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Arthropod immunity : characterisation of the humoral immune responses in two species of arthropods - the Kalahari millipede Triaenostreptus Triodus (Attems) and the scorpion Opisthopthalmus Latimanus (Koch).

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1998

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ARTHROPOD IMMUNITY: CHARACTERISATION OF THE HUMORAL IMMUNE RESPONSES IN TWO SPECIES OF ARTHROPODS - THE KALAHARI MILLIPEDE *TRIAENOSTREPTUS TRIODUS* (ATTEMS) AND THE SCORPION *OPISTHOPTHALMUS LATIMANUS* (KOCH)

Etienne van der Walt

A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science.

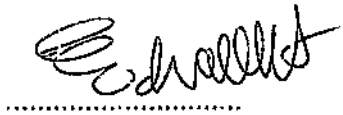
Johannesburg 1998

ABSTRACT

This is the first report comparing the inducible humoral immune responses of two long-lived arthropods. Inducible humoral immune responses were detected in two arthropods namely, the scorpion *Opisthopthalmus latimanus* (Arachnida) and the millipede *Triaenostreptus triodus* (Diplopoda: Spirostreptidae). These anti-bacterial activities were elicited by live gram-negative and gram-positive bacteria. A dramatic hemocytopenia was demonstrated in both the millipede and scorpion after experimental infection, suggesting the possible release of a hemocyte depletion factor. The anti-bacterial humoral responses of the millipede and the scorpion were similar in magnitude to those that have been reported for a large variety of short-lived insects. I also provide further characterisation of the anti-bacterial defence protein(s) of the Kalahari millipede. My results suggest that the humoral immune response of arthropods may have a long and conserved phylogeny.

DECLARATION

I declare that this is my own, unaided work. It is being submitted for the degree of Master of Science (Physiology and Medical Biochemistry) in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.



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22nd Day of April 1998

ACKNOWLEDGEMENTS

I wish to thank the trustees of the National Parks board and Mr. Elias Le Riche, Head Game Warden of the Kalahari Gemsbok Park, for his assistance with this study (Park Collection Permit CKL/I/63/y). This study has been sponsored by MRC and FRD grants to Dr E. McClain.

I would like to extend my sincere appreciation to my supervisors during this project: to Dr Liz McClain, for her devoted support and guidance during this project as well as taking an interest in the character development of her students, to: Prof Nerina Savage for her unending patience, encouragement and inspiration.

DEDICATION

To Lenette with love...

PREFACE

Parts of this manuscript has been published as follows:

Van der Walt, E., McClain, E.L., Puren, A. and Savage, N. (1990). *Arthropod Immunity:* An Inducible Humoral Response in the Kalahari Millipede, *Triaenostreptus triodus*, (Attems), *Naturwissenschaften*, **77**, 189 - 190

Van der Walt, E. and McClain, E.L. (1991). Humoral Immune Response of the Kalahari Millipede: *Triaenostreptus triodus* (Attems), Proceedings of the Physiological Society of South Africa, 1988, Cape Town, South Africa. *South African Journal of Science.*, **86**, 97-98.

CONTENTS

SECTION PAGE

PREFACE v

LIST OF ABBREVIATIONS USED 18

CHAPTER 1: INTRODUCTION 19

1.1 PREAMBLE 19

1.2 BACKGROUND TO THIS STUDY 20

1.3 AIM OF THIS STUDY 38

CHAPTER 2:

THE INHIBITION ZONE ASSAY (PLATE ASSAY): PRIMARY ANTI-BACTERIAL

DETECTION TECHNIQUE: Comparisons between millipedes, scorpions and other insect species ... 40

2.1 INTRODUCTION 40

2.2 MATERIALS AND METHODS 41

2.2.1 Collection and Housing of Animals: 41

2.2.2 Collection of Hemolymph. 44

2.2.3 Bacteria used. 46

2.2.4 Obtaining Bacteria 48

2.2.5 Transformation of bacteria 50

2.2.6 Assays for Anti-bacterial Activity. 52

2.2.4.1 Inhibition Zone Assay (Plate Assay). 52

2.3 PHYSICAL CHARACTERISATION OF ANTI-BACTERIAL PROTEINS 55

2.3.1 Determination of Thermal Stability of Anti-Bacterial Activity from Vaccinated Hemolymph 55

2.3.2 Determination of Salt-out Precipitation Levels .. 56

2.3 RESULTS AND DISCUSSION 58

2.3.1 Antibacterial activity. 58

2.3.1.1 Inhibition Zone Assay (Plate Assay) 58

2.3.2 Phenoloxidase activity (PO) 62

2.3.3 Determination of thermal stability 73

2.3.4 Ammonium sulphate salt-out point determination 76

CHAPTER 3: CHARACTERISATION OF ANTI-BACTERIAL PROTEINS 79

3.1 BACKGROUND AND INTRODUCTION 79

3.2 MATERIALS AND METHODS 82

3.2.1 Assay for Hemocyte Depletion. 82

3.2.2 Statistical procedures 84

3.2.3 Protein Concentration determination 85

3.2.3.1 Micro protein assay 85

3.2.4 Sodium Dodecyl Sulphate - Polyacrylamide Electrophoresis (SDS - PAGE). 87

3.2.5 Assays for Antibacterial Proteins 91

3.2.5.1 Reverse Field Acidic Zone Electrophoresis (RF-AZE) 92

3.2.5.2 Reverse Field Dual Gradient Iso Electric Focussing (RF-DG-IEF) 93

3.2.5.3 Gel Overlay of Reverse Field Iso-Electric Focussing (RF-IEF) and RF-DG-IEF Gels 96

3.3 RESULTS AND DISCUSSION 98

3.3.1 Hemocyte Depletion 98

3.3.2 SDS-PAGE 101

3.3.3 GEL OVERLAY (Activity Determination of Separated Proteins). 103

3.3.3.1 Reverse Field Acidic Zone Electrophoresis: Gel Overlay (RF-AZE) 103

3.3.3.2 RF-IEF and gel overlay 111

3.3.3.3 Reverse Field Dual Gradient Iso-Electric Focussing (RF-DG-IEF) 113

3.4 CONCLUSIONS 120

REFERENCES: 128

LIST OF FIGURES

Figure 1 The Malabari Millipede *Triaenostreptus triodus*. Ruler scale given in centimetre graduations. 59

Figure 2 Plate assay seeded with 10^5 cfu *E. coli*.ml^{-1} and photographed after 21 days storage at 4 °C on dark background in order to demonstrate transparency of the bacterial / nutrient agar medium which serves as a bacterial control and indicates that no bacterial growth occurred during the storage period (refer figure 3, which was photographed under identical circumstances for comparison of bacterial growth). 60

Figure 3 Representative anti-bacterial activity against *E. coli* plate assay. Wells 1 - 4: hemolymph samples from millipede 24 hours post infection with 10^9 cfu *E. coli*.g^{-1}. Wells 5 and 6 - hemolymph samples from millipedes 48 hours post infection 10^9 cfu *E. coli*.g^{-1}. Wells 7 and 8 hemolymph samples from saline injected millipedes. Anti-bacterial activity present in hemolymph sample taken from millipede 48 hours post *E. coli* vaccination (well 6). Well 4 shows slight anti-bacterial activity. Loaded 5 $\mu\ell$ hemolymph in all wells. PO activity is present in wells 1, 2, and 3. In order to gain better clarity of PO activity, also compare figures 4 and 5, which represent the same plate assay (fig. 3), photographed on white illuminated background (fig. 4) and

figure 5 - photographed in colour on dark background, to aid visual conceptualisation of actual PO activity. ... 61

Figure 4 Photograph of *E. coli* plate assay used in figure 3, taken on white illuminated background to illustrate darking areas of phenoloxidase activity. Phenoloxidase activity present on *E. coli* plate assay (wells 1,2,3). Nutrient agar seeded with 10^5 cfu *E. coli*.mℓ^{-1}. Legend same as for Figure 3. Also compare colour photograph in figure 5, which depicts the same plate assay. 62

Figure 5 Photograph of *E. coli* plate assay used in figure 3, taken in colour on dark background to illustrate diffuse, brownish and darking areas of phenoloxidase activity. Phenoloxidase activity present on *E. coli* plate assay (wells 1,2,3). Nutrient agar seeded with 10^5 cfu *E. coli*.mℓ^{-1}. Legend same as for Figure 3. 63

Figure 6 Representative *E. coli* plate assay of millipede (wells noted by M) and scorpion hemolymph samples (wells noted by S) collected immediately after experimental infection with 10^9 cfu *E. coli*.g^{-1} (time 15 minutes). No anti-bacterial activity present. Loaded 5 $\mu\ell$ hemolymph per well. 64

Figure 7 Representative *E. coli* plate assay. Anti-bacterial activity present in scorpion hemolymph sample (wells denoted by S) taken 24 hours after vaccination with 10^9 cfu. g^{-1} *E. coli*. Millipede hemolymph sample (well denoted by M) 24 hours

after vaccination with 10^9 cfu. g_{-1} *E. coli* showed no anti-bacterial activity. 65

Figure 8 *E. coli* plate assay. Anti-bacterial activity present against *E. coli* in millipede hemolymph sample collected 96 hours after experimental infection with 10^9 cfu *E. coli*.g^{-1}. Loaded 5 $\mu\ell$ hemolymph per well. Scorpion (well S) died due to haemorrhage 48 hours post vaccination, therefore hemolymph could not be loaded. Refer results and discussion section regarding haemorrhage in scorpions. 66

Figure 9 Representative *S. aureus* plate assay. Anti-bacterial activity exhibited against *S. aureus* in millipede hemolymph sample collected 72 hours after experimental infection with 10^9 *E. coli* cfu. g^{-1} (wells - left). Wells to the right - no anti-bacterial activity present in hemolymph samples from scorpion 48 hours post vaccination with 10^9 *E. coli* cfu. g^{-1}. Nutrient agar was seeded with 10^5 *S. aureus* cfu.$m\ell^{-1}$. Loaded 5 $\mu\ell$ hemolymph per well. 67

Figure 10 *E. coli* plate assay. Lanes 1,2,3 and 4 - hemolymph samples from four millipedes, 72 hours post vaccination with 10^9 *E. coli* cfu. g^{-1}. Anti-bacterial activity is present in two of the four animals (lanes 3 and 4) with no evidence of PO activity. Lanes 1 and 2 demonstrated PO activity with the absence of anti-bacterial activity. Twenty five micro litres of hemolymph were loaded in

each lane. Compare visual PO activity in figures 3 to 5. 68

Figure 11 Representative thermal inactivation plate assay (*E. coli*). Anti-bacterial activity present in vaccinated millipede hemolymph samples collected 72 hours post infection with *E. coli* and loaded on plate assay after samples were incubated for 15 min @ 50°C (wells 1 and 6). No thermal inactivation evident. Wells 2 and 7: anti-bacterial activity of millipede hemolymph after incubation for 30 min @ 50°C, negligible thermal inactivation of anti-bacterial activity; wells 3 and 8: millipede hemolymph after 50 min incubation @ 50°C - marked thermal inactivation of anti-bacterial activity exhibited after 50 min exposure to heat (50°C); well no 5: anti-bacterial activity not present in millipede hemolymph after 75 min incubation @ 50°C and, well no 4: control millipede hemolymph. Loaded 5 μl hemolymph per well. 69

Figure 12 Determination of thermal stability of anti-bacterial activity. Denaturation of millipede hemolymph anti-bacterial proteins in samples collected 72 hours post infection with *E. coli* and, exposed to a temperature of 50°C. Graph shows anti-bacterial activity remained stable for ± 30 min @ 50°C. 70

Figure 13 Graph of comparative plate assay inhibition zone diameters between millipede and scorpion after injection of 10^9 cfu *E. coli*.g^{-1}. Only two scorpions survived 48 hours post vaccination, none were alive at 72 hours - due to haemorrhage of injection and sampling wounds. 73

Figure 14 Determination of salt-out point of antibacterial proteins. Representative ammonium sulphate (NH_4SO_4) precipitation fractions screened on *E. coli* plate assay. Hemolymph sample taken from millipedes 72 hours post *E. coli*, Well no 1 - untreated hemolymph sample from vaccinated millipede; 2 - renatured precipitate of 40% NH_4SO_4 fraction; 4 - 35% NH_4SO_4 fraction; 6 - 15% NH_4SO_4 fraction; 7 - 25% NH_4SO_4 fraction; 11 - 30% NH_4SO_4 fraction; 5 and 8 - no activity present in the resultant supernatant fractions of wells 4 (35%) and 11 (30%) respectively indicating complete precipitation of anti-bacterial component; wells 9 and 12 - 10 $\mu\ell$ of a 50% saturated NH_4SO_4 solution (negligible response on plate assay); well no 10 - 10% NH_4SO_4 precipitate fraction and well no 13 - resultant supernatant from 60% NH_4SO_4 fraction. Ammonium sulphate precipitate and supernatant renatured with 10 $\mu\ell$ 0.1 M Phosphate buffer (pH 7.4). Five micro litres of renatured

precipitate solution loaded per well. (% - percent saturated NH_4SO_4 solution) 74

Figure 15 Determination of salt-out point of antibacterial proteins. Ammonium sulphate precipitation fractions screened on *E. coli* plate assay. Salt-out point occurs at approximately 35% saturated NH_4SO_4 of antibacterial proteins precipitated from hemolymph 72 hours post infection). .....

Figure 16 Comparative hemocyte depletion between millipede and scorpion after injection of pyrogen free saline (control) and 10^9 cfu *E. coli*/g. Results are expressed as mean percentage change ± SD (millipede N=4, scorpion N=4) that occurred before and after injection. Line "0" on y-axis indicates level where no change in hemocyte population occurs. Decrease in number of scorpions were due to haemorrhage after sampling. Wounded and control (saline injected) data are depicted as the solid line at zero on the y-axis. 107

Figure 17 Reverse field acidic zone electrophoresis (RF-AZE) *E. coli* gel overlay. Anti-bacterial activity against gram-negative *E. coli* is present in wells 1, 2, 4 and 5 (arrowed). Anti-bacterial activity was not present in control (non injected) millipede hemolymph (well 3). Wells 1, 4 and 2, 5 - hemolymph samples from two millipedes collected 48 hours post experimental infection

with 10^7 cfu *E. coli*.g^{-1}. Gel A) and Gel B) are duplicate gels where gel A was overlayed with nutrient agar seeded with 10^5cfu *E. coli*.$m\ell^{-1}$ and Gel B) was stained with PAGE Blue and then with silver in order to visualise individual protein bands. Two hundred and fifty micrograms of total hemolymph protein was loaded in each well in order to obtain detectable anti-bacterial activity on the gel overlay (Gel A) as well as on the stained gel (Gel B). Proteins were separated on a 10% acrylamide gel ran towards the cathode i.e. from top to bottom. (figure taken from Van der Walt *et al*, 1990) 109

Figure 18 *E. coli* overlay of a 10% reverse field acidic zone electrophoresis (RF-AZE) gel. Lane 1 - control millipede hemolymph; lanes 2 and 3 - millipede hemolymph samples taken 72 hours post vaccination with 10^9cfu *E. coli*.g^{-1}; lanes 4 and 5 - millipede hemolymph samples taken 48 hours post infection with 10^9 cfu.*E. coli*.g^{-1}; lane 6- control hemolymph. Loaded 350 μg total protein per well. There was a more than three-fold increase (= 337.85% SD ± 88.9%, N = 4, by manual measurement of surface area) in anti-bacterial protein synthesis in hemolymph samples collected between 48 h and 72 hours post vaccination with *E. coli*. 110

Figure 19 Silver stained 10% RF-AZE gel. Lane 1 - control scorpion hemolymph; lanes 2, 3 and 5 - hemolymph sample collected from scorpions 48 hours post injection of 10^9cfu *E. coli*.g^{-1}; lane 4 - millipede hemolymph sample taken 48 hours after experimental infection with 10^9cfu *E. coli*.g^{-1}. No anti-bacterial activity was detected in scorpion hemolymph samples when performing *E. coli* gel overlay..... 112

Figure 20 *E. coli* overlay of 10% RF-AZE gel. Hemolymph samples collected from millipedes 72 hours after injection of 10^6 cfu *E. coli*.g^{-1}. Loaded 50 $\mu\ell$ of hemolymph in each well. Lanes 1, 2, 3 and 4 - hemolymph sampled from 4 millipedes 72 hours post infection with 10^9 cfu *E. coli*.g^{-1}; lane 5 - hemolymph sample from control millipede 72 hours post injection of pyrogen-free saline (0.7% w/v NaCl). Strong anti-bacterial activity is present in lanes 1 to 4. Saline injected hemolymph sample weakly positive after 72 hours. The of anti-bacterial protein activities reflect relative differences despite the use of a constant hemolymph volume for each individual animal. .. 113

Figure 21 *E. coli* overlay of 12% to 22% linear gradient RF-AZE duplicate gel (A) and PAGE Blue and silver stained gel (B). Lanes A)1 and B)1 - 5 μg Cytochrome C; Lanes A) 2, 3, 5 and B) 2, 3, 5 - millipede hemolymph samples

collected 48 hours after vaccination with 10^8 cfu *E. coli*.g^{-1}; lanes A) 4 and B) 4 - control millipede hemolymph sample injected with pyrogen-free saline (0.7% w/v NaCl). Anti-bacterial activity of the PAGE Blue and silver stained anti-bacterial proteins in gel B (B:2,3 & 5), is reflected on *E. coli* overlayed gel (A:2,3 & 5)(arrowed). Loaded 250 μg of total protein per well. 114

Figure 22 SDS - Page of vaccinated and control hemolymph samples from millipedes and scorpions. Silver stained linear gradient gel 10 - 20% poly acrylamide, Lanes: 1,10 - Molecular mass standards: The gels were calibrated using six molecular mass calibration markers (BDH Chemicals, Germany) namely : (A) Ovotransferrin - 76 to 78.0 kD, (B) Albumin - 66.25 kD, © Ovalbumin - 42.7 kD, (E) Carbonic anhydrase - 30.0 kD, (F) Myoglobin - 17.2 kD and (G) Cytochrome c - 12.3 kD; Lane 2: Chicken egg white lysozyme 13.8 kD; 3 and 5: Control millipede; 4 and 6: Millipede hemolymph 96 hours post injection of 10^9 cfu *E. coli*. g^{-1}; 7 and 8; Scorpion hemolymph sample 24 hours post injection of 10^9 cfu *E. coli*. g^{-1}; 9: Control scorpion hemolymph sample. Loaded 100 μg of total protein per well. . 115

Figure 23 Reverse field isoelectric focusing (RF-IEF) gel overlay (*E. coli*) (A) and B) PAGE Blue and silver stained gel

focussed under the same conditions. A) 1, 2 and 3 - Hemolymph samples taken from millipede 48 hours post injection of 10^9 cfu *E. coli*.g^{-1}, loaded 150 μg, 250 μg and 300 μg of total protein in lanes A) 1, 2 and 3 respectively. Anti-bacterial activity against *E. coli* present at the bottom of the gel (arrowed) indicating inadequate pH range for proper resolution of antibacterial proteins. Gel B) lane 3 - Cytochrome C focussed at the bottom of the gel; Lanes B) 1 & 2 - 10 μg Chicken egg white Lysozyme, poorly focussed. 116

Figure 24 Silver stained reverse field dual gradient iso-electric focussing (RF-DG-IEF) and concomitant Gel overlay of Millipede hemolymph proteins 72 hours post injection of 10^9 cfu *E. coli*. g^{-1}. Plus (anode) and minus (cathode) sign indicate direction of current. Lane A: Antibacterial activities (short and long arrow) of hemolymph proteins against *E. coli* Gel Overlay; Lanes 1 and 2 - PAGE Blue and silver stained anti-bacterial proteins from millipede hemolymph. Lane 3 - Iso-electric point marker (Cytochrome C: pI = 11). Multi layer focussing areas due to fractionation caused by repeated freezing and thawing of Cytochrome C sample. Loaded 500 μg of total protein per well. 117

LIST OF TABLES

TABLE I Comparison of antibacterial r sponses between millipede and scorpion to different doses of gram-negative and gram-positive bacteria on *E. coli* plate assay. 60

TABLE II Comparative Inhibition zone diameters reported for other the millipede and scorpion as well as other arthropod species against *E. coli.* 61

LIST OF EQUATIONS

EQUATION 1

. 84

LIST OF ABBREVIATIONS USED

APS	**Ammonium persulphate**
cfu	**Colony forming units**
d	**Relative molecular mass (in dalton)**
HDF	**Hemocyte depletion factor**
IHIR	**Inducible humoral immune response**
kd	**Relative molecular mass (in kilo dalton)**
Pi	**Iso electric point (pH)**
PO	**Phenoloxidase**
RF-AZE	**Reverse field acidic zone electrophoresis**
RF-DG-IEF	**Reverse field dual gradient iso-electric focussing**
RF-IEF	**Reverse field iso-electric focussing**
SDS-PAGE	**Sodium dodecyl sulphate polyacrylamide electrophoresis**
TEMED	**Tetramethylethylenediamine**

CHAPTER 1: INTRODUCTION

1.1 PREAMBLE

Invasion of the body by foreign micro organisms and the lethal risk of infection resulting from such an invasion, is a threat that all living organisms have to face (Herried, 1977). The mechanisms used to combat such an occurrence have been developed over many thousands of years of evolution (Ravindranath, 1981) and are almost as equally varied in their complexity and origin as the numb[illegible] of different forms of life that [illegible]st today.

Strategies employed by arthropods to survive potentially lethal invasion by foreign organisms range from basic, structural barriers to prevent entry of micro organisms into the body e.g. the cuticle, calcium carbonate exoskeleton and the internal peritrophic membrane of invertebrates

(Herried, 1977) to the more complex cellular and acellular processes e.g. phagocytic (cellular) type reactions present (Lackie, 1986b; Napp. and Carton, 1986; Dularay and Lackie, 1987; Brookman *et al*, 1988) in vertebrates as well as in invertebrates and sophisticated acellular complement processes found in various organisms (Söderhall and Smith, 1984). There is then a remarkably varied "immunological repertoire" from which the present spectrum of immunological defences arose.

1.2 BACKGROUND TO THIS STUDY

The use of the term "immunity" is defined as "the ability of an organism to resist diseases". The use of this term in in ct immunity is justified for the following reasons: a) The term has been widely used in the literature and has been incorporated in the computerised citation of scientific documents, b) Alternative expressions i.e. "defence reactions of in.ects"

can be misleading as it also refers to reactions by which insects defend themselves against predators eg. the external chemical secretions of millipedes and other insects, c) the term insect immunity is in agreement with the historical use of this term and also with the definition of "immunity" given in recent encyclopaedias as "an organism's resistance, natural or acquired, to the onset of pathological conditions from infection, natural or artificial, by micro organisms or their products" (Henderson, 1979; Götz and Boman, 1985).

The first and most well known anti-bacterial protein was discovered by accident in 1922 when the renowned Sir Alexander Fleming was working with bacterial agar plates while suffering from a cold. A drop from his nose fell onto one of the agar plates containing bacteria. Later, he observed that the bacterial growth in the area surrounding where the drop had fallen caused fading of bacterial growth and Fleming concluded that

it could only be the result of an "agent" that killed the bacteria - and "eureka" lysozyme was discovered (Osserman *et al*, 1974). Subsequent studies focussing on lysozyme has shown it to be almost ubiquitous in the animal kingdom and is found among other places in cartilage, genital secretions, eggs of fish as well as in birds (Wilson and Prager, 1974). Soon after, Fleming discovered Penicillin, the miracle antibiotic drug under almost identical circumstances when yet another agar plate containing bacteria was contaminated with a fungus.

Investigation of the arthropod immune response was, more recently, conducted along similar lines when Mohrig and Messner (1968a,b) reported the first discoveries of lysozyme from the hemolymph of insects. The discovery of a protein (lysozyme) that exists in humans as well as in insects triggered a myriad of investigations in order to determine if indeed

arthropods have an humoral immune system which is in any way similar to that found in humans (Götz and Boman, 1985; Dunn, 1986).

Work on arthropod immunity has been conducted by two different groups of scientists characterised by very different attitudes toward invertebrate immunity (Lackie, 1986b). The first group of investigators utilised experimental methods and terminology gleaned from vertebrate immunology. In the pursuit of this endeavour they used transplants, heat-killed bacteria and unfractionated and denatured venoms as immunological challenges. The information obtained in this fashion can only lead to final conclusions once further molecular analysis has been performed. The use of terms from mammalian immunology may be misleading in that a certain understanding is implied that does not exist. The second group takes their departure point from the assumption that there are fundamental differences between the immune system of

vertebrates and invertebrates. Experiments by investigators of this group are designed to simulate natural infections arthropods are likely to encounter. They use controlled doses of live bacteria in order to explain results in terms of a possible survival value to the animal. Although injection is considered artificial, it most likely corresponds to an injury. Therefore, it is important to compare immune responses with controls in which an equal injury is performed (Götz and Boman, 1985).

Insects are considered to be some of the most ancient and successful animals on earth. Part of the reason for their success is the fact that they have adapted to occupy a vast number of different ecological habitats (Crawford and McClain, 1983). Many of these niches are satiated with pathogenic and parasitic organisms that maintain a serious threat of infection (Bulla *et al*, 1975). The presence of these infectious organisms may have provided a strong selection pressure for those insects species

that have developed immune systems that are effective against infection (Boman and Steiner, 1981). The pursuit of this field of study may lead to development of more effective and environmentally friendly pest control chemicals. The modern species of insects found today therefore represent the most comprehensive summary of successful defensive immune strategies in the animal kingdom.

The initial response of insects to invasive foreign organisms, is elicited via circulating hemocytes. Insect hemocytes are very capable of removing foreign particles such as bacteria, fungi and nematodes from the hemocoel by phagocytic, nodule forming and encapsulating processes (Salt, 1970; Mohrig *et al*, 1979a,b; Anderson *et al*, 1981; Götz *et al*, 1981; Dularay and Lackie, 1985; Götz *et al*, 1987). The phagocytic process normally provides an effective defence against invading bacteria below a certain threshold level. This threshold differs for each insect species. When this

threshold level of bacterial infection is exceeded, nodule formation is initiated in order to supplement phagocytosis (Horohov and Dunn, 1983; Ratcliffe and Walters, 1983a). During nodule formation, hemocytes and bacteria become entangled in an extra cellular matrix which is later melanized resulting in aggregates that adhere to the inner walls of the hemocoel (Ratcliffe and Walters, 1983b). Encapsulation is another cellular defence process whereby multi-layered cellular encasements are formed to surround foreign particles which are also melanized (Götz, 1986a,b). The study by Ratcliffe and Rowley (1979) identified two types of hemocytes, granulocytes and plasmatocytes, that are implicated as the mediating hemocytes of the cellular and possibly the humoral immune responses.

Experimental bacterial injection into the hemocoel of insects, elicits profound and rapid changes in the population of circulating hemocytes.

The surface of the hemocytes become more adhesive to each other and to other tissue surfaces (Wago, 1980). Studies performed on the wax moth, *Galleria mellonella*, showed that a dramatic hemocytopenia was produced just 5 minutes after experimental bacterial infection (Gagen and Ratcliffe, 1976; Ratcliffe and Rowley, 1979; Chain and Anderson, 1982; Chain and Anderson, 1983). The number of circulating hemocytes return to their pre-treatment levels several hours after infection. However, in the tobacco horn worm, *Manducca sexta*, the opposite reaction was observed after experimental bacterial infection (Horohov and Dunn, 1982) and resulted in a marked increase in the number of circulating hemocytes. The increase in hemocyte number could be due to the mobilisation of sessile hemocytes as a result of bacterial challenge. However, a later study by Geng and Dunn (1989) showed that injections of bacteria and bacterial cell walls in *M. sexta* larvae elicited a depletion of hemocytes from circulation. This finding is contradictory to an earlier report (Horohov and

Dunn, 1982) where an increase in hemocyte numbers was recorded in *M. sexta* after bacterial [illegible]tion. In order to explain this phenomenon Geng and Dunn (1989) performed differential hemocyte counts and reported that plasmatocytes, specifically, were depleted. The depletion in the numbers of circulating hemocytes are believed to be mediated through the release of one or more hemocyte depletion factor(s) (HDF)(Chain and Anderson, 1983).

Insects respond to a wide variety of biotic and abiotic substances and leads one to ask, how do insects recognise foreign particles?. Lackie (1981) proposed a dual process of recognition of foreign particles by insect hemocytes: Firstly, recognition of abiotic particles seem to be transduced through detection of physicochemical properties such as surface charge and secondly, that the detection of biotic particles or organisms be mediated by specific cell surface receptors. A paucity in

information exists regarding the specific receptor mediated recognition hypothesis. There is however some evidence to suggest that lectins (agglutinins) may play a role in recognition of foreignness (Lackie, 1986a; Dularay and Lackie, 1987).

The enzyme phenoloxidase (PO) is present in the hemolymph of all arthropods as well as in the hemocytes of insects (Iwama and Ashida, 1986) and catalyses the oxidation of tyrosine to 3,4-dihydroxyphenylalanine which initiates melanisation during nodule formation and encapsulation. Under normal circumstances PO is found in its inactive precursor form, prophenoloxidase (proPO) which is activated by proteolysis in the presence of foreign material or organisms (Ashida and Ohnishi, 1967; Dohke, 1973; Ashida and Dohke, 1980; Ashida *et al*, 1983; Ashida and Yoshida, 1988). Pye (1974), Brookman *et al* (1988) and Brookman (1989) have shown that proPO is activated by

bacterial cell walls to produce active PO. Prophenoloxidase can also be activated by fungal β 1,3-glucans (Söderhall, 1981; Söderhall *et al*, 1988). Söderhall (1982) and Leonard *et al* (1985b) reported that PO in insects could play a role in recognition of foreign organisms present in the hemocoel. The exact role of PO in the cellular immune response is still uncertain at this stage, given the current information contained in the literature. This fact is compounded further in that experimental injection of LPS (lipopolysaccharide) was shown not to activate PO but, stimulated enhanced phagocytic activity (Ratcliffe *et al*, 1984). It is postulated that there might be more than one PO cascade functioning in the insect immune response (Gupta, 1979, Horohov and Dunn, 1982).

Injection of viable bacteria into insects elicit a cell free anti-bacterial immune response (Boman *et al*, 1974). This humoral immune response results in the appearance of several "new" proteins in the hemolymph of

vaccinated insects (Faye *et al*, 1975; Boman *et al*, 1986). Amongst these proteins is the enzyme lysozyme, an enzyme which hydrolyses bacterial cell walls, and, proteins which are capable of killing gram-negative bacteria, as well as other proteins which as yet have no discernable biological function (Powning and Davidson, 1973; Jarosz, 1979; Jolles *et al*, 1979; Hultmark *et al*, 1980; Hoffmann *et al*, 1985; Boman, 1986; Dunn, 1986). The synthesis of new hemolymph proteins have been demonstrated in various developmental stages in a variety of insect species (Faye and Wyatt, 1980; Hoffmann, 1980; Boman and Steiner, 1981; De Verno *et al*, 1984; Hurlbert *et al*, 1985; Keppi *et al*, 1986; Flyg *et al*, 1987; Götz *et al*, 1987). Lysozyme(s) have been discovered and purified from several vaccinated lepidopteran larvae and were found to be small (15,300-16,200 d) heat stable and basic proteins whose characteristics are similar to that of chicken egg white lysozyme (Mohrig and Messner, 1968a and 1968b; Powning and Davidson, 1973; Jolles *et*

al, 1979; Hultmark *et al*, 1980). The partial amino acid sequences of four insect lysozymes have been determined and demonstrate significant sequence homology to each other as well as to chicken egg white lysozyme (Jolles *et al*, 1979; Engström *et al*, 1985). Hemolymph of native (non-vaccinated) insects, does not have bactericidal properties and thus provides a nutrient rich environment for bacterial growth (Bulla *et al*, 1975; Horohov and Dunn, 1982). However, after bacterial injection, the hemolymph of insects become bactericidal as a result of the synthesis of various anti-bacterial proteins. Inducible hemolymph bactericidal factors that kill gram-negative bacteria were first discovered by Stevens (1962) and Hink and Briggs (1968), in larvae of the wax moth *G. mellonella*. Another study by Boman *et al*, 1972, reported an inducible anti-bacterial system in the fruit fly, *Drosophila melanogaster*. Individual anti-bacterial proteins were first purified by Boman and co-workers (Boman *et al*, (1972); Boman and Hultmark, 1981; Boman and Steiner, 1981) from the

hemolymph of *Hyalophora cecropia* after vaccination with the bacterium, *Enterobacter cloacae* and resulted in the discovery of two families of anti-bacterial proteins namely the cecropins and attacins. Consequently three major and four minor cecropins have been purified and their amino acid sequence determined (Hultmark *et al*, 1980; Steiner *et al* , 1981; Hultmark *et al*, 1982). Cecropins are small (3,500-4,000 Da) basic peptides. Cecropins A and B present a helical conformation in the presence of hydrophobic environments and is believed to aid the activities of these two cecropins (Steiner, 1982). Two similar bactericidal peptides were discovered and isolated from *Antheraea pernyi* pupae (Qu *et al*, 1982) and six similar cecropin-like bactericidal peptides were discovered in six other lepidopteran species (Hoffmann *et al*, 1981). Cecropin-like peptides have also more recently been reported in larvae of the tobacco horn worm, *M. sexta* (Hughes *et al*, 1983; Dunn *et al*, 1985) and other insect species (Kaaya *et al*, 1987). The second group of insect

bactericidal proteins that kill gram-negative bacteria, the attacins, consists of six similar proteins (A to F) with a molecular mass range of between 20 kD and 23 kD (Hultmark *et al*, 1983). Attacins can be divided into two groups according to their physical charge, I) a basic group, attacins A to D and ii) an acidic group which comprises attacins E and F (Kochum *et al*, 1984). Attacin- and cecropin-like anti-bacterial proteins have been discovered in various insect species (Götz and Boman, 1985; Kaaya *et al*, 1987). Each of these groups of anti-bacterial proteins has a unique bactericidal specificity for certain types of bacteria (Hultmark *et al*, 1982), however no evidence exists for specific synthesis of anti-bacterial proteins in response to different bacterial types (gram positive / negative). The coordination of the cellular (phagocytosis and stress responses) and humoral immune responses (anti-bacterial proteins and inflammation) seem to be, among others, under hormonal control (Jones *et al*, 1993).

The origin of anti-bacterial protein- as well as lysozyme -synthesis has been reported in the isolated fat body of *H. cecropia* (Faye and Wyatt, 1980) and *M. sexta* (Dunn *et al*, 1985). Zachary and Hoffmann (1984) reported lysozyme synthesis in granular hemocytes of the migratory locust, *Locusta migratoria*. Although most hemocytes and hemocoelic tissues appear to produce lysozymes, synthesis of anti-bacterial proteins that kill gram-negative bacteria have only been reported in the fat body of insects (Dunn, 1986; Trenczek and Faye, 1988). Recently, immune proteins, different and unrelated to attacins and cecropins, have been reported in *L. migratoria* (Boigegrain *et al*, 1993) that have lectin type properties.

Anti-bacterial protein synthesis can be elicited by a multitude of substances and micro organisms. Lysozyme activity in *G. mellonella* increased 50-fold, 24 hours after injection of live and killed cells of

Micrococcus luteus, *Bacillus cereus*, *B. s[illegible]* and *Pseudomonas aeruginosa* (Mohrig and Messner, 1968a). In the same study, similar responses were observed after injection of sterile distilled water, Ringer's solution, methylene blue, and Chinese ink. However, all studies working on lysozyme in insects, reported that maximum release of lysozyme occurred after injection of live bacteria (Mohrig and Messner, 1968a; Powning and Davidson, 1973; Jarosz, 1979; Schneider, 1985; Dunn, 1986). This was also found in the giant silk moth *H. cecropia* after injection of UV irradiated *Enterobacter cloacae*, which retain their surface properties, and produced a response no larger than the injury reaction caused by a sham injection but strong anti-bacterial activity was produced after injection of live *E. cloacae* (Faye *et al*, 1975).

Bacteria which are naturally pathenogenic to insects seem to avoid or suppress induction of immunity, and this may in fact be one main reason

for pathogenicity of some bacteria (Chadwick, 1975; Flyg and Xanthopoulos, 1983; Berg , 1988).

1.3 AIM OF THIS STUDY

In order to obtain a clearer understanding of the phylogeny of the arthropodian humoral immune response and indeed, the evolution of human immune responses, it is necessary to establish whether inducible humoral immune response (IHIR) occurs in the more primitive and long-lived species of arthropods and also if it is similar to that demonstrated for short-lived insects. We recently produced and reported the first evidence of an inducible anti-bacterial protein(s) in a long-lived arthropod namely, the Kalahari millipede *Triaenostreptus triodus* (Van der Walt, 1988; Van der Walt *et al*, 1990; Van der Walt and McClain, 1991). The purpose of this study is to develop methodology in order to provide data to further characterise the anti-bacterial proteins by their various chemical and physical properties and to compare this information to another long-lived

arthropod, the scorpion *Opisthopthalmus latimanus* and also to anti-bacterial proteins reported in short-lived insects.

CHAPTER 2:

THE INHIBITION ZONE ASSAY (PLATE ASSAY): PRIMARY ANTI-BACTERIAL DETECTION TECHNIQUE: Comparisons between millipedes, scorpions and other insect species

2.1 INTRODUCTION

We have previously developed a modified assay (Hoffmann, 1980) for the detection of anti-bacterial activity from nated hemolymph samples (Van der Walt, 1988; Van der Walt *et al*, 1990 and Van der Walt & McClain, 1991). This technique, the plate assay, allows the anti-bacterial components of experimentally infected animals, to react with live bacteria that are suspended in soft agar, in order to demonstrate both the presence

and intensity of anti-bacterial activity. This technique is flexible and can be modified to detect anti-bacterial activity against different species of bacteria (gram-negative or gram positive).

2.2 MATERIALS AND METHODS

2.2.1 Collection and Housing of Animals:

Millipedes of the species, *Triaenostreptus triodus* were collected at Nossob in the Kalahari Gemsbok Park (20°20'.0 S; 25°30'.0 E, Park collection permit no. CKL/I/63/y) shortly after seasonal rains when they emerge from underground to feed on the newly sprouted desert vegetation. The millipedes were kept at room temperature (23°C) in glass aquaria on native Kalahari sand. They were fed on a diet of carrots and lettuce leaves and received water *ad lib.* in the form of a fine mist spray. *T.*

triodus is an ideal animal in which to study immune responses, as large quantities of hemolymph can be sampled from the same individual without

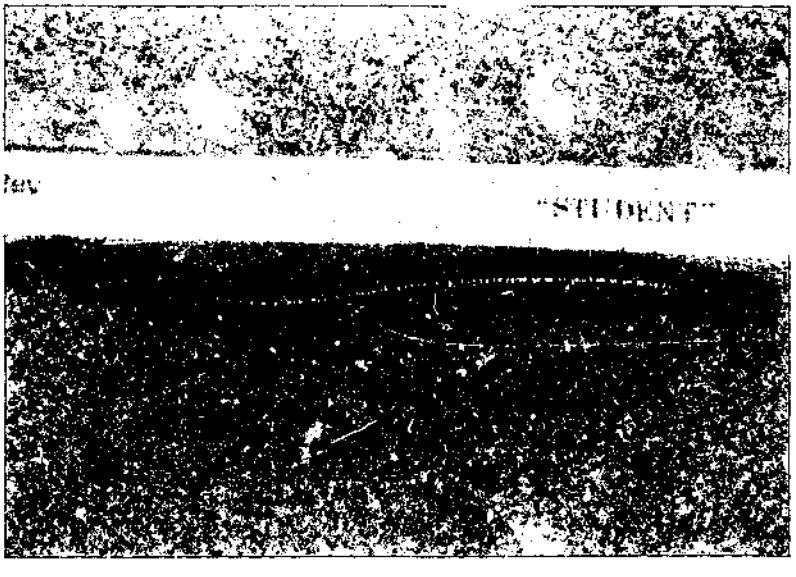


Figure 1 **The Kalahari millipede *Triaenostreptus triodus*. Ruler scale in centimetre graduations.**

harm (see **Figure 1**).

Scorpions of the species, *Opisthopthalmus latimanus* were exhumed from their burrows (± 16 cm deep) underneath flat rocks on north to north-west facing hill sides near Johannesburg (26°08'.67 S, 28°04'.27 E). The scorpions were kept separate in plastic petri dishes (i.d. 150 mm × 20 mm). This was done in order to prevent cannibalism. A damp piece of cotton wool was placed in the dishes to prevent dehydration. The scorpions were fed meal worms once a week. The millipedes and scorpions were kept under a 12 hour light/dark cycle. The millipedes used weighed on average 55 grams (±SD 6.83 g; n=10) and were up to 30.5 cm (±SD 4.06 cm; n=10) in length, while the average mass for the scorpions were 2.5 g (±SD 1.79 g; N = 10) and 8.03 cm (±SD 1.82 cm; N = 10) in length. Care was taken in handling the scorpions to avoid the poison of the stinger.

2.2.2 Collection of Hemolymph.

Aseptic techniques were used during injection and sampling in order to avoid contamination. Hemolymph from millipedes was collected as follows: a sterile 25G hypodermic needle (0.45 mm) was inserted into the mid-dorsal region in between adjacent segments (± 2 cm from the head). The millipede was turned over and the hemolymph drained into a sterile 1.5 mℓ polypropylene micro centrifuge tube that was kept on ice. A total of ± 3 mℓ of hemolymph could be sampled without any detrimental effect to the millipede. Sampling volumes from millipedes for time course experiments were approximately 200 µℓ of hemolymph at each interval. After experimental infection the millipedes were kept singly in plastic containers that were covered with cheese cloth. Scorpions were bled as follows: a sterile 30G needle (0.3 mm) was inserted laterally through the intersegmental membrane of the cuticle of the last abdominal segment.

until half of the bevelled edge was submerged (± 0.7 mm) causing the hemolymph to leak out. Fi.. micro litres of hemolymph was then aspirated with a 200 $\mu\ell$ adjustable pipette and the hemolymph transferred to a sterile 1.5 mℓ polypropylene micro centrifuge tube kept on ice. A separate 5 $\mu\ell$ aliquot was taken immediately after sampling from both millipedes and scorpions for hemocyte counting (see Assay for Hemocyte Depletion). Cells and debris were removed from the hemolymph samples by l gh speed centrifugation (8000 g for 5 min. at 0 °C). Samples were taken at various time intervals namely: before injection 0 hours (control); 4 h; 12 h; 24 h; 48 h; 72 h and 96 hours after injection of either pyrogen free insect saline (0.7% w/v NaCl, control), or bacteria (see Bacteria used). Each animal served as its own control. Additional controls were prepared by wounding the animal with a sterile hypodermic needle without injection. This was done in order to take into account the effect wounding might have on the immune response.

2.2.3 Bacteria used.

In this study live bacteria was used for the following reasons: I) under natural conditions arthropods are infected by live bacteria; dead bacteria can not pose a real threat to arthropods, ii) natural infections occur with rather low numbers of bacteria and such low levels can only be detected by colony forming unit counts, iii) the argument against using heat killed bacteria is that their surface proteins are denatured during the heat process and therefore do not represent a natural threat. Bacteria are divided into two main categories based on the structure and composition of the lipopolysaccharide outer membrane. These categories are: gram positive- or gram negative-bacteria. Severe immune reactions may occur to each of the two types of bacteria. Some organisms respond more severely to a specific type of bacteria i.e. either gram-negative or gram-positive. It is known that primates exhibit fever only when a gram positive bacterial

infection is present. Humans, however, produce fever responses when infections of either these bacterial types are present. It is therefore important to determine whether these selected long lived arthropods exhibit immune responses to both gram positive and gram negative bacterial infection.

The gram negative bacterium *Escherichia coli* K12 and the gram positive *Staphylococcus aureus* (ATCC # 12600) were employed as our standard test bacteria, for both induction of the humoral immune response as well as for detection of anti-bacterial activity. This allowed us to determine which type(s) of bacteria could elicit a humoral immune response and, if a specific humoral immune response was elicited towards a specific category or type of bacteria.

2.2.4 Obtaining Bacteria

Bacteria were obtained from the Department of Microbiology, University of the Witwatersrand, Johannesburg, South Africa. Bacteria were grown in Nutrient broth (Biolab) and agitated (40 oscillations per minute) at 37 °C until log phase growth, which was determined spectrophotometrically as an exponential increase in absorbance units (A 600 nm). The bacteria were harvested by centrifugation (8000 g for 5 min.), washed and suspended in pyrogen free saline (0.7% w/v NaCl) and used for experimental injection. The number of viable bacteria were counted as the number of colony forming units (cfu) and expressed as number of cfu per mℓ. The counting procedure is briefly as follows: 100 $\mu\ell$ aliquots of serially diluted bacterial suspensions (i.e. $1:10^{5}$ - $1:10^{15}$) were plated out on nutrient agar plates and incubated at 35 °C for 24 hours and the number of colony forming units (cfu) counted. The bacterial suspensions were

then diluted with pyrogen free saline (0.7% w/v NaCl) in order to obtain concentrations of 10^9 and 10^{12} cfu. $m\ell^{-1}$. These doses of bacteria were determined during LD_{50} experiments and were designed to produce the minimum and maximum immune responses that were non lethal. The apparent mass differences between millipedes and scorpions necessitated adjustment of the injection volumes in order to maximise accuracy as a result of high viscosity of bacterial suspensions. The adjustments were as follows: Millipedes received a 2 $\mu\ell.g^{-1}$ injection of either pyrogen free saline or bacterial suspension. Scorpions received 15 $\mu\ell.g^{-1}$ injections of 7.5 × times diluted bacterial suspensions or pyrogen free saline. The reason for the dilution was to increase the transfer efficiency of the bacterial dose in scorpions due to the small hemolymph volume. The final doses of bacteria used were 10^6, 10^8, 10^9 and 10^{12} cfu. g^{-1}. Animals were injected using sterile pyrogen free 25G and 30G hypodermic needles. The

cuticle was disinfected with a cotton swab impregnated with 70% (w/v) propanol before each injection and sampling event.

2.2.5 Transformation of bacteria

Bacteria used for assay purposes were transformed in order to obtain ampicillin resistant bacteria. This was done in order to reduce interfering growths of non assay microbes during the plate assay and gel overlay incubation procedures. All plate assays and gel overlays contained 3% (w/v) ampicillin unless stated otherwise. Only naive bacteria were used for injection purposes.

The bacterial transfection were performed according to Davis *et al*, (1986) and is briefly as follows: assay bacteria were grown in LB medium (Biolab) at 37°C until log phase growth (at an O.D. of 0.3 to 0.5 at 600

nm). Bacteria were harvested by centrifugation (2,500 x g for 5 min) and resuspended in 50 mM $CaCl_2$ and incubated for 30 min on ice. The bacteria were centrifuged for 5 min. at 2,500 x g @ 4°C and resuspended in one-tenth of original volume with ice cold 50 mM $CaCl_2$. Two hundred microliters of suspension was mixed with ± 0.1 μg of ampicillin resistant plasmid (plasmid, pBR322, obtained from Dept. of Medical Biochemistry, University of the Witwatersrand) and incubated on ice for 30 min. The bacteria were heat shocked for 2 min. @ 42°C after which 1 mℓ of LB medium was added. The mixture was then spread on nutrient agar plates containing 3% (w/v) ampicillin (Boehinger Mannheim) and incubated in inverted fashion for 12 hours. The subsequent colonies were scraped off with a heat sterilised spatula and suspended in sterile tubes containing 2 mℓ nutrient broth (Biolab) to which 3% (w/v) ampicillin was added. The tubes were incubated under continuous agitation at 37°C until cloudy. Twenty percent (v/v) glycerol was added and mixed with the

suspension and stored in 1.5 ml Eppendorf tubes until use @ minus 70°C. Bacteria were recovered by thawing tubes and plating bacteria onto nutrient agar plate containing 3% (w/v) ampicillin.

2.2.6 Assays for Anti-bacterial Activity.

In order to detect the presence of anti-bacterial activity, it is necessary to demonstrate this activity against live bacteria in a visual manner. The inhibition zone plate assay is the method of choice for detection and verification of anti-bacterial activity.

2.2.4.1 Inhibition Zone Assay (Plate Assay).

Plate assays were performed according to a modified method of Hoffmann (1980) using nutrient agar. The method is as follows: nutrient agar

(Biolab) was autoclaved at 121°C for 20 min and kept at 42°C in a circulating water bath to prevent premature solidification of the agar. The liquid agar was seeded with bacteria by mixing saline suspensions of either *E. coli* or *S. aureus* into the agar solution resulting in a final bacterial concentration of ± 10^5 cfu. $m\ell^{-1}$. Five millilitres of the agar/bacterial mixture was poured into sterile petri dishes (9 cm O.D.) and left to solidify. After solidification the bacterial agar plates were clear and transparent (**Figure 2**). When these plates were put in an incubator at 35 °C, bacterial growth became evident after 24 hours as a milky and opaque discolouration (**Figure 3**, **Figure 7** to **Figure 12** and **Figure 14**). The bacterial agar plates were prepared every two weeks and were stored at 4 °C. The bacterial agar plates remained viable at least 21 days after pouring and means that plate assays could be prepared in bulk, which also increased accuracy of comparisons over time. Wells 3 mm in diameter were made in the agar into which 2 $\mu\ell$ to 10 $\mu\ell$ hemolymph samples from

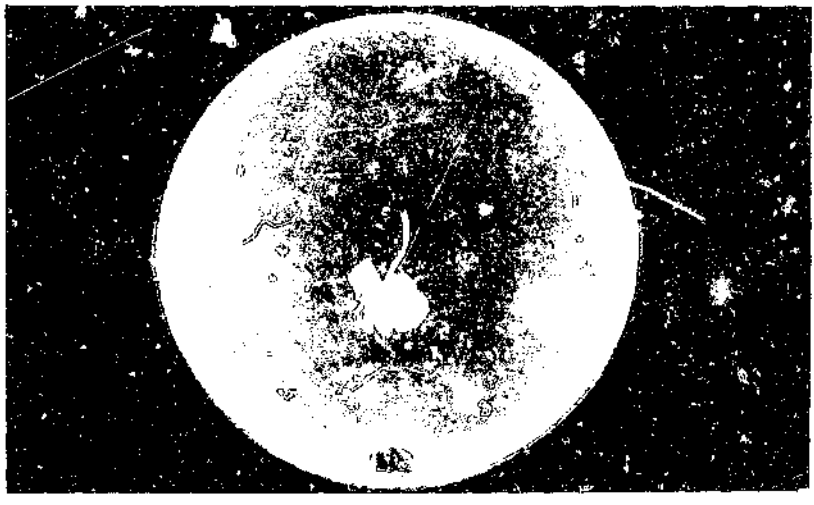


Figure 2 **Plate assay seeded with 10^6 cfu *E. coli*.ml^{-1} and photographed after 21 days storage at 4 °C on dark background in order to demonstrate transparency of the bacterial / nutrient agar medium, which serves as a bacterial control and indicates that no bacterial growth occurred during the storage period (refer Figure 3, which was photographed under identical circumstances for comparison of bacterial growth).**

control, saline injected and vaccinated (0 h; 24 h; 48 h; 72 h; and 96 hours post injection) millipedes and scorpions were loaded. The plates were incubated in a moist environment at 35°C for 24 hours to allow for the development of zones of bacterial inhibition. The diameter of the

inhibition zones (including well diameter, 3 mm) were measured using a Vernier calliper (eg. see **Figure 3** to **Figure 5**, **Figure 12** and **Figure 14**).

2.3 PHYSICAL CHARACTERISATION OF ANTI-BACTERIAL PROTEINS

2.3.1 Determination of Thermal Stability of Anti-Bacterial Activity from Vaccinated Hemolymph

Determination of the thermal stability of the anti-bacterial component from vaccinated animals is an important property in characterising anti-bacterial proteins and is widely used in the literature in order to determine similarities between anti-bacterial proteins from different sources (Osserman *et al*, 1974; Jingwei and Ziran; 1980).

The procedure was as follows: 100 µl hemolymph samples from control and vaccinated millipedes (72 hours post infection with 10^9 cfu *E. coli*) were collected in 1.5 ml polypropylene centrifuge tubes. The hemolymph samples were placed in a circulating water bath kept at 50°C by means of a circulating thermostat (Julabo PC, Labotec). Five micro litre aliquots of hemolymph samples were taken 15 min, 30 min, 50 min and 75 min after heat exposure and loaded on an *E. coli* plate assay to develop zones of inhibition (**Figure 12** and **Figure 13**).

2.3.2 Determination of Salt-out Precipitation Levels

The salt-out point of proteins is an important physical characteristic of proteins (Götz and Boman, 1985).

This method was used in order to determine the salt out point of the anti-bacterial protein(s). The salt-out point can also assist in partially purifying the anti-bacterial protein(s) of millipedes and the scorpions.

The method is briefly as follows: hemolymph samples from vaccinated animals were added to varying volumes of saturated solution of ammonium sulphate (NH_4SO_4, Merck, Germany) resulting in a stepwise precipitation of hemolymph proteins. The precipitate was then screened on the plate assay to detect anti-bacterial activity. The results were plotted as percentage saturated NH_4SO_4 vs amount of anti-bacterial activity present (**Figure 14** and **Figure 15**).

2.3 RESULTS AND DISCUSSION

2.3.1 Antibacterial activity.

2.3.1.1 Inhibition Zone Assay (Plate Assay)

Antibacterial activity was detected in both the millipede and scorpion after experimental infection with 10^{9} *E. coli* cfu. g^{-1} (**Figure 3**, **Figure 9** and **Figure 10**). The inducible antibacterial activity from millipede hemolymph was found to be active against both gram-negative and gram-positive bacteria (*E. coli* and *S. aureus*) (**Figure 3** to **Figure 7** , **Figure 10** and **Figure 11**). Injection of 10^{12} *S. aureus* cfu. g^{-1} elicited antibacterial activity in the millipede but failed to do so in the scorpion (Table I). This dose was near lethal for the millipede. It seems therefore

that the scorpion has a higher inherent resistance to gram positive bacteria than millipedes (Table 1).

TABLE I Comparison of antibacterial responses between millipede and scorpion to different doses of gram-negative and gram-positive bacteria on *E. coli* plate assay.

	Gram-positive *S. aureus*		**Gram-negative** *E. coli*	
	10^8 cfu. g^{-1}	**10^{12} cfu. g^{-1}**	**10^6 cfu. g^{-1}**	**10^9 cfu. g^{-1}**
Millipede	-	+	+	++
Scorpion	-	-	-	+

-: No antibacterial activity was detected on *E. coli* Plate Assay (0 mm inhibition zones); +: Limited anti-bacterial activity detected on *E. coli* Plate assay (0 to 5 mm inhibition zones); ++: Maximum antibacterial activity occurred against *E. coli* (inhibition zone greater than 5 mm). This method was also used to detect cross (gram positive and gram negtive) release of immune proteins by either bacterial type.

TABLE II Comparative Inhibition zone diameters reported for other the millipede and scorpion as well as other arthropod species against *E. coli*.

Arthropod or Protein	Inhibition zone (mm)	Time post infect. (h)	Bacteria used	Reference
Millipede *T. triodus*	**9[a]**	**96**	***E. coli* K12**	**This study**
Scorpion	**6[b]**	**24**	**"**	**This study**
Cecropin A	**14.3**	**192**	**"**	**Boman *et al* (1981)**
Cecropin B	**12**	**192**	**"**	**"**
Tsetse fly (adult)	**8**	**72**	**"**	**Kaaya *et al* (1987)**
Cecropia	**8[c]**	**192**	**"**	**"**
***M. sexta* (larvae)**	**9**	**120**	**"**	**Dunn *et al* (1985)**
Millipede *(Chicobolus)*	**none**	**120**	**"**	**Xylander & Nevermann (1990)**
C tenans	**4.5**	**192**	**"**	**Götz *et al* (1981)**

All inhibition zone diameters were measured using the plate assay technique with 3 mm wells. [a]- Millipede response measured 96 hours post vaccination with 10^9 cfu *E. coli*.g^{-1}; [b]- Scorpion response measured 24 hours post injection of 10^9 cfu *E. coli*.g^{-1}; [c]- Inhibition zone diameter measured for vaccinated cecropia hemolymph.

Maximal antibacterial activity in the millipede occurred between 72 h - 96 hours after infection and after 48 hours in the scorpion (**Figure 8**). The antibacterial activity in vaccinated millipedes seemed to be slightly more potent than those in vaccinated scorpions when comparing their respective inhibition zone diameters (Table I). The potency of the antibacterial response from millipedes and scorpions appears to be similar to those that have been reported for various short lived insect species (Table II). Saline injected millipedes and scorpions were sometimes weakly positive on the plate assay a fact also reported by other workers (Hoffmann, 1980), and is probably due to the wounding response.

2.3.2 **Phenoloxidase activity (PO)**

Vaccinated millipede hemolymph samples exhibited PO activity (**Figure 3**, **Figure 4**, **Figure 5** and **Figure 6**) and this is usually not present together with anti-bacterial activity as shown in **Figure 6**, well 5 versus wells 1 and 2. The colour photograph in **Figure 5**, taken from the same

plate assay as in **Figure 3** and **Figure 4**, demonstrates PO activity as the diffuse and brownish areas, substantially evident surrounding wells 1, 2 and 3, and slightly in well 4. Th s finding has also been reported by other workers (Kreil *et al*, 1980; Söderhall, 1981; 82; Söderhall and Smith, 1984; Dunn *et al*, 1985; Leonard *et al*, 1985a,b). No evidence of *in vitro* PO activity was found in vaccinated scorpion hemolymph.

The colour photograph (**Figure 5**) is shown in order to illustrate the difficulty in photographing anti-bacterial activity which presents itself as clear and transparent areas of inhibition which have, by definition - no colour properties. In order to visualise these activities, plate assays were photographed o black background so that the clear areas will reflect the black background directly and, so, visualising the effect as a dark circle of inhibition. However, areas of bacterial growth scatters the light from below which, results in the white, opaque areas described previously.

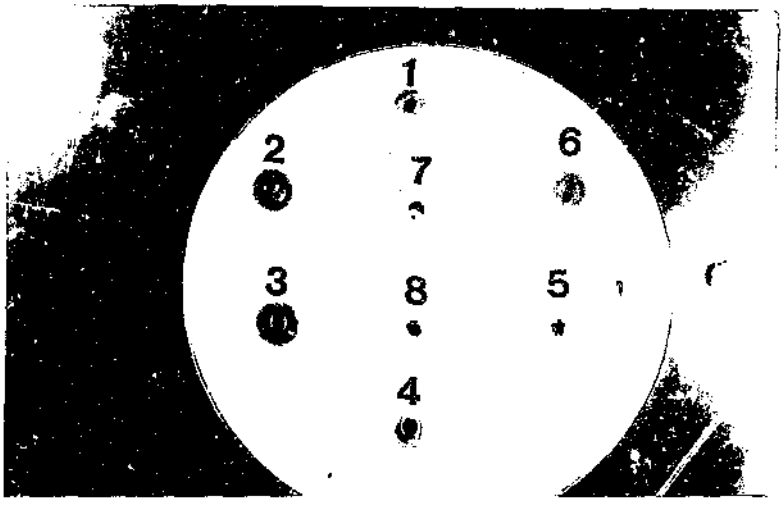


Figure 3 Representative anti-bacterial activity against *E. coli* plate assay. Wells 1 - 4: hemolymph samples from millipede 24 hours post infection with 10^9 cfu *E. coli*.g^{-1}. Wells 5 and 6 - hemolymph samples from millipedes 48 hours post infection 10^9 cfu *E. coli*.g^{-1}. Wells 7 and 8 hemolymph samples from saline injected millipedes. Anti-bacterial activity present in hemolymph sample taken from millipede 48 hours post *E. coli* vaccination (well 6). Well 4 shows slight anti-bacterial activity. Loaded 5 $\mu\ell$ hemolymph in all wells. PO activity is present in wells 1, 2, and 3. In order to gain better clarity of PO activity, also compare Figure 4 and Figure 5, which represent the same plate assay (this figure), photographed on white illuminated background (Figure 4) and Figure 5 - photographed in colour on dark background, to aid visual conceptualisation of actual PO activity.

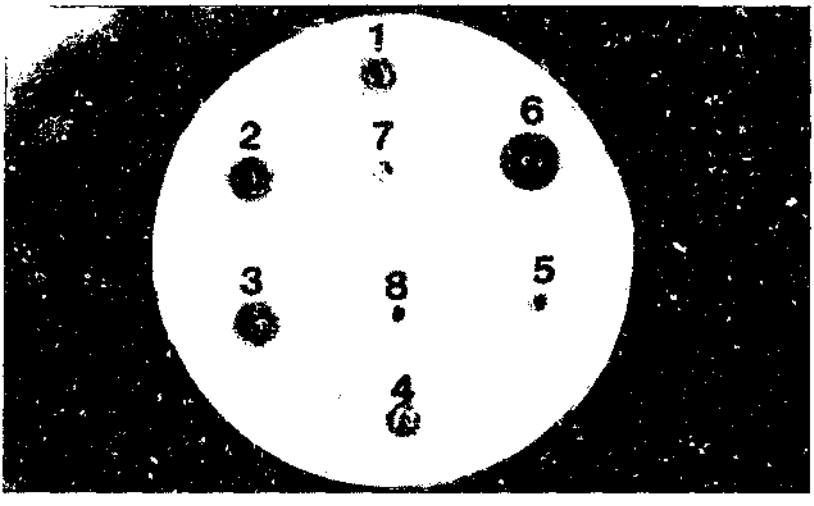


Figure 4 Photograph of *E. coli* plate assay used in Figure 3, taken on white illuminated background to illustrate darking areas of phenoloxidase activity. Phenoloxidase activity present on *E. coli* plate assay (wells 1,2,3). Nutrient agar seeded with 10^5 cfu *E. coli*.ml^{-1}. Legend same as for Figure 3. Also compare colour photograph in Figure 5, which depicts the same plate assay.

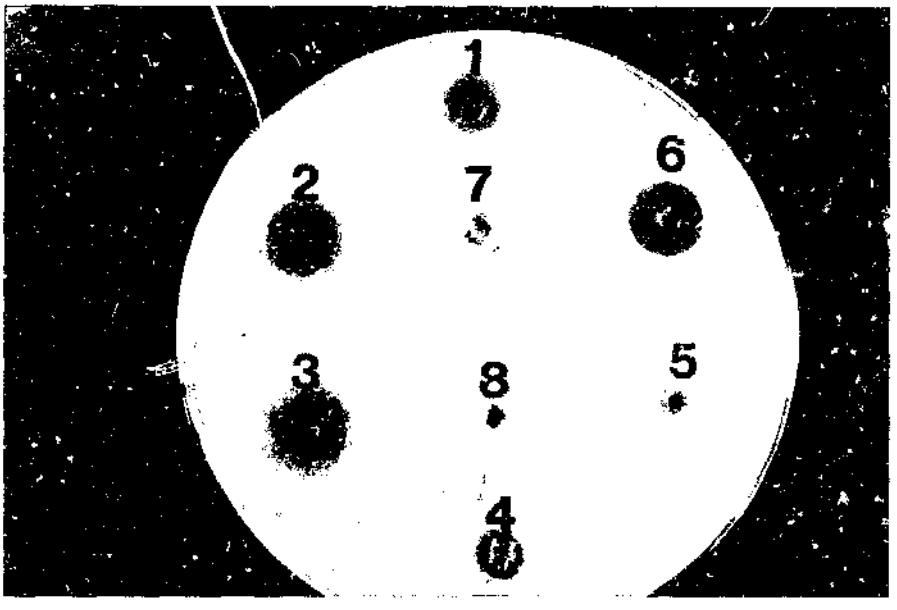


Figure 5 Photograph of *E. coli* plate assay used in Figure 3, taken in colour on dark background to illustrate diffuse, brownish and darking areas of phenoloxidase activity. Phenoloxidase activity present on *E. coli* plate assay (wells 1,2,3). Nutrient agar seeded with 10^5 cfu *E. coli*.mℓ$^{-1}$. Legend same as for Figure 3.

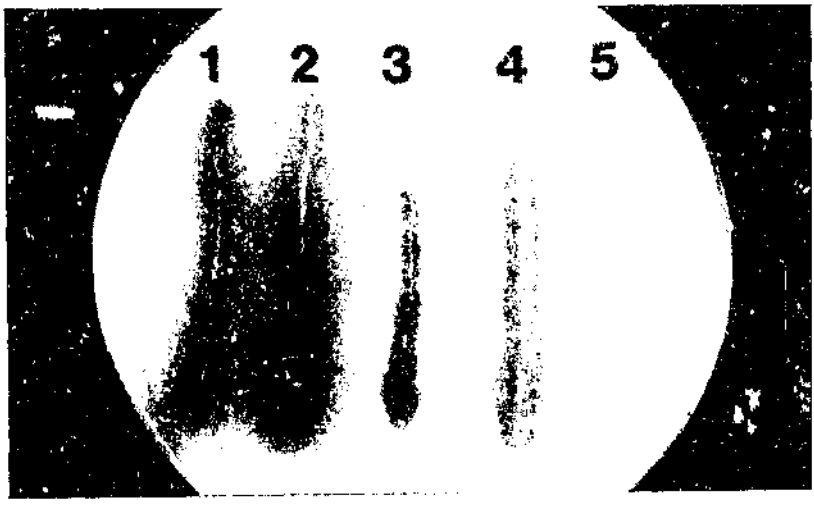


Figure 6 ***E. coli*** **plate assay. Lanes 1,2,3 and 4 - hemolymph samples from four millipedes, 72 hours post vaccination with 10^9** ***E. coli*** **cfu. g^{-1}. Anti-bacterial activity is present in two of the four animals (lanes 3 and 4) with no evidence of PO activity. Lanes 1 and 2 demonstrated PO activity with the absence of anti-bacterial activity. Twenty five micro litres of hemolymph were loaded in each lane. Refer to visual PO activity in Figure 3, Figure 4, Figure 5.**

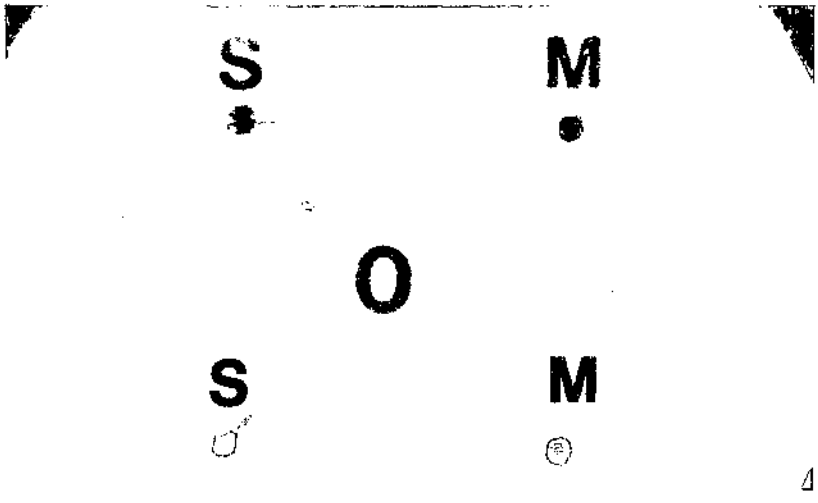


Figure 7 Representative *E. coli* plate assay of millipede (wells noted by M) and scorpion hemolymph samples (wells noted by S) collected immediately after experimental infection with 10^9 cfu *E. coli*.g^{-1} (time 15 minutes). No anti-bacterial activity present. Loaded 5 $\mu\ell$ hemolymph per well.

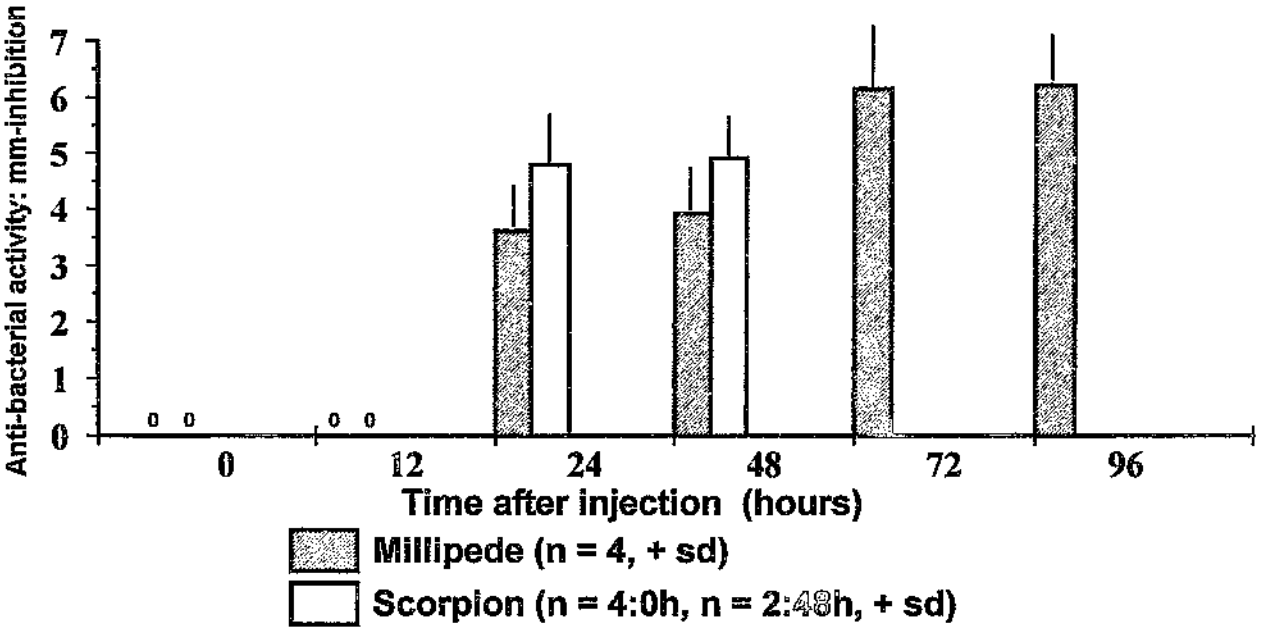


Figure 8 Graph of comparative plate assay inhibition zone diameters between millipede and scorpion after injection of 10^9 cfu *E. coli*.g^{-1}. Only two scorpions survived 48 hours post vaccination, none were alive at 72 hours - due to haemorrhage of injection and sampling wounds

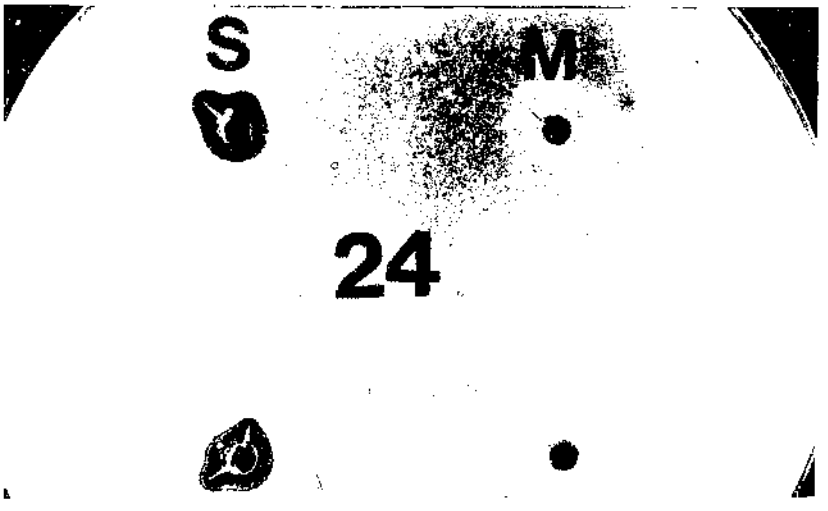


Figure Representative *E. coli* plate assay. Anti-bacterial activity present in scorpion hemolymph sample (wells denoted by S) taken 24 hours after vaccination with 10^9 cfu. g_{-1} *E. coli*. Millipede hemolymph sample (well denoted by M) 24 hours after vaccination with 10^9 cfu. g^{-1} *E. coli* showed no anti-bacterial activity due to severity of infection (millipede died after sampling).

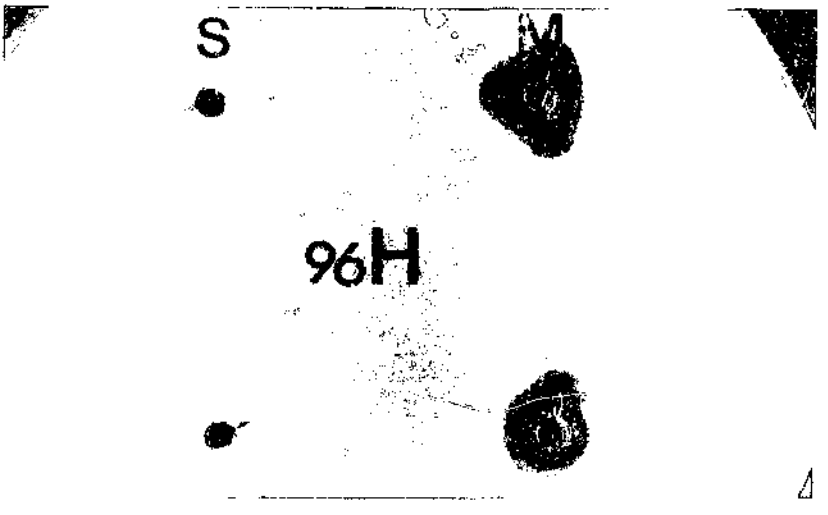


Figure 10 ***E. coli*** **plate assay. Anti-bacterial activity present against *E. coli* in millipede hemolymph sample collected 96 hours after experimental infection with 10^9 cfu *E. coli*.g^{-1}. Loaded 5 μl hemolymph per well. Scorpion (well S) died due to haemorrhage 48 hours post vaccination, therefore hemolymph could not be loaded. Refer results and discussion section, regarding haemorrhage in scorpions.**

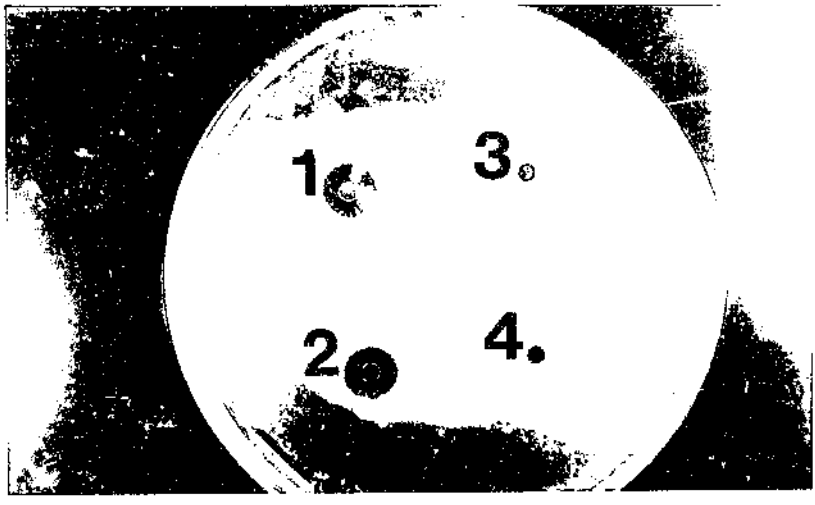


Figure 11 Representative *S. aureus* plate assay. Anti-bacterial activity exhibited against *S. aureus* in millipede hemolymph sample collected 72 hours after experimental infection with 10^9 *E. coli* cfu. g^{-1} (wells - left). Wells to the right - no anti-bacterial activity present in hemolymph samples from scorpion 48 hours post vaccination with 10^9 *E. coli* cfu. g^{-1}. Nutrient agar was seeded with 10^6 *S. aureus* cfu.$m\ell^{-1}$. Loaded 5 $\mu\ell$ hemolymph per well.

2.3.3 Determination of thermal stability

Anti-bacterial activity from infected millipede hemolymph samples collected 72 hours post vaccination with 10^5 cfu. g^{-1} of *E. coli*, remained active at 50 °C for ± 30 min. (**Figure 12** and **Figure 13**), where after anti-bacterial activity was inactivated. Results of thermal inactivation of millipede anti-bacterial proteins show that more than 95% of anti-bacterial activity was still present after incubation of vaccinated millipede hemolymph for 30 minutes at 50 °C (**Figure 13**).

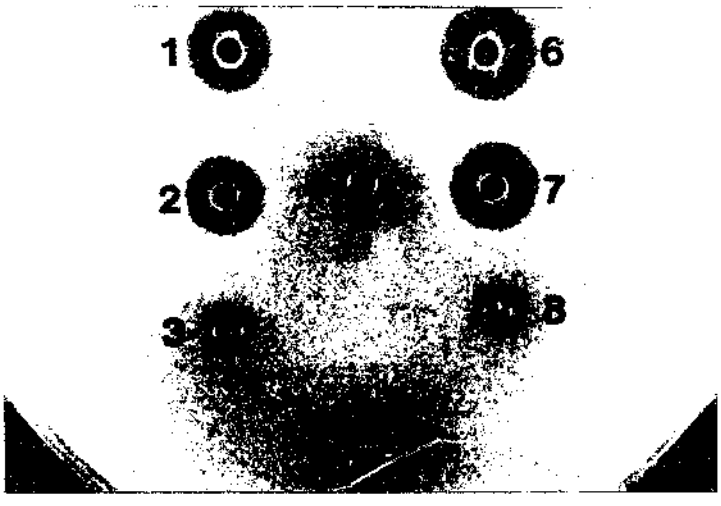


Figure 12 Representative thermal inactivation plate assay (*E. coli*). Anti-bacterial activity present in vaccinated millipede hemolymph samples collected 72 hours post infection with *E. coli* and loaded on plate assay after samples were incubated for 15 min @ 50°C (wells 1 and 6). No thermal inactivation evident. Wells 2 and 7: anti-bacterial activity of millipede hemolymph after incubation for 30 min @ 50°C, negligible thermal inactivation of anti-bacterial activity; wells 3 and 8: millipede hemolymph after 50 min incubation @ 50°C - marked thermal inactivation of anti-bacterial activity exhibited after 50 min exposure to heat (50°C); well no 5: anti-bacterial activity not present in millipede hemolymph after 75 min incubation @ 50°C and, well no 4: control millipede hemolymph. Loaded 5 $\mu\ell$ hemolymph per well.

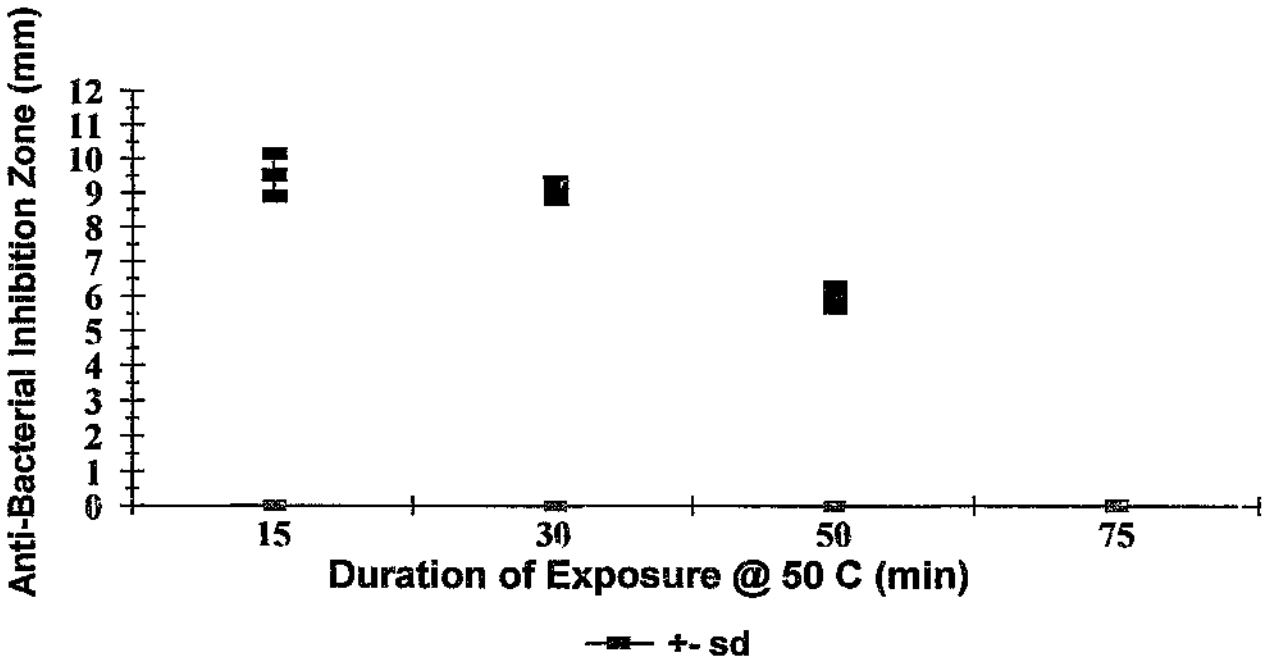


Figure 13 **Determination of thermal stability of anti-bacterial activity. Denaturation of millipede hemolymph anti-bacterial proteins in samples collected 72 hours post infection with *E. coli* and, exposed to a temperature of 50°C. Graph shows anti-bacterial activity remained stable for ± 30 min @ 50°C.**

2.3.4 Ammonium sulphate salt-out point determination

The largest fraction of anti-bacterial activity of vaccinated millipede hemolymph collected 72 hours post *E. coli* infection, precipitated at ± 35 % to 40 % saturated ammonium sulphate (NH_4SO_4)(**Figure 14** and **Figure 15**). However some activity was also removed at lower ammonium sulphate concentrations. This information can assist in characterising and partially purifying the anti-bacterial proteins from other hemolymph proteins.

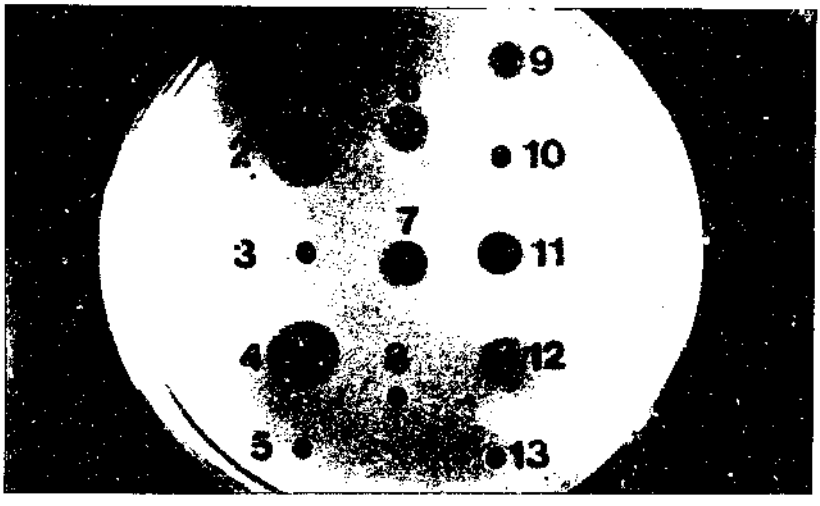


Figure 14 Determination of salt-out point of antibacterial proteins. Representative ammonium sulphate (NH_4SO_4) precipitation fractions screened on *E. coli* plate assay. Hemolymph sample taken from millipedes 72 hours post *E. coli*, Well no 1 - untreated hemolymph sample from vaccinated millipede; 2 - renatured precipitate of 40% NH_4SO_4 fraction; 4 - 35% NH_4SO_4 fraction; 6 - 15% NH_4SO_4 fraction; 7 - 25% NH_4SO_4 fraction; 11 - 30% NH_4SO_4 fraction; 5 and 8 - no activity present in the resultant supernatant fractions of wells 4 (35%) and 11 (30%) respectively indicating complete precipitation of anti-bacterial component; wells 9 and 12 - 10 $\mu\ell$ of a 50% saturated NH_4SO_4 solution (negligible response on plate assay); well no 10 - 10% NH_4SO_4 precipitate fraction and well no 13 - resultant supernatant from 60% NH_4SO_4 fraction. Ammonium sulphate precipitate and supernatant renatured with 10 $\mu\ell$ 0.1 M Phosphate buffer (pH 7.4). Five micro litres of renatured precipitate solution loaded per well. (% - percent saturated NH_4SO_4 solution)

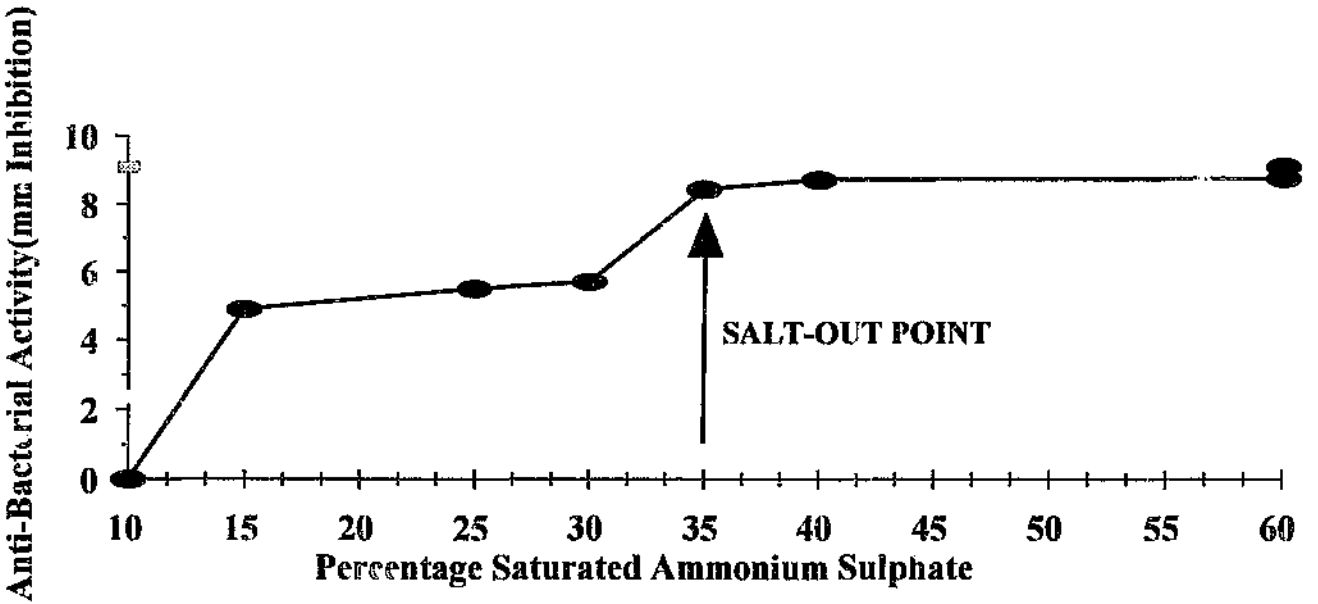


Figure 15 Determination of salt-out point of antibacterial proteins. Ammonium sulphate precipitation fractions screened on *E. coli* plate assay. Salt-out point occurs at approximately 35% saturated NH_4SO_4 (> 95% of antibacterial proteins precipitated from vaccinated hemolymph 72 hours post infection).

CHAPTER 3: CHARACTERISATION OF ANTI-BACTERIAL PROTEINS

3.1 BACKGROUND AND INTRODUCTION

As discussed in Chapter 1, Section 1.2, the immune responses of insects exhibit two major features when foreign organisms gain access to the hemolymph 1) a rapid change in the population of circulating hemocytes (Chain and Anderson, 1983) and 2) an induced synthesis of new hemolymph proteins (Hoffmann, 1980; Hughes *et al*, 1983; De Verno *et al*, 1984). The enzyme lysozyme was the first antibacterial factor to be identified from the hemolymph of insects (Hink and Briggs, 1968; Stevens, 1962). This enzyme is a major factor in conferring immunity to insects during bacterial infection (Powning and Davidson, 1973; Anderson and Cook, 1979; Jarosz, 1979; Jolles *et al*, 1979; Engström *et al*, 1985; Schneider, 1985; Boman *et al*, 1986, Boman 1986; Brehelin, 1986; Dunn,

1986). Two new families of anti-bacterial proteins were isolated and purified from various insect species, the attacins and cecropins (Hultmark *et al*, 1980; Hoffmann *et al*, 1981; Merrifield *et al*, 1982; Qu *et al*, 1982; Andreu *et al*, 1983; Hultmark *et al*, 1983; Lee *et al*, 1983; Kockum *et al*, 1984; Andreu *et al*, 1985; Götz and Boman, 1985; Boman *et al*, 1986a). These studies focussed on short-lived, hemimetabolis and holometabolis insect species. Anti-bacterial proteins from a wide range of other invertebrates have subsequently been isolated and purified i.e. from Crustaceans, Gastropods etc. (Lackie, 1986b). In order to obtain a better understanding of the phylogeny of arthropod anti-bacterial activity, it is necessary to characterise these anti-bacterial proteins discovered in the more primitive and long-lived species of arthropods (millipede and scorpion) in order to make accurate comparisons to anti-bacterial proteins reported in a variety of insects and other arthropods species and to interpret the similarities and differences that may exists so that the phylogeny of these immune responses may become clear.

We recently produced and reported the first evidence of an inducible antibacterial protein(s) in a long-lived arthropod namely, the Kalahari millipede *Triaenostreptus triodus*)(Van der Walt, 1988; Van der Walt *et al*, 1990 and Van der Walt & McClain, 1991). The present study reports on the comparative characterisation of these immune response between the scorpion *O. latimanus* and the Kalahari millipede *T. triodus* and those responses in other arthropods. Both of these animals have a life span of between two and seven years or longer (Attems, 1928; Crawford and McClain, 1983), and are considered to be amongst the more primitive living members of the arthropoda. We also compared the humoral responses of these two long lived arthropods to the humoral responses that have been reported for various species of insects in the literature by employing RF-AZE and IEF techniques (Hultmark *et al*, 1980; Hoffmann *et al*, 1981). The iso-electrical point (pI) range has been determined for the antibacterial protein(s) that have been found in the Kalahari millipede by using novel and unique reverse field dual gradient iso-electric focusing (RF-DG-IEF).

3.2 MATERIALS AND METHODS

3.2.1 Assay for Hemocyte Depletion.

One of the immune responses of insects is a rapid change in the population of circulating hemocytes when foreign organisms gain access to the hemolymph (Chain and Anderson, 1983). This phenomenon has been reported for various short-lived insects (Ratcliffe and Rowley, 1979; Götz and Boman, 1985). We decided to investigate whether hemocyte depletion was part of the immune response of long lived arthropods, and if this depletion was due to the release of a hemocyte depletion factor. Also, if the depletion was as intense and rapid as that which have been reported in the literature (Chain and Anderson, 1983; Geng and Dunn, 1989). This was investigated by employing total hemocyte counts before and after treatment in controls and infected millipedes and scorpions.

Five micro litre aliquots were taken from hemolymph samples from wounded, control, saline-injected and vaccinated millipedes and scorpions. The hemolymph was diluted 2 × times with sterile saline (0.7% w/v NaCl). The 5 $\mu\ell$ sample was spread out on a clean microscope slide (in an area of ± 2 cm^2). The total number of hemocytes were then counted under a phase contrasting microscope (Zeiss, Germany) using a manual numeric counter. Considerable differences existed in the number of total hemocytes between individual millipedes and scorpions and therefore, the results are expressed as the average relative percentage difference and/or change in total hemocyte counts in each individual taken before and after treatment.

The volume changes due to the injections that could affect the accuracy of the total hemocyte counts, were controlled for by adjusting the results according to those changes that occurred in control animals. As an example the results for 24 hours post injection time period were calculated according to the formula given below:

$$\% \text{ Change} = \left(\sum_{N=1}^{4} \left(\frac{M_{N_{T=24h}} - M_{N_{T=0h}}}{M_{N_{T=0h}}} \times \frac{100}{N} \right) \right) - \left(\sum_{N=1}^{3} \left(\frac{C_{N_{T=24h}} - C_{N_{T=0h}}}{C_{N_{T=0h}}} \times \frac{100}{N} \right) \right)$$

where: N = Number of animals; M = Experimental observation, after bacterial infection; C = Control observation and T = Time period after injection (4 h; 12 h; 24 h; 48 h and 72 hours). The calculated result represents the percentage change, positive or negative, that occurred in total hemocyte number (see **Figure 17**)

3.2.2 Statistical procedures

The significance of the results was determined by using a t - test (two tailed) employing the Bonferroni correction(s) as required for recurring group testing protocols (Glantz, 1992). The level of significance tested was $p < 0.05$ (95%) between comparisons of millipede and scorpion responses.

3.2.3 Protein Concentration determination

When comparing anti-bacterial protein responses between individuals of the same species over time, as well as comparing between different species, it is important to compare equal amounts of protein in order to increase accuracy of such comparisons.

3.2.3.1 Micro protein assay

Hemolymph protein concentrations were determined using the Micro protein assay method by Bradford (1976). This method was used in preference to the Lowry (1951) method as it is a rapid (2 min development time) and extremely sensitive (1 μg to 10 μg.0.1 $m\ell^{-1}$) assay with a very wide range linear curve. The protein dye solution consisted of 0.01% (w/v) PAGE blue G 90 (Electran, BDH Chemicals, Germany and was used as a substitute for Coomassie Brilliant Blue G-250), 4.7% (v/v) absolute ethanol and 8.5% (v/v) phosphoric acid. During the preparation of the protein dye solution, care should be taken to add the phosphoric acid to the PAGE Blue dye first and then the ethanol required.

This procedure avoids interfering colour development of the dye (PAGE Blue). The protein dye solution was then filtered through a Whatman no 1 filter paper before storage. The protein dye solution is stable for at least 60 days at room temperature, making this the method of choice over the labourious Lowry (1951) method.

Bovine serum albumin (BSA, SIGMA, U.S.A.) was used as a protein standard in the construction of the protein concentration standard curve. Hemolymph samples (5 $\mu\ell$) were diluted with 1 mℓ deionised water and were used for protein determination. Hemolymph samples were appropriately diluted when their absorbancy values were below or above those of the standard curve. Colour development was complete after 2 min and was stable for at least 90 min. The absorbances were read on a spectrophotometer (PYE UNICAM SP8-100 UV-VS, U.S.A.) at a wavelength of 595 nm. The results were plotted as $A_{595\,nm}$ versus protein concentration. All samples were prepared in duplicate.

3.2.4 Sodium Dodecyl Sulphate - Polyacrylamide Electrophoresis (SDS - PAGE).

The plate assay technique (Chapter 2.) showed that an anti-bacterial factor(s) was present in the hemolymph of the millipede and the scorpion. In order to find out what exactly conferred the anti-bacterial activity to the vaccinated hemolymph samples further investigations were conducted to identify and characterise these factor(s). I decided to pursue this matter by looking at total hemolymph protein profiles of millipedes and scorpions in order to detect new or altered proteins that could be synthesised in response to experimental bacterial infections.

Hemolymph proteins from millipedes and scorpions were separated using the Laemmli (1970) discontinuous stacking gel buffer system. Proteins were separated on 1,5 mm thick 12cm x 14 cm, 12 - 20% linear gradient polyacrylamide gels (Hoefer SE 600, Scientific Associates S.A.). A 30%T (w/v) acrylamide (29.6% (w/v) acrylamide : 0.4% (w/v) bisacrylamide, BDH Chemicals, Electran) stock solution was used in gel preparations. A 4% (w/v) acrylamide 1.5 cm stacking gel was used and

contained 0.125 M Tris-HCl pH 6.8 and 0.1% (w/v) SDS (sodium dodecyl sulphate, BDH). The linear gradient separating gel consisted of a 12 cm 10% to 20% (w/v)(top to bottom) acrylamide gel containing 0.375 M Tris-HCL pH 8.8, 0.1% (w/v) SDS. Gradients were poured by peristaltic pump and gradient mixer (manufactured in the Dept. of Physiology - Workshop. Univ. of the Witwatersrand). The gradient was produced by mixing two acrylamide solutions of differing concentrations i.e. light acrylamide 30% (w/v) stock and heavy acrylamide 30% (w/v) Stock. The heavy acrylamide stock contained 70% (v/v) glycerol. Light acrylamide was used to make a 10% (w/v) solution and heavy acrylamide was used to make a 20% (w/v) solution. The light and heavy acrylamide solutions were poured into the two gradient mixer containers which were connected by a small channel (1 mm I.D.) On the bottom. One of the containers (heavy acrylamide) was linked to the peristaltic pump. TEMED (N,N,N',N'- tetramethylethylenediamine, Sigma) in a final concentration of 0.033% (v/v), and ammonium persulphate (APS, freshly prepared, Merck) in a final concentration of 0.025% (w/v) was added to initialise polymerisation. Polymerisation was completed after ± 40 min. During gradient pouring, the heavy acrylamide solution was agitated by

a magnetic stirrer in order to mix with the light acrylamide entering the container. The electrode buffer (5 ℓ) contained 0.025 M Tris-HCL pH 8.3, 0.192 M Glycine and 0.1% (w/v) SDS.

Gels were run under constant current, 15 mA (milliampere) for stacking gel and 35 mA for separating gel. Gels normally ran between 6 and 10 hours and were kept at 10°C by means of a circulating waterbath which was connected to the electrophoresis heat exchanger (Labcon, model CPE, Labotec). The electrode buffer was stirred continuously by a magnetic stirrer. Hemolymph samples from millipedes and scorpions were denatured in sample buffer containing 62.5 m M Tris-HCL pH 6.8, 2% (w/v) SDS, 10% (v/v) Glycerol, 5% (v/v) 2-β mercaptoethanol (BDH) or 3% (w/v) dithiothreitol (DTT, Boerhinger), and 1 mg % (w/v) bromophenol blue (tracking dye) for 5 min at 50°C.

After electrophoresis the gels were stained with either PAGE-blue 83 (BDH) and / or silver stained as follows: gels were fixed overnight in 45% (v/v) methanol and 15% (v/v) glacial acetic acid, then stained in solution containing 0.2% (w/v) PAGE-blue 83, 45% (v/v) methanol and 10% (v/v)

glacial acetic acid, and destained in 45% (v/v) methanol and 7% (v/v) glacial acetic acid (which was constantly replaced as necessary) until gel background was clear. The destaining solution was recovered by passing solution through activated charcoal (powdered, Holpro Analytical).

Silver staining was performed according to Guilian *et al*, (1983) and is as follows: After electrophoresis gels were fixed overnight in 45% (v/v) methanol and 15% (v/v) glacial acetic acid and washed three times (15 min each) with 10% (v/v, absolute) ethanol and 5% (v/v) glacial acetic acid. Oxidisation was done in a solution containing 3.2 mM nitric acid and 3.4 mM potassium dichromate. Excess oxidising agent was washed off three times (5 min each) in distilled water. Gels were then impregnated with 20 mM silver nitrate ($AgNO_3$, J.M. Chemicals, Eng.) for 30 min. Gels were rinsed once with distilled water and developed, under close scrutiny with 0.28 M sodium carbonate (Na_2CO_2, Merck) and 0.0037% (v/v) paraformaldehyde. The developing solution was constantly replaced with new developing solution when the developer became cloudy. Care was be taken to avoid over staining of gels. This process was continued until adequate protein staining occurred.

Developing was stopped by adding 5% (v/v) glacial acetic acid. Gels were photographed on illuminated white background and stored in 50% (v/v) methanol.

The gels were calibrated using six molecular mass calibration markers (BDH Chemicals, Germany) namely : Cytochrome c - 12.3 kD; Myoglobin - 17.2 kD; Carbonic anhydrase - 30.0 kD; Ovalbumin - 42.7 kD; Albumin - 66.25 kD, Ovotransferrin - 76 to 78.0 kD and Chicken egg white lysozyme - 13.8 kD Normally between 80 μg to 100 μg of total protein were loaded per lane.

3.2.5 Assays for Antibacterial Proteins

New methods were developed whereby we could expose each of the hemolymph proteins separated by various techniques to bacteria in order to determine which of these proteins had anti-bacterial properties. In order to do this, the proteins have to be separated and be kept viable (native) in order to prevent denaturation or inactivation of their anti-

bacterial properties. The proteins could then be exposed to live bacteria and allowed to demonstrate their anti-bacterial properties, in order to be identified and characterised. The three methods described below, were used for this purpose.

3.2.5.1 Reverse Field Acidic Zone Electrophoresis (RF-AZE)

Reverse field acidic zone electrophoresis was carried out on vaccinated and control hemolymph samples from millipedes and scorpions according to modified methods described by Gordon (1971), Gabriel (1971) and Hultmark *et al* (1980). In this method the proteins are allowed to migrate down to the cathode, instead of using the usual non-denaturing procedure where proteins migrate to the anode. The reason for adapting the method was the basic nature of the proteins under investigation.

Hemolymph samples from millipedes and scorpions were electrophoresed under reverse field conditions (i.e. towards the cathode) in 1.5 mm thick 8 cm x 10 cm acidic (pH 4) 10% (w/v) or 12% to 22% (w/v) linear

gradient polyacrylamide gels using a cooled mini slab gel electrophoresis unit (Hoefer SE 230 Mighty Small-Mini gel, Scientific Associates). The resolving gel consisted of 0.14 M glacial acetic acid and buffered to pH 4 with 2 N KOH and containing either 10% or 12% to 22% polyacrylamide (30%T - 29.6% Acrylamide : 0.4% Bis acrylamide Stock).

The electrode buffer contained 0.035 M β alanine, 0.14 M glacial acetic acid adjusted to pH 4 with 2 N KOH. Hemolymph samples from millipedes and scorpions were mixed with 0.5 M acetic acid, 15 % (v/v) glycerol and 1 mg% Methyl green as the tracking dye of the sample buffer. The measured pH of the sample buffer solution was pH 1.2. Gels were run at 4°C at a constant current (10 mA per gel). Gradient formation was performed as in section 3.2.4.

3.2.5.2 Reverse Field Dual Gradient Iso Electric Focussing (RF-DG-IEF)

Reverse field iso-electric focussing in this and the following section, was performed in order to establish the iso-electric point(s) (pI) of the anti-

bacterial proteins. The pI is an important indicator of protein identity and is widely quoted in the literature for comparisons of anti-bacterial proteins from different arthropod species (Hultmark *et al*, 1980; Dunn, 1986). Gels were prepared in the cooled mini slab electrophoresis unit described in section 3.2.5.1.

Hemolymph proteins from control, saline injected and vaccinated millipedes and scorpions were electrophoresed towards the cathode in 0.75 mm thick, 8 cm x 10 cm, 4%T/4%C (Bis acrylamide) polyacrylamide gels at 4°C. Cytochrome c (Boehringer Mannheim, Germany) was used as a visual indicator of pI (red appearance) and relative position in the gel . The focussing was stopped when cytochrome c was focussed after ± 16 hours (current zero). The IEF gel was poured with an increasing concentration gradient of cadaverine (5 mM - 20 mM, Sigma) and a decreasing gradient of arginine (20 mM - 5 mM, Boehringer Mannheim) from the anode towards the cathode respectively (dual ampholine gradient). These two compounds arrange themselves, on focussing, in an increasing order of dissociation constants resulting in a pI range of ± 10.8 - 12.8 (Righetti, 1983). The gradient formation (of the solutes) was performed similar to the acrylamide gradients used in section 3.2.4.

Arginine and cadaverine were included in the gel in order to extend the basic pH range to accommodate the high alkaline properties of the antibacterial proteins. One molar glacial acetic acid was used as the anolyte and 0.1 M NaOH was used as the catholyte. The gel also contained 20% (w/v) sucrose (BDH Chemicals), 3% (v/v) Ampholine (pH 9,5 - 10, Pharmacia). The sample buffer contained 0.1 M glacial acetic acid, 5 mM cadaverine, 20 mM arginine (BDH Chemicals), 3% (v/v) Ampholine (pH 9.5 - 10, Pharmacia) and 20% (w/v) sucrose. The anti-bacterial activity remained stable in the extremely acidic sample buffer (pH 1.5) for over 12 hours. All iso-electric focussing gels were run under constant wattage (16 Watts). The initial voltage was 1250 V and it increased to 2500 V on completion of focussing. One half of the gel was used for silver staining according to the method of Guilian *et al* (1983)(see section 3.2.4) and the other identical half was used in the gel overlay for detection of anti-bacterial activity as described in the section below.

3.2.5.3 Gel Overlay of Reverse Field Iso-Electric Focussing (RF-IEF) and RF-DG-IEF Gels

This represents a combined method according to Gabriel (1971), Hultmark *et al* (1980) and Gordon (1971). Hemolymph proteins from control, saline injected and vaccinated millipedes and scorpions were focussed towards the cathode in a 4%T/4%C (Bis acrylamide) polyacrylamide gel at 4°C. Cytochrome c (Boehringer Mannheim, Germany) was used as an pI indicator and the focussing was stopped when cytochrome c was focussed after ± 16 hours.

For the detection of the antibacterial activity it was necessary to remove the ampholines from the gel. For this reason the following modifications were made to the gel overlay procedure (Hultmark *et al*, 1980): the gel was incubated in 200 mM sodium phosphate buffered nutrient broth (pH 7.4) for 45 minutes (4°C) and in 50 mM phosphate buffered nutrient broth (pH 7.4) for 15 minutes. The gel was then placed in a warm (35°C) 15 cm sterile glass petri dish and overlaid with liquid nutrient agar (42°C) that had been seeded with ± 10^3 cfu .$m\ell^{-1}$ of *E. coli* (refer section 2.2.4.1

for details of agar seeding). The overlay was incubated at 35°C for 24 hours to develop zones of bacterial inhibition that corresponded to specific antibacterial proteins present in the hemolymph of vaccinated millipedes and scorpions. The gel overlay was also performed in a similar manner for the detection of anti-bacterial activity in RF-DG-IEF gels (3.2.5.2).

3.3 RESULTS AND DISCUSSION

3.3.1 Hemocyte Depletion

My results showed that a dramatic decrease in the number of circulating hemocytes occurred in both the scorpion and the millipede as early as 4 hours, or earlier, post injection of 10^6 cfu. *E. coli*.g^{-1} (**Figure 16.**). Twelve hours after vaccination, the decrease in the number of hemocytes was as much as 90% less compared to the number of hemocytes present in the hemolymph before injection of *E. coli*. Although the scorpion received a relatively larger volume dose during injection (see collection of hemolymph, section 2.2.1), the decrease in hemocyte numbers due to dilution from the injected volume, was less than 7% when compared to samples taken from scorpions injected with an equal volume of pyrogen free saline. The percentage decrease due to dilution was accordingly controlled for in the vaccinated scorpions. The dilution due to injection in millipedes was negligible (< 1%). The number of circulating hemocytes collected 72 hours after infection, from vaccinated scorpions and millipedes, remained 60% to 90% lower compared to their respective

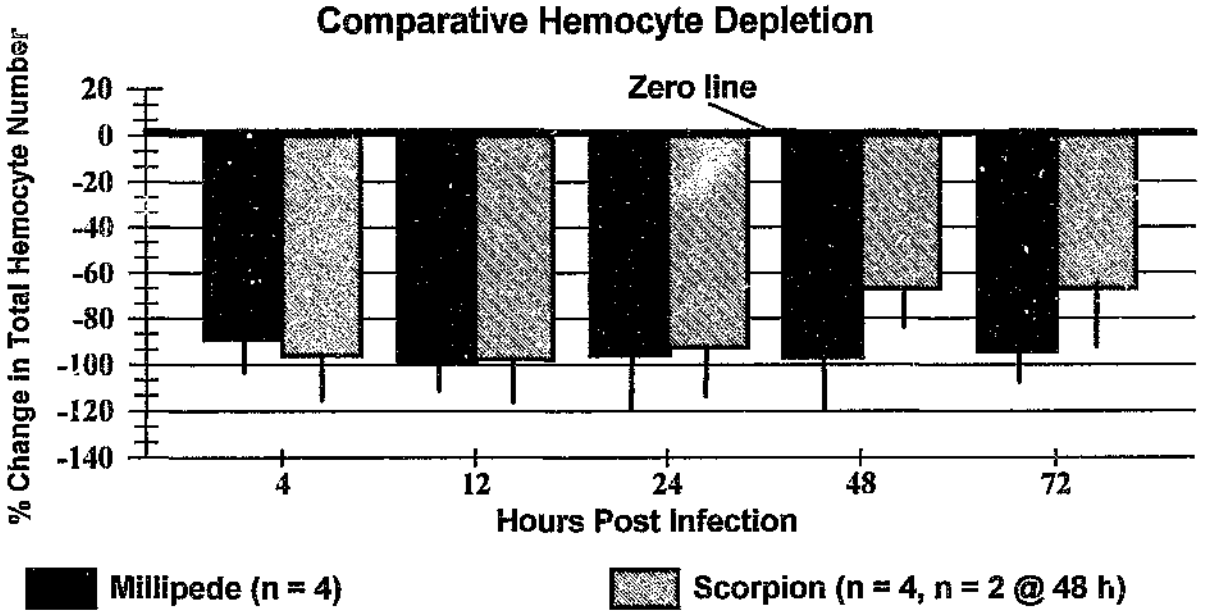


Figure 16 Comparative hemocyte depletion between millipede and scorpion after injection of pyrogen free saline (control) and 109 cfu E. coli.g^{-1}. Results are expressed as mean percentage change ± SD (millipede n=4, scorpion n=4) that occurred before and after injection. Line "0" on y-axis indicates level where no change in hemocyte population occurs. Decrease in number of scorpions were due to haemorrhage after sampling. Wounded and control (saline injected) data are depicted as the solid line at zero on the y-axis.

saline injected / wounded controls (depicted as the solid line at zero on the y axis, **Figure 16**). Hemocyte depletion after *S. aureus* infection is not shown.

This dramatic hemocytopenia (after infection with *E. coli*), especially in the millipede, can be induced by both gram negative and gram positive bacteria. These results therefore indicate the possible release of a hemocyte depletion factor into the hemolymph of millipedes and scorpions after bacterial infection. This bacterial-induced hemocytopenia seems similar to that which have been reported for various short-lived insect species (Anderson, 1981; Chain and Anderson, 1983; Götz and Boman, 1985; Boman, 1986; Boman *et al*, 1986; Dunn, 1986; Lackie, 1986b; Boman and Hultmark, 1987).

In the wax moth *G. mellonella* hemocytes return to their pre-treatment levels within several hours after bacterial infection (Chain and Anderson, 1983). However, in the millipede *T. triodus* as well as the scorpion *O. latimanus*, hemocyte levels remain depleted for at least three days after experimental infection.

3.3.2 SDS-PAGE

Denaturing polyacrylamide gel electrophoresis (PAGE) of control, saline injected and vaccinated hemolymph proteins from millipedes and scorpions showed good separation of proteins was achieved by linear gradient electrophoresis. Qualitative similarities, but quantitative differences were observed in the protein patterns of the control and experimental samples (particularly in scorpion hemolymph samples lanes 7 & 8, **Figure 17**). The qualitative differences between control and infected protein samples have also been reported by other workers (Götz, 1981).

Scorpion hemolymph proteins separated on a 10 - 20% gradient gel, (**Figure 17**, lanes 8 and 9, vaccinated and control respectively) revealed the relative increase in a 11.8 kD protein band as well as the emergence of a new 11.3 kD protein (indicated by small arrows near the bottom of well 7). The position of chicken egg white lysozyme (13.8 kD), a well known bactericidal protein, is shown for comparison (**Figure 17**: lane 2). However it seems unlikely that the proteins in the scorpion for which differences were shown, belong to the lysozyme family of bactericidal

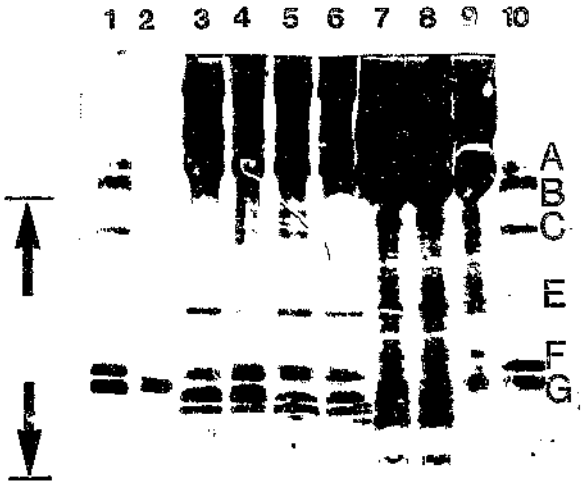


Figure 17 **SDS - Page of vaccinated and control hemolymph samples from millipedes and scorpions. Silver stained linear gradient gel 10 - 20% poly acrylamide, Lanes: 1,10 - Molecular mass standards: The gels were calibrated using six molecular mass calibration markers (BDH Chemicals, Germany) namely : (A) Ovotransferrin - 76 to 78.0 kD, (B) Albumin - 66.25 kD, © Ovalbumin - 42.7 kD, (E) Carbonic anhydrase - 30.0 kD, (F) Myoglobin - 17.2 kD and (G) Cytochrome c - 12.3 kD; Lane 2: Chicken egg white lysozyme 13.8 kD; 3 and 5: Control (saline injected) millipede; 4 and 6: Millipede hemolymph 96 hours post injection of 10^9 cfu *E. coli.* g^{-1}; 7 and 8; Scorpion hemolymph sample 24 hours post injection of 10^9 cfu *E. coli.* g^{-1}; 9: Control (saline injection) scorpion hemolymph sample. Loaded 10 µg of total protein per well.**

proteins as molecular mass differences exist between the protein bands of the scorpion and lysozyme. Visual analysis of separated millipede proteins revealed differences in the relative protein quantities which

occurred in the 30 - 12 kD range (large arrows on the left of **Figure 17**). However, no distinct conclusions can be made with regards to the exact locatic. of millipede anti-bacterial protein(s) in these protein profiles at this time.

3.3.3 GEL OVERLAY (Activity Determination of Separated Proteins).

3.3.3.1 Reverse Field Acidic Zone Electrophoresis: Gel Overlay (RF-AZE)

Anti-bacterial activity was evident when vaccinated millipede hemolymph samples were separated on 10 % RF-AZE gels and subjected to *E. coli* gel overlay (**Figure 18**: lanes 1,2,4,5; **Figure 19**: lanes 2,3,4,5; **Figure 20**: lanes 1,2,3,4 and weakly in lane 5). Unlike the SDS-electrophoresis procedure, this method showed the presence of new protein bands after vaccination of millipedes when compared to controls. These bands corresponded to activity (see **Figure 18**).

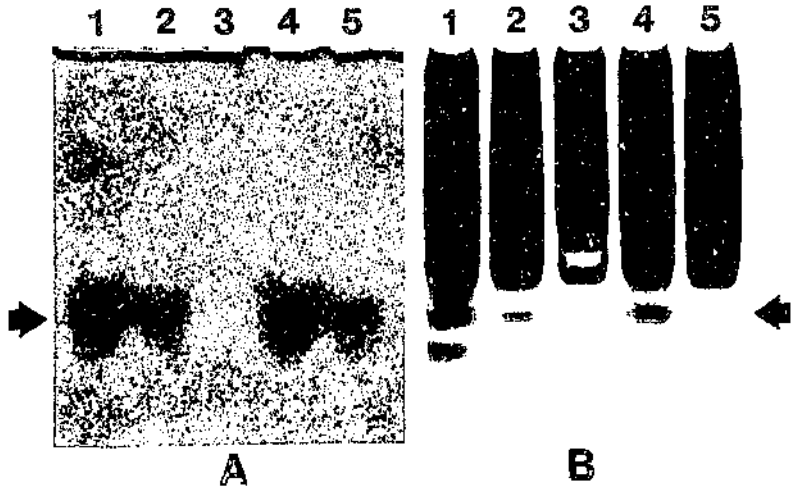


Figure 18 **Reverse field acidic zone electrophoresis (RF-AZE) *E. coli* gel overlay. Anti-bacterial activity against gram-negative *E. coli* is present in wells 1, 2, 4 and 5 (arrowed). Anti-bacterial activity was not present in control (non injected) millipede hemolymph (well 3). Wells 1, 4 and 2, 5 - hemolymph samples from two millipedes collected 48 hours post experimental infection with 10^7 cfu *E. coli*.g^{-1}. Gel A) and Gel B) are duplicate gels where gel A was overlayed with nutrient agar seeded with 10^6cfu *E. coli*.ml^{-1} and Gel B) was stained with PAGE Blue and then with silver in order to visualise individual protein bands. Two hundred and fifty micrograms of total hemolymph protein was loaded in each well in order to obtain detectable anti-bacterial activity on the gel overlay (Gel A) as well as on the stained gel (Gel B). Proteins were separated on a 10% acrylamide gel ran towards the cathode i.e. from top to bottom. (figure taken from Van der Walt *et al*, 1990)**

The anti-bacterial protein activity in hemolymph samples of millipedes collected 72 hours post vaccination with *E. coli* (Figure 19 - lanes 2,3),

showed a more than three-fold increase (manual measurement of surface area), when compared to the anti-bacterial protein activity in millipede hemolymph samples taken 48 hours post *E. coli* infection (**Figure 19** - lanes 4,5). The anti-bacterial activity remained for at least 96 hours. Anti-bacterial protein activity against *E. coli* could not be detected on RF-AZE gel overlays of vaccinated scorpion hemolymph samples drawn 48 hours post infection. This could be due to protein denaturation during the preparation of samples. The representative, silver stained gel is shown in **Figure 22**: lanes 2,3 and 5. I have been unable to develop a suitable non-denaturing separation protocol for scorpion anti-bacterial proteins, although differences in protein pattern can be seen between control samples (**Figure 22**: lane 1) and vaccinated scorpion hemolymph samples (**Figure 22**: lanes 2,3 and 5). Slight differences can also be observed between vaccinated- millipede (**Figure 22** -lane 4) and -scorpion hemolymph (**Figure 22** - lanes 2,3,5). It would appear that the antibacterial protein(s) of the scorpion is(are) more labile than the corresponding millipede proteins.



Figure 19 ***E. coli*** **overlay of a 10% reverse field acidic zone electrophoresis (RF-AZE) gel. Lane 1 - control millipede hemolymph; lanes 2 and 3 - millipede hemolymph samples taken 72 hours post vaccination with 10^9cfu *E. coli*.g^{-1}; lanes 4 and 5 - millipede hemolymph samples taken 48 hours post infection with 10^9 cfu.*E. coli*.g^{-1}; lane 6- control hemolymph. Loaded 350 μg total protein per well. There was a more than three-fold increase ($\bar{x}$ = 337.85% SD ± 88.9%, N = 4, by manual measurement of surface area) in anti-bacterial protein synthesis in hemolymph samples collected between 48 h and 72 hours post vaccination with *E. coli*.**

When loading equal hemolymph volumes on 10% RF-AZE gels, from different vaccinated millipedes, marked differences were observed between individual millipede hemolymph anti-bacterial protein activities as shown on the 10% RF-AZE *E. coli* gel overlay (**Figure 20**: lanes 1 to 4). Hemolymph samples collected from control millipedes 72 hours post pyrogen free saline injection, exhibited slight anti-bacterial activity on the *E. coli* gel overlay and is probably due to wounding. However, anti-bacterial protein activities appear to have similar anti-bacterial intensities, in each millipede, when expressed relative to total hemolymph protein concentration as can be seen in **Figure 19**, lanes 2 and 3 as well as lanes 4 and 5 - samples taken from two individuals respectively, and, also in **Figure 21** A: lanes 2,3 & 5 and on the duplicate stained gel (**Figure 21** B: lanes 2,3 &5) after loading equal amounts of total protein. Anti-bacterial protein activity is present in the 12% to 22% linear gradient RF-AZE gel overlay (**Figure 21**: Gel A: lanes 2,3 and 5) and the anti-bacterial proteins are reflected on the duplicate silver gel in **Figure 21** B: lanes 2,3 and 5 indicated by arrow. Neither activity nor protein is evident in control millipede hemolymph (**Figure 21** A: lane 4). The artifact on gel overlay

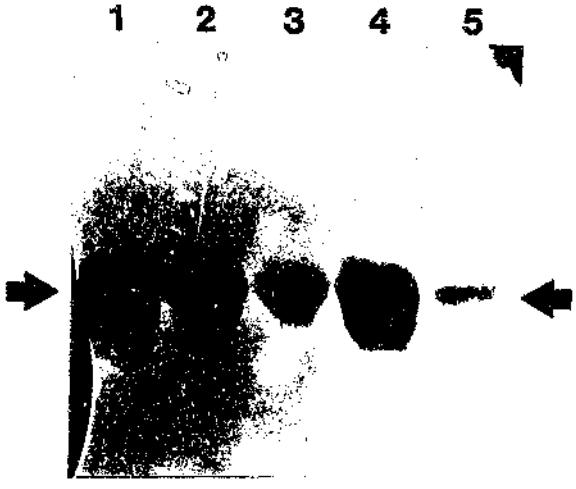


Figure 20 ***E. coli*** **overlay of 10% RF-AZE gel. Hemolymph samples collected from millipedes 72 hours after injection of 10^6 cfu *E. coli*.g^{-1}. Loaded 50 $\mu\ell$ of hemolymph in each well. Lanes 1, 2, 3 and 4 - hemolymph sampled from 4 millipedes 72 hours post infection with 10^9 cfu *E. coli*.g^{-1}; lane 5 - hemolymph sample from control millipede 72 hours post injection of pyrogen-free saline (0.7% w/v NaCl). Strong anti-bacterial activity is present in lanes 1 to 4. Saline injected hemolymph sample weakly positive after 72 hours. The of anti-bacterial protein activities reflect relative differences despite the use of a constant hemolymph volume for each individual animal.**

shown in **Figure 21** A, was caused by uneven shrinking of nutrient agar on the linear gradient polyacrylamide gel surface.

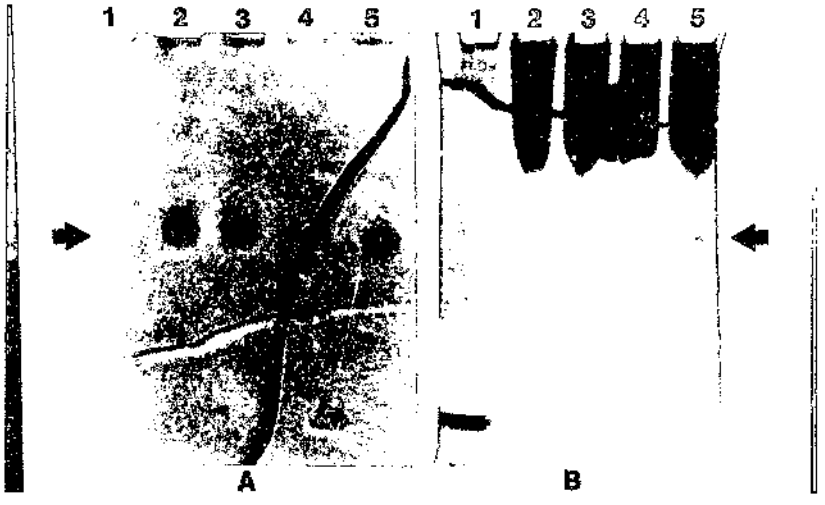


Figure 21 ***E. coli*** **overlay of 12% to 22% linear gradient RF-AZE duplicate gel (A) and PAGE Blue and silver stained gel (B). Lanes A)1 and B)1 - 5 μg Cytochrome C; Lanes A) 2, 3, 5 and B) 2, 3, 5 - millipede hemolymph samples collected 48 hours after vaccination with 10^8 cfu *E. coli*.g^{-1}; lanes A) 4 and B) 4 - control millipede hemolymph sample injected with pyrogen-free saline (0.7% w/v NaCl). Anti-bacterial activity of the PAGE Blue and silver stained anti-bacterial proteins in gel B (B:2,3 & 5), is reflected on *E. coli* overlayed gel (A:2,3 & 5)(arrowed). Loaded 250 μg of total protein per well.**



Figure 22 Silver stained 10% RF-AZE gel. Lane 1 - control scorpion hemolymph; lanes 2, 3 and 5 - hemolymph sample collected from scorpions 48 hours post injection of 10^9cfu *E. coli*.g^{-1}; lane 4 - millipede hemolymph sample taken 48 hours after experimental infection with 10^9cfu *E. coli*.g^{-1}. No anti-bacterial activity was detected in scorpion hemolymph samples when performing *E. coli* gel overlay.

3.3.3.2 RF-IEF and gel overlay

Under normal field iso-electric focussing conditions, hemolymph samples are loaded at the cathode. The alkaline environment at the cathode seemed to denature anti-bacterial protein activity. However, anti-bacterial activity in vaccinated millipede hemolymph samples was unaffected by very low pH ranges (0.1 M glacial acetic acid, pH 1.5) utilised during RF-AZE electrophoresis as previously described in section 3.2 - Materials and Methods. I decided to perform IEF under reverse field conditions to allow loading of hemolymph samples at the anode (acidic conditions). Iso-electric focussing of anti-bacterial proteins from millipede hemolymph performed under these conditions remained active.

Anti-bacterial protein activity was present in the RF-IEF gel overlay (**Figure 23**: Gel A lanes 1,2 and 3) in millipede hemolymph samples taken 72 hours post experimental infection with 10^9cfu *E. coli*.g^{-1}. However this method resulted in poor focussing of millipede anti-bacterial proteins and was also demonstrated in **Figure 23** B, lane 3 - Cytochrome C, used as pI indicator protein, was focussed on bottom edge of the gel

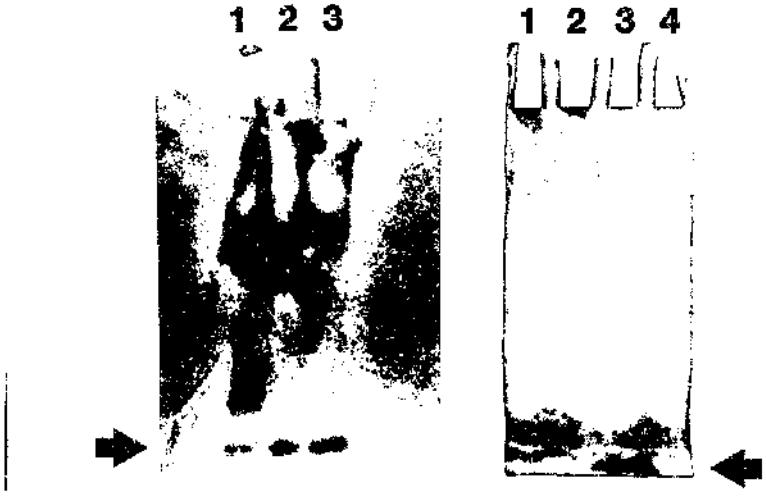


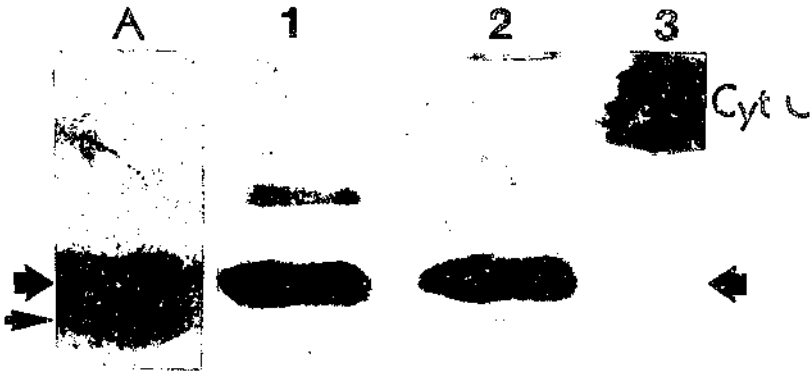
Figure 23 **Reverse field isoelectric focusing (RF-IEF) gel overlay (*E. coli*) (A) and B) PAGE Blue and silver stained gel focussed under the same conditions. A) 1, 2 and 3 - Hemolymph samples taken from millipede 48 hours post injection of 10^9 cfu *E. coli*.g^{-1}, loaded 150 μg, 250 μg and 300 μg of total protein in lanes A) 1, 2 and 3 respectively. Anti-bacterial activity against *E. coli* present at the bottom of the gel (arrowed) indicating inadequate pH range for proper resolution of antibacterial proteins. Gel B) lane 3 - Cytochrome C focussed at the bottom of the gel; Lanes B) 1 & 2 - 10 μg Chicken egg white Lysozyme, poorly focussed.**

and resulted in inaccurate iso-electric point determination. An improved and further modified IEF system is described in the following section.

3.3.3.3 Reverse Field Dual Gradient Iso-Electric Focussing (RF-DG-IEF)

The poor focussing shown in RF-IEF gels was remedied by incorporating an inverse dual gradient of arginine and cadaverine together with 2% ampholyte (pH 9 to 10.5) in order to extend the basic focussing region (**Figure 24**). Iso-electric focussing in the absence of these compounds using the most basic, commercially available ampholines, resulted in a focussed antibacterial activity zone that was close to the cathodic edge of the gel. The RF-DG-IEF method allowed a more accurate measurement of the pI (Iso-electric point) range of millipede anti-bacterial proteins. The anti-bacterial proteins were unstable in an alkaline buffer and the problem was overcome by reversing the electrodes of the electrophoresis equipment to allow loading of the protein samples from the anode. The pI of the anti-bacterial protein(s) was determined by cutting the indicated area of gel out and placing it in an Eppendorf centrifuge tube with 1 ml

- Anode



- Cathode

Figure 24 **Silver stained reverse field dual gradient iso-electric focussing (RF-DG-IEF) and concomitant Gel overlay of Millipede hemolymph proteins 72 hours post injection of 10^9 cfu *E. coli.* g^{-1}. Plus (anode) and minus (cathode) sign indicate direction of current. Lane A: Antibacterial activities (short and long arrow) of hemolymph proteins against *E. coli* Gel Overlay; Lanes 1 and 2 - PAGE Blue and silver stained anti-bacterial proteins from millipede hemolymph. Lane 3 - Iso-electric point marker (Cytochrome C: pI = 11). Multi layer focussing areas due to fractionation caused by repeated freezing and thawing of Cytochrome C sample. Loaded 500 μg of total protein per well.**

de-ionised water. The tube was agitated for 4 hours and the pH of the solution measured (Corning, Ion Analyser).

Dramatic anti-bacterial protein(s) activity was demonstrated in RF-DG-IEF of millipede hemolymph samples collected 72 hours post injection of 10^9 cfu *E. coli*.g^{-1}, when subjected to the *E. coli* gel overlay (**Figure 24**: lane no. A, Short and long arrow). Although two distinct bands of anti-bacterial activities can be seen on the RF-DG-IEF gel overlay, the silver stain procedure on the other identical half of the RF-DG-IEF gel: Lanes 1 and 2, failed to detect the presence of the faster moving component. This could be because detection of "bands" of activity on the gel overlay is the result of a dynamic process between the growth of live bacteria versus the presence of anti-bacterial protein(s), which halt bacterial growth, and, because of this dynamic process, the effect of extremely low quantities of anti-bacterial protein, will be amplified over time. It is possible that this might be a precursor or fractionated protein.

All chemicals (except acetic acid) used in the RF-DG-IEF gel, were also subjected to the plate assay in order to detect any possible inherent antibacterial activity. None of the chemicals tested showed any signs of

antibacterial activity. From the RF-DG-IEF gel shown in **Figure 24**, the pI range of the antibacterial activity was determined to be between 11.8 and 12.5. This result is close to the range of other families of antibacterial proteins (cecropins and attacins) found in other insects species i.e. basic attacins: pI range 9 to 10.5, and cecropins pI range between 8 and 11 (Pye and Boman (1977); Qu *et al*, 1982; Steiner, 1981; Steiner, 1982; Shiba *et al*, 1984; Engström *et al*, 1984a, b, Geng and Dunn, 1989).

Results from my study are somewhat contradictory to the only other report of work, Xylander and Nevermann, (1990), which was performed on the immune reactions of other millipede species . Their report states that no anti-bacterial activity could be induced in *Chicobolus sp.* and *Rhapidostreptus virgator* (Diplopoda) as well as in *Lithobius forficatus* and *Scolopendera indurata* (Myriapoda) against the gram-negative bacterium (*E. coli* K12), but, that all species tested exhibited anti-bacterial activity against gram-positive bacteria (*Micrococcus luteus*). However, results from this study and also previous reports (Van der Walt, 1988; Van der Walt *et al*, 1990; Van der Walt and McClain, 1991) demonstrated inducible anti-bacterial activity in *T. triodus* against gram-negative *E. coli* K12.

The anti-bacterial activity reported by Xylander and Nevermann (1990) was inactivated after 15 min exposure at 55°C which is dissimilar to results for thermal stability experiments performed in this study, where, anti-bacterial activity in the Kalahari millipede *T. triodus* was inactivated after 30 min exposure to 50°C (Chapter 2, **Figure 12** and **Figure 13**). These slight differences in exposure time as well as the two temperatures (50°C vs 55°C) used, do not allow full comparison between the two studies. However both indicate the thermal instability of the antibacterial proteins. Xylander and Nevermann (1990) also reported inactivation of anti-bacterial activity in all four species after cooling at -15°C and is similar to a previous study by Van der Walt (1988) which reported the inactivation of anti-bacterial activity in vaccinated *T. triodus* hemolymph samples after cooling to -20°C.

In this study, PO activity was demonstrated in the *E. coli* plate assay of vaccinated hemolymph of *T. triodus*. Phenoloxidase activity was also present in all four vaccinated species of millipedes used by Xylander and Nevermann, 1990.

The number of bacteria required to exceed the threshold level for anti-bacterial protein synthesis was found to be 10^9 cfu *E. coli*.g^{-1} in *T. triodus* as well as *O. latimanus* (Table 7). However, the threshold in *Chicobolus sp.* was 10^5 cfu *E. cloacae*. g^{-1} (Xylander and Nevermann, 1990).

Unfortunately, no electrophoretic data on the antibacterial proteins (acidic mobility, pI or SDS-PAGE) detected in four other millipede species have been reported (Xylander and Nevermann, 1990) and therefore co comparisons to this study can be performed.

Our study is the first report of the existence of antibacterial proteins in millipede hemolymph that have such a high iso electric point range (10.8 - 12.8 ,**Figure 24**).

I have as yet been unable to separate active scorpion hemolymph proteins using the RF-DG-IEF technique. However, results from vaccinated scorpion hemolymph samples tested positive on the *E. coli* plate assay (**Figure 9**). RF-AZE results show that differences in protein existed between control and vaccinated scorpion samples collected 48 hours after *E. coli* infection (**Figure 22**). SDS-electrophoresis also showed the

appearance of new protein bands in vaccinated scorpion samples (**Figure 17**, lanes 7 & 8) Differences also existed between vaccinated scorpion hemolymph samples and vaccinated millipede (**Figure 17**).

3.4 CONCLUSIONS

My study has demonstrated inducible humoral responses in two long-lived arthropods, a millipede and a scorpion. These inducible substances are proteins and are active against both gram-negative (millipede and scorpion) and gram-positive bacteria (not ascertained in case of scorpion), *E. coli* and *S. aureus* respectively. Furthermore, these humoral anti-bacterial responses were found to be similar to the anti-bacterial activities that are documented for more ephemeral insects (Table II).

My results also suggest the possible existence of a hemocyte depletion factor as part and parcel of the immune response of these long-lived arthropods. There is also evidence to suggest that the inducible humoral immune response of millipedes is transduced through a phenoloxidase pathway similar to reports on other arthropod species (Mohrig *et al*, 1979a, 79b; Bohn *et al*, 1981; Ashida *et al*, 1983; Brookman, 1989). Evidence of PO activity was found in vaccinated millipedes as demonstrated in the plate assay technique (**Figure 3** to **Figure 5** as well as **Figure 6** and **Figure 12**). Similar PO results were obtained in another

study focussing on other millipedes (Xylander and Nevermann, 1990). PO activity was elicited by both gram-positive and gram-negative bacteria (Chapter 2: **Figure 3**, **Figure 6** and **Figure 12**). In almost all instances PO activity was absent in the presence of anti-bacterial activity suggesting that when PO activity initiates anti-bacterial activity, melanisation actions in vaccinated millipede hemolymph are diverted into the production of anti-bacterial proteins and this could be the threshold sensitive trigger which routes the immune response into either a cellular response or synthesis of anti-bacterial protein. However this is speculation at this stage.

It is still not clear if the PO system is involved in eliciting the release of a hemocyte depletion factor which could cause the severe hemocytopenia observed in vaccinated millipedes and scorpions. This study demonstrated a hemocytopenia in both millipedes and scorpions after bacterial infection which was still unabated after 3 days which is different to hemocyte depletion reports in insects where hemocyte depletion abates several hours post bacterial infection (Geng and Dunn, 1989). The anti-bacterial proteins in *T. triodus* showed similar heat- and cold -sensitivity

to those that have been reported in another study (Xylander and Nevermann, 1990).

At this stage it is not certain whether the proteins detected in the millipede and scorpion are related to the three major families of anti-bacterial proteins that have been shown to exist in a variety of short lived insect species i.e. lysozymes, attacins and cecropins (Faye and Wyatt, 1980; Hultmark *et al*, 1982; Flyg *et al*, 1987). The anti-bacterial activity of millipedes and scorpions against gram-negative bacteria (*E. coli* K12) precludes these proteins from the lysozyme family of anti-bacterial proteins (Phillips, 1974; Schneider, 1985) as the antibacterial proteins are active against gram negative bacteria (*E. coli*).

The results from reverse field dual gradient isoelectric focusing indicate that the antibacterial protein(s) from the millipede are extremely basic, more so than any other proteins so far reported for insects (Götz and Boman, 1985).

Differences in anti-bacterial protein responses may exist within various millipede species when comparing results from this study to other studies (Xylander and Nevermann, 1990). This study provides further information to support the theory (Hoffman *et al*, 1995) that arthropods have an ancient and conserved phylogeny of immunity. Furthermore the response to infection is rapid and as potent or more so than demonstrated for the more recently evolved insects.

Further research needs to be done in order to determine the amino acid sequence of the millipede and scorpion anti-bacterial protein(s) as this would assist in verifying the degree of homology between anti-bacterial proteins from the more primitive classes of arthropods to those of insects and even vertebrates.

The process from recognition of foreign particles to the actual synthesis of anti-bacterial proteins through the phenoloxidase cascade, also needs to be investigated more closely to establish, if indeed these ancient arthropods use a mechanism similar to that which exists in short lived insects for combatting bacterial infection.

The corner stones of the human immune response are the existence of lymphocytes and immunoglobulins. In addition to these, a febrile response is often elicited in reaction to the presence of foreign material like endotoxins (from gram-negative bacteria and cell wall fragments from gram positive bacteria), in blood (Donaldson, 1981). The febrile response of the human immune system is evoked whether the source of endotoxin is live or dead. The actual mechanism of fever production in vertebrates has recently been uncovered and is mainly transduced through endotoxin induced synthesis of cytokines which include Interleukin 1 (IL 1 - a peptide produced by B lymphocytes, monocytes and macrophages, which acts as a cytokine on a specific receptor which are members of the immunoglobulin superfamily) (Dinarello *et al* 1989). It has also been shown that this cytokine binds to specific neurons whose function is temperature control (set-point neurons) and are located in the hypothalamus (Dinarello and Wolff, 1982). Interestingly it was discovered that the hemocytes of the horse-shoe crab (*Limulus sp.*) react strongly even to very low concentrations of endotoxins a fact exploited for the assay for the presence of endotoxins (Limulus amoebocyte lysate assay)(Levin and Bang, 1968). Arthropods on the other hand are

considered to be cold-blooded animals whose body temperature is equal to that of ambient temperature. However, recent studies have demonstrated that some cold-blooded vertebrates (lizards) and arthropods (insects), demonstrate a behavioural-fever when subjected to experimental bacterial infection (Hacker *et al*, 1981). These animals normally raise their body temperature by basking in sunlight for longer periods of time after microbial challenge. The biochemical mechanism by which some cold-blooded animals are enabled to produce a behavioural-fever remains unexplained. It is postulated that behavioural-fever in hetero therm animals could be elicited by the synthesis of a peptide compound, similar to that which exists in humans (Beck *et al*, 1989).

First studies, investigating the homology (amino acid sequences) of immunological molecules between arthropods and the mammalian immune system, reported the existence of a new immune protein from the wax moth *H. cecropia* namely Haemolin. This study also reported that a high degree of similarity/homology existed between this protein and the human immunoglobulin super family (Hultmark, 1994, Hoffman *et al*, 1995).

The field of comparative immunology has already resulted in preliminary medicinal applications for example initial treatment of human cancer patients with purified cecropins (Jayes, 1989). The action of cecropin P4 (also from *H. cecropia*) has been characterised as an ionophore which produces "holes" in the membranes of mammalian cells. Cecropin P4 acts by eventually destroying the cytoskeleton of cells. At the correct dc. cecropin P4 can selectively destroy cancer cells by disrupting their cytoskeleton. This is made possible because cancer cells by their virtue, have much higher mitotic rates compared to normal cells and as a result of this phenomenon, cancer cells form slightly less adequate cytoskeletons which can easily be destroyed by cecropin P4.

Other comparative aspects of arthropod and human immunity is mechanisms for immuno suppression. Studies (Asgari *et al*, 1997) have shown mechanisms of immuno suppression in insects which are similar to those that occur in humans.

n the discussion above, it appears obvious that endeavours in the field of arthropod immunity has led to some interesting discoveries.

I hope that this field of research will receive ongoing support in this country, not only for the purposes of phylogenetic clarity and development of more effective bio-pesticides for insect control, but also because comparative immunology is reaffirming the potential implications that basic research could have in medicine and society as a whole.

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Author:Van der Walt, Etienne.
Name of thesis:Arthropod immunity: characterisation of the humoral immune responses in two species of arthropods • the Kalahari millipede

PUBLISHER:
University of the Witwatersrand, Johannesburg
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