.1.2. Rabbits:

New Zealand white rabbits were provided by the ICIPE Animal Rearing Quarantine Unit. They were kept in separate metallic cages and offered standard rabbit cubes and water *ad libitum*.

3.1.3. Parasitoids:

Tick hymenopteran parasitoids, *Ixodiphagus hookeri*, were

supplied by Dr E. Mwangi of livestock pest research programme (LPRP). Female parasitoids were placed singly in 3x1 cm vials with engorged nymphs of *A. variegatum* and kept in 21x21 cm tins containing 40% Potassium chloride (for a constant 85% humidity).

3.1.4. Bacteria:

Non-pathogenic bacteria e.g. *Escherichia coli*, *Enterobacter cloacae* and *Bacillus thuringiensis* were used to induce the immune responses.

3.1.5. Fungi:

Entomopathogenic fungi, *Beauveria bassiana* and *Metharizium anisopliae* were, like bacteria, used to induce tick immune responses. They were provided by Dr Kaaya of L.P.R.P.

3.1.6. Microscope:

A Nikon Phase contrast microscope was used to identify and count tick haemocytes and bacteria. The same type of microscope was also used to observe different stages of the tick parasitoid. Pictures of tick haemocytes and parasitoids were taken using a Wild phase contrast microscope fitted with a Wild MPS 55 camera.

A Philips CM 12 electronique microscope was used to observe

different types of tick haemocytes and the types of haemocytes that play a role in encapsulation and nodule formation.

3.2. Haemocyte studies:

3.2.1. Morphology:

Nymphs and adult ticks were bled by cutting the leg(s) at coxa level with a pair of forceps and the haemolymph was forced out by applying gentle pressure. The haemolymph was put on microscope slide or aspirated into a graduated 5 μl micropipette, diluted to a required volume by aspirating in a certain quantity of ice cold phosphate buffer saline (PBS), mixed gently and placed on a microscope slide. In both preparations, haemocytes were examined using phase contrast microscope (Kaaya et al. 1982).

3.2.2. Total and differential haemocyte counts (THC and DHC):

Total and differential haemocyte counts were determined by aspirating 1-2 μl of haemolymph into a 10 μl micropipette, and then diluting to a required volume by aspirating a further quantity of ice cold PBS (Kaaya et al. 1982). The mixture was gently mixed, placed on a haemocytometer and the haemocyte concentration was determined. Differential haemocyte count (DHC) was determined from the same preparation by counting 100-200 different haemocytes from each specimen (Kaaya et al. 1982).

Twenty ticks were used in each experiment.

3.2.3. Cellular immune reaction:

Immunized ticks were bled and the haemolymph examined for phagocytosis and free bacteria at given time interval (Kaaya et al. 1987). Haemolymph smears were examined for nodule formation using phase contrast microscope.

3.2.3.1. **Antibacterial activity studies**:

3.2.3.1.1. **Serological procedures and antibacterial assays**:

3.2.3.1.1.1. Haemolymph collection: Adults of *A. variegatum* and *R. appendiculatus* were injected with different concentrations (10^3, 10^4, 10^5) of bacteria suspended in 2-5 μl of PBS and haemolymph was collected and placed in glass or plastic vials containing a few crystals of phenylthiourea (PTU). Haemolymph samples were analyzed directly after collection or were pooled and stored in ice-cold eppendorf tubes at -20°C until required for use (Kaaya et al. 1987).

3.2.3.1.1.2. **Antibacterial activity**:

Antibacterial activity was tested as explained by Postlethwait et al. (1988). In some experiments, antibacterial activity was

tested by the inhibition zone assay for the determination of Lysozyme and other antibacterial factors. In this test, factors diffuse from a well cut in an agar plate (diameter 2-2.5mm) seeded with the test bacteria (e.g. *E. coli*) and dead lyophilized *Micrococcus luteus* for lysozyme activity. For all haemolymph samples in this investigation antibacterial and lysozyme activity were determined by measuring diameters of inhition zones from wells. Each experiment was run thrice and mean diameters were calculated compared using the General linear model (GLM) procedure. A standard curve prepared using known concentrations of egg-white lysozyme in *Micrococcus luteus* plates were used for the estimation of lysozyme concentration in haemolymph samples (Kaaya and Darji, 1988).

3.2.3.1.1.3. In other experiments, haemolymph proteins were separated by electrophoresis on polyacrilamide gels at PH 4. To detect antibacterial activity after electrophoresis, the gels were overlaid with phosphate buffered agar seeded with *E. coli* and regions with antibacterial factors were visualized for any bacterial growth.

3.2.3.1.2. <u>Induction parameters</u>:

From insects inoculated with different bacterial concentrations, haemolymph was collected at different time

intervals and assayed for antibacterial activity as described above. This test gave two fold results. It was used to determine the number of bacterial cells needed to raise the antibacterial activity above that of wounded controls, and also to determine the time course of antibacterial activity, especially the time taken to mount an immune response (Postlethwait et al., 1988).

CHAPTER IV

Characterization and comparison of different morphological types of haemocytes in *Amblyomma variegatum* and *Rhipicephalus appendiculatus* as well as total and differential haemocytes counts in bacteria, fungi and parasitoid infected and unfected ticks.

Very few records exist on tick haemocytes. Dolp (1970) found 3 types of haemocytes: prohaemocytes, plasmatocytes (early and advanced stages) and spherule cells (early and advanced stages) in *Hyalomma anatolicum excavatum* and *H. dromedarii*. Bunnington and Burgdorfer (1971) identified in *Dermacentor andersoni* prohaemocytes, plasmatocytes and 4 subtypes of spherule cells. The most striking finding in these two studies is that no granulocyte was found. While Balashov (1979) identified two types of granulocytes, prohaemocytes and plasmatocytes in *H. asiaticum*. There is no record on haemocyte types of *A. variegatum* and *R. appendiculatus*.

4.1. Materials and methods:

4.1.1. Characterization of tick haemocytes:

Ticks were applied to the ears of rabbits as described by Bailey (1960) and Irwin and Brocklesby (1970). Ear bags were attached to rabbits ear (one per ear) with leukoplast. A round

colar was attached around the neck of the rabbit behind the ears preventing it from scratching the ticks off. Fifty pairs of *R. appendiculatus* or 15 pairs of *A. variegatum* were placed in ear bag of each ear of the rabbit. Ticks were collected after engorgement and bled directly on Neubauer slide (for counting) and ordinary slide (for staining). The bleeding was done using the method described earlier. Other legs could be used but the first pair was found to give the best amount. Engorged *A. variegatum* and *R. appendiculatus* always give higher amount of haemolymph when punctured in the area behind the capitulum using a microcapillary tube sharpened using a vertical pipette puller, Model 700c, David Kopf Instruments. The collected haemolymph was dropped immediatly on 5μl of Percoll gradient (Mead et al., 1986) on Neubauer haemocyte count chamber and covered with a coverslip. A phase contrast microscope was used to identify cell types one or two minutes after the slide was mounted.

Haemolymphs from engorged females of both tick species were also collected directly in an eppendorf tube containing 3% glutaraldehyde in 0.1M phosphate buffer (pH 7.2) and centrifuged at 3000g in a megafuge 1.0 Heraeus Spartech. The pellet was collected and prepared for electronic microscopy according the method described by Ratcliffe and Gagen (1977). The pellet was transfered in a fixative solution consisting of 2.5% glutaraldehyde in 0.05 M sodium cacodylate at pH 7.2 containing 0.15 M sucrose (20 mn) then postfixed in 1% Osmium tetroxyde (20 mn), immersed in 1 % aqueous

uranyl acetate (30 minutes), dehydrated in graded methanol, and embedded in Araldite. Ultrathin sections were cut using an LKB3 Ultramicrotome. They were then mounted on copper grids, stained in 305 uranyl acetate in methanol (7 minutes), and then in lead citrate (7 minutes), and were examined using a TM Philip CM12 electron microscope.

4.1.2. Haemocyte count:

Two methods of haemocyte counts were used for both infected and uninfected ticks.

4.1.2.1. Uninfected ticks:

4.1.2.1.1. Total haemocyte count (THC):

a. Wet count: Tick haemolymph was diluted in a 5 μl capillary tube with percoll gradient to 1:4. The diluted haemolymph was put on a Neubauer haemocyte count chamber and haemocytes were counted. The following formula was used to determine the Total haemocytes number.

$$THC = \frac{\text{No cells x dilution}}{\text{Volume counted}}$$

ie. For 80 small squares the volume counted is = 0.02 μl

b. Smear:

A drop of haemolymph was put on a grease-free microscope slide

and a smear was made by drawing a second slide across the first one at a 45° angle. The smear was air-dried and stained using the modified Pappenheim method (1914) which is as follows: (1) flood air-dried smear with wright (instead of May Grunwald) stain (0.3 mg/100 ml) solution and allow to remain on slide for 3 min; (2) add distilled water so that a layer is formed and allow to stand for 2 min; (3) discard the wright-distilled water layer; (4) flood slide with Giemsa solution (I part concentrated giemsa solution to 40 part distilled water) and allow to dry; (5) wash slide in running tap water and allow to dry.

Smears were examined under immersion oil, and at least 200 haemocytes were differentiated. By this method of staining, azurophilic material appears purple red; chromatin, reddish violet; and basic protoplasm, blue (Pappenheim, 1914).

4.1.2.1.2. Differential haemocyte count:

Differential haemocyte count was done after leaving the slide on the bench for 2 to 5 minutes to let cells settle. At least 100 to 200 cells per tick were counted

4.1.2.2. Infected ticks:

Bacterial infection: The following bacteria were used, *Escherichia coli*, *Enterobacter cloacae* and *Bacillus thuringiensis*. They were provided by Dr Kaaya.

Bacteria were grown in Nutrient broth then centrifuged 3 times at 3000g for 15 min. The supernatant was every time poured of and replaced by PBS Ph 7.2. Then the concentration was determined using the method described by Dacie and Lewis (1984) which consist of counting cells in 4 or 8 horizontal rectangles of 1 mm x 0.05 mm (80 or 160 small squares) or in 5 or 10 groups of 16 small squares, including the cells which touch the bottom and left-hand margins of the small squares. The formula used was described previously.

Bacteria were injected at a dose of $2x10^3$, 10^4 or $10^5/\mu l$ per tick using the method described in chapter III. Haemolymph was collected after 1, 3, 6 12, 24 and 48 hours using the method described earlier.

Total and differential haemocyte count (THC and DHC):
THC and DHC on ticks infected with bacteria was done using the method described in 4.1.2.1.1.

4.2.3. Fungal infection:

A preliminary study was done to determine the time taken by fungi to get access to tick haemolymph when applied topically. It was found that blastospores don't reach tick haemolymph before 4 to 5 day post-infection. Also ticks give enough haemolymph if they are bled the day they are removed from the host or the day after. It was therefore decided to undertake intrahaemocelic injection of spore for the present study.

Fungal spores (provided by Kaaya) were seeded in petri dishes containing Sabouraud Dextrose Agar (SDA). The petri dishes were sealed with parafilm and left on the bench for the fungi to grow. Fourteen day old hyphae were collected from the culture and centrifuged 3 times in a megafuge at 3000g for 15 minutes. The supernatent was poured of after every centrifugation and replaced by Pbs (ph 7.2). The concentration of the fungal solution was then determined using a phase contrast microscope and a Neubauer slide.

Engorged females of *A. variegatum* and *R. appendiculatus* were injected with 2 x 10^8 (or 10^9)/μl of *entomopathogenic fungi Metarhizium anisopliae* and *Beauveria bassiana* using the method described in 4.1.2.2.1. The haemolymph was collected in eppendorf tubes and kept in a freezer for different experiments.
Total and differential haemocyte count were done using the method described in 4.1.2.1.1. Haemolymphs were monitored for other cellular reactions.

4.1.2.4. **Parasitization**:

Engorged *Amblyomma* nymphs were put singly in vials along with mated female parasitoids (one parasitoid/nymph/vial) and monitored until the parasitoid laid its eggs inside the tick. Haemolymph was collected after 1, 3, 6, 12, 24, 48 hours from parasitized ticks and non-parasitized nymphs and stored in aliquot at -20°C immunological work (gel). Total haemocyte counts were done using

the method described earlier.

4.2. RESULTS:

Most of the works in this study were done on fed (engorged) ticks because they provide more haemolymph and there were less viscosity and turbidity caused by cells compared to haemolymphs in unfed ticks. *Rhipicephalus appendiculatus* was not used in some of the experiments because of its fragile cuticle and it provided less haemolymph. It was almost impossible to collect haemolymph from *R. appendiculatus* at 1, 3 up to 24 hours post infection. The time required for the wound in *A. variegatum* required a shorter time. They could be bled at one hour post-infection without any risk of bursting.

4.2.1. Characterisation:

Seventy five adults of *R. appendiculatus*, 50 adult *A. variegatum* and 50 nymphs of *A. variegatum* were bled. The following cell types were identified:

a) Plasmatocytes: (Fig. 4.3 - 4.7)

Medium to big cells, the average diameter was 15.3 ± 1.3 μm, nucleus 9.3 ± 1.5μm. The nucleus was round and generally centrally located. Plasmatocytes had few smaller granules compared to granular haemocytes and spherule cells. The nucleus appeared reddish-purple with granular chromatin. The blue staining indicates the presence of vacuoles as mentionned earlier. The nucleus:cytoplasmic ratio fairly high (Fig. 4.3 and 4.4).

Plasmatocytes were found either to be rounded or spindle shaped and appeared dark blue when stained using modified Pappenheim method. Filopodia were sometimes present.

Ultrastructurally PLs presented an irregular surface with presence of cytoplasmic projections (Fig. 4.5 - 4.7). The nucleocytoplasmic ratio was fairly low. The cytoplasm was dense and contained various shapes of RER and mitochondria. There were several lysozomes-like structures visible as dense small granules.

b) Granular haemocytes: (Fig. 4.8 - 4.10)

Granular haemocytes were mostly spherical or ovoid with numerous granules. They had very fragile membrane. They resembled granular haemocytes 2 found in *Locusta migratoria* (Drif and Brehelin, 1993). In phase-contrast microscopy, they were easily recognized by the presence of numerous granules. The nucleus:cytoplasmic ratio was low (Fig. 4.8 and 4.9). The diameter was 16.7 ± 2.7 μm, nucleus 8.3 ± 1.6 μm. They appeared reddish when stained using modified Poppenheim method.

Ultrastructurally Grs presented various shapes (round and oval) of granules (Fig. 4.10). Some granules were electron dense. The cytoplasm also contains lipid-like droplets. The presence of deep invaginations and cytoplasmic projections.

c) Oenocytoids: (Fig. 4.13 and 4.14)

Large to medium size, they were characterized by the absence of granules. Oenocytoids appeared violet when stained (Poppenheim

stain). They were medium to large cells and appeared darkish in wet mount (Fig. 4.13). The diameter 18.2 ± 2.3 μm, nucleus 8.1 ± 1.2 μm.

Ultrastrucrurally the nucleus presented an amorphous band of chromatin at its periphery (Fig. 4.14). Several mitochondria and free and attached ribosomes were present in the plasma. Very few ganules were present. The RER was scattered all other the body of the cell body.

d) <u>Prohaemocytes</u>: (Fig. 4.1 and 4.2)

Small to medium size, they lucked granules. The nucleus was generally centrally-located with a homogenous chromatin. The nucleus:cytoplasmic ratio was high (Fig. 4.1). Prohaemocytes were generally smaller than other cells and could be confused with big granules. The diameter was 9.7 ± 1.6 μm; nucleus 7.3 ± 1.3 μm.

Ultrastructurally the nucleocytoplasmic ratio was high (Fig. 4.2). Heterochromatin material was distributed as densely staining amorphous patches in a lightly reticulated and moderately electron-dense nucleoplasm. The rough endoplasmic reticulum (RER) was constituting a narrow band varying in electron density around the nucleus. There was presence of mitochondria.

e) <u>Spherule cells</u>: (Fig. 4.11, and 4.12)

Giant cells with big granules, spherule cells were easily broken especially when making a slide (Fig. 4.12). Spherules stained reddish purple and the nucleus light pink with granular

chromatin. The nucleus:cytoplasmic ratio was very low. The diameter was 19.5 ± 2.6 *μm; nucleus* 9.3 ± 1.5 *μm*.

Ultrastructurally SPs were characterized by the presence of several spherules (Fig. 4.11). Some spherules were electron dense. The nucleus was containing amorphous heterochromatin located at the periphery. Between the spherules there were RER, mitochondria, and free ribozomes. There was also presence of short filopodial extensions.

4.2.2. Haemocyte count:

4.2.2.1. Uninfected ticks:

a. Total haemocyte count:

Haemocytes from 50 adults of *A. variegatum*, 75 adults of *R. appendiculatus* and 50 nymphs of *A. variegatum* were counted. Table 1 shows THC on infected and non-infected. Although not significantly different, the mean THC in the nymph of *A. variegatum* were higher than that of adult *A. variegatum* and *R. appendiculatus* (Table 4.1a).

b. Differential haemocyte counts:

Among the 5 types of haemocytes present in both species. spherulocytes outnumbered others (Table 4.2), plasmatocytes come second and granular cells, oenocytoids and prohaemocytes were in lower numbers in both ticks.

4.2.2.2. Infected ticks:

4.2.2.2.1. Bacterial infection:

a. Total haemocyte count:

Tick haemocytes reacted to bacterial infection. Table 4.1 shows THC in all the ticks after 24 hours of infection were higher than THC in uninfected ticks. The number of haemocytes in ticks infected with *E. coli* 10^5 was lower than that of *E. coli* 10^4 (Table 4.1c). This can be explained by the fact that bacterial concentrations of 10^5 or higher caused cell lysis very fast; it was difficult to do haemocyte count after 6 hours post-infection (Table 4.3).

b. Differential haemocyte count:

DHC of ticks infected with *E. coli* 10^4 were not significantly different from that of non infected ticks. Total number of each type of haemocytes were more or less equal in all the species (Table 4.2).

4.2.2.2.2. Fungal infection:

a. Total haemocyte count:

THC in ticks infected with entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* are shown in table 4.1b. They were lower to THC of ticks infected with *E. coli* and non infected ticks.

b. Differential haemocyte count (DHC):

Table 4.3 shows the effect of fungal infection on DHC of ticks infected by fungi. The percent number of haemocytes, especially granulocytes and spherule cells, were lower in infected ticks than in uninfected ticks.

4.2.2.2.3. Parasitoid infection:

The effect of parasitoids on haemocytes of the nymph of *A. variegatum* is shown in figure 4.1. Haemocyte numbers increased after 12 hr of parasitization and started decreasing after 24 hours.

4.3. DISCUSSION:

4.3.1. Characterisation:

Description of haemocytes in this study was based on morphological classification. Five haemocytes were found in both *A. variegatum* and *R. appendiculatus*; prohaemocytes, plasmatocytes, granulocytes, oenocytoids and spherule cells.

Prohaemocytes were rarely found in this study because only engorged female ticks were used. This phenomenon was also observed in *Hyalomma a. excavatum* and *H. dromedarii* by Dolp (1970) and in *Glossina austeni, G. m. morsitans, Calliphora erythrocephala, Stomoxys calcitrans, Lucilia sericata, Aedes aegypti and Culex quinquefasciatus* by Kaaya and Ratcliffe (1982). Dolp (1970) found PRs more common in immatures and young adults in conformity with the statement by Jones (1975) saying that PRs are more common in early stages of insect development. These observations also confirm the view that PRs are stem cells (Lai-Fook, 1973) which transform into Plasmatocytes (Jones 1956) and may be other haemocytes (Arthur and Richards, 1964). In the present study it was difficult to differentiate Prs and Pls. The same difficulty was observed by Gupta (1979) in *Periplaneta migrata* and *Locusta migratoria* and (Kaaya and Ratcliffe, 1982) in *Glossina austeni, G. m. morsitans, Calliphora erythrocephala, Stomoxys calcitrans, Lucilia sericata, Aedes aegypti and Culex quinquefasciatus*. Prohaemocytes in *A. variegatum* and *R. appendiculatus* were similar to the one found in *H.a. excavatum* and *H. dromedarii* (Dolp, 1970), *D. andersoni*

(Brinton and Burgdorfer, 1971), and *H. asiaticum* (Balashov, 1979), based on the typical high nuclear cytoplasmic ratio and the intensely basophilic cytoplasm, ultrastructurally by the presence of rough endoplasmic reticulum around the nucleus, and mitochondria in the cytoplasm.

Plasmatocytes also showed some characteristics like the presence of pseudopods which were also found in plasmatocytes reported in *H.a. excavatum* and *H. dromedarii* (Dolp, 1970), *D. andersoni* (Brinton and Burgdorferi, 1971) and *H. asiaticum* (Balashov, 1979). Pleomorphism reported in some insects (Jones, 1965; Arnold and Salked, 1967) was observed in *A. variegatum* and *R. appendiculatus* in this study as well as in *D. andersoni* (Brinton and Burgdorferi, 1971) and *H. asiaticum* (Balashov, 1971). Ameboid movements and presence of mitochondria at the base of filopodia were observed implying a phagocytic capacity of the cell as mentionned by Dolp (1970).

Granulocytes are polymorphic cells with several round or oval granules of different sizes. Those features were observed in type I granulocytes in *H. asiaticum* by Balashov (1979) and and type IV spherule cells in *D. andersoni* by Brinton and Burgdorfer (1971). Cytoplasmic projections or filopodia thought to be chacteristic of phagocytic haemocytes (Crossley, 1975) were observed. Filopodia were also oberved in young granulocytes in *H. asiaticum* (Balashov, 1979).

Spherule cells identified in this study resemble spherule cells type III in *D. andersoni* (Brinton and Burgdorfer, 1971) because of the presence of several spherules sometimes pushing the nucleus at the periphery. Spherule cells described by Dolp (1970) in *H.a. excavatum* and *H. dromedarii*, especially the ones she called advanced spherule cells, also showed some features like the size of the cells and the purple staining of the nucleus similar to the one found in this study. Dolp (1970) used unfed and fed ticks while only fed ticks were used in this study, this may explain why early spherule cells were not found in *A. variegatum* and *R. appendiculatus*.

Oenocytoids were scarcely seen. They were medium to large cells more or less similar to the ones described by Brinton and Burgdorfer (1971), epecially the eccentric position of the nuclei and the existence of large gray-staining granules. But further investigations are necessary, especially the use of electron microscopy and cell lines, to ascertain different types of haemocytes found in ticks.

4.3.2. Total haemocyte count:

Total haemocyte count (THC) of engorged females of both *A. variegatum* and *R. appendiculatus* were 3,588.1 ± 55.108 and 3450 ± 54.309 respectively. They were more or less equivalent to THC of engorged females of *H. a. excavatum* (4,921 ± 3250) but not that of

unfed males (22,343) and females (36,941) of the same species Dolp (1970). Dolp in her study found THC of unfed males and females much higher than THC of engorged females. More investigations are necessary to understand that phenomenon.

Total haemocyte count in ticks infected with bacteria were higher than the THC in uninfected ticks. This implies a certain reaction of tick haemocytes to bacterial infection. The low THC in ticks infected with *E. coli* 10^5 (Table 4.1) may be due to a high degree of haemocytes lysis and involvement of nodule formation. Bacterial concentrations of 10^5 were too high. There were some cases of melanization at 24 hours post-infection; haemocytes could not be counted (Table 4.3, Fig. 4.15).

Fungi appear to be more damaging because of the lower THC (Table 4.1) and melanization in 3 days. Toxic substances, destruxins A, B, C, D, and desmethyl destruxin B in *Metarhizium anisopliae* (Kodaira, 1961; Roberts, 1969; Suzuki et al., 1970; Suzuki and Tamura, 1972) and Beauverin from *Beauveria bassiana* (Hamill et al., 1969; Dorschner and Lardy, 1968) probably induced lysis of tick haemocytes. Further investigations are necessary to explain this phenomenon.

4.3.3. <u>Differential haemocyte count (DHC)</u>:

Spherule cells compared to other haemocyte types, were more

prevalent in both species. This observation was found by Dolp (1970) in engorged females of both *H.a. excavatum* and *H. dromedarii*; the percent number of spherule cells increased by 2 fold with engorgement (from 25 to 50%). While in unfed ticks especially in *H.a. excavatum*, plasmatocytes and prohaemocytes were more prevalent.

Infection of ticks with bacteria or fungi did not cause any major change in the percentage of each type of haemocytes (Table 4.3); spherule cells remained more prevalent in all infected ticks. There is a possibility that a massive production of haemocytes in reaction to infections occured. This again raises the issue of haemocytes origins in insects and acari. The existence of Haemopoietic organ tissues for the production of haemocytes in immature stages of several insects and acari have been demonstrated (Hoffman et al., 1979). After pupation the organ remains in adult exopterygota and continues to provide haemocytes especially when the immune system is challenged or when the haemocyte population is depleted. In contrast, it disappears in endopterygota. The production of haemocytes is insured by circulating haemocytes. They divide through mitosis and maintain the level of population (Lackie, 1988). The latter case is likely to happen in ixodid ticks; some binucleated and extended haemocytes were noticed in haemolymph of *H.a. excavatum* and *H. dramedarii* (Dolp, 1970) and *Boophilus decoloratus (George, 1987)*. Dolp (1970) have observed some pro-, ana-, meta- and prophases in haemocytes of some ixodid

and argas.

Table 4.1
Total haemocyte counts in *A. variegatum* and R. appendiculatus

a) Non infected ticks

	Means THC	Number of ticks used	STD	Range 95 % m ± STD	Coefficient of variation (CV), %
Avf	3588.1	75	55.10869	3532.991 - 3643.208	1.5
Avn	3614.29	50	55.30944	3558.980 - 3669.599	1.5
Raf	3450	50	54.03776	3395.962 - 3504.037	1.5

Avf = Female of *A. variegatum*
Avn = Nymph of *A. variegatum*
Raf = Female of *R. appendiculatus*

b) Ticks infected with fungi

	Means THC	Number of ticks used	STD	Range 95 % m ± STD	Coefficient of variation (CV), %
Avma7	3311.9	20	52.94518	3258.954 - 3364.845	1.5
Avma8	4116.67	20	59.02837	4057.641 - 4175.698	1.4
Rama7	4276.19	20	60.16117	4216.028 - 4336.351	1.4
AvBb7	3140.48	20	51.55678	3088.923 - 3192.036	1.6

Avma7 = *A. variegatum* infected with *Metarhizium anisopliae* (10^7)
Avma8 = *A. variegatum* infected with *Metarhizium anisopliae* (10^8)
Rama7 = *R. appendiculatus* infected with *Metarhizium anisopliae* (10^7)
AvBb7 = *A. variegatum* infected with *Beauveria bassiana* (10^7)

c) Ticks infected with bacteria

	Means THC	Number of ticks used	STD	Range 95 % m ± STD	Coefficient of variation (CV), %
AvEco3	2185.71	20	43.01145	2142.698 - 2228.721	1.9
AvEco4	5233.33	20	66.55441	5166.775 - 5299.884	1.2
AvEco5	4511.9	20	61.79702	4450.102 - 4573.697	1.3

AvEco3 = *A. variegatum* infected with *Escherichia coli* (10^3)
AvEco4 = *A. variegatum* infected with *Escherichia coli* (10^4)
AvEco5 = *A. variegatum* infected with *Escherichia coli* (10^5)

Table. 4.2
Effect of bacteria on Total haemocytes of *Amblyomma variegatum* (thousand/μl)

Time (post-infect.)	E. cloacae			B. t.		S. marsecens		
	10^3	10^4	10^5	10^3	10^4	10^3	10^4	10^5
1 hour	3.52	3.37	3.87	3.75	3.62	3.87	3.62	3.25
3 hours	3.25	2.87	2.17	3.62	4.12	4.37	3.87	1.62
6 hours	37.5	25	1.87	30	3.37	5.22	4.62	*
24 hours	42	2.25	*	4.75	20	4.87	2.62	**

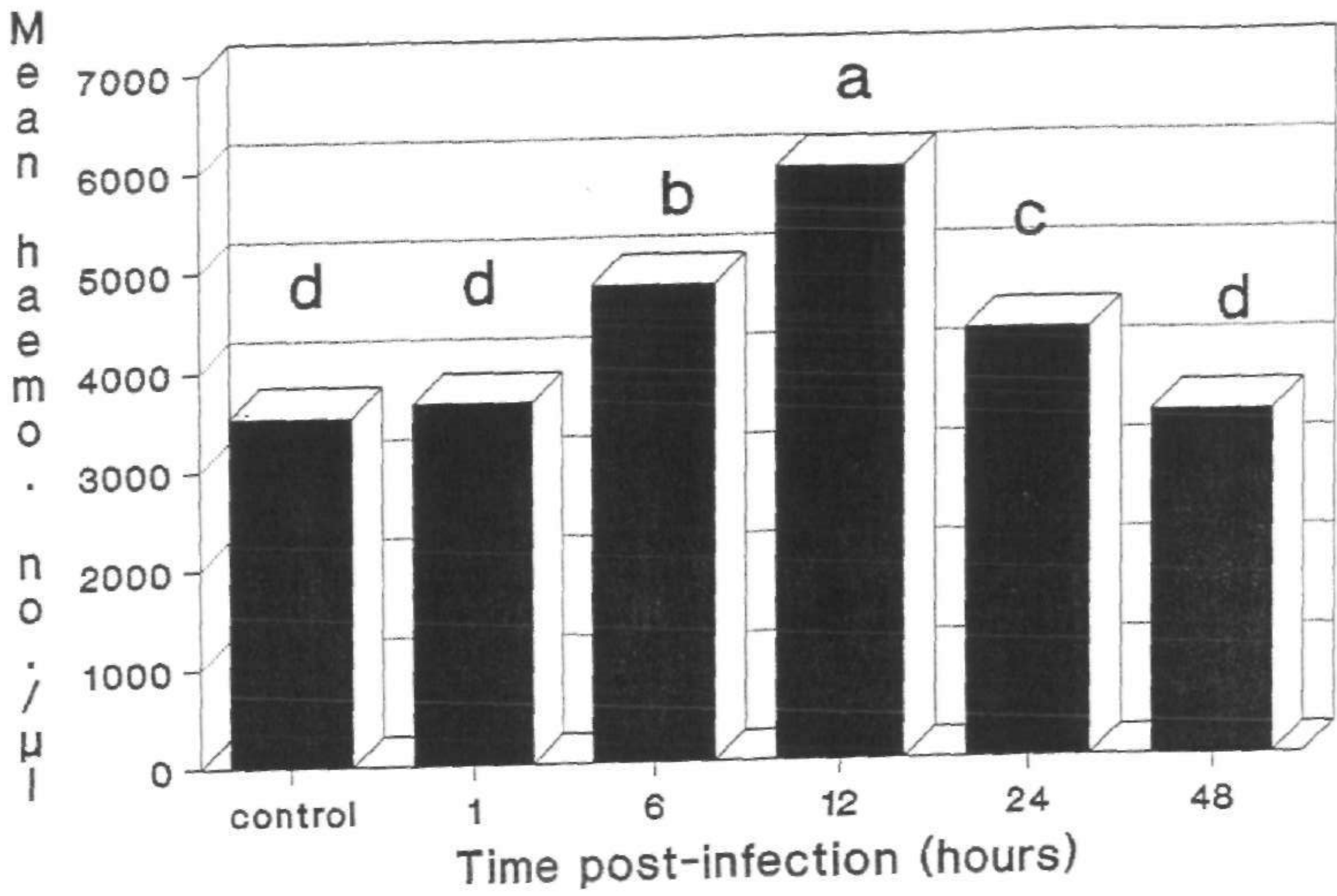
* Cells could not be counted because of nodules and darkening

** Melanization

Table. 4.3
Percent differential haemocyte count on infected and uninfected ticks. Haemocytes of infected ticks were counted at 24 hours post-infection.

		Haemocyte types				
		SP	PL	GR	OE	PR
A. var.	Control	44.54	31.34	16.17	0.98	6.97
	E. coli 10^3	42.42	30.65	16.88	1.08	8.97
	M. anisopliae 10^8	42.75	30.33	14.83	1.87	10.22
	B. bassiana 10^8	39.48	33.62	16.37	1.19	9.34
R. app.	Control	40.8	35.65	15.58	0.75	7.22
	E. coli 10^8	43.5	31.45	12.98	0.68	11.39
	M. anisopliae 10^3	37.64	30.87	13.87	1.45	15.17
	B. bassiana 10^8	39.93	31.67	16.05	1.83	10.52

A. var.: *A. variegatum*
R. app.: *R. appendiculatus*
E. coli: *Escherichia coli*
M. anisopliae: *Metarhizium anisopliae*
B. bassiana: *Beauveria bassiana*



* Means of the same letters are not significantly different

Fig. 4.2. Electron micrograph of a prohaemocyte with high nucleocytoplasmic ratio. Rough endoplasmic reticulum (RER) forms a narrow band varying in electron density around the nuclei. Filopodia (f), heterochromatin (hc), mitochondrion. (X 19500).

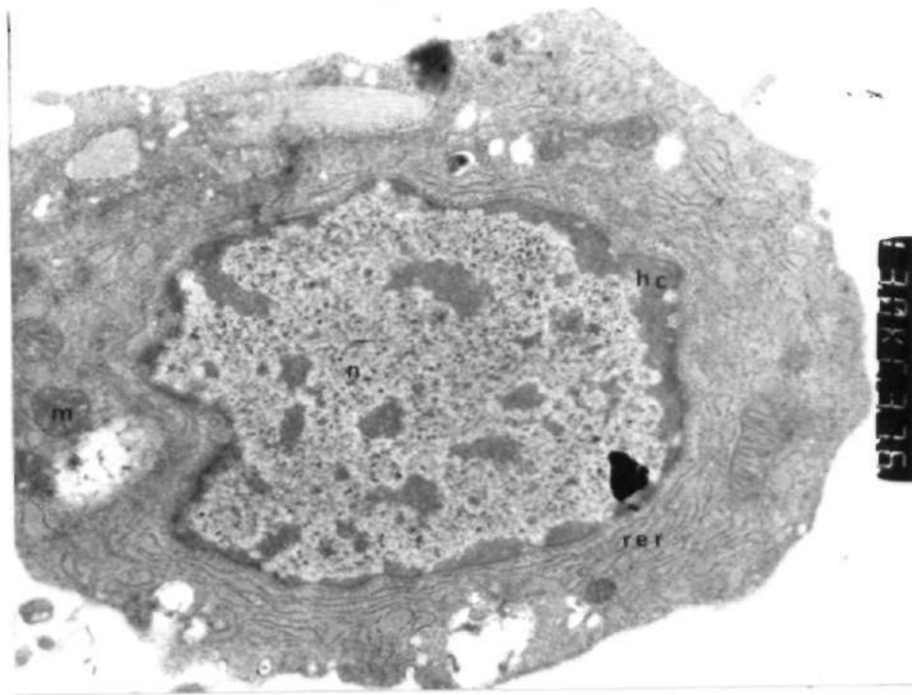

hc
n
m
rer

Fig. 4.3. Light micrograph of a plasmatocyte (spindle shape). Modified pappenheim stain. (X 1,500)

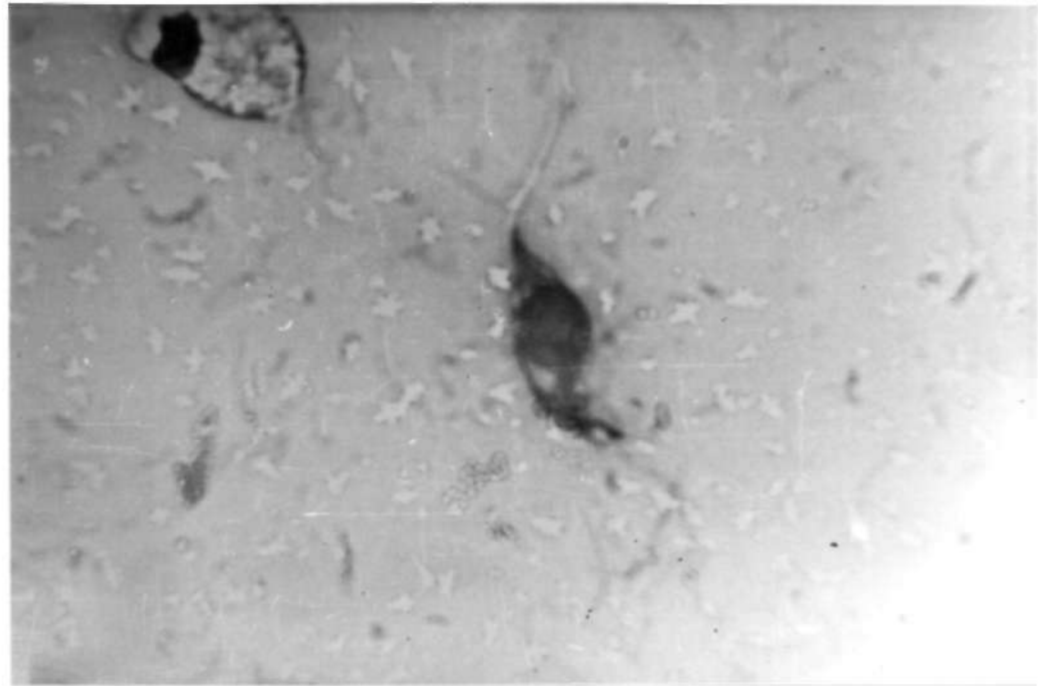


Fig. 4.4.

Light micrograph of a plasmatocyte, round shape. Modified Pappenheim stain. (X 1,500).

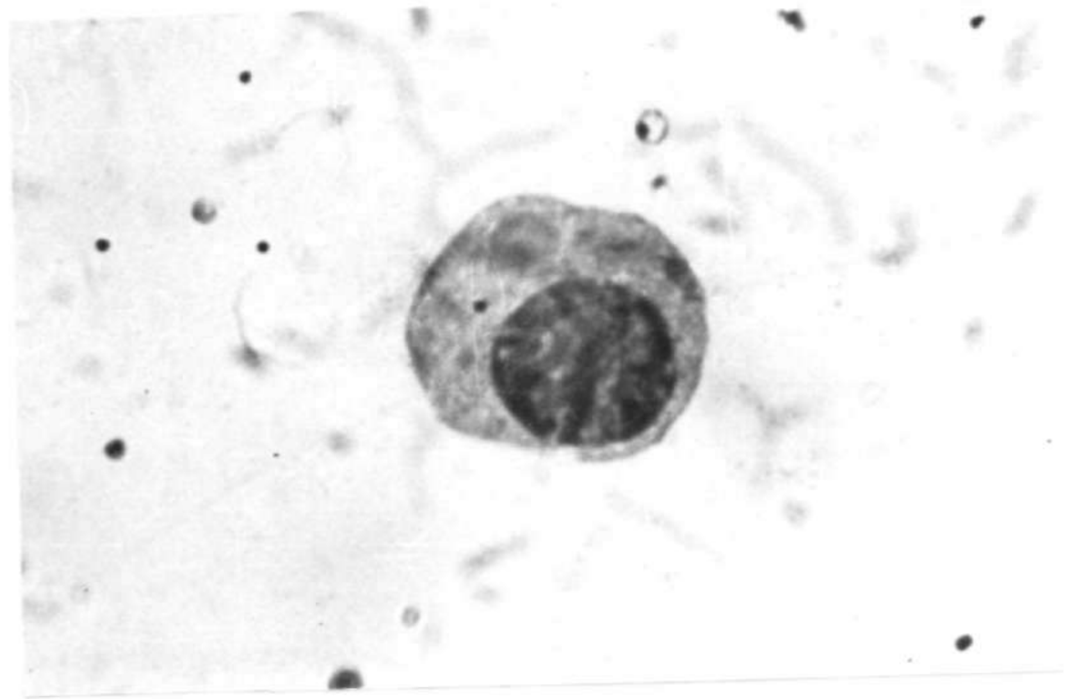


Fig. 4.5. Electron micrograph of a spindle shaped plasmatocyte showing filopodial extension (f), mitochondria (m). Note rer around the nucleus and on the side, (X 12000).

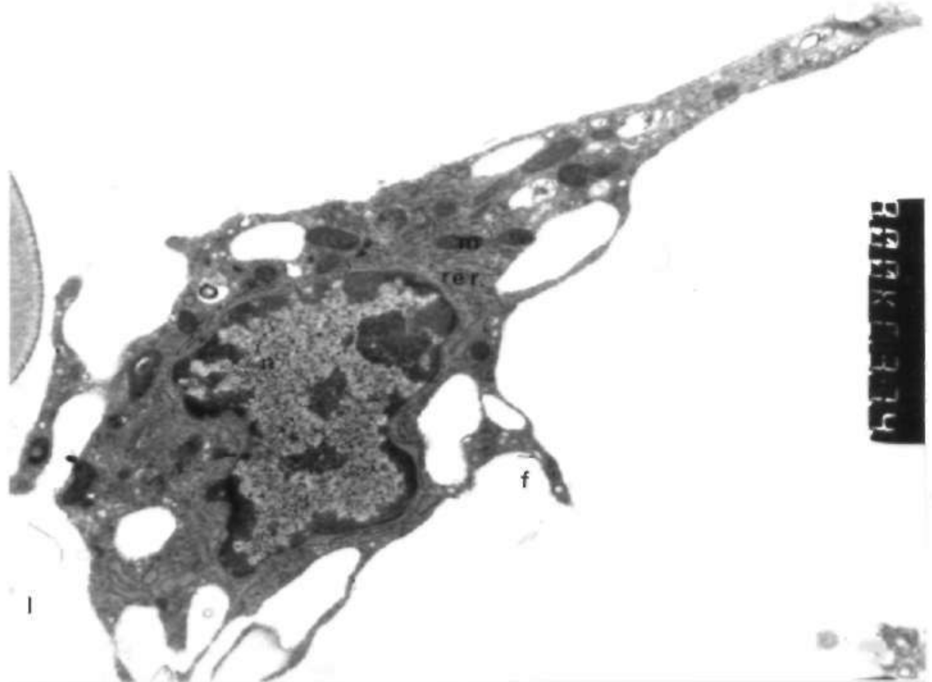

m
rer.
f

Fig. 4.6. Electron micrograph of an oval shaped plasmatocyte showing filopodial extension (f), mitochondria (m). Note taht the rer is well developped around the nuclei and on the side. (X 19500).

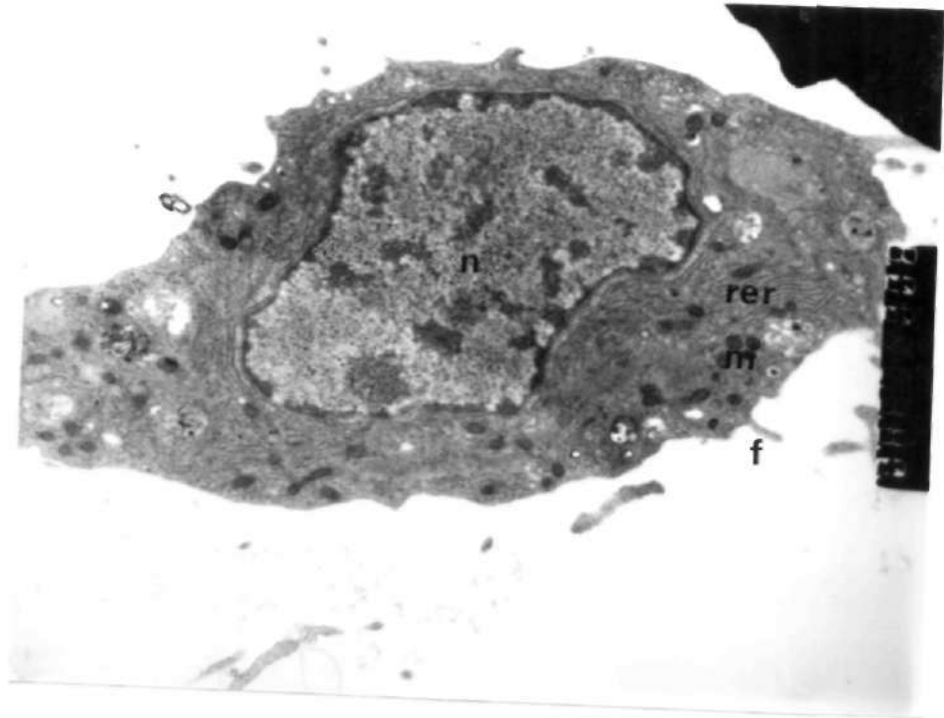

n
rer
m
f

Fig. 4.7.

Electron micrograph of a round shaped plasmatocyte showing filopodial extension (f), mitochondria (m), rough endoplasmic reticulum (rer), phagocytic particle (p), (X 19500).

Fig. 4.8.

Granulocyte in *A. variegatum*, 1500X. Modified Pappenheim stain.

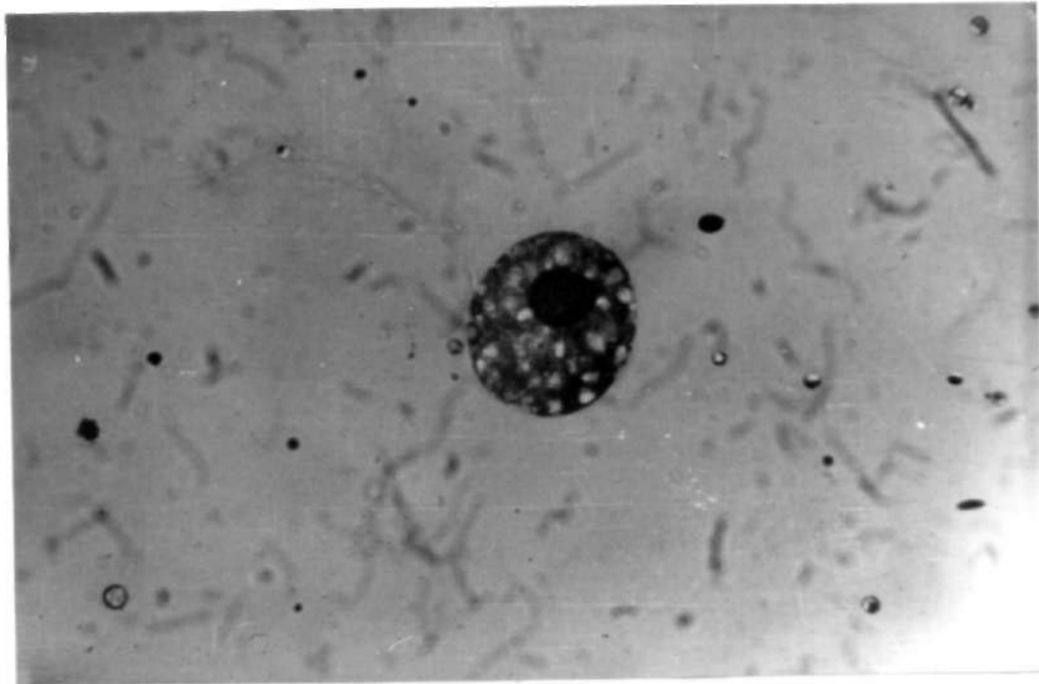


Fig. 4.9.

Granulocyte (spindle shape) in *A. variegatum*, 1500X. Modified Pappenheim stain.

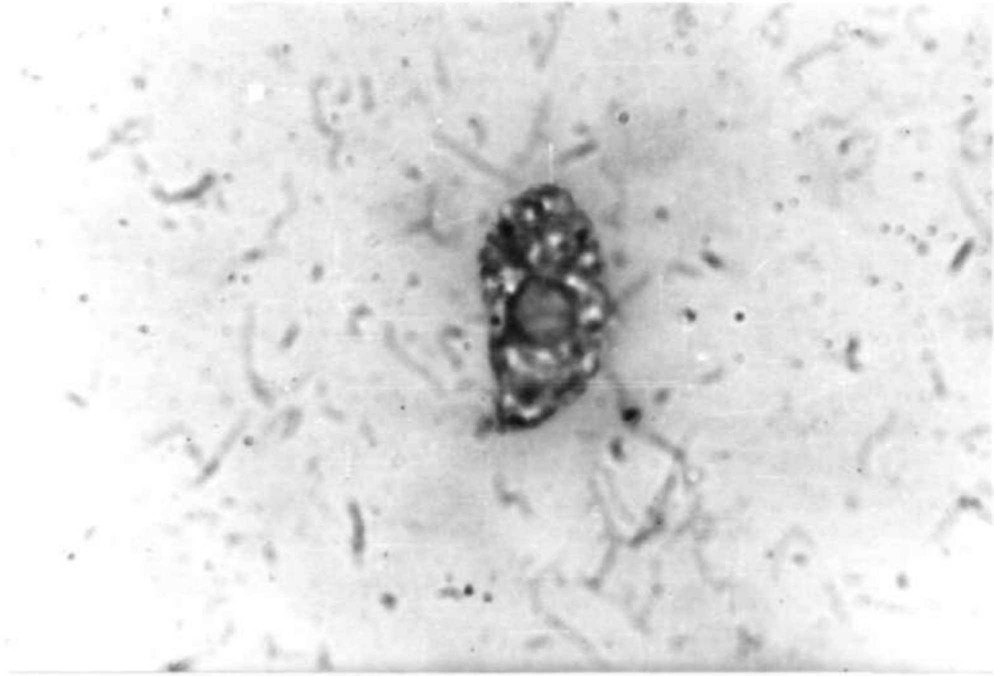


Fig. 4.10. Electron micrograph of an oval shaped granulocyte showing round and oval granules. Note that some of the granules are electron dense, filopodia (f), (X 15000).

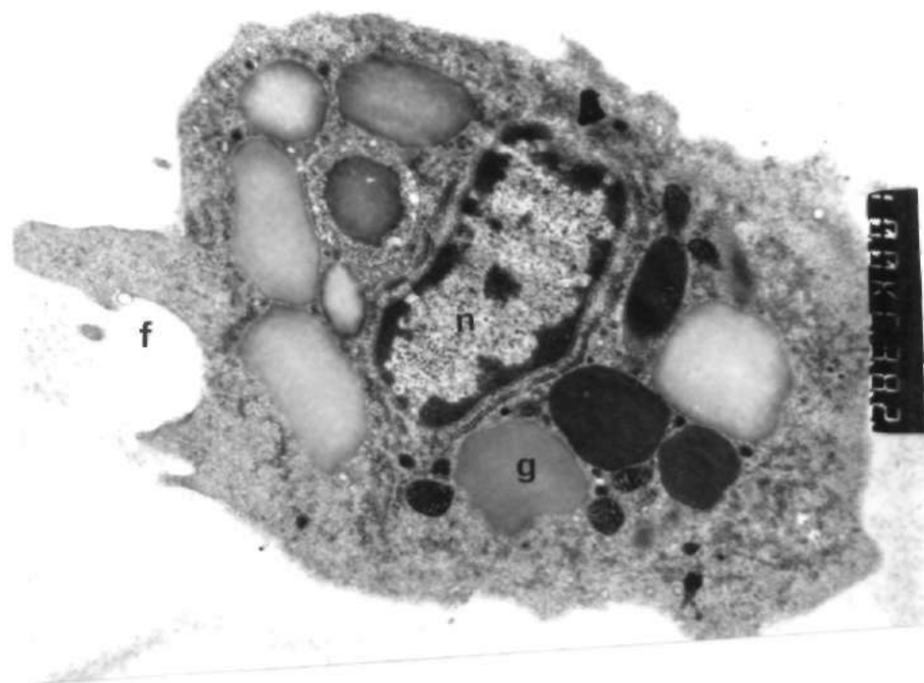

f
n
g

Fig. 4.11. Electron micrograph of a round sperule cell showing several uniform granules. Rough endoplasmic reticulum (rer) around nucleus. Mitochondria (m), (X 12000),

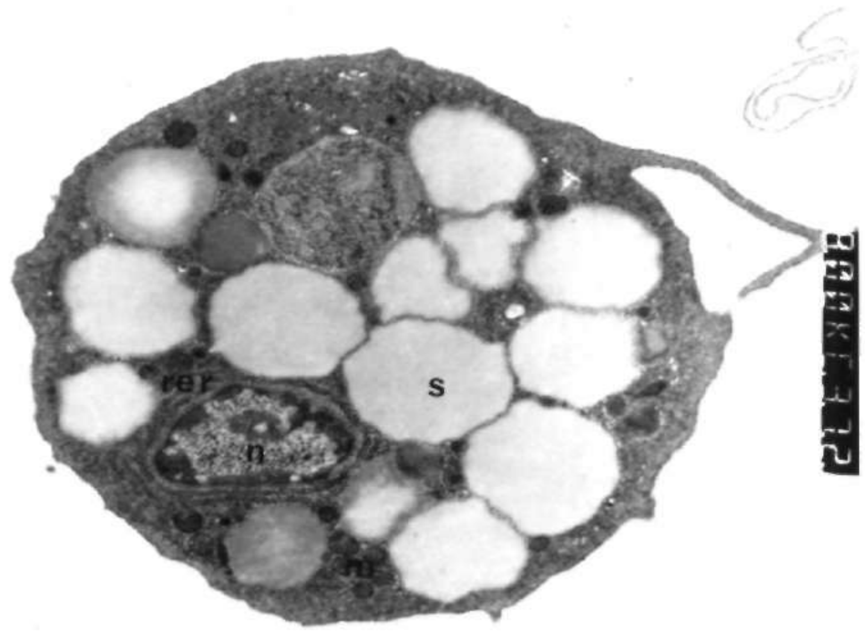

rer
s
n
m

Fig. 4.12. Sperule cell, X 1500. Modified Pappenheim method.

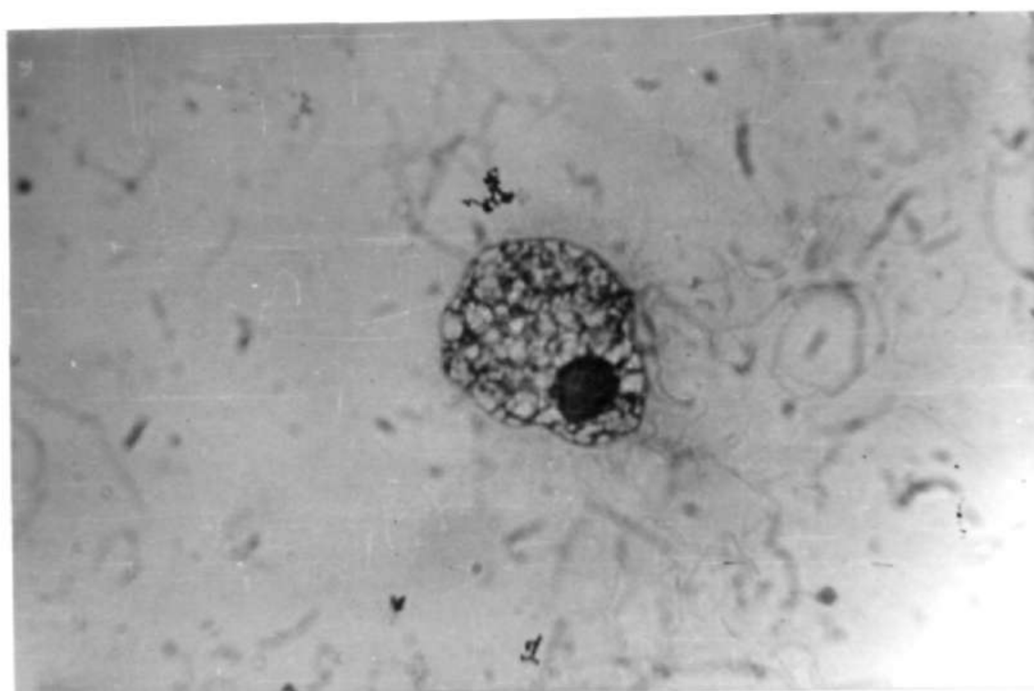


Fig. 4.13.

Oenocytoid, X 1500. Modified Pappenheim method.

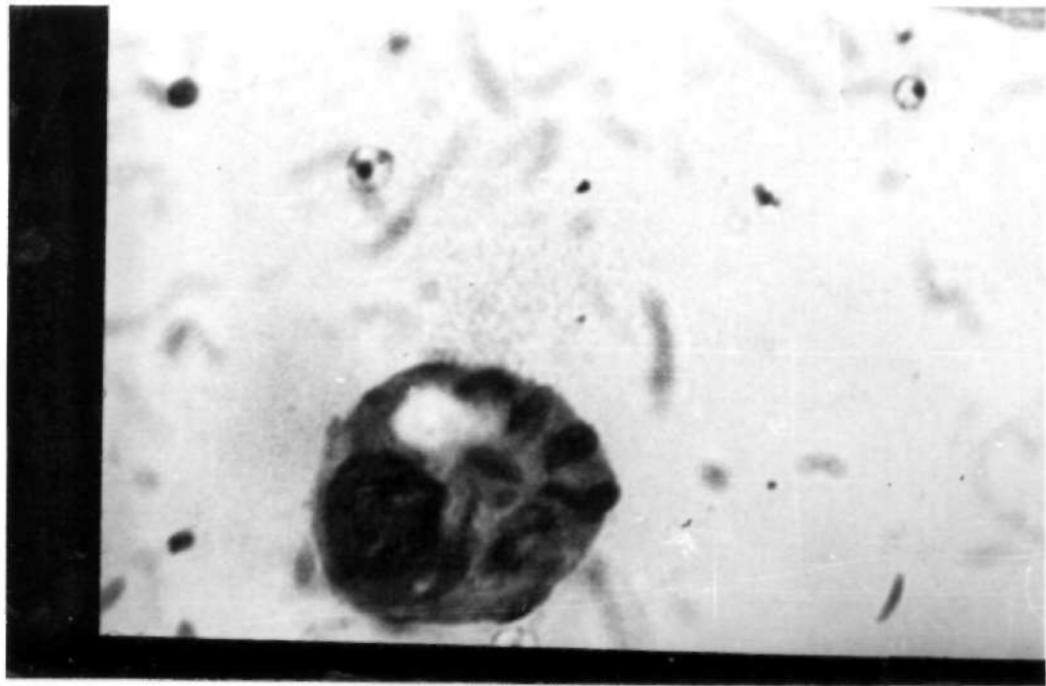


Fig. 4.14. Electron micrograph of an oenocytoid showing very few granules. Nucleus (n) and mitochondria (m), (X 19500).

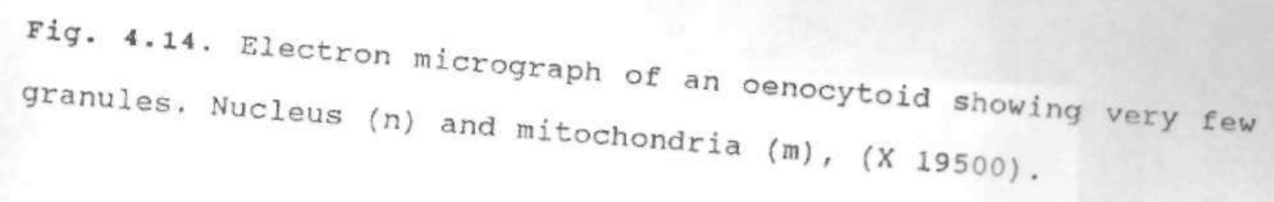
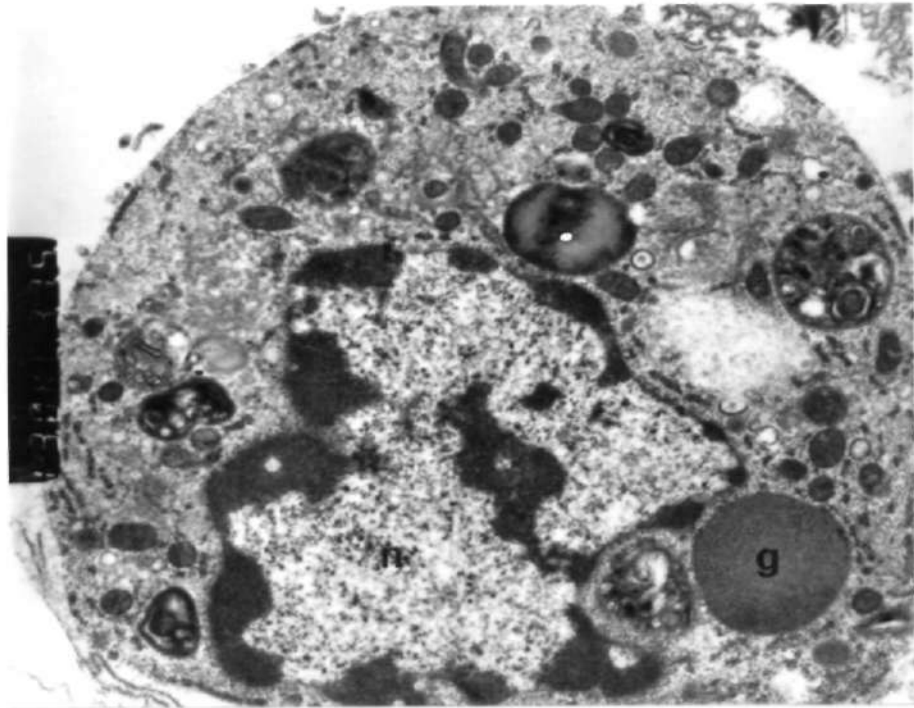
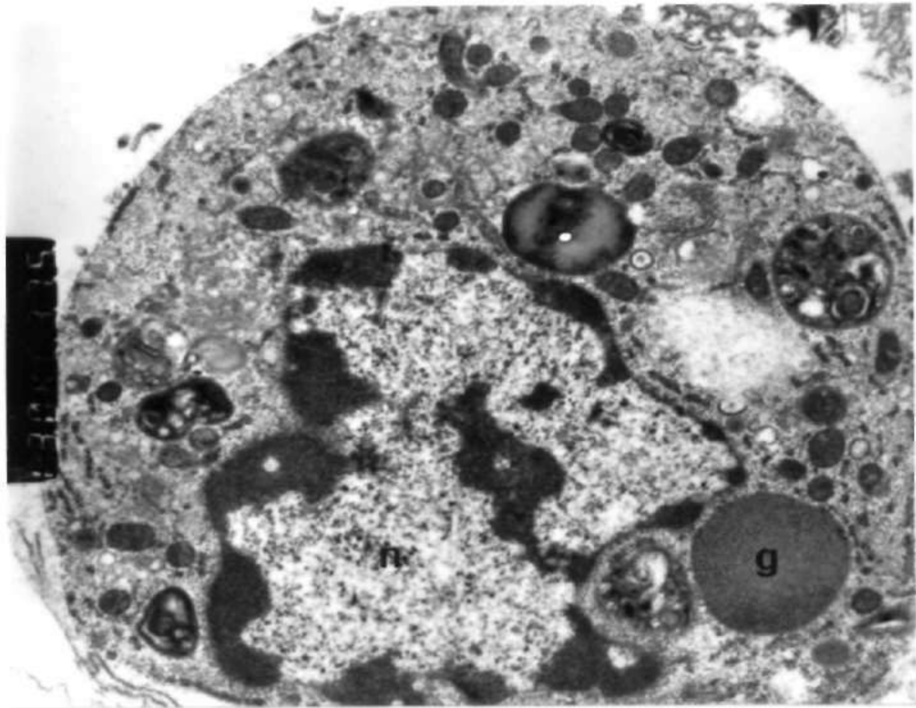
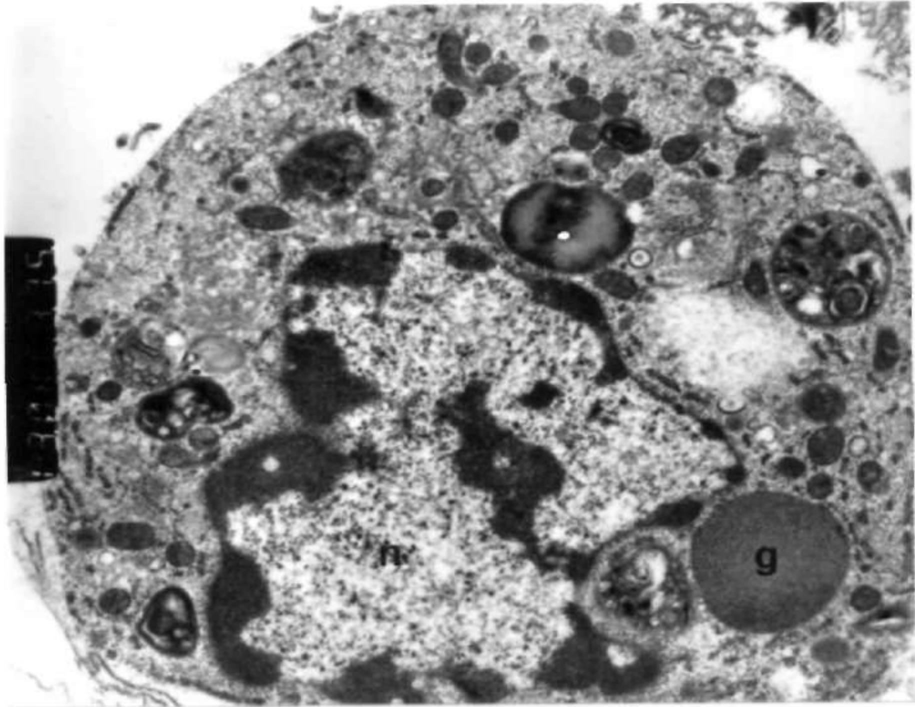


Fig. 4.14. Electron micrograph of an oenocytoid showing very few granules. Nucleus (n) and mitochondria (m), (X 19500).


g


n
g


n
g

CHAPTER V

Comparison of cellular and humoral immune reactions in different sexes and ages of ticks of both species, the level of pathogens that induce antibacterial activity and the type of humoral antibacterial factors produced.

Defence against the invasion of haemolymph by foreign bodies through cellular and humoral responses have been demonstrated in insects (Salt 1970; Lackie, 1988). Insect blood cells have been shown to ingest viruses, bacteria, protozoans and fungi both *in vitro* and *in vivo* (Jones 1962; Salt 1970; Whitcomb, Shapiro et al., 1974). It has also been demonstrated that humoral factors like lysozyme, cecropins, diptericins and others intervene in combination with haemocytes to defend insect against invadors (Ratcliffe, 1986). There is little information on tick immune system. Do they have cellular and humoral immune system? How do they react to the presence of foreign bodies? The objective of this study was an attempt to answer some of these questions.

5.1. MATERIALS AND METHODS:

5.1.1. Cellular immune response:

5.1.1.1. Bacterial infection:

Engorged ticks were collected and injected with 10^3, 10^4 or 10^5

each in 2μl of different bacteria (*Enterobacter cloacae*, *Escherichia coli* and *Bascillus thuringiensis*) using the method described earlier. After 1, 3, 6, 12, and 48 hours post-infection haemolymphs were collected in eppendorf tubes containing PTU (phenylthiourea) and kept at -20°C. Some of the haemolymph was used immediately to make a wet mount to assess cellular reactions like nodule formation.

5.1.1.2. **Encapsulation**:

- A piece of sharpened microcapillary tube was inserted inside each of 30 engorged females of *A. variegatum* and *R. appendiculatus*. Five ticks of each species were dissected after 1, 6, 24, 48 hours, 1 week and 2 weeks respectively. The end of the microcapillary tube was observed for any sign of encapsulation.

- Twenty five females of both *A. variegatum* and *R. appendiculatus* were each inserted a piece of araldite (1mm wide and approximately 4mm) as implant. Five ticks of each species were dissected at 24, 48, 72, 96 and 120 hours post-implantation. The pieces of araldite were immediately fixed in a 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and processed, according to the method described in chapter IV, for the Transmission electronique microscope.

5.1.2. **Humoral reactions**:

5.1.2.1. **Lysozyme activity**:

5.1.2.1.1. **Inhibition zone assay**:

Haemolymph collected from infected and non infected *A. variegatum* and *R. appendiculatus* adults as well as parasitized and non parasitized nymphs of *A. variegatum* were subjected to lysozyme test along with egg white lysozyme in agar plate seeded with dead *Micrococcus luteus*. The technique, similar to the one described by Kaaya (1993), was as follows: Six ml of 1% agar in 0.1 M phosphate buffer, pH 6.4, containing 1 mg/ml lyophilyzed Micrococcus luteus (Sigma chemicals) was placed in sterile disposable 9-cm petri dishes. After solidification, 2.5 mm diameter wells were made using a 2.5 mm well punch and 2.5 μl test samples were placed in each well. Simultaneously, samples of serial dilutions of chicken egg-white lysozyme with known concentration were placed in wells on the same plate. Plates were kept at 37°C 80% RH and checked after 3, 24, 48 and 72 hours. Diameters of clear zones around the wells were recorded. A standard curve using diameters of the clear zones around the wells containing the chicken egg-white lysozyme was prepared, from which lysozyme concentration in the samples were determined.

5.1.2.1.2. Electrophoresis:

a. Protein determination:

The protein content of each haemolymph sample was determined according to Smith et al. (1985) using the Pierce Bicinchoninic acid (BCA). The standard used was the Pierce Bovine Serum Albumin. Samples were read on a Beckman DU-50[R] spectrophotometer, USA at 562 nm.

b. Sodium-Dodecyl-Sulphate Polyacrylamide gel electrophoresis (SDS-PAGE):

Sixteen microgrammes (16μg) of protein of each sample were subjected to the gel electrophoresis according to the method described by Laemmli (1970). The sample gel buffer consisted of 0.5 M Tris-Hcl, pH 6.8 (1 ml), glycerol (0.8 ml), 10% (w/v) SDS (1.6 ml), 2 ß-mercaptoethanol (0.4 ml) and 0.05% (w/v) bromophenol blue (0.2ml). The haemolymph sample gel buffer mixtures were heated in boiling water for 5 min and centrifuged. Hundred microliters of each mixture were applied in each well of 2.5 mm thick 12.5% SDS-PAGE gels. Gels were run at constant 70 v until the trucker dye just ran off the gels (3 hours).

5.1.2.2. Antibacterial activity:

a. Inhibition zone assay:

The modified method of Hoffmann et al. (1981) by Kaaya (1993)

was used. Plates were prepared by adding log-phase *E. coli* D31 to 7 ml of 1% nutrient agar at 45°C, containing 100 μg streptomycin/ml, to a final concentration of 10^5 bacteria/ml. After mixing, 6 ml of agar was placed in a 9 cm plastic disposable petri dish and left for 30 mn to solidify. Two and half-milimeter diameter wells were made and 2.5 μl of the test haemolymph were placed in each well. Petri dishes were placed in the incubator at 37°C and monitored after 24 hours. Diameters of the growth inhibitor zones were recorded for the test sample.

b. <u>Acid gel: Sodium-dodecyl-sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)</u>: (pH 4.5)

Samples of immune haemolymph were acified by adding 0.5 vol. of 0.1 M acetic acid (Hultmark et al., 1980). Fifteen percent (15%) polyacrylamide gels (pH 4) using a discontinuous buffer system (Gabriel, 1971) but omitting the stacking gel were used (Hultmark et al., 1980). The acrylamide and bisacrylamide ratio was 40:0.43. Gels were run at 200v until the tracker dye (Methyl green) runs off the gel (≈2.5 hours).

The method described by Hultmark et al. (1980) in order to localize bands with antibacterial activity was used. Gels were incubated in a rich bacterial medium which contained 0.2 M sodium phosphate pH 7.4, and streptomicin, 100μg/ml. Gels were then overlaid with 5ml of melted agar, 0.6% in the same medium, containing about $2x10^5$ viable *E. coli* cells, strain D31. On top of

this was poured another layer of agar without bacteria, and the gel was incubated at 37°C overnight.

Data were analyzed using SAS analysis, General Linear Model (GLM).

5.2. RESULTS:

5.2.1. Cellular reactions:

5.2.1.1. Phagocytosis and nodule formation:

Some plasmatocytes and granulocytes were observed flattening or extending filopodia in haemolymphs of ticks infected with bacteria (e.g *B. thuringiensis* and *E. coli*) and parasitoid eggs immediately after infection. Haemolymphs collected after 24 hours to 1 week showed some cell masses looking like granuloma (Fig. 5.3). Plasmatocytes were also seen flattening and sticking onto the glass (slide).

5.2.1.2. Encapsulation:

- **Light microscopy:** Some Haemocytes were observed flattened around sharpened microcapillary tubes 3 hours after they were inserted into the tick. After 3 to 4 days the end of the microcapillary tube was completely covered with a withish capsule in both *A. variegatum* and *R. appendiculatus* (Fig. 5.6).
- **Transmission electron microscopy:** Two morphologically distinct types of haemocytes were found to participate in the formation of capsule in this study, plasmatocytes and granulocytes. At 24 hr after implantation, the surface of the implant was covered with a dense amorphous matrix (Fig. 5.7). Some regions of the implant had few plasmatocytes and degranulating granulocytes while others had

none. Some haemocytes appeared attracted by the matrix, they had loose contact with it. Electron dense granules were observed in a layer mainly formed with plasmatocytes and granulocytes.

At 48 h, some more haemocytes had flattened on top of the first layer (Fig. 5.8a). Cells on top of the capsule appeared loosely connected to the capsule. Degranulation was still continuing. There was presence of binucleated plasmatocytes (Fig 5.8c1), mitochondria and granulocytes with electron dense granules (Fig. 5.8c2). The inner layer of the capsule appeared to be composed of particles of haemocytes (Fig. 5.8b). After 72 h, the capsule looked more compact. There was no intercellular space (Fig 5.9a). Gap junctions were observed (Fig 5.9b). After 96 hours the capsule is complete. It looks like there is no additional flattening of haemocytes. The outermost region of the capsule was formed with loose haemocytes. There is presence of rough endoplasmic reticulum and mitochondria. The capsule appeared to be disintegrating, after 120 hours. Some melanized portions of haemocytes were observed in different parts of the capsule. The capsule was mainly comprised of three regions. The three regions were more or less similar to the ones described by Ratcliffe and Rowley, (1979): 1) an inner microtic region with characteristic granulocytes remnants close to the implant; 2) a middle region with extremely flattened plasmatocytes and 3) an outer region consisting of unflattened plasmatocytes.

5.2.2. **Humoral reactions**:

5.2.2.1. **Lysozyme activity**:

5.2.2.1.1. **Nymphs of *A. variegatum***:

Results of the three trials have shown a mean diameter of clear zone around the well less than 2 mm in both parasitized and unparasitized ticks.

5.2.2.1.2. **Female of *A. variegatum* and *R. appendiculatus***:

Haemolymphs from ticks infected with different concentrations of bacteria were assessed for the presence of lysozyme. Figures 5.1 and 5.2 show the existence of lysozyme in both *A. variegatum* and *R. appendiculatus*. Although all the bacteria used in the experiments induced lysozyme activity, *B. thuringiensis* gave a better result (Fig. 5.1 and 5.2). The bacterial concentration and time after infection enhanced lysozyme production. Haemolymphs of ticks infected with bacteria gave higher inhibition depending on post-infection time and bacterial concentration.

5.2.2.2. **Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)**:

Fifteen percent (15%) SDS gel were ran to compare haemolymphs

from females of *A. variegatum* infected with *M. luteus* and *R. appendiculatus* infected with *E. ccli* and *M luteus*. There is a band on gels common to haemolymphs of infected ticks and non infected ticks but it is darker in haemolymphs of infected ticks (Fig. 5.5).

5.2.2.3. Antibacterial factor: inhibition zone

Among all the bacteria (*E. coli, E. cloacae, S. marcescens* and *M. luteus*) used in the experiment only *M. luteus* provoked some activities. A mean diameter of 1.5 mm of diffusion in agar seeded with live *M. luteus* was observed 24 hours after the haemolymph was put inside wells.

Acid gels (ph 4.5) were run to counter check the presence of antibacterial factors. No inhibitory effect was seen on bacterial growth.

5.3. DISCUSSION

5.3.1. Nodule formation:

Nodule formation is a defence reaction which operates against large doses of particulate materials which invade or are injected into the haemocel of insects, and which cannot be cleared by phagocytosis alone. Nodules may be formed within 1-5 mn in insects and the initial stage involves mainly the GRs, which clump together, discharge granules and entrap the foreign particules (e.g. bacteria) in a clot like gelatinous material. Within 5-60 min, melanization of the degenerating haemocytes and bacteria begins and compaction of the necrotic, melanized mass takes place. The second phase begins from 1-6 hours post-bacterial injection when Pls start attaching to the melanized mass and flatten on its surface forming a multicellular sheath (Ratcliffe and Gagen, 1977).

In this study some nodules were observed in the haemolymph of ticks of both species injected with bacteria. The nodules start forming at 3 to 4 hours post-infection and were still visible after 48 hours (Fig. 5.3). In some cases when the bacterial concentration is high there is melanization in addition to nodule formation (Fig. 5.4).

Nodules were not dissected to study their composition but they appeared to be composed of granular haemocytes and plasmatocytes

because of the presence of granules. Some adults *Glossina m. morsitans*, injected bacteria were observed entrapped in nodules formed by the GRs and PLs (Kaaya et al. 1986). The nodules were observed in tsetse haemolymph by 3 hour post-infection, but were relatively small compared to those formed by lepidopterans, e.g. *G. mellonella*.

5.3.2. Encapsulation:

Encapsulation is the reaction by which insects defend themselves against foreign bodies that enter the haemocel and that are too large to be phagocytized by individual haemocytes. In this study it was found that microcapillary tubes were covered by witish layers (Fig. 5.6) probably made of haemocytes but more studies are necessary to certify if the capsule is due to a cellular or humoral reaction.

It has been proved by the use of electronmicroscopy that capsules in invertebrates are formed with different types of haemocytes. The types of haemocytes so far implicated were listed by Ratcliffe and Rowley (1979): amoebocytes (Tinberlake, 1912), leucocytes (Hollande, 1920), Lymphocytes (Schneider, 1950), macronucleocytes (Paillot, 1928), micronucleocytes (Boese, 1936), proleucocytes (Lartschenko, 1933), spherule cells (Meyer, 1926), and granulocytes (Brehélin et al., 1975) and thrombocytoids (Zachary et al., 1975). In this study the capsule was mainly

formed by plasmatocytes like in *Dermacentor variabilis* (Eggenberger et al., 1990) and *Schistocerca gregaria* (Schmit and Ratcliffe, 1977). Granulocytes and spherule cells also participated but were mainly involved in degranulation especially near the implant. Eggenberger found the same type of phenomenon in *D. variabilis.* Cells in the middle of the capsule appeared extremely modified. This phenemenon was observed in many insects eg. *S. gregaria*. The degranulation observed in *A. variegatum* and *R. appendiculatus* in this study and in others arthropods may produce a proteinaceous material (Misko, 1972) which probably plays a role of holding cells together (Grimstone et al., 1967). Cell particules like RER, mitochondria, granules were also observed in *D. variabilis*, *S. gregaria* and many other insects. The most striking observation was that capsules formed in ticks in this study are containing three main regions like in *S. gregaria* and other insects.

5.3.3. Lysozyme activity:

Lysozyme a bacteriolytic enzyme, is released consecutively by both live and dead bacteria. The presence of lysozyme has been demonstrated in *Galleria mellonella* (Powning and Davidson, 1976), *Bombix mori*, *Locusta* (Zachary and Hoffmann, 1984), tsetse (*Glossina m. morsitans*) (Kaaya, and Darji, 1988). Figures 5.1 and 5.2 show activity of lysozyme in both *A. variegatum* and *R. appendiculatus*. Although normal insect haemolymph contains low levels of lysozyme, the presence of lysozyme in controls may be due to clumping and

breakdown of granular haemocytes in response to bacterial injection as mentioned by Kaaya et al. (1986) or discharge of granules which may contain lysozyme (Zachary and Hoffmann, 1984). Haemolymph of saline-injected ticks produced lysozyme activity slightly superior to non injected ticks, but significantly different ($P<0.05$). The same reaction was observed in haemolymph of pregnant *G.m. morsitans* and their larvae injected with saline.

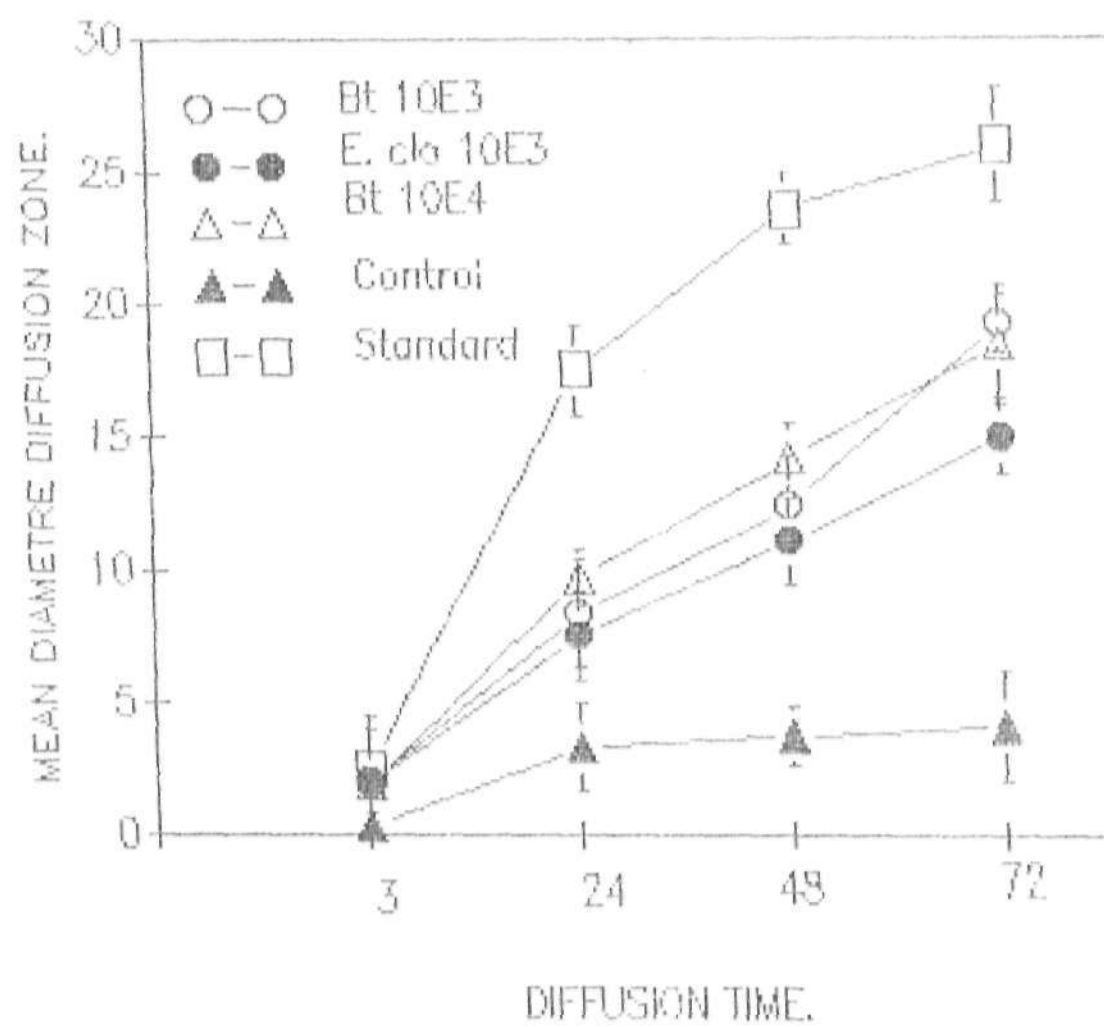
Kaaya and Darji reported that inoculations of *E. coli, E. cloacae, B. subtilis, M. luteus* and *Acinetobacter calcoaceticus* stimulated lysozyme activity with higher activity due *E. coli*. In this study *B. thuringiensis, E. coli* and *E. cloacae* produced the same effects. *Bacillus thuringiensis* produced the highest activity. Lysozyme activities increase after one hour post-inoculation, reach the peak after 3 hours.

The band representing proteins which production is enhanced by the presence of bacteria in tick haemolymphs is may be a lysozyme but more studies are needed to identify the type of proteins in the band.

Although present in some invertebrates like *Galleria mellonella, Hyalophora cecropia* and *Glossina m. morsitans* (Kaaya et al. 1987) antibacterial factors do not seem be important in ticks. In agreement Kaaya et al. (1992) have failed to demonstrate the existence of antibacterial factors in *R. appendiculatus*.

Fig. 5.1.

Lysozyme production in haemolymph of *Amblyomma variegatum* infected with bacteria compared to haemolymph of non-infected ticks and standard egg white lysozyme.



30
25
20
15
10
5
0
MEAN DIAMETRE DIFFUSION ZONE.
Bt 10E3
E. clo 10E3
Bt 10E4
Control
Standard
3
24
48
72
DIFFUSION TIME.

Fig. 5.2.

Lysozyme production in haemolymph of *Rhipicephalus appendiculatus* infected with bacteria compared to haemolymph of non-infected ticks and standard egg white lysozyme.

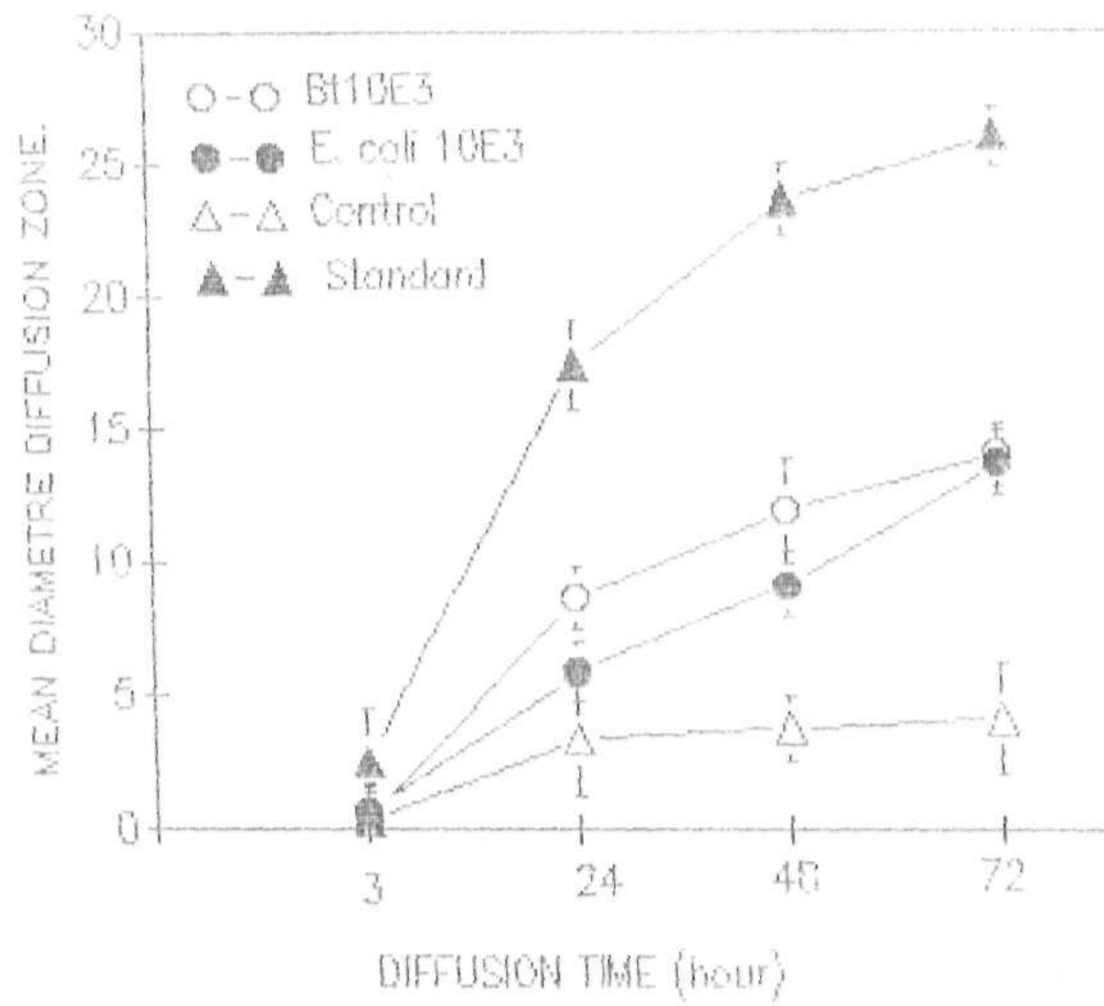

O-O BI10E3
●-● E. coli 10E3
△-△ Control
▲-▲ Standard
MEAN DIAMETRE DIFFUSION ZONE.
0
5
10
15
20
25
30
3
24
48
72
DIFFUSION TIME (hour)

Fig. 5.3.

Cell mass looking like granuloma indicating cell reaction and degranulation in *A. variegatum infected with* E. coli 2 x 10^4, (X 1500).

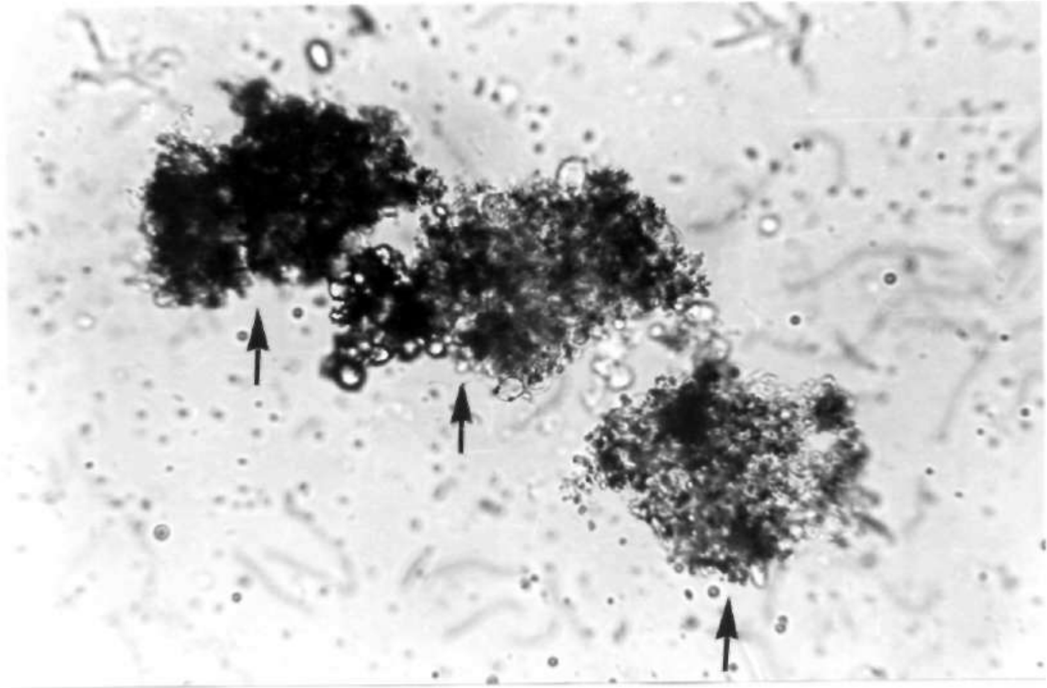


Fig. 5.4.

Melanisation and cell reaction (arrows) in *A. variegatum* infected with *Bacillus thuringiensis* 2 x $10^5\mu l$. (X 1500).

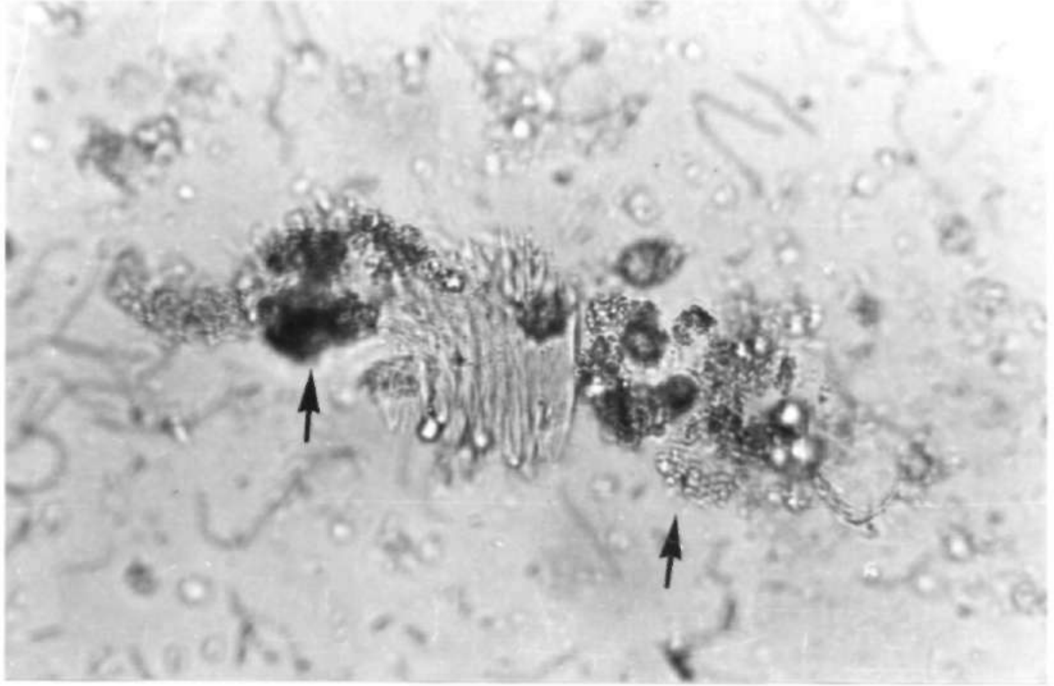


Fig. 5.5.

Haemolymph of ticks collected at 24 hours post-infection. 1. Non infected *Amblyomma variegatum*, 2. *A. variegatum* infected with *Micrococcus luteus*, 3. Non-infected *Rhipicephalus appendiculatus*, 4. *R. appendiculatus* infected with *M. luteus*, 5. *R. appendiculatus* infected with *E. coli*.

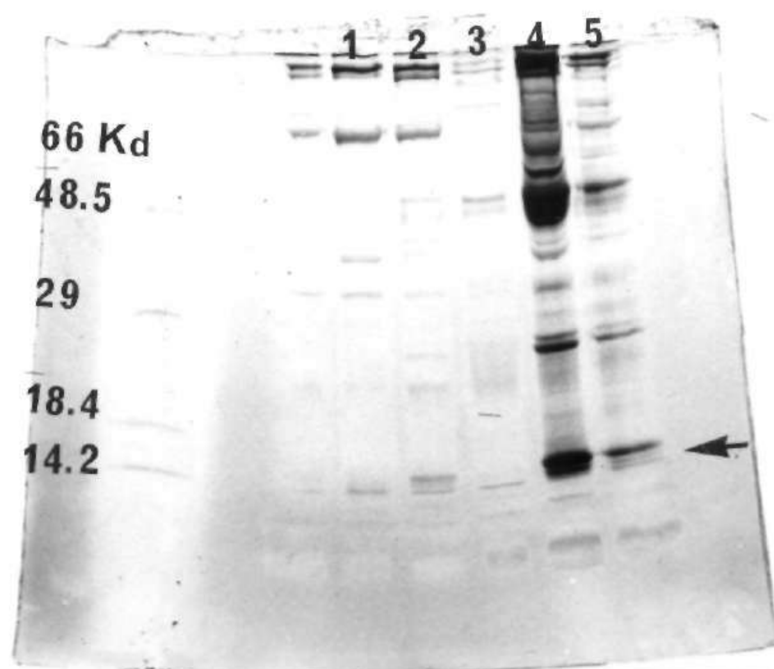

1
2
3
4
5
66 Kd
48.5
29
18.4
14.2

Fig. 5.6.

Tip of microcapillary tube covered with a withish capsule 4 days after being inserted in an engorged female of R. appendiculatuus. (X 900).

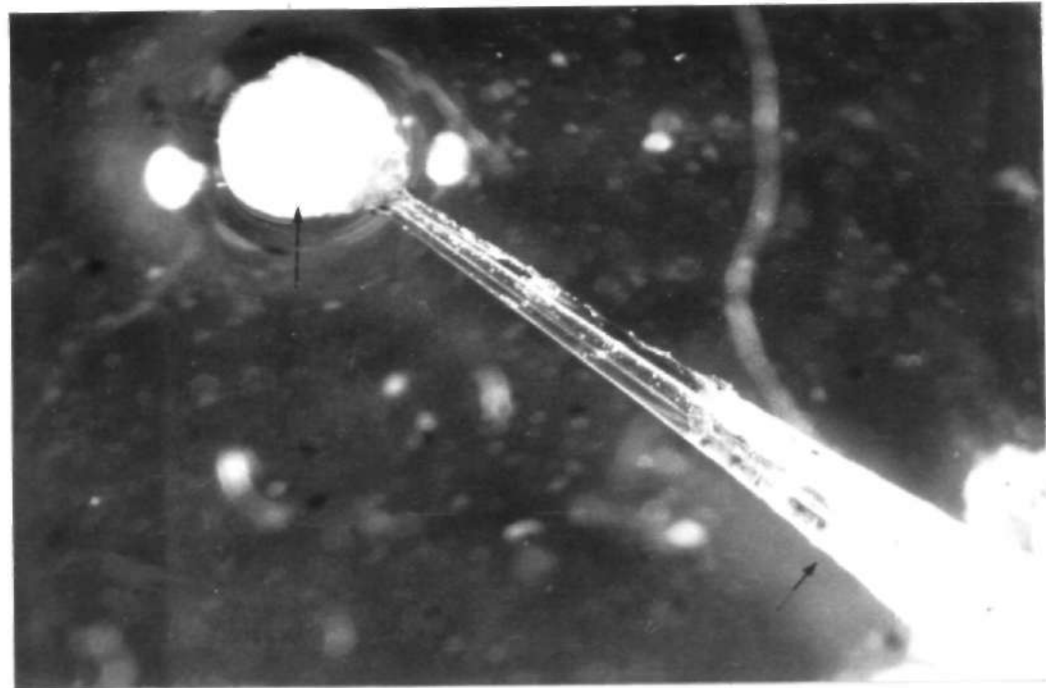
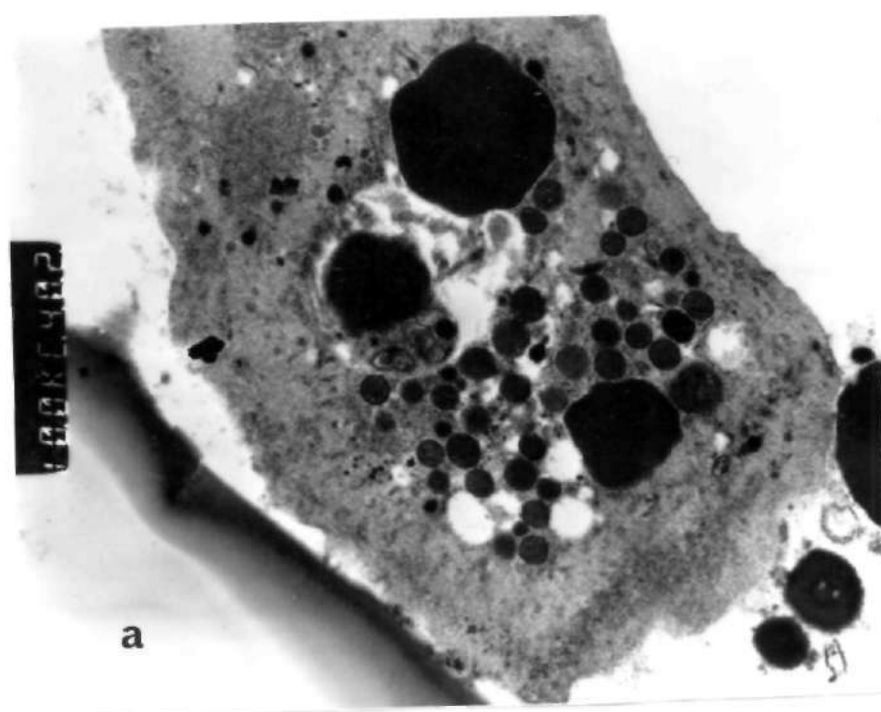


Fig 5.7.

Encapsulation, 24 hours after implantation, beginning of capsule formation. Deposition of melanized amorphous matrix (arrow) on the surface of the araldite (a), haemocytes have loose contact with the surface of the implant, (X 15,000).


a

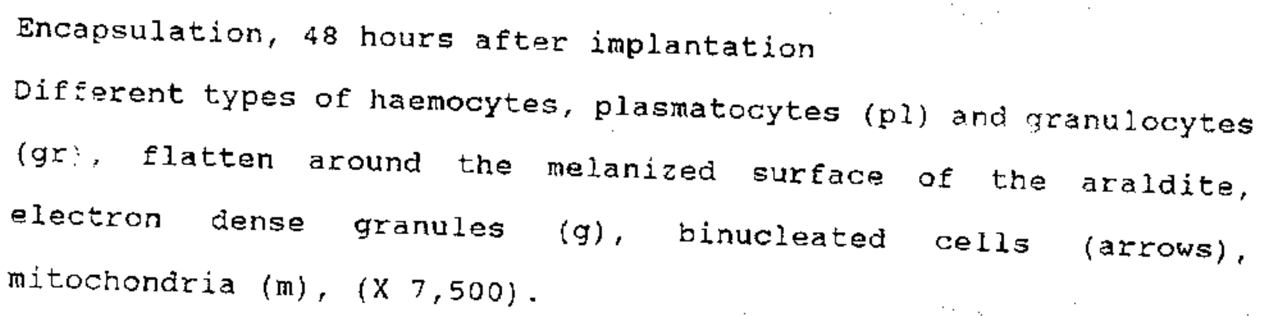


Fig 5.8a

Encapsulation, 48 hours after implantation

Different types of haemocytes, plasmatocytes (pl) and granulocytes (gr), flatten around the melanized surface of the araldite, electron dense granules (g), binucleated cells (arrows), mitochondria (m), (X 7,500).

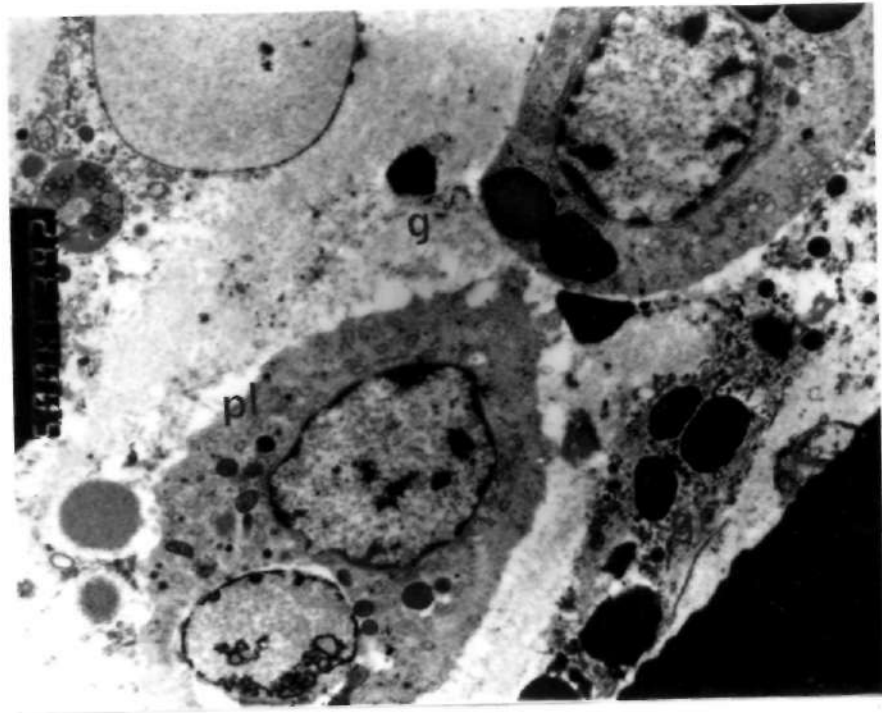

g
pl

Fig. 5.8b.

Encapsulation, 48 hours after implantation

Inner layer of the capsule composed of fragments of haemocytes, (X 7,500).

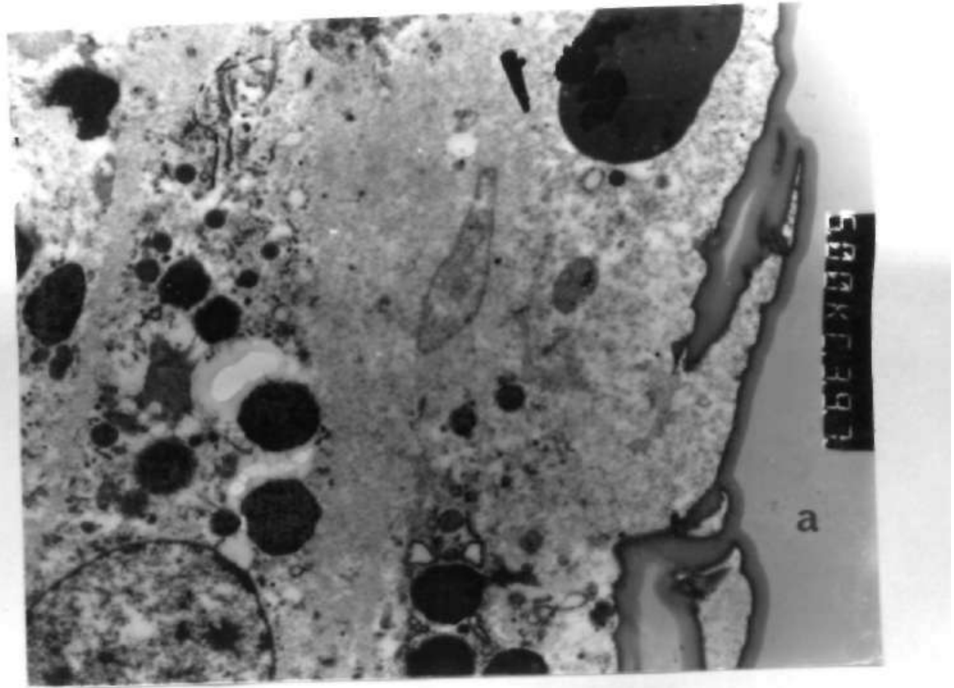

a

Fig. 5.8c1.

Encapsulation, 48 hours after implantation

Haemocytes participating in the formation of capsule, binucleated plasmatocyte, granule (g), structured granules (sg), (X 15,000).

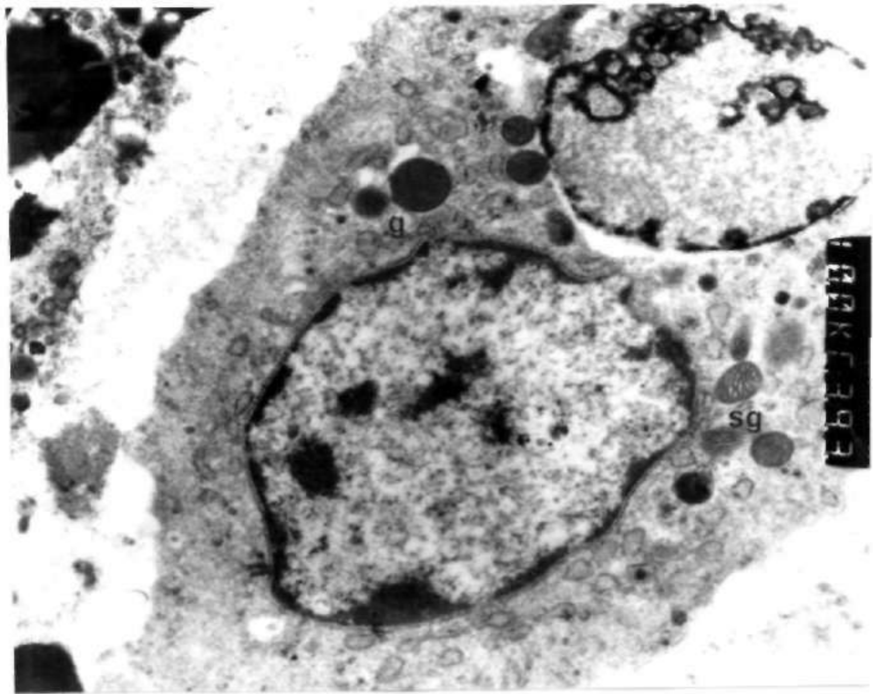

g
sg

Fig. 5.8c2.

Encapsulation, 48 hours after implantation

Haemocytes participating in the formation of capsule, granulocyte with electron dense granules, (X 15,000).

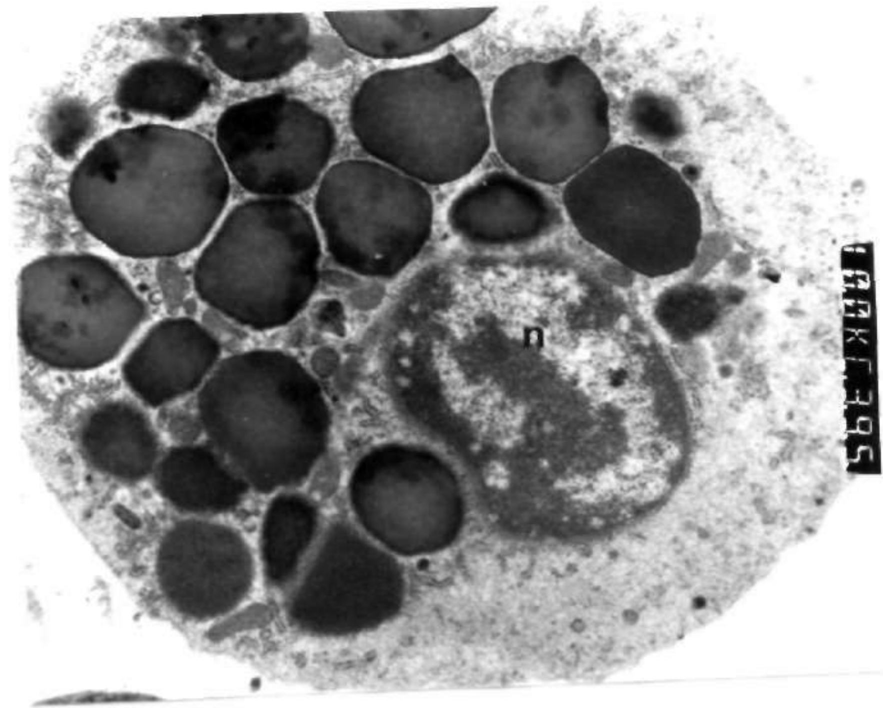


Fig. 5.9a.

Encapsulation, 72 hours after implantation.

Note flatten haemocytes, gap junctions (arrows), (X 7,500).

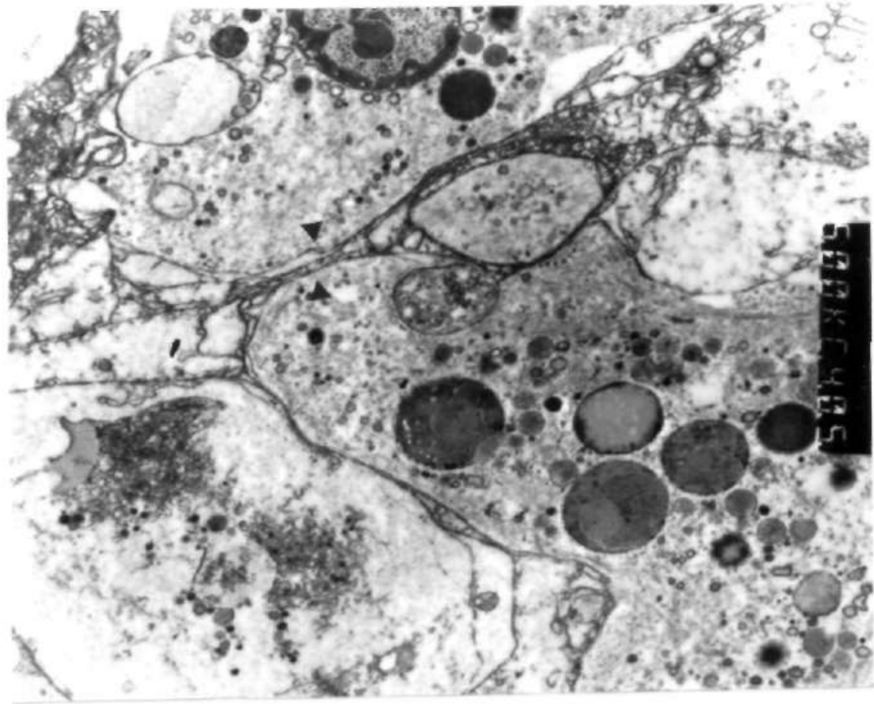
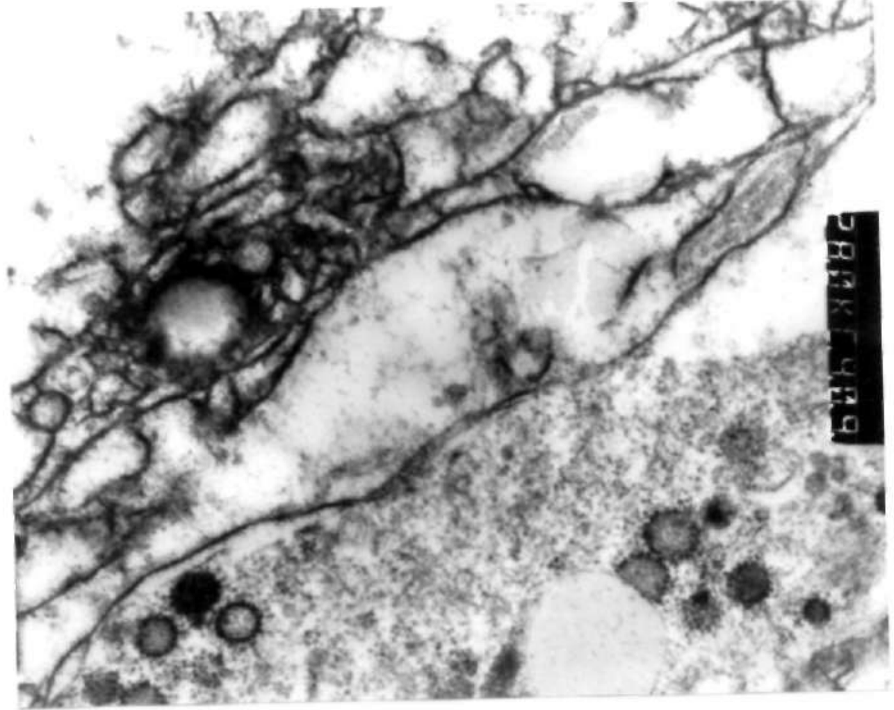


Fig. 5.9b.

Encapsulation, 72 hours after implantation.

Higher magnification of gap junctions, (X 42,000).



CHAPTER VI

The immune status of ticks infected with the fungi *B. bassiana, M. anisopliae* and the hymenopteran tick parasitoid *I. hookeri*.

It is generally recognized that insects are infected by entomopathogenic fungi through the cuticle but not the digestive truct. Zacharuk (1970) demonstrate that mycelia penetrate insect cuticle by the means of mechanisms of physical or enzymatic nature. Once inside the insect body the mycelium buds in blastospores (Ferron, 1975) and start the colonisation process. There is reaction of the insect immune system. Vey (1971) described the reaction of the larvae of *Galleria mellonella* to the fungi *Aspergillus niger*; there is formation of granulosoma, mainly by plasmatocytes, around hyphae.

It has been demonstrated that insects show some cellular and humoral reactions to parasitoid infections. Guzo and Stoltz (1987) demonstrated some cellular and humoral reactions of the tussock moth, *Orgyia leucostigma*, larvae to the braconid wasp *Cotesia melanoscela*. An increase of haemocyte number was shown as well as encapsulation. The purpose of this study is to demonstrate the existence of cellular and humoral reacions in ticks vis a vis the fungi and the parasitoid.

6.1 MATERIALS AND METHODS:

6.1.1. Fungal infection:

Fungal spores were seeded in petri dishes containing SDA (Sabouraud Dextrose Agar). The petri dishes were sealed with parafilm and left on the bench for the fungi to grow. Fourteen day old hyphae were collected from the culture and centrifuged 3 times in a megafuge at a 3000g for 15 minutes. The concentration of the solution was determined using a phase contrast microscope and a Neubauer slide.

Engorged females of *A. variegatum* and *R. appendiculatus* were injected with 2 x 10^8 (or 10^9)/μl of entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* using the method described in chapter V. The haemolymph was collected at 1, 2 and 3 day post-infection and observed directly under light microscopy for cellular reactions.

6.1.2. Parasitization:

6.1.2.1. Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis:

Haemolymphs collected from engorged *Amblyomma* nymphs infected with the parasitoid were used for the experiment. They were

collected after 1, 3, 6, 12, 24, 48 and 72 hours according to the method described earlier.

a) **Sodium-dodecyl-sulphate Polyacrylamide gel (SDS-PAGE)**:

Some equal amount of protein contained in all the samples were run in gels using the method described in chapter 5.

b) **Staining**:

Protein bands were visualized by silver stain.
Periodic acid schiff (PAS) reagent (Kapitany and Zebrowski, 1973) was used for the presence of carbohydrates.

6.1.2.2. Development of the parasitoid:

The development of *Ixodiphagus hookeri* was monitored by introducing the parasitoid to fed and unfed females and nymphs of *A. variegatum* and to fed and unfed females of *R. appendiculatus*. Female parasitoids were given opportunity to lay eggs in ticks (all stages). Some of the ticks were dissected daily while some (unfed) were put on rabbits and others kept on the bench for the development of the parasitoid. Ticks were dissected in a petri dish containing Pbs pH 7.2. A drop of the mixture Pbs-tick contents was observed using a Leitz phase contrast microscope. Pictures of eggs, larval and pupal stages were taken using a Wild MPS 55 camera. The size of different stages was measured using an occular micrometer.

6.2. <u>RESULTS</u>:

6.2.1. <u>Reaction to fungal infection</u>:

Ticks injected with conidia of entomopathogenic fungi *M. anisopliae* and *B. bassiana* died within 5 to 7 days. Haemolymph collected at 24 and 48 hours post-infection showed some cellular reactions like nodule formation (Fig 6.1) and melanization. Attempts to collect haemolymph at 3 to 4 day post-infection were fruitless; ticks had started darkening.

6.2.2. <u>Parasitization</u>:

Cell masses or capsules were observed in nymphs and adults of *A. variegatum* and *R. appendiculatus* when parasitized by the tick parasitoid *I. hookeri* (Fig. 6.4).

6.2.2.1. <u>Production of a parasitism-specific protein</u>:

Fifteen percent (15%) sample gels comparing haemolymphs collected at different time post-parasitization showed that a parasitism protein is produced in reaction to the parasitoid eggs inside the host (Fig. 6.11). It appears in the tick haemolymph from 3 hours post-parasitization but the band on the gel appears clearly after 48 hours. The molecular weight is approximately 33 KD.

The periodic acid Schiff (PAS) having tested negative, it was

therefore concluded that the protein is not a carbohydrate.

6.2.2.2. Development of the parasitoid:

a. Parasitization of tick:

Parasitoids use a variety of cues to determine suitability including host size, texture, shape, movement, and the presence or absence of internal or external chemical (Arthur, 1981; Vinson, 1985). If there is any external cue that attracts females of the tick parasitoid *I. hookeri* to the host it must be common to all the stages of *A. variegatum* and *R. appendiculatus*. The parasitoid laid eggs (Fig. 6.2) in all of them. Larvae of the parasitoid were observed moving freely in the haemolymph of larvae of *A. variegatum* as well as nymphs of both species. Parasitoids completed their cycle only in *A. variegatum* but not in larvae and nymphs of *R. appendiculatus* and larvae of *A. variegatum*. Parasitoids did not also succeed to emerge from adults of *A. variegatum* and *R. appendiculatus*. Eggs or embryos of parasitoids were observed trapped in cell masses one or two days post-parasitization (Fig. 6.5). No parasitoid (egg or larvae) was observed in tick haemolymphs after 1 week.

b. Development of parasitoids inside the host:

Once the eggs of parasitoids (Fig. 6.3) are laid the content of the distal end is transferred into the main part and the embryo is formed then the first instar larvae is seen in the tick haemolymph within a day (Fig. 6.6).

The first instar larvae do not seems to feed. They may be leaving on the reserve from the egg. They are not very active. First instar larvae live in the host for one week. In contrast the second instar larvae (Fig. 6.7) are very active. This stage is very voracious; larvae are seen moving in the haemolymph of the host chewing everything around, including haemocytes. They are seen in the haemolymph during the second and third weeks of post-parasitization; that is the time yellow spots start appearing on the scutum of the host.

The third instar larvae appear at the end of the third week post-parasitization (Fig. 6.8). They are not very active and do not feed as there is nothing (internal organs) remaining inside the tick after 3 weeks. This stage was seen in the host for 3 days to one week then they migrate to one side (anterior, posterior, left or right) leaving a space for the feces. They pupate (Fig. 6.9) molt and adults emerge generally through one hole Fig. 6.10). The cycle, from the time eggs are laid to the day adults emerge, takes 4 to 6 weeks.

6.3. DISCUSSION:

6.3.1 Fungal infection:

The potential of entomopathogenic fungi as mycoinsecticides has not been realised because of problems like specificity, mass production, stability in storage, speed to kill and efficacy under adverse environmental conditions (Moore and prior, 1993). Germination and infection process by fungi are slowed down due to biotic and abiotic factors. For example, the germination rate of *M. anisopliae* was increased when the beetle *Hylobius pales* was surface-sterilized (Schabel, 1976). Once inside the host fungi also face the reaction of the immune system. Vey and Fargues (1977) observed some formation of granulommas and some slight melanized blastospores in haemolymph of *Leptotarsa decemlineta* larvae infected with *B. bassiana*. In this study some debut of formation of granulomma were observed. It was unfortunately impossible to collect haemolymph from ticks after 4 days post infection; only darken blood was collected. This may be due to toxins produced by the fungi.

6.3.2. Parasitization:

6.3.2.1. Parasitization of ticks:

Parasitoids use a variety of cues to determine suitability

including host size, texture, shape, movement, and the presence or absence of internal or external chemical (Arthur, 1981; Vinson, 1985). If there is any external cue that attracts females of the tick parasitoid *I. hookeri* to the host it must be common to all the stages of *A. variegatum* and *R. appendiculatus*. The parasitoid laid eggs in all of them.

Larvae of the parasitoid were observed moving freely in the haemolymph of larvae of *A. variegatum* as well as nymphs of both species. Parasitoids completed their cycle only in *A. variegatum* but not in larvae and nymphs of *R. appendiculatus* and larvae of *A. variegatum*. The failure of parasitoids to emerge is mostly due to the size of the mentioned ticks, therefore the lack of nutrients.

Parasitoids did not also succeed to emerge from adults of *A. variegatum* and *R. appendiculatus*. Eggs of parasitoids were observed trapped in cell masses one or two days post-parasitization. No parasitoid (egg or larvae) was seen in tick haemolymphs after 1 week.

6.3.2.2. Development of parasitoids inside the host:

Once the eggs of parasitoids are laid the content of the distal end is transferred into the main part and the embryo is formed. The first instar larvae is then seen in the tick haemolymph within a day. The phase between the egg and the first instar larvae

seems to be the most vulnerable stage because some eggs and embryo were seen trapped inside egg masses. How do the parasitoid survives? Is it due to a virus that was injected with the eggs like in other parasitoids of the same family as mentioned by Vinson (1993)? It was proved that an indigenous virus exists in the ovaries of all females of several species of parasitoid wasps belonging to the family Ichneumonidae e.g. *Campoletis sonorensis* (Edson et al, 1981) and endoparasitic braconids (Vinson, 1993).

The first instar larvae do not seem to feed. They may be living on the reserve from the egg. They are not very active. First instar larvae live in the host for one week. In contrast the second instar larvae are very active. This stage is very voracious; larvae are seen moving in the haemolymph of the host chewing everything around, including haemocytes. They are seen in the haemolymph during the second and third weeks of post-parasitization; that is the time yellow spots start appearing on the scutum of the host. Later on the whole body of the tick becomes yellow or brown. The change of the colour of the cuticle and the developmental arrest of the tick suggests that *I. hookeri* belongs to the group Beckage (1990) described as "host regulators"; parasitoids which induce drastic alterations in the host hormone titers and metabolic pathways.

The third instar larvae appear at the end of the third week post-parasitization. They are not very active and do not feed as

there is nothing (internal organs) remaining inside the tick after 3 weeks. This stage was seen in the host for 3 days to one week then they migrate to one side (anterior, posterior, left or right) leaving a space for the feces and pupate. Adults parasitoids emerge, generally, through one whole. The cycle, from the time eggs are laid to the day adults emerge, takes 4 to 6 weeks.

Fig. 6.1.

Beginning of a Nodule formation in the haemolymph of *A. variegatum* infected with *M. anisopliae* (10^8), (X 1500).

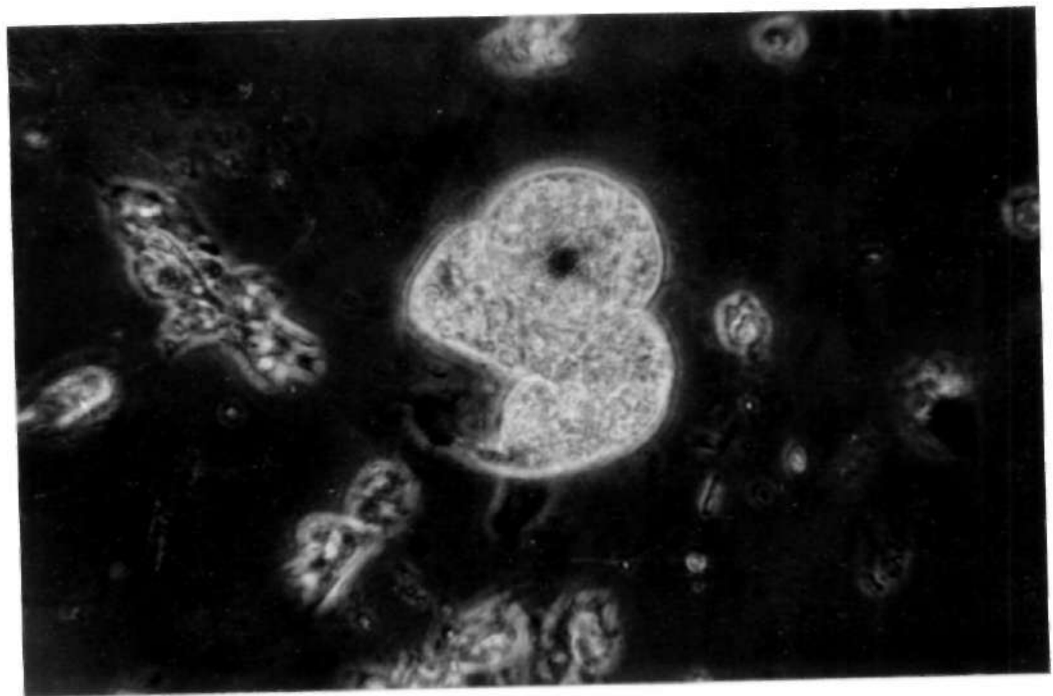


Fig. 6.2. A female parasitoid laying eggs inside a nymph of *Amblyomma variegatum*. (X 187).

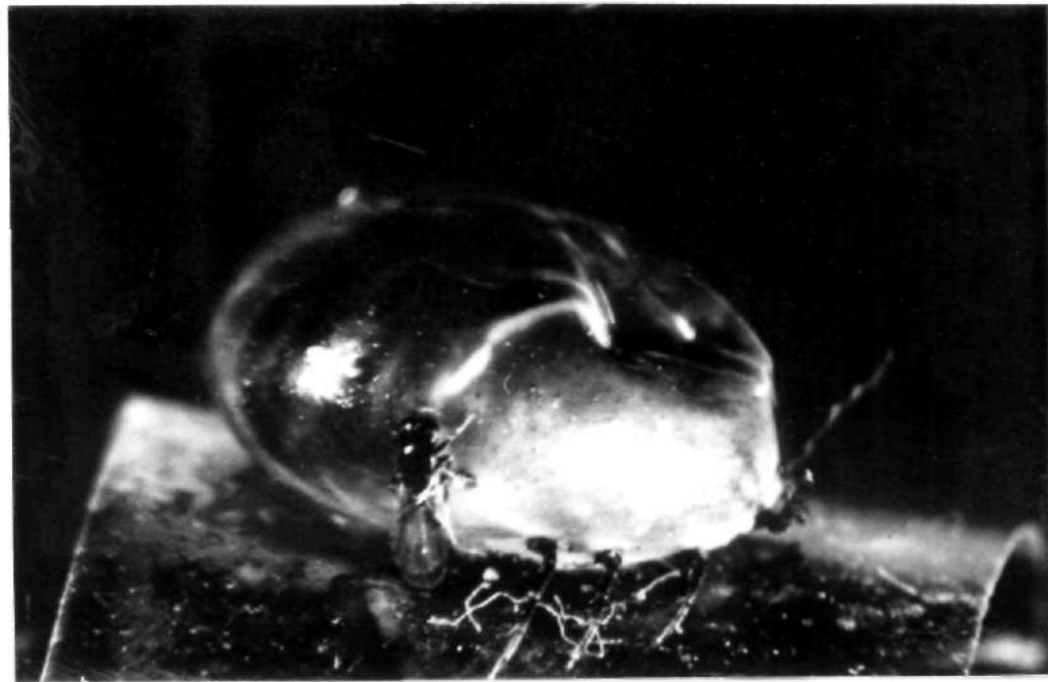


Fig. 6.3. Eggs of the tick parasitoid, 3 hours post-infestation, (X 1500).

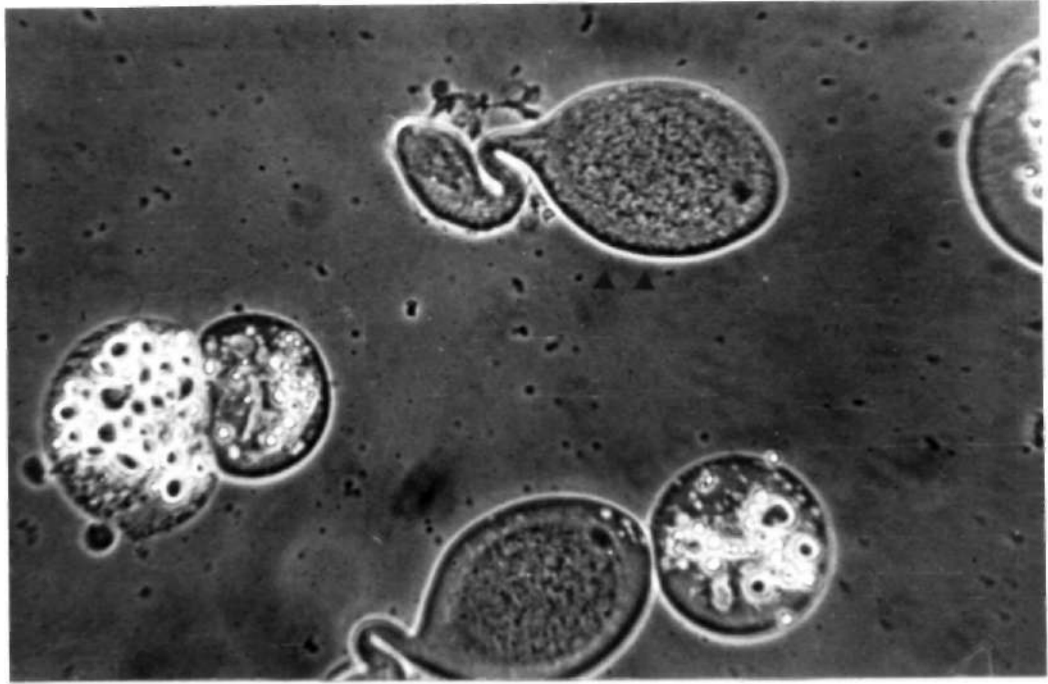


Fig. 6.4. Immune reaction of the nymph of *A. variegatum* to the parasitoid, formation of a cell mass, (X 1500), wet mount.

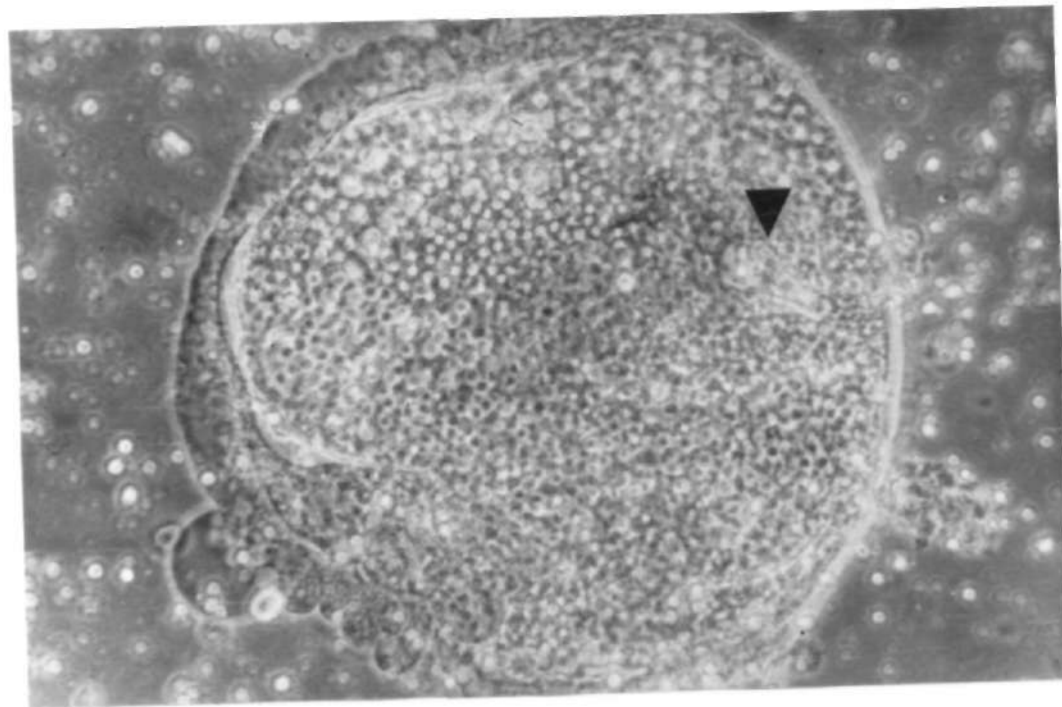


Fig. 6.5. Embryo in cell mass at 3 hours post-infection, (X 600).

Fig. 6.6. First instar larvae, 3 days post-infestation, (X 240).

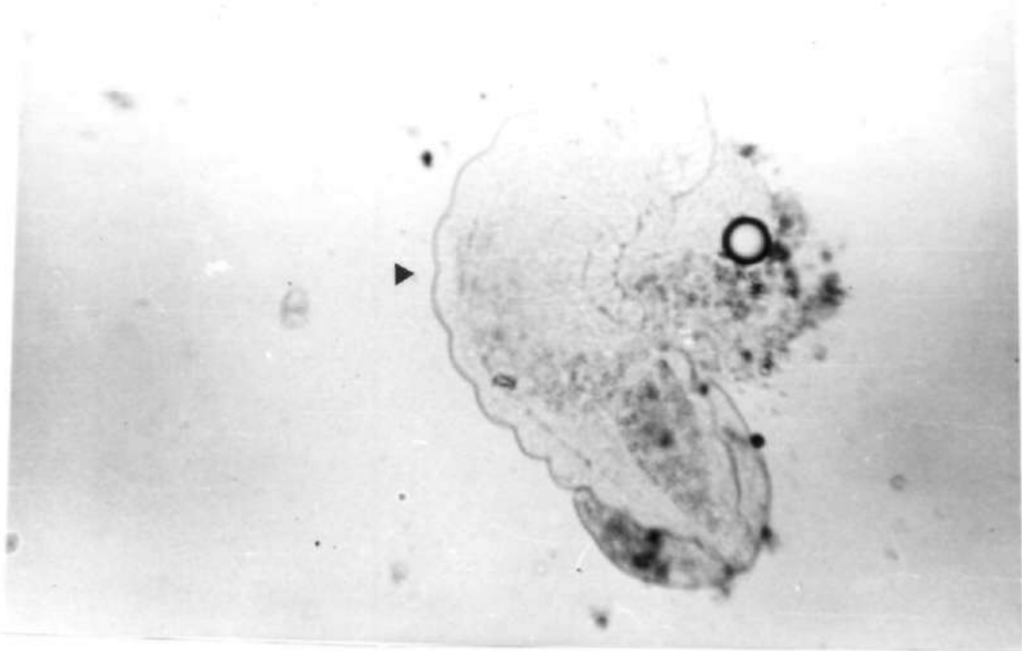


Fig. 6.7. Second instar larvae, 4 days post-infestation, (X 150).
Note the trachea and the digestive system.

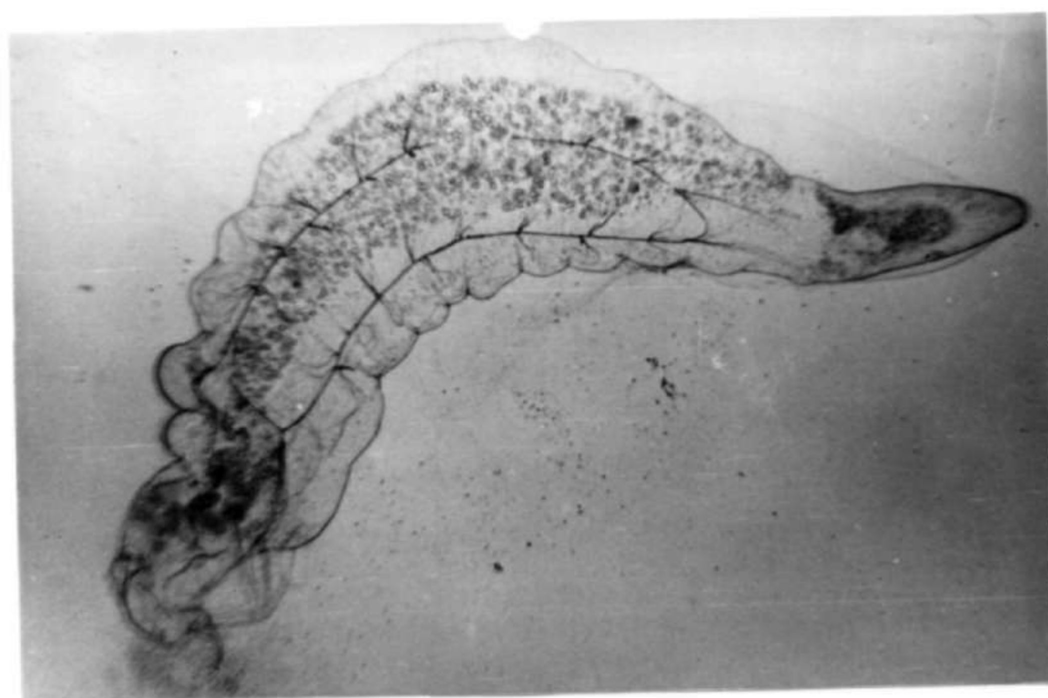


Fig. 6.8. Third instar larvae of the tick parasitoid at 13 days post-infestation, (X 60).

Fig. 6.9. Parasitoid, inside the nymph, pupating (white) on one side and leaving their feces (dark) on the other side, (X 240).

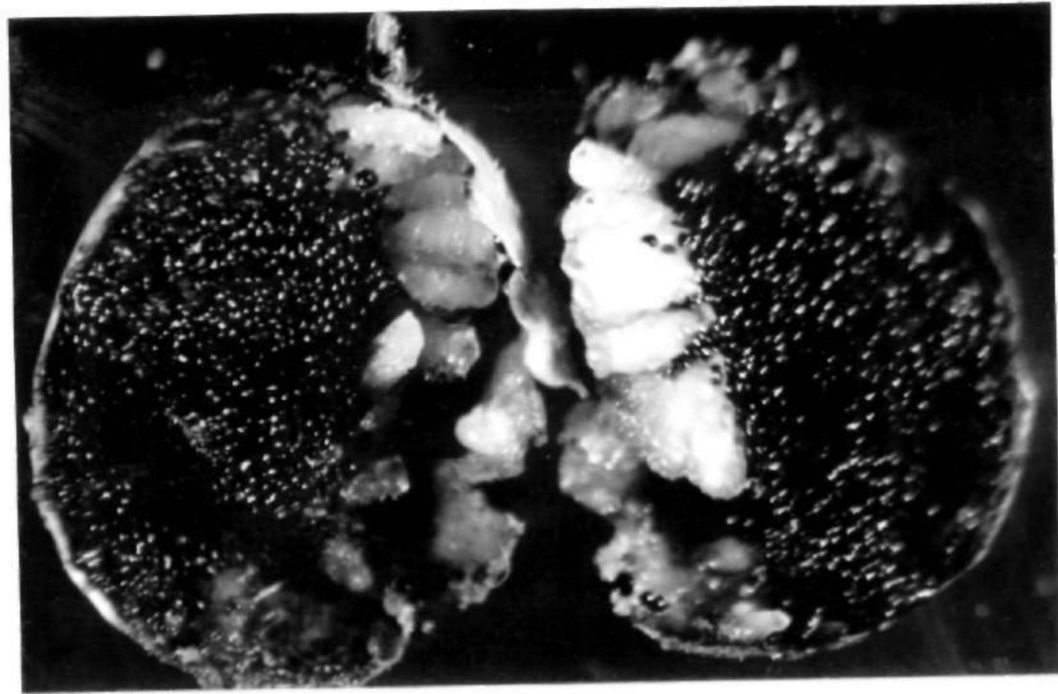


Fig. 6.10. The hole (arrow) through which adult parasitoids emerge from the body of the engorged nymph of *A. variegatum*, (X 90).

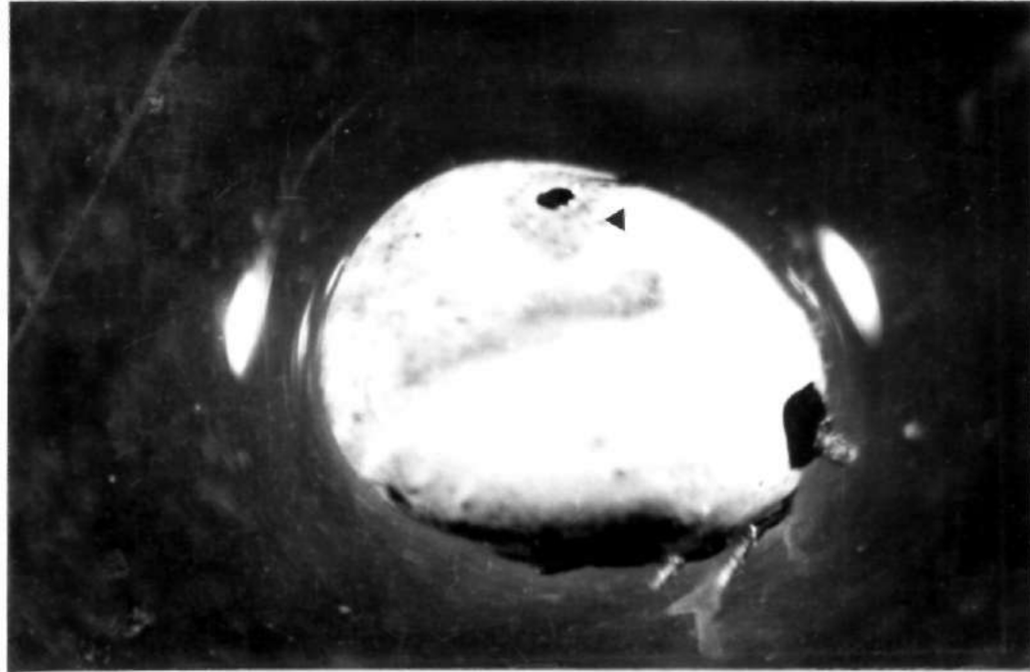
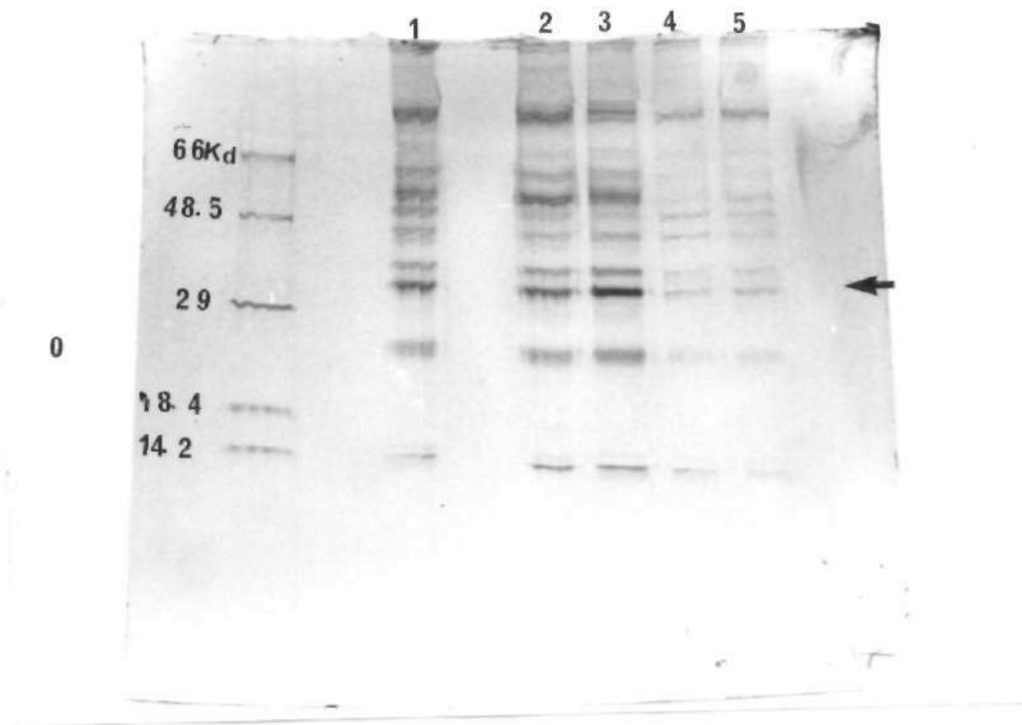


Fig. 6.11. Haemolymph of nymphs of *A. variegatum* parasitized by *Ixodiphagus hookeri*. Haemolymph collected at 12 hours (1), 24 hours (2), 48 hours post-parasitization (3). Haemolymph from non infected nymphs and adult *A. variegatum* (4 and 5).


1
2
3
4
5
66Kd
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29
18.4
14.2
0

CHAPTER VII

GENERAL DISCUSSION AND CONCLUSION

7.1 DISCUSSION

Ticks are annoying pests and surpass all other arthropods in number and variety of diseases they transmit to man and his domestic stock (Arthur, 1962). Millions of dollars have been spent to control ticks and tick-borne diseases for years. Recent development of resistance of some ticks to acaricides have led to the development of alternative control methods like the use of resistant cattle, predators, parasitoids, pathogens and immunomanipulation. The present study examines the immune reaction of ticks to pathogens like bacteria, fungi and parasitoids.

Studies on circulating haemocytes of ticks and the role they play have indicated the existence of different types of haemocytes. Dolp (1970) has identified 3 types of haemocytes: prohaemocytes, plasmatocytes (early and advanced) and spherule cells (early and advanced) in *Hyalomma a. excavatum*. Brinton and Burgdorfer (1971) on the other hand identified 4 types: prohaemocytes, plasmatocytes, spherule cells (4 types) and oenocytes in *Dermacentor andersoni*. Balashov (1979) in contrast has reported prohaemocytes, plasmatocytes and 2 types of granulocytes in *Hyalomma asiaticum*. The present study indicated the existence of 5 types of haemocytes

in *A. variegatum* and *R. appendiculatus*: prohaemocytes, plasmatocytes, granulocytes, spherule cells and oenocytes. One common thing about all these findings is the presence of prohaemocytes and plasmatocytes. The presence or absence of haemocyte types in a species or the other may be due to species diversity (which is unlikely) or differencies in identification techniques. A more consistent study dealing with phase contrast and electron microscopy and if necessary cell cultures will give more tangible results.

Total circulating haemocytes counts (THC) varies remarkably. Dolp (1970) obtained a range 5000 - 37000 per mm^3 in *H. a. excavatum* and 10,000 - 35,000 per mm^3 in *H. dromedarii*. While the present study shows a THC ranging between 3532.9 - 3643.2 and 3395.9 - 3504.03 in *A. variegatum* and *R. appendiculatus* respectively. Dolp (1970) also recorded a THC from different stages of *Argas persicus* ranging from 20,000 to 53,000 per mm^3 and from *A. arboreus* between 13,000 - 73,000 per mm^3. In agreement with Jones (1962), Dolp (1970) believes that THCs in arthropods are greatly affected by their nutritional state. Jones (1962) demonstrated that THC in *Rhodnius* decreases after a blood meal but increased 6 to 9 days later. Dolp (1970) found the highest concentration in unfed argasidae and the lowest in fed ones. In Ixodid ticks the highest concentrations were found in unfed ticks. While in engorged ticks the concentration was very low (Dolp, 1970). This study was done on engorged tick female, that is why THC in *A. variegatum* (3588.75) and *R. appendiculatus*

(3450) are not much different from the THC in engorged female of *H. a. excavatum* (4921). The degree of change of THC in engorged ticks may be influenced by the increase of haemolymph volume. Measurements of the total volume of tick haemolymph under the mentionned conditions may be instructive. Mean THC in ticks infected with bacteria were very high at 24 hours post-infection compared with non infected ticks (Table 1). This denotes the existence of a cellular immune system.

Although not observed during this study, some mitotic figures were observed in *H. a. excavatum* and *H. dromedarii* (Dolp, 1970) and in *Babesia* infected *Boophilus decoloratus* (George, 1987). These cells, identified as early plasmatocytes and spherule cells by Dolp (1970), undergo mitosis to supply enough haemocytes (especially plasmatocytes) to destroy foreign bodies. The ability to extend filopodia and the existence of cell debris in plasmatocytes obesrved in this study denotes their phagocytic ability. This is in agreement with Tsvilenova (1959) who demonstrated the ability of plasmatocytes to move and sequester indian ink. The role of spherule cells seems to be similar to that of granulocytes but a cell line would be a better way of differentiating the two cell types and defining the role they play in tick immune system.

The presence of lysozyme an antibacterial factor was demonstrated in several arthropods. It has been found in the gut of several insects (Morhig and Messner, 1968; Ribeiro and Pereira,

1884), in the haemocytes of *Spodoptera eridana* (Anderson and Cook, 1979) and Locusta (Zachary and Hoffman, 1984), in haemocyte cell line (Landeru, 1976). Kaaya et al. 1986 induced lysozyme activity in *Glossina m. morsitans* by inoculating live *E. coli* and *E. cloacae*. Injection of *E. coli* and *E. cloacae* as well *B. thuringiensis* have induced lysozyme activity in *A. variegatum* and *R. appendiculatus* in this study. It is generally known that lysozyme is antibactericidal but how does it act on bacteria? Does it remove murein sacculus of bacteria like it is the case in *Hyalophora cecropia* (Engström et al., 1984)? Contrary to lysozyme the antibacterial factor does not seem to exist in ticks or if so it must insignificant. Its existence has been demonstrated in insects like *H. cecropia* (Hultmark et al., 1980) and *G.m. morsitans* (Kaaya et al 1987).

Nodule formation and encapsulation are some of the methods used by arthropods to combat foreign bodies. Both phenomenon have been observed in this study. Nodules were formed in reaction to bacterial like *E. coli*, *E. cloacae* and *B. thuringiensis* also to fungal infection. While encapsulation was demonstrated in reaction to parasitoid and implants of microcapillary tubes and araldite. Ultrastructure of capsules formed against araldite showed that they are composed of plasmatocytes, granulocytes and spherule cells. Further investigations are necessary to determine the composition of nodules. It will certainly be more or less the same as that of the capsule; it is difficult to distinguish structures of nodules

and capsules (Shapiro, 1970; Ratcliffe & Rowley, 1979). Ratcliffe and Rowley (1979) described mature nodules in *G. mellonella* as composed of (1) an outer region of newly attaced cells, (2) a middle region of extremely flattened cells and (3) an inner region of partially flattened containing melanized inclusions. This structure is more or less similar to the one of capsules described in this study.

Entomopathogenic fungi *M. anisopliae* and *B. bassiana* appeared to be very pathogenic to ticks once inside the body, but how and when do they invade the tick body? It has been demonstrated that ticks react to fungal infection by the formation of nodules but how do fungi evade ticks's immune system? A study on the effect of fungi toxins on ticks will probably answer the latter question.

Despite the immune reaction (encapsulation) of the nymph of *A. variegatum* the tick parasitoid *I. hookeri* complete its development through 3 larval stages and a pupal stage in 4 to 6 weeks. How do the parasitoid succeeds against the tick's immune system? Further investigations are needed to prove whether the success of the parasitoid depends on the virus in which the egg is coated when laid in the host as it was demonstrated in most of the parasitoids (Vinson, 1985).

7.2 CONCLUSION:

There are 5 types of haemocytes in *A. variegatum* and *R. appendiculatus*. They are prohaemocytes, plasmatocytes, granulocytes, spherule cells and oenocytoids. Ticks use means of cellular and humoral immune systems to combat foreign bodies like bacteria, fungi and parasitoids. It has been demonstrated that haemocytes like plasmatocytes and granulocytes participate in encapsulation and nodule formation as cellular reactions to foreign bodies while there is production of lysozyme as humoral immune reaction. Finally the foreign bodies will either be destroyed as it was shown with parasitoid eggs in adults of *A. variegatum* and *R. appendiculatus* or the tick immune system will be overwhelmed by the foreign bodie e.g. the parasitoid in the nymph of *A. variegatum*, and the tick will eventually die.

7.3 **SUGGESTIONS**:

Further investigations are needed in the following areas:

1. The role played by tick haemocytes in nodule formation and encapsulation. This could be done by establishing a cell culture.
2. Why do some ticks transmit some diseases others don't? Tick haemocytes could be playing a role? Ticks could be fed artificially with infected blood and pathogens monitored from the gut to the salivary gland.
3. How do pathogenic fungi infect ticks? I believe the easiest way is through openings like the gonopore, the spiracles and the anal area. Some histopathological studies are necessary to follow the progression of the fungi through the cuticle of the tick. It would also be interesting to study the effect of toxins produced by fungi, e.g. destruxin A and others, on ticks.
4. The tritrophic relationship between the parasitoid, the tick and the cow. How do the parasitoid avoid the tick immune system? The 33 Kd protein found in parasitized nymphs could be used, if characterized, to develop an ELISA which could eventually be utilized to establish the distribution of the tick parasitoid *Ixodiphagus hookeri*.

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