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Expression patterns of immune associated genes in Euoniticellus intermedius and characterization of the embryonic cell line

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Expression patterns of immune associated genes in *Euoniticellus intermedius* and characterization of the embryonic cell line

Mohamed Alaouna

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

Johannesburg, 2012

Declaration

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of MSc in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



Mohamed Alaouna

~~26~~ 31 Day of ~~March~~ octoben 2012

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_____

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_____ _26_____Day of____March_____2012

Abstract

As bacteria are becoming resistant to conventional antibiotics, researchers are looking for new ways to combat microbial infection. We have begun to adopt genetic and functional genomic approaches to define the molecular determinants of pathogen resistance in the dung beetle, *Euoniticellus intermedius.* This dung beetle survives microbe-rich environments such as dung. This ability makes it a potential model for the study of infectious agents and ecological damage. To date, *E. intermedius* has not been studied at the molecular level. In this study, a range of complimentary analytical techniques were used to characterize the *E. intermedius* embryonic cell line established in our laboratory. These techniques characterize morphology, growth characteristics, karyotype, isoenzyme patterns and embryonic development. Complete characterization of the *E. intermedius* cell line is essential for the cell banks and for the regulatory requirements in biopharmaceutical production.

This study followed gene sequences and their comparisons for both adult and cell line to confirm that the *E. intermedius* (EISA08) cell line is originated from the embryonic *E. intermedius* dung beetle. cDNA was synthesized from mRNA isolated from *E. intermedius* adult beetles and cell line (EISA08) was sequenced using GS (FLX) technology by a commercial facility, Inqaba Biotechnical Industries (Pty) Ltd, South Africa. In addition to characterization of the cell line, two genes, namely *hopscotch* and ribosomal protein S9 (RpS9), were selected from the Flylab genome data base. The *E. intermedius* database is a web-based system for the genome and transcriptome of the dung beetle to evaluate the immune system of the dung beetle (http://Flylab.wits.ac.za/). *hopscotch* was selected because it is believed to be involved in the JAK-STAT signalling pathway for anti-viral response, embryonic development and cell growth. Rsp9 was chosen as a loading control because it is expected to be a housekeeping gene. The conserved molecular signalling

pathway JAK-STAT is used by *E. intermedius* (as in other insects and humans) for immune defence and early embryonic development. The project followed *hopscotch* and Rsp9 gene expression in all the *E. intermedius* life cycle developing stages; adult, pupae, larvae, embryo, and cell line cell growth, life cycle developing stages and embryonic development has was monitored.

E. intermedius embryonic development is described as short germ-band. *E. intermedius* embryogenesis is regarded as basal and is observed in most arthropods. The study revealed that *E. intermedius hopscotch* is over expressed in the early developing stages, embryo, larvae, and pupae and in the newly established cell line EISA08. The results from this study lead to the suggestion that *E. intermedius* JAK-STAT pathway is activated early and has an important role in embryonic development, cell proliferation and immune defence. Studies of *E. intermedius* could provide more insight into the properties and evolution of innate immunity and embryonic development.

Dedication

This dissertation is dedicated to my parents

Seek knowledge from the cradle to the grave

Acknowledgements

I would like firstly to thank Dr Yasien Sayed for the advice, encouragement and support. I would like to thank Dr M. Ntwasa for giving me an opportunity to work in his lab. His patience, support and guidance have given me enough strength to complete this course. My sincere gratitude goes to Prof. Stefan F T Weiss, Prof. Rob Veale, Prof. M.E.C. Rey, Prof. Ballim, Prof. Marcus Byrne, Rodney Hull, members of the Fly Lab in recent years and to all those people in the school of molecular and cell biology (Technical staff, fellow students and staff members) for all their assistance. I would like to acknowledge the University of the Witwatersrand. Lastly a big thank you to my parents and my friend Abdel Alhafid for being there for me all the time, thank you and God bless you all.

Publications

Hull, R., Alaouna, M, Khanyile, L., Byrne, M. and Ntwasa, M. (2012) 'Lifestyle and host defense mechanisms of the dung beetle *Euoniticellus intermedius (*Coleoptera: *Scarabaeidae*). The Toll signaling pathway, (in preparation).

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Abbreviations

AMP	Antimicrobial peptide
AST	Aspartate amino transferase
bp	base pair
cDNA	Complementary DNA
C-terminal	Carboxy-terminal
DAP	Diaminopimelic acid
DD	Death domain
Dif	Dorsal-related immunity factor
Dome	Domeless
DNA	Deoxyribonucleic acid
Dredd	Death-related ced-3/Nedd2-like protein
Dro	Drosocin
EISA08	*Euoniticellus intermedius* South Africa 2008
EtOH	Ethanol
FADD	Fas (TNFRSF6)-associated via death domain
G6PD	Glucose-6-phosphate dehydrogenase
GlcNAc	N-acetylglucosamine
IKK	*I- kappa β* kinase kinase
Iκβ	*I- kappa β*
IMD	Immune Deficiency
IRAK	IL-1 receptor-associated kinase
JAK	Janus kinase

JNK	Jun kinase
kbp	kilo base pair
LB	Lysogeny Broth
LD	Lactate dehydrogenase
LPS	Lipopolysaccharide
Lys	Lysine
MD	Malate dehydrogenase
MPI	Mannose 6-phosphate isomerase
mRNA	messenger Ribonucleic Acid
MyD88	Myeloid Differentiation factor 88
MurNAc	N-acetylmuramic acid
NF-κB	Nuclear factor kappa B
NP	Nucleoside phosphorylase
N-terminal	Amino-terminal
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase Chain Reaction
PGN	Peptidoglycan
PEP B	peptidase B
PGRP	Peptidoglycan Recognition Protein
PGRP-SA	Peptidoglycan Recognition Protein-SA
PGRP-SD	Peptidoglycan Recognition Protein-SD
PRR	Pattern Recognition Receptor
Psh	Persephone

OD	Optical Density
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SCF	Skp-l/Cull/F-box
SH2	Src Homology 2
SMH	Shai-Matsuzaki-Huang
SPE	Spätzle processing enzyme
Spz	Spätzle
STAT	Signal Transducer and Activator of Transcription
TAK1	TGF-beta Activated Kinase I
TCT	Tracheal Cytotoxin
TEPs	Thioester-containing Proteins
TLR	Toll-like Receptor
Tot	Turandot
Upd	Unpaired

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Chapter 1

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1 Introduction

With around one million and 10^{18} individuals insects represent one of the most successful groups (Vilmos and Kurucz, 1998). Fifty-five percent (55%) of total biodiversity (Chernysh *et al.*, 2002). During evolution, insects developed effective innate immune systems which differed from the adaptive immune system of vertebrates (Vilmos and Kurucz, 1998). The innate immunity of insects can cope with an extremely large variety of pathogens.

Insect defence mechanisms develop very rapidly lasting upto a few days. Insects also have powerful resistance to microbial infections (Vilmos and Kurucz, 1998). Recent experiments provide phenomenological evidence that specific memory exists in invertebrates, which solely rely on innate defence (Kurtz and Franz, 2003; Little *et al.*, 2003; Witteveldt *et al.*, 2004). Activation of the innate immune system leads to the expression of antimicrobial peptides that attack micro-organisms (Hoffmann *et al.*, 1999).

Antimicrobial peptides are evolutionarily ancient weapons; mostly small cationic molecules that are very active against gram-positive and gram-negative bacteria (Casteels *et al.*, 1989; Bulet *et al.*, 1991). The microbial pathogens do not acquire resistance to these cationic peptides even though they have often been exposed to them for a very long time (Zaltoff, 2002). In contrast to the currently available antibiotics, bacterial strains have developed ways to develop resistance (Hancock, 2001). In *Staphylococcus aureus*, more than 95% of the strains are resistant to the antibiotic penicillin (Sackton *et al.*, 2007). For this reason, there is an increased commercial interest in developing cationic peptides as potential antimicrobial therapeutics (Zaltoff, 2002).

A thorough understanding of this system of immune defence is important not only in terms of potential applications to the biological pest control of pest insects, but also because insects are useful models for studying immune systems (Wells, 2008). Therefore, insect immunity is being

increasingly studied, especially in *Drosophila*, which has become an important model for innate immunity examination over the past 10 years (see Figure 1) (Hoffmann, 2003).

1.1 Dung beetles (*Scarabaeinae*: Coleoptera)

Dung beetles are members of the subfamily *Scarabaeinae* within the family *Scarabaeidae*, with 5000 species distributed into 12 tribes and grouped in 234 genera (Hanski and Camberfort, 1991). The order, coleopteran, is the most diverse on earth thus representing the major group of the superfamily, *Scarabaeidae* with approximately 350 000 known species (Stork, 1993).

Dung beetles are especially divergent and plentiful in tropical rain forests (Hanski and Camberfort, 1991). They display a great variety of morphology and nesting behaviour. They play a significant role in tropical ecologic systems by contributing to the recycling process of organic matter. They are also excellent bioindicators (Cambefort and Hanski, 1991; Halffter and Favila, 1993). Dung beetle species feed on the microorganism-rich liquid organic of mammalian dung and use the more fibrous material to brood their larvae (Halffter and Edmonds, 1982). They minimize the number of parasites acquired by cattle and significantly reduce the population of pestilent flies. The tunnelling activity of dung beetles increases the soil's ability to absorb and hold water (Bang *et al.*, 2005). They stimulate a series of ecosystem functions. Many of these ecological functions provide valuable ecosystem services (De Groot *et al.*, 2002). The economic value of their ecological services for the USA is estimated to be $380 million annually (Losey and Vaughan, 2006) Based on relocation of dung used for breeding, most dung beetles use one of four broad nesting strategies. Paracoprid (tunneler) species bury brood balls for feeding and breeding in vertical chambers. Telocoprid species transport balls horizontaly away before burial beneath the soil. Endocoprid species brood their young inside the dung mass itself (Halffter and Edmonds, 1982) (Figure 2). Kleptocoprids utilise dung collected and buried by other dung beetles (Nielsen, 2007) (Figure 2).

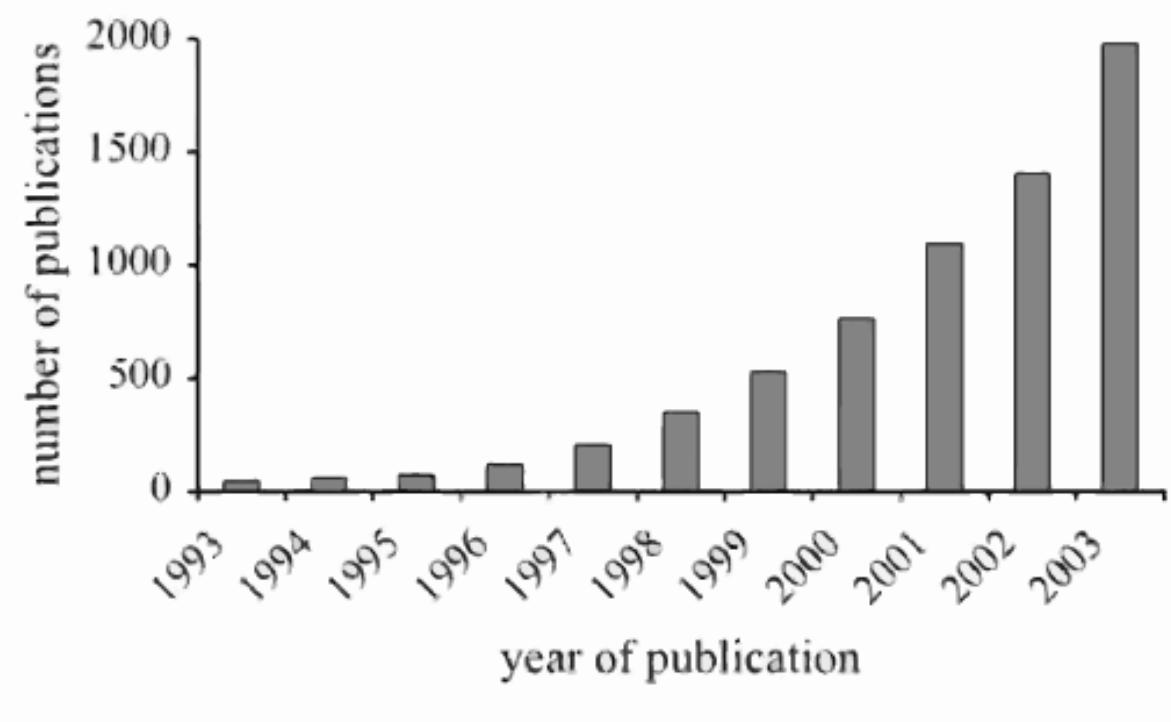


Figure 1: Innate immunity publications over a 10 years period. Data was obtained from ISIWeb of Science®, searching for the terms “immun*” and “innate”, from the article title, keywords, or abstract (Kurtz, 2004).

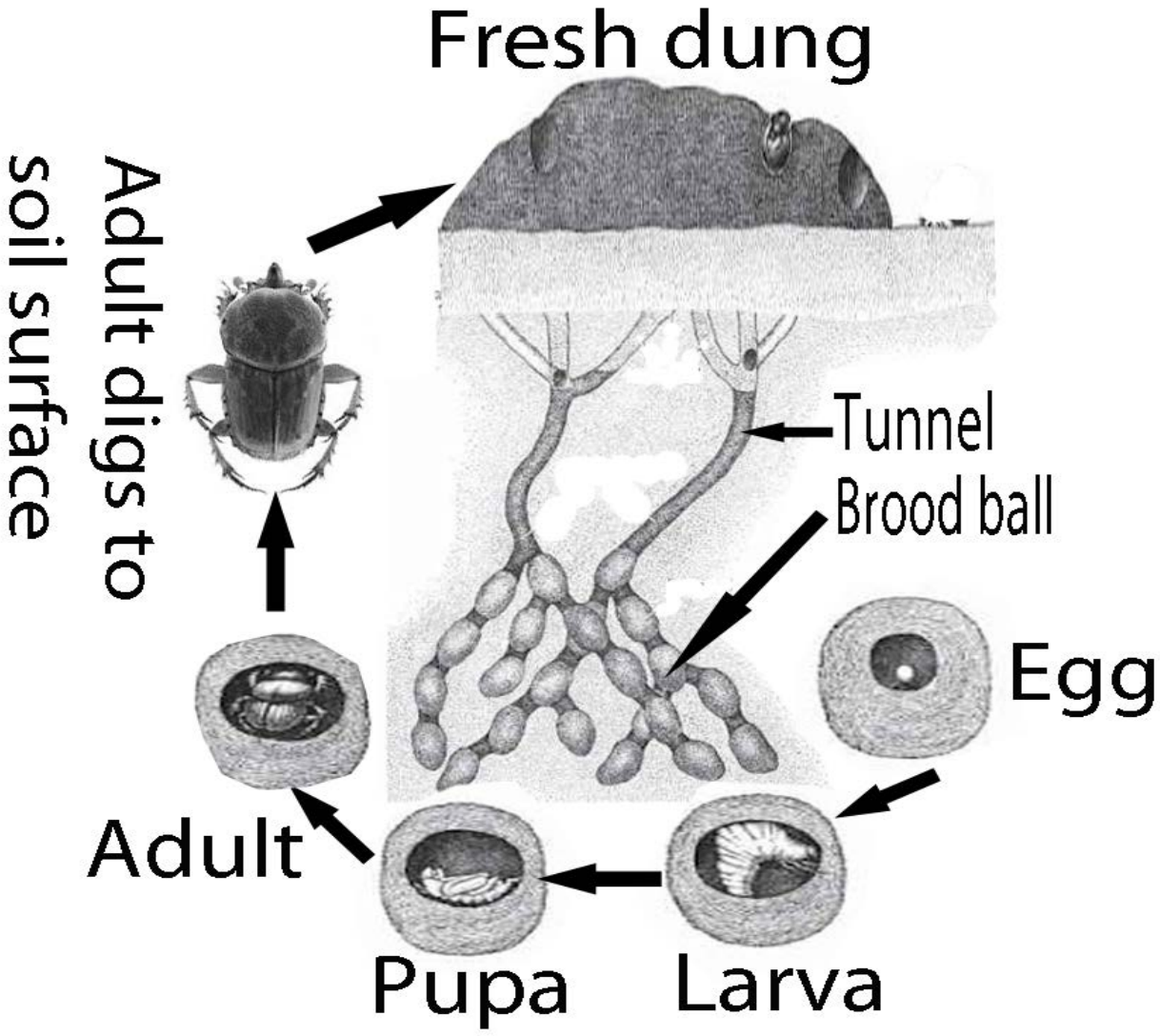


Figure 2: The tunnelling dung beetle *E. intermedius* life cycle.

1.1.1 Dung beetle *E. intermedius*

The tunnelling species dung beetle, *E. intermedius* (Reiche) (Family *Scarabaeidae*, Subfamily *Scarabaeinae*, Tribe *Coprini*) (Wood and Kaufman, 2008) (Table 1) are small (7-9 mm in length) and yellow-brown or straw-colored. Males have a curved, blunt horn on the head. They are active from spring to autumn and during warm spells (de Oca and Halffter, 1998). They are widespread and are found in countries from South Africa to Arabia (Halffter and Arellano, 2002) and they feed mostly on cow dung. Female beetles dig into the soil beneath the dungpat and drag dung into the burrow to produce brood balls into each of which they lay an egg (Figure 2). Females are able of laying about two eggs per day (Halffter and Arellano, 2002). The eggs hatch into larvae after two days and adults emerge after about one month (de Oca and Halffter, 1998).

1.2 The insect innate immune system

The insect innate immune responses are particularly represented by four signal transduction pathways. Each pathway contains many factors and plays important roles in the production of effectors that eliminate infectious microorganisms. The signal pathways are the Toll, Immune Deficiency (IMD), JNK and Janus kinase (JAK)-signal transducer (STAT) pathways (Evans *et al.*, 2006). The Toll pathway is activated by gram-positive bacteria, fungi (Hoffmann and Reichhart, 2002; Tzou *et al.*, 2002) and viruses (Xi *et al.*, 2008); whereas the IMD pathway is activated by gram-negative bacteria (Hoffmann and Reichhart, 2002; Tzou *et al.*, 2002). JNK signalling via the IMD pathway has been linked to wound repair and stress responses (Aggarwal and Silverman, 2008) and required for antimicrobial response via the IMD pathway. The JAK-STAT pathway is required for multiple developmental events and regulates

the cellular immune response (Zeidler *et al.*, 2000a).

Innate immunity is the first line of defence that is used to combat infectious microorganisms and depends on interactions between molecular structures existent on the surface of pathogens and host proteins called pattern-recognition receptors (PAMPs) (Medzhitov and Janeway, 1997). The insect innate immune response includes physical barriers and both cellular and humoral responses (Wells, 2008). The humoral responses are induced in the fat body and the cellular responses are induced in haemocytes. Phagocytosis and encapsulation are cellular immune responses. Antimicrobial peptides, cell adhesion molecules, lysozyme, lectins and the propenol-oxidase system are humoral immune responses (Hoffmann, 2003; Kanost *et al.*, 2004) (see Figure 3).

Generally if the pathogen is able to overcome the insect's first line of defence (Cuticule), or cross the gut wall, coordinated responses of immune cells in haemolymph are initiated (Ashida and Brey, 1995). This involves detection by pattern recognition proteins (PRPs) present in the heamolymph and the surfaces of haemocytes in the haemolymph (Gillespie et al., 1997). Haemocytes can respond tominfection by trapping invading microorganism within aggregates of many haemocytes called nodules (Gillespie et al., 1997). Detection of a pathogen by PRP induced proteolytic cascades were they produce blood clotting and melanisation(Gillespie et al., 1997). Finally, the fat body produces a large set of inducible effector molecules, such as antimicrobial peptides (AMPs), stress response proteins (Zeidler et al., 2000b).

The components of the insect immune system that originate from the mesoderm are the fat body, the lymph gland and the hemocytes (Tepass *et al.*, 1994; Hartenstein *et al.*, 1992) (Figure 4). The fat body is connected to the internal surface of the cuticle and made up of adipose tissue. It is a large biosynthetic organ which corresponds functionally to the mammalian liver (Søndergard, 1993) and is responsible for the synthesis of antimicrobial peptides (Trenczek and Faye, 1988; Samakovlis *et al.*, 1990) in response to infection (Figure 5 and Figure 6).

Table 1: *Euoniticellus intermedius* classification.

Kingdom	Animalia (Animals)
Phylum	Arthropoda (Arthropods)
Class	Insecta (Insects)
Order	Coleoptera (Beetles)
Suborder	*Polyphaga*, (Water, Rove, Scarab, Longhorn, Leaf and Snout Beetles)
Superfamily	*Scarabaeoidea* (Scarab, Stag and Bess Beetles)
Family	*Scarabaeidae* (Scarab Beetles)
Subfamily	*Scarabaeinae* (Dung Beetles)
Genus	*Euoniticellus*
Species	*intermedius* (*Euoniticellus intermedius*)

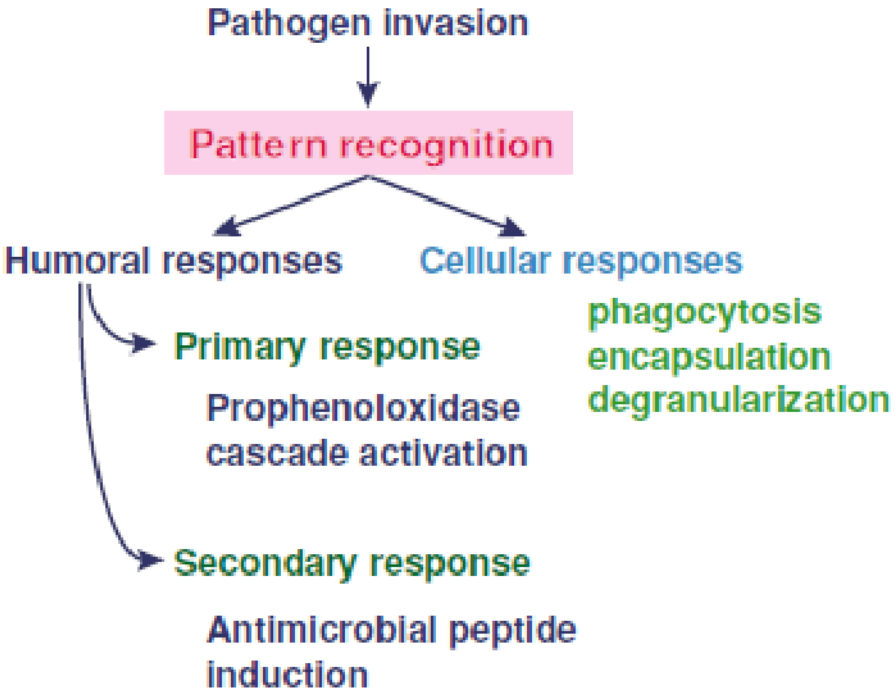


Figure 3: Activation of insect host defence system (Kurata *et al.*, 2006).

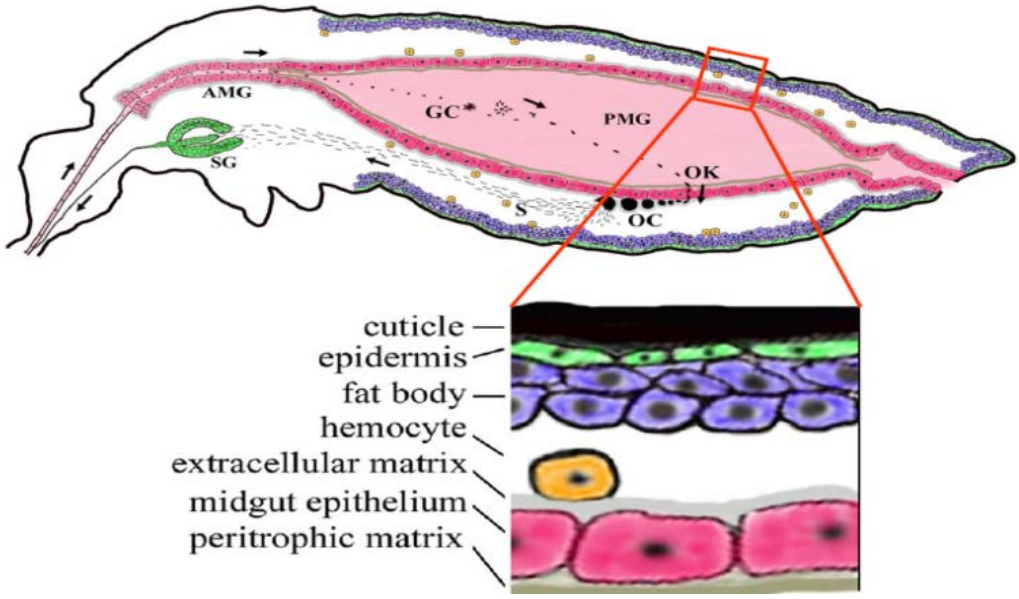


Figure 4: Insect defence: barriers and immune tissues. Organs and cell types involved in *Plasmodium* interaction and immune response, and the parasites life cycle. Gametocytes (GC) enter the posterior midgut (PMG) through the anterior midgut (AMG), fertilize and develop into an ookinete (OK) that traverses the peritrophic matrix and midgut epithelium to form an oocyst (OC) under the basal lamina. After maturation, sporozoities (S) translocate from the oocyst (OC) to the salivary glands (SG). Drawings of organs and cells are not proportional (Dimopoulos, 2003).

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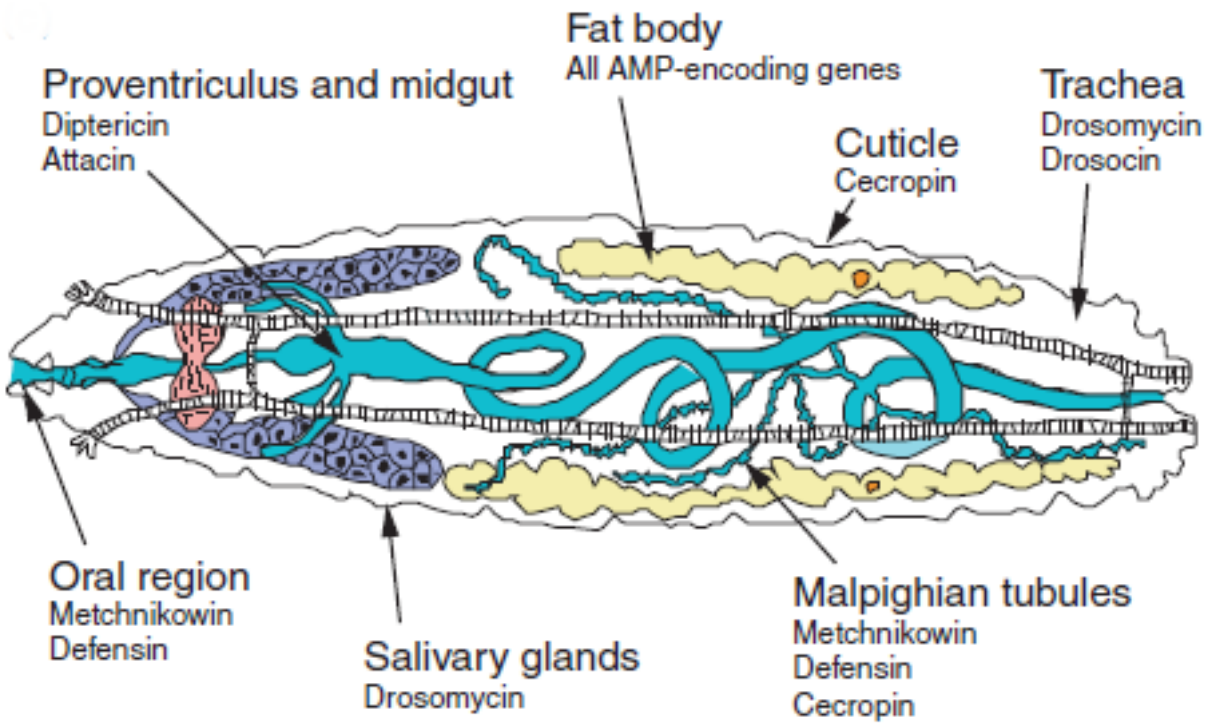


Figure 5: The site of expression of antimicrobial peptides (AMPs) in the larvae (Zeng *et al.*, 2001).

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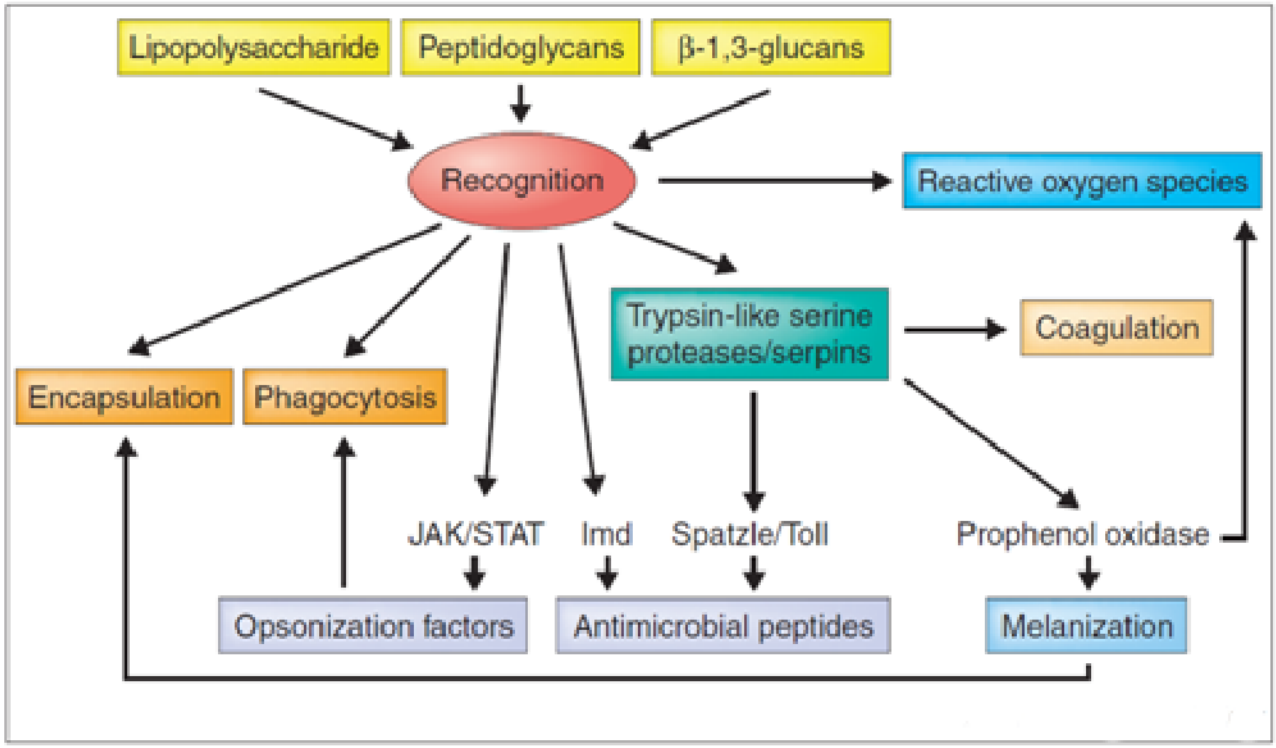


Figure 6: Schematic overview of the insect host defence system (Drolet *et al.*, 2005)

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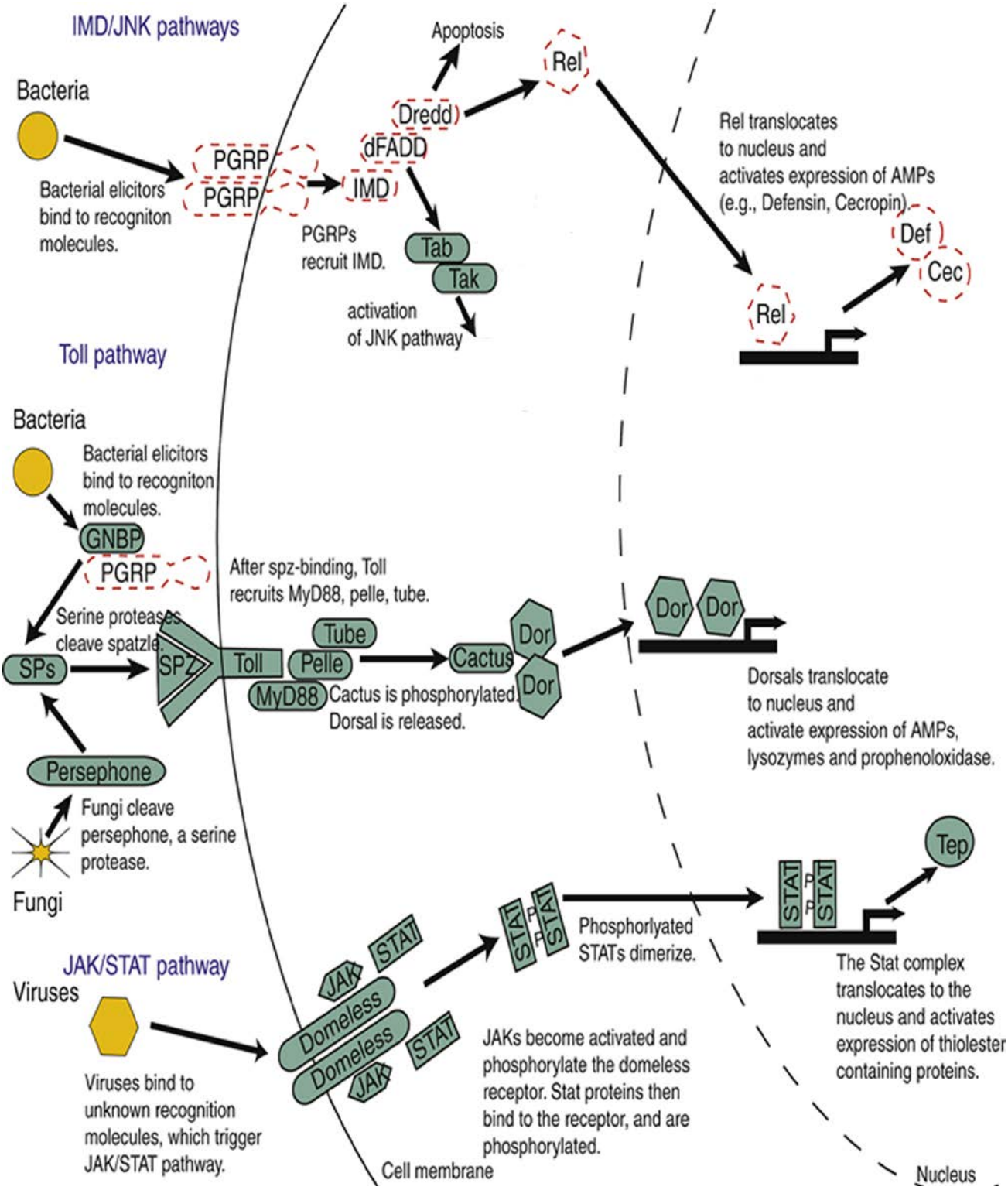


Figure 7: Model of Toll, IMD and JAK-STAT pathways activation (Arbouzova and Zeidler, 2006).

1.2.1 The Toll pathway

The Toll signalling pathway responds to fungal and gram-positive infections (Lemaitre *et al.*, 1996; Lemaitre *et al.*, 1997). It also has a role in establishing the dorsal-ventral pattern of the early embryo (Lemaitre *et al.*, 1996). Toll is a cytokine receptor, activated by the pro-protein, with a disulfide-linked dimeric structure cytokine called Spätzle.

In order to activate the Toll pathway, pathogens first activate serine protease cascades that conclude in Spätzle cleavage, releasing the mature Toll ligand (Weber *et al*., 2003; Hu *et al*., 2004; Kambris *et al*., 2006)**.** Gram-positive bacteria are detected through the detection of lysine-containing peptidoglycan (PGN) by PGRP-SA and PGRP-SD (Michel *et al.*, 2001; Bischoff *et al.*, 2004)**.** Fungal infections are recognized by two additional detection systems. First, fungal betaglucans are detected by theGNBP-3 (Gottar *et al.*, 2006) and second, by the pathway that involves a serine protease persephone (PSH).

PGRP-SA/GNBP1, PGRP-SD, GNBP3 or PSH lead to Spätzle cleavage by activating serine protease cascades that converge on two chymotrypsin-like serine proteases, Spirit and the Spätzle processing enzyme (SPE) (Jang *et al.*, 2006; Kambris *et al.*, 2006). SPE cleaves Spätzle directly, while Spirit cleaves and activates SPE. The GNBP3/fungal pathway upstream protease(s) are not yet identified (Gottar *et al.*, 2006; Kambris *et al.*, 2006). Spätzle binds the Toll receptor and induces dimerization. Toll receptor dimerization recruits a Myd88/Tube complex that further recruits the kinase Pelle (Towb *et al*., 1998; Sun *et al*., 2002; Tauszig-Delamasure *et al*., 2002).(Figure 8).

Pelle kinase activation leads to the phosphorylation and ubiquitin/protesome-mediated degradation of Cactus (Fernandez *et al.*, 2001). The degradation of Cactus frees DIF (and Dorsal) to translocate to the nucleus (Belvin *et al*., 1995; Bergmann *et al*., 1996; Groisman, 1998; Wu and Anderson, 1998), and induces immune responsive gene transcription (Irving *et al.*, 2001)

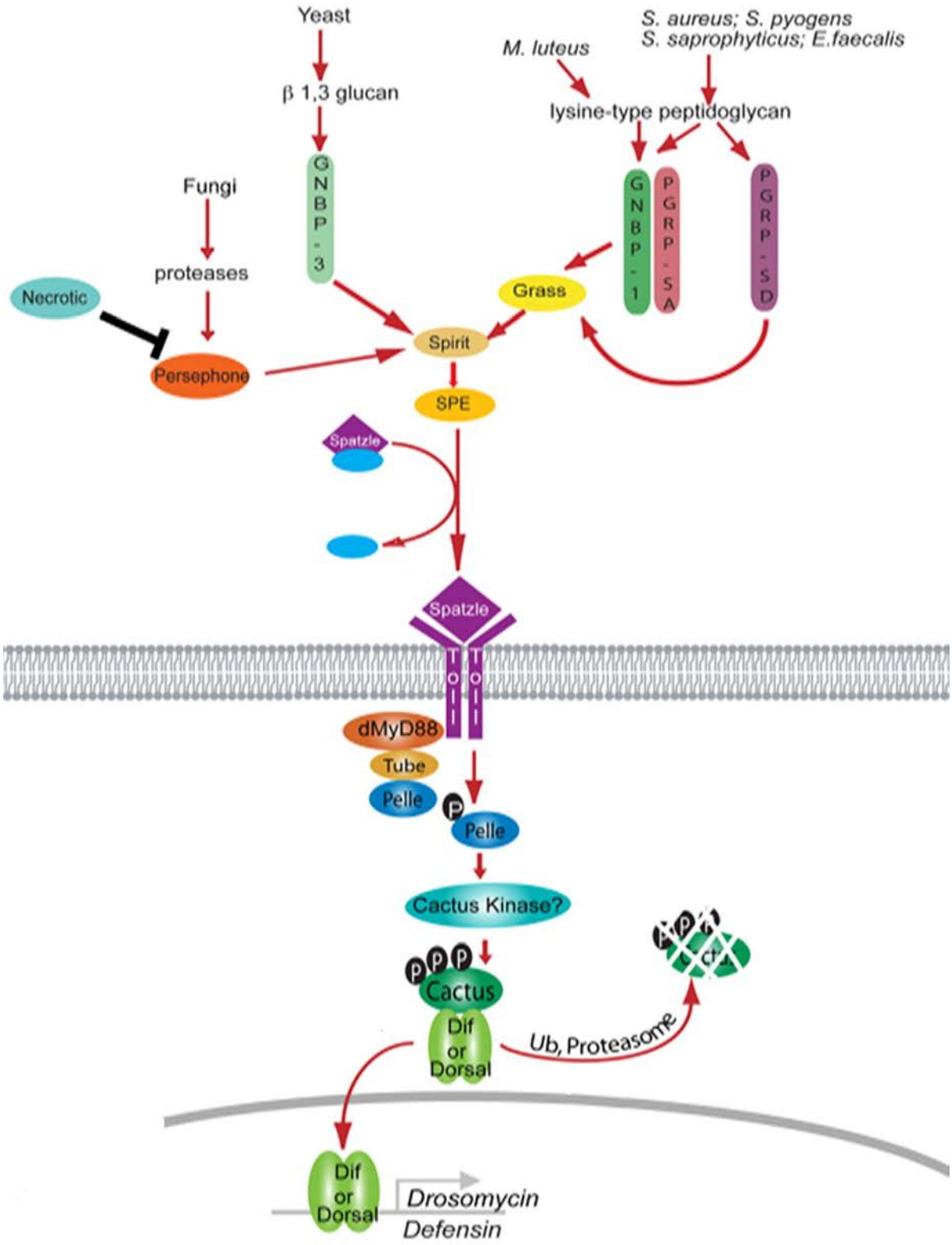


Figure 8: Schematic representation of the Toll pathway (Aggarwal and Silverman, 2008).

1.2.2 The IMD pathway

The IMD pathway is activated by membrane-bound recognition proteins, unlike the Toll pathway, which is triggered by circulating recognition proteins (Choe *et al.*, 2002; Gobert *et al.*, 2003). The IMD pathway is activated by mesminopimelic acid (DAP)-type PGN derived from gram-negative bacteria and certain gram-positive bacteria. DAP-type PGN recognition involves the receptors PGRP-LC and PGRP-LE (Takehana *et al*., 2002; Leulier *et al*., 2003; Kaneko *et al*., 2004).

PGRP-LC encodes three spliced transcripts PGRP-LCa, -LCx, -Lcy (Werner *et al*., 2000). PGRP-LE encodes only one protein that lacks a signal sequence and a transmembrane domain. PGRP-LCx recognizes polymeric PGN. PGRP-LCa and PGRP-LCy are required for monomeric fragment of a DAP-type PGN recognition is a tracheal cytotoxin (TCT) in cells (Kaneko *et al*., 2004; Hetru and Hoffmann, 2009). TCT recognized PGRP-LC or PGRP-LE (Kaneko *et al.*, 2006; Takehana *et al.*, 2004).

Once the pathway is activated, the recognition proteins activate membrane-bound IMD (Choe *et al*., 2005; Kaneko *et al*., 2006), dFADD binds which in turn binds Dredd (Hu and Yang, 2000). Dredd binds and cleaves *relish*, the NF-κB transcription factor in the IMD pathway (Stoven *et al.*, 2000). *Relish* is first phosphorylated and cleaved by the IKK signalling complex (Groisman, 1998). The IKK complex is activated by TAK1 and TAB2 in an IMD dependent manner (Groisman, 1998) (Figure 9).

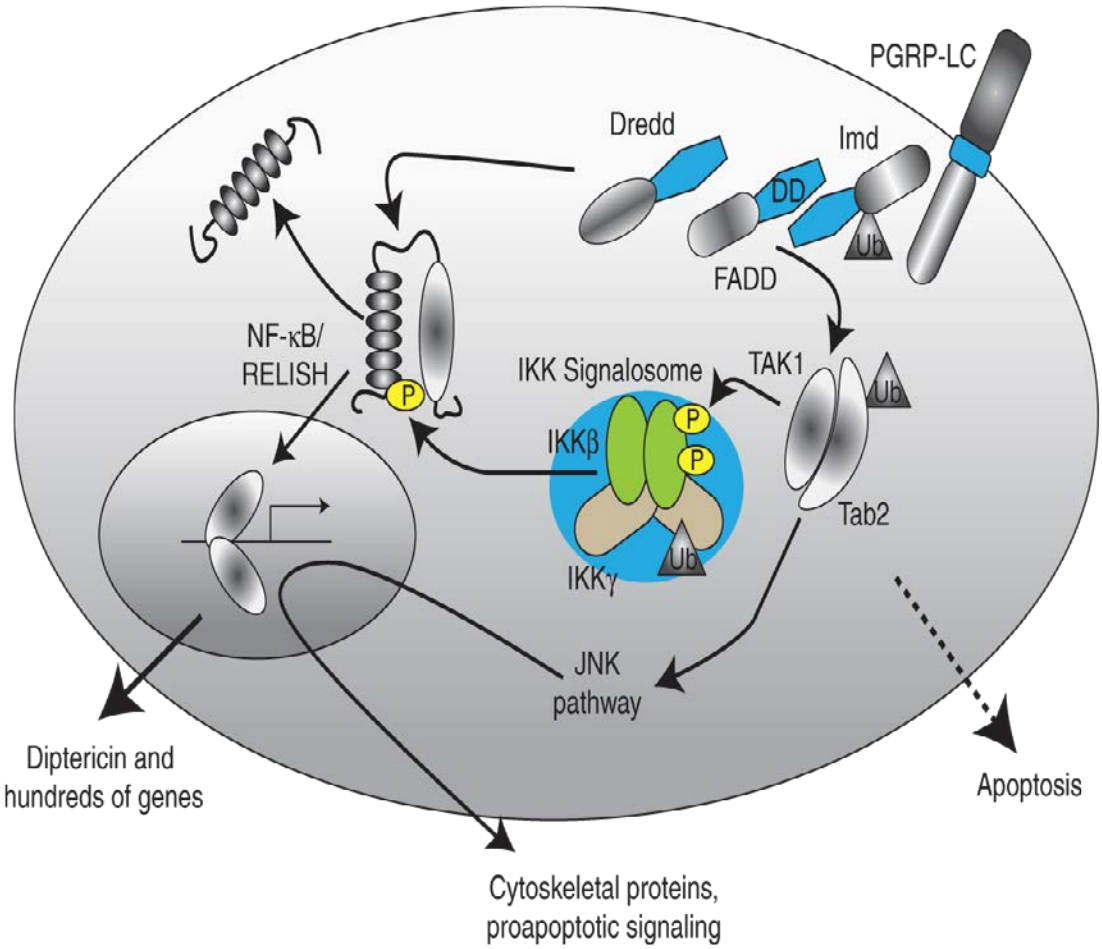


Figure 9: Schematic representation of the IMD pathway (Hetru and Hoffmann, 2009).

1.2.3 JNK signalling pathway

The c-Jun N-terminal Kinase (JNK) is a group of stress activated protein kinases or MAP kinases that respond to stress signals including heat shock, osmotic stress and pro-inflammatory cytokines, growth factors and virus infection (Mittelstadt *et al.*, 2005). The pathway is also involved in the negative feedback control of antimicrobial peptide genes (Kim *et al.*, 2005). The pathway is required in thenegative feedback control of antimicrobial peptide genes. The pathway is also involved in the negative feedback control of antimicrobial peptide genes as defensin D and cecropin A, B and C genes(Mizutani et al., 2003; Kim et al., 2005; Uvell, 2006) and MAP4K3, *β*-Tubulin60D and pims (Kallio et al., 2005).

Activation is by dual phosphorylation by MAPK kinase 4 (MKK4) and MKK7 (Kaneko *et al*., 2004). The MKKs upstream are MAPKKKs, which include MEKKs 1-4, a member of the mixed-lineage kinases (MLK3) and ASK-1 (Kaneko *et al*., 2004). Successively, these are activated by GTP-binding proteins of the Rho family (Ishizaki *et al.*, 1996). The JNKs dimerize and translocate to the nucleus where they phosphorylate transcription factors including c-Jun, ATF-2, Elk-1, and p53. The JNK signalling activation generally results in apoptosis and is involved in inflammation (Kaneko *et al*., 2004) (Figure 10).

1.2.4 The JAK-STAT (*hopscotch*) signalling pathway

The JAK-STAT pathway is characterized by its role in embryonic segmentation (Binari and Perrimon, 1994; Agaisse and Perrimon, 2004). The pathway plays a role in wound healing, viral defence, germ cell sex determination and morphogenesis (Wawersik, 2006). Insect immune response was first identified in *Anopheles gambiae* when a Ag-STAT, a member of the STAT (signal transducer and activator of transcription) protein family and homologous to *Drosophila* STAT92E, was shown to accumulate in nuclei of cells upon immune challenge of the mosquito (Barillas-Mury *et al.*, 1999).

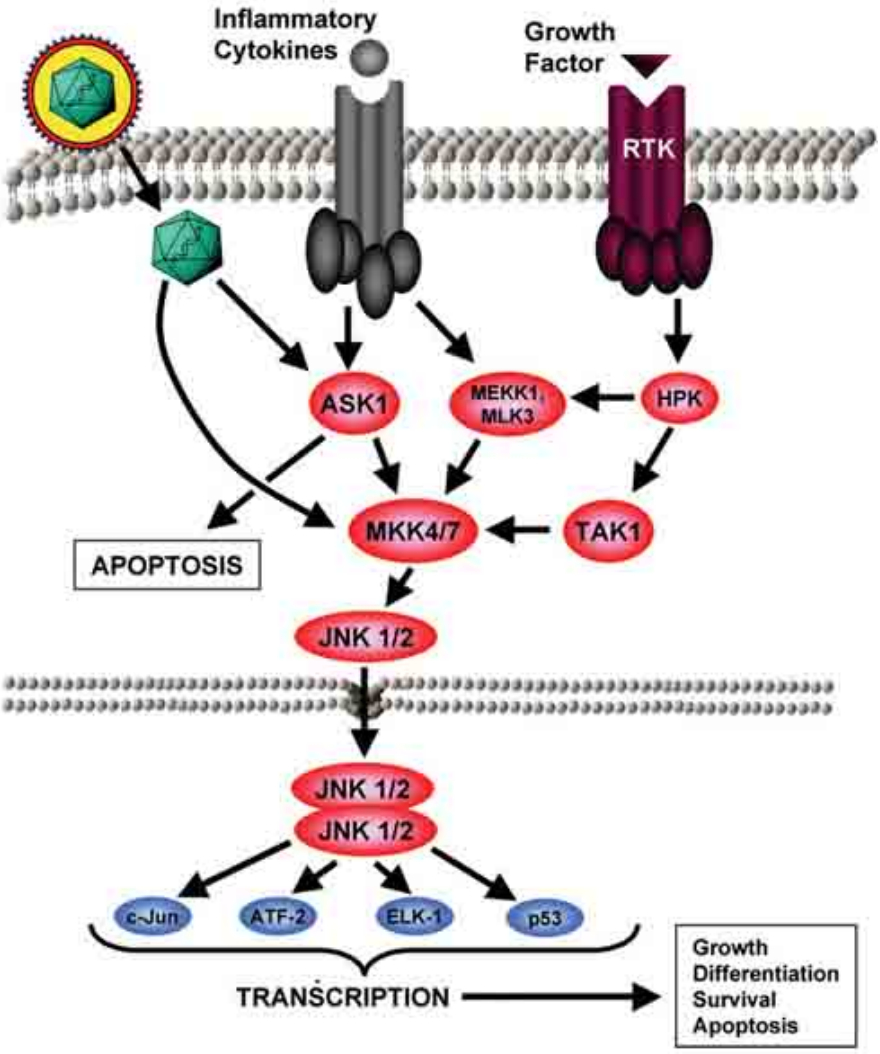


Figure 10: Schematic representation of the JNK pathway (Aurelian, 2005).

The hemocyte releases cytokine Unpaired-3 (*Unpaired (Upd)*-3). Thus JAK-STAT pathway activates by *Unpaired (Upd)*-3 binding to the fat body Domeless (Dome) receptor in cells upon an immune challenge (Agaisse *et al.*, 2003). The binding of an extracellular ligand to a transmembrane receptor results in the activation of the receptor-associated JAKs. These tyrosine kinases phosphorylate themselves and their associated receptors to generate docking sites for the SH2 domains of STATs. STATs are present in the cytoplasm as inactive monomers before recruitment to the receptor/JAK complex. STATs shuttle between the cytoplasm and nucleus before being retained in the nucleus following activation (Vinkemeier, 2004) where it binds to a palindromic region of promoters of downstream genes to activate their transcription (Agaisse and Perrimon, 2004). STAT molecules are phosphorylated and dimerize once bound to the receptor JAK complex. Dimers, stabilized between the SH2 domain of one molecule and the phospho-Tyr of the other molecule, translocate to the nucleus where they bind to a palindromic DNA sequence of the pathway target genes to activate transcription (Tauszig-Delamasure *et al*., 2002).

The JAK-STAT pathway controls expression of TEP1, part of a four-member family of thioester-containing proteins (TEPs) (Lagueux *et al.*, 2000). TEPs appeared early in animal evolution and have been identified in nematodes, insects, molluscs, fish, birds and mammals. In vertebrates, these proteins play important roles in immune responses (Nonaka, 2000). TEPs are a component of the innate immune response of insects to invading microbes (Werner *et al*., 2000).

TEP1 functions as an opsonin, promoting phagocytosis (Levashina *et al.*, 2001). TotA is part of the Turandot (Tot) protein family produced by the larval fat body and has been shown to be upregulated in response to various stress conditions and regulated by JAK-STAT signalling (Agaisse *et al.*, 2003) CG11501 proteins have been shown to be regulated by the JAK-STAT signalling pathway (Boutros *et al.*, 2002) (Figure 11).

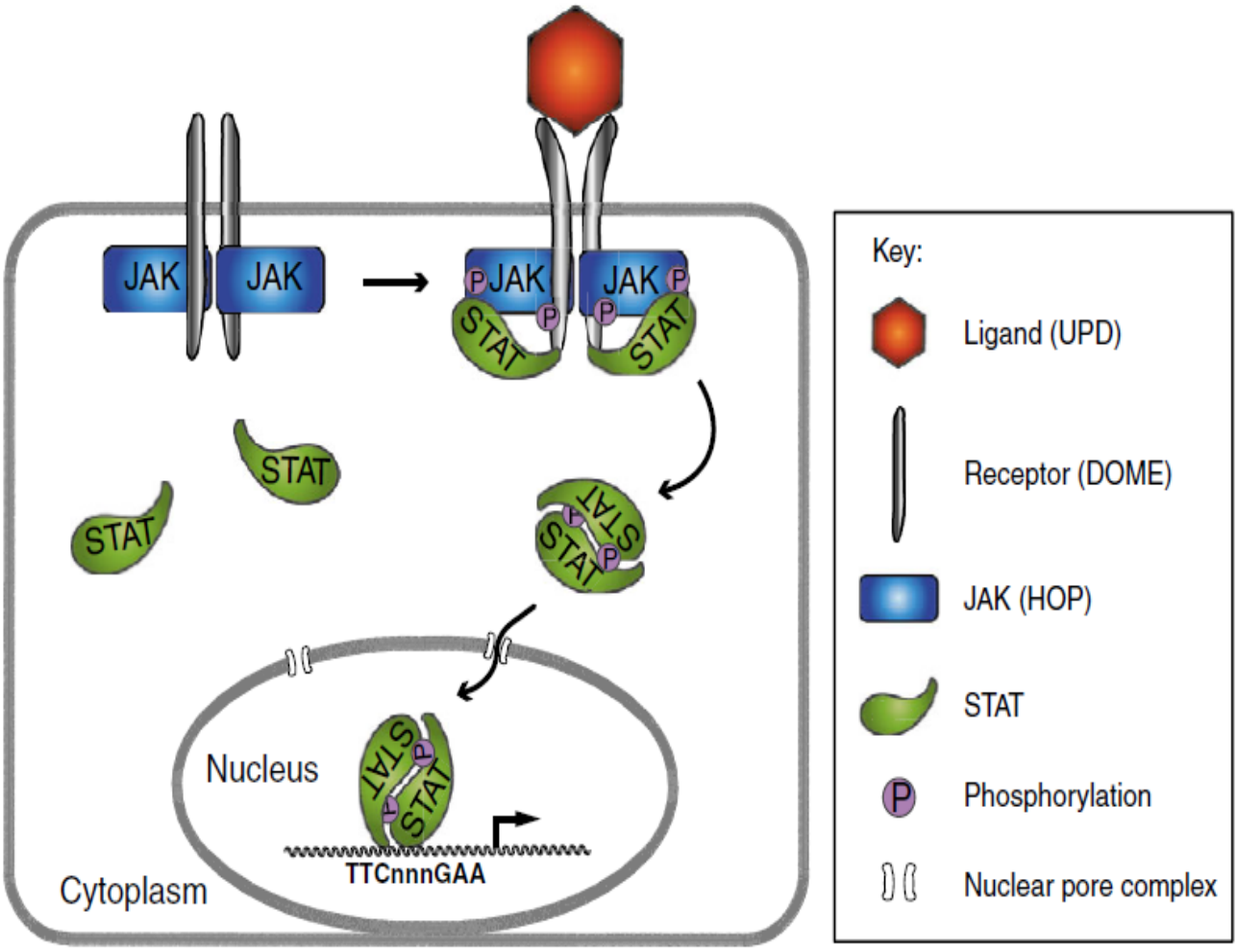


Figure 11: The canonical model of JAK-STAT signalling (Natalia and Martin, 2006).

1.3 Bacterial recognition by PGRPs

Bacterial cell wall pathogen-associated molecular patterns (PAMPs), trigger innate immune responses in the host. Recognition of PAMPs is achieved by germ-line-encoded receptors and soluble proteins (Medzhitov and Janeway, 2002). Two bacterial peptidoglycans (PGs) recognition systems are present in insects, one for the induction of antimicrobial peptides and the other for activating the prophenoloxidase cascade leading to melanization (Michel *et al.*, 2001; Bischoff *et al.*, 2004). Recognition of Lys-type PG by the PG recognition complex activates the serine protease (SP) cascade, leading to the processing of Spätzle (Spz) and subsequent activation of the Toll signalling pathway (Kim et al., 2008a) (Figure 12).

1.4 Antimicrobial peptides

Antimicrobial peptides (AMPs) are produced by the fat body in response to infection. They are small molecules (<10 kDa), with the exception of the 25 kDa Attacin (Imler and Bulet, 2005). Diptericin, Drosocin and Attacin are effective against gram-negative bacteria (Asling *et al.*, 1995). Defensin is active against gram-positive bacteria (Friedrich *et al.*, 2000) and Drosomycin and Metchnikowin are antifungal agents (Ekengren and Hultmark, 1999). Cecropin A1 acts against both bacteria and some fungi (Kylsten *et al.*, 1990) (Figure 13 and Figure 14).

Antimicrobial peptides targete the fundamental difference in the membranes of microbes and multicellular animals (Zasloff, 2002). The bacterial membranes outermost leaflet of the bilayer is covered by lipids with negatively charged phospholipid headgroups. In contrast, the outer leaflet of the membranes of plants and animals is composed mostly of lipids with no net charge. Most of the lipids with negatively charged headgroups are segregated into the inner leaftet, facing the cytoplasm (Matsuzaki, 1999).

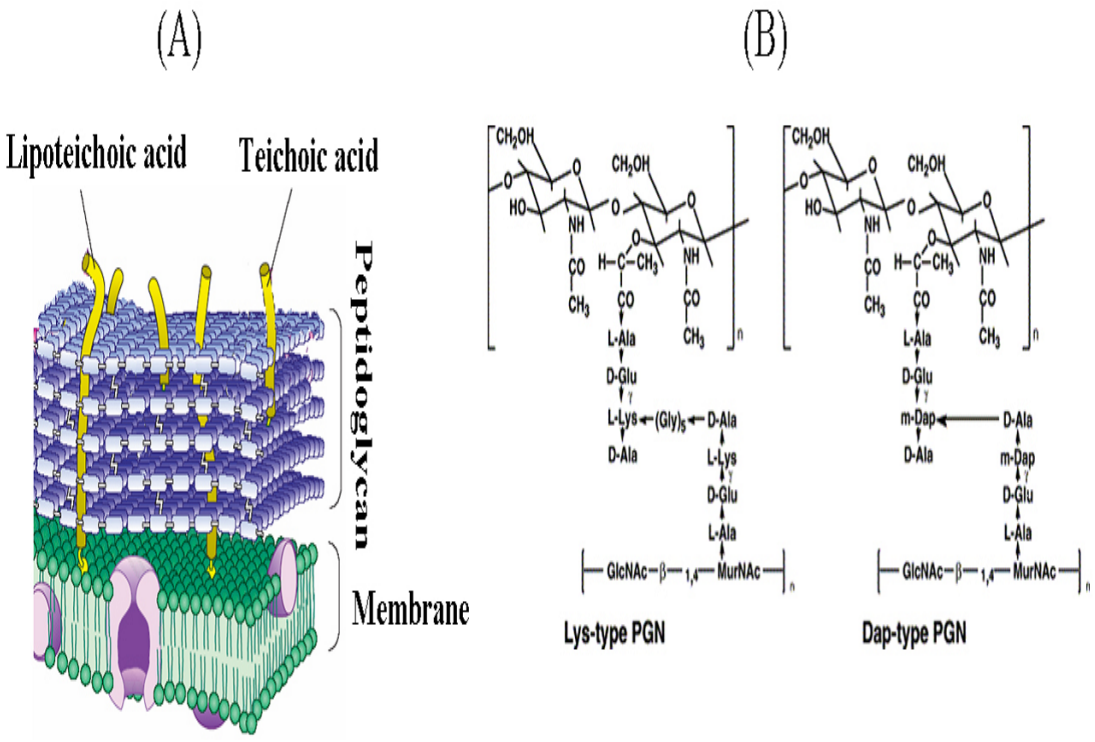


Figure 12: Gram-positive bacteria cell wall (A) and primary structures of Lys-type and DAP-type PG (B) (Kim *et al.*, 2008b).

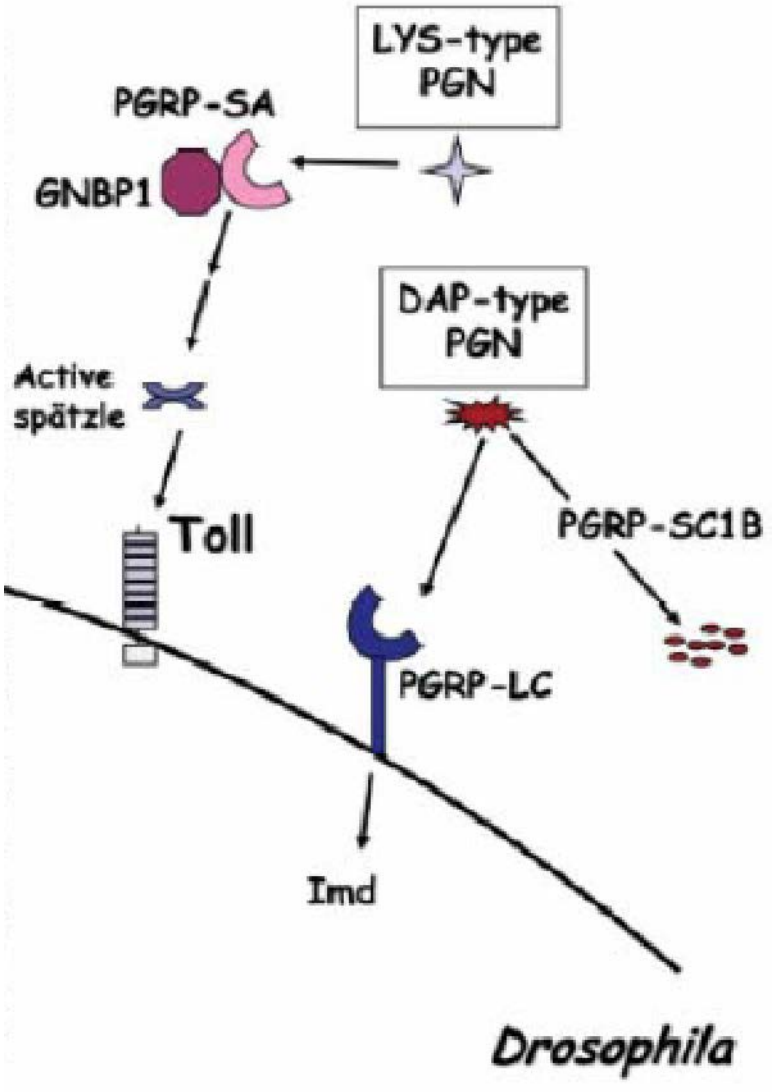


Figure 13: Innate immune recognition of peptidoglycan by *Drosophila* relies on specific detection of DAP type or LYS-type peptidoglycan (PGN) (Girardin and Philpott, 2004).

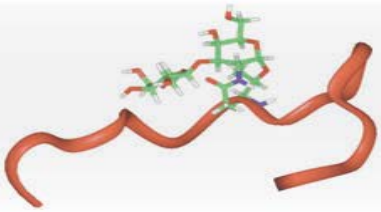
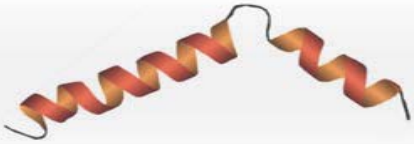
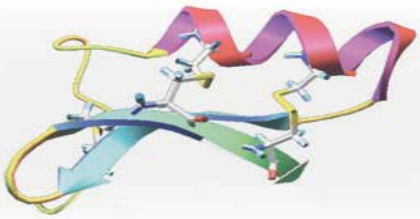
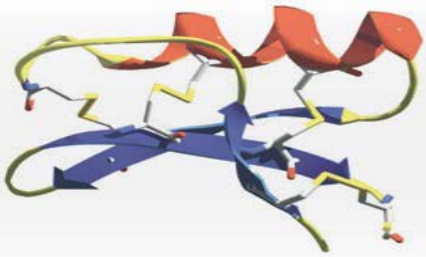
Peptides	# of genes	Main activity	Concentration	3-D structure
Diptericin	2	Gram-negative bacteria	0.5 µM	nd
Attacin	4	Gram-negative bacteria	nd	nd
Drosocin	1	Gram-negative bacteria	40 µM	
Cecropin	4	Gram-negative bacteria	20 µM	
Defensin	1	Gram-positive bacteria	1 µM	
Drosomycin	7	Fungi	100 µM	
Metchnikowin	1	Fungi	10 µM	nd

Figure 14: *Drosophila* antimicrobial peptides. Name, number of genes in the genome, antimicrobial activities, estimated concentration in the hemolymph after bacterial injection, and 3-D structure (Imler and Bulet, 2005).

How do antimicrobial peptides actually kill microbes? Many hypotheses have been presented, which include: fatal depolarization of the normally energized bacterial membrane (Westerhoff *et al.*, 1989); the creation of physical holes that cause cellular contents to leak out (Shai, 1999); the activation of deadly processes such as induction of hydrolases that degrade the cell wall (Bierbaum and Sahl, 1985). The scrambling of the usual distribution of lipids between the leaflets of the bilayer, resulting in disturbance of membrane functions (Matsuzaki, 1999); and the damaging of critical intracellular targets after internalization of the peptide (Kragol, 2001).

The gram-negative bacteria outer membrane is composed of lipopolysaccharides (LPS) which are held together by magnesium and calcium ions that bridge negativelycharged phosphosugars. Addition of cationic peptides results in displacement of the metal. Thus damaging the outer membrane allowing entry of additional molecules from the exterior (Groisman, 1998) . Peptides that have gained access to the periplasmic space integrate into the cytoplasmic membrane (Zasloff, 2002). Cationic peptides are able to interact electrostatically with the negatively charged head groups of bacterial phospholipids and then insert into the model membranes of planar bilayers or liposomes, forming transient channels or pores (Christensen *et al.*, 1988; Silvestro *et al.*, 1997). The channel leads to the leakage of cell contents and eventually cell death (Matsuzaki *et al.*, 1998) (see figure 15).

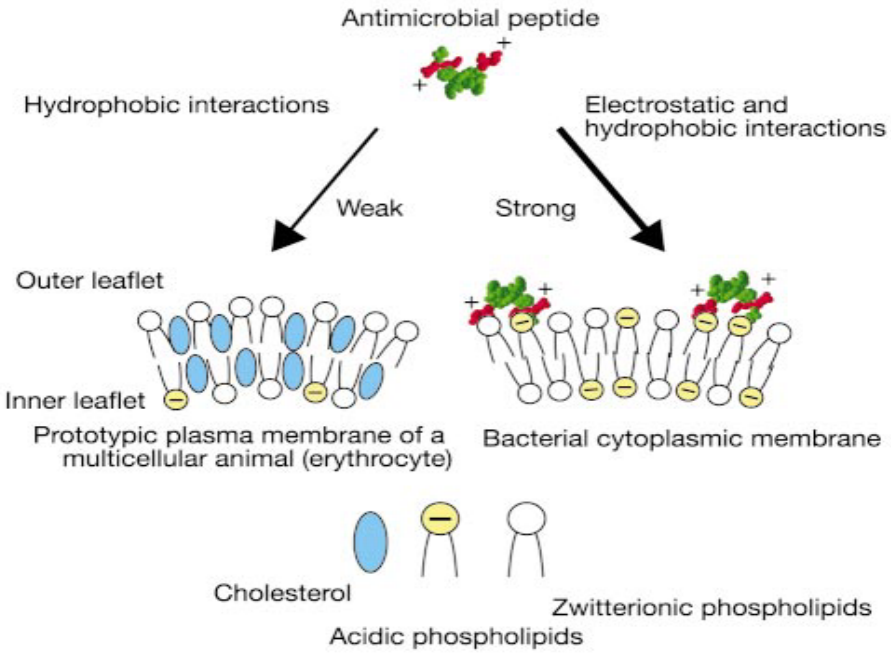


Figure 15: The large positive charges of the antimicrobial peptides enable selective binding to bacterial membranes through electrostatic interactions. Cationic peptides bind to bacterial membranes with abundant acidic phospholipids by the aid of electrostatic interactions. The outer leafets of cell membranes are exclusively composed of zwitterionic phospholipids, for which the peptides show only low affinity. The cholesterol contributes to the resistance of the membranes against the peptides (Matsuzaki, 1999).

1.5 Objectives

The primary objective of this study is to characterize the newly established cell line *Euoniticellus intermedius* (SAEI08) using PCR techniques followed by expression patterns of immune associated gene in all *E. intermedius* life cycles, adult, pupae, larvae, embryo and the embryonic cell line (EISA08). In addition, the study will investigate parameters relating to embryonic and larval development, karyotyping, cell morphological, growth and isoenzyme analysis.

E. intermedius hopscotch and the Ribosomal 9 protein gene (Rps9) genes will be cloned and sequenced in both adult and the embryonic cell line folowed by bioinformatic studies. cDNA will be synthesized for both adult beetles and the cell line (SAEI08). *hopscotch* was selected because it is role in the JAK-STAT signalling pathway and Rsp9 was chosen as a loading control as housekeeping gene

Chapter 2

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2 Materials and Methods

2.1. Materials

Check Appendix 1 for the materials section page 100.

2.2 Methods

2.2.1. Maintenance of dung beetles

Beetles were bred in plastic containers (160 mm x 130 mm x 130 mm) that are three-quarters filled with compact, moist, sandy soil. Each container was supplied with three breeding pairs of *E. intermedius* (three males and three females). Every 3-4 days, fresh cow dung was placed inside the container. The contents of the buckets were sieved after every seven days and the brood balls (each containing one egg) removed.

The brood balls were placed in large plastic containers (400 x 300 x 200 mm). These were covered with damp sand. The box was covered with a wet sponge to keep it moist. Once the beetles emerge (about 3 weeks), small plastic dishes with dung were used as traps to capture them. If the breeding pair survived, they were placed in a new container with fresh soil and dung.

2.2.2 Cell culture

2.2.2.1 Maintaining the embryo *E. intermedius* cell line

In order to further characterise the genome of *E. intermedius*, an embryonic cell line was established by Mr Rodney Hull (personal communication, Flylab), and grown in 25 cm^2 tissue culture flasks (Falcon) containing 5 mL of Schneider medium (purchased from Sigma) for the monolayer culture. Schneider's medium has been found to support the rapid growth of both primary and established cultures of cells derived from *Drosophila* melanogaster. The medium has been used for the growth and maintenance of the cell lineoriginally derived by Schneider from *Drosophila* embryos as well as the culture of cells from other dipteran species (see Table 12, Appendix 1, page 100). Cells were

maintained at an appropriate temperature of 25 ºC with a gas mixture of 5% CO_2 in a cell culture incubator. Schneider *Drosophila* medium was used for routine maintenance. The medium was supplemented with 10% of foetal bovine serum (FBS) as a source of embryonic growth promoting factors and 1% of Penicillin-Streptomycin purchased from Sigma-Aldrich Co. LLC (Solution stabilized, with 10,000 units penicillin and 10 mg streptomycin/mL, suitable for cell culture applications at 10 ml/L.). Medium replacement was done every second day. Cells grow exponentially between $5x10^5$ and 10^7 cells/ml in flasks and were healthiest if kept within this range.

2.2.2.2 Maintaining the embryonic *Drosophila* S2 cell line

The embryonic cells were grown in 25 cm^2 tissue culture flasks (Falcon) containing 5 mL of medium for the monolayer Schneider medium (purchased from Sigma). Cells were maintained at a temperature of 25 ºC and a gas mixture of 5% CO_2 in a cell culture incubator. Schneider *Drosophila* medium was used for routine maintenance sees (see Table 12, Appendix 1, Page 104). The medium was supplemented with 10% of foetal bovine serum (FBS) as a source of embryonic growth promoting factors and 1% of Penicillin-Streptomycin purchased from Sigma-Aldrich Co. LLC (Solution stabilized, with 10,000 units penicillin and 10 mg streptomycin/mL, suitable for cell culture applications at 10 ml/L.). Medium replacement was done every Second day.

2.2.2.3 Cell count

The cell line of the embryonic *E. intermedius* (SAIE08) were grown in five 25 cm^2 tissue culture flasks (Falcon) containing 5 mL of Schneider medium only. Cells were counted using the Trypan blue exclusion method. Cells were washed with PBS pH 7.2 (Lonza, USA), treated with 0.25% trypsin, 0.02% Versene (EDTA) (Lonza, USA) and the clumps dispersed by gentle pipetting. A 1:10 dilution of the cell suspension was made in 0.4% Trypan blue (Sigma, Germany) and loaded

into a haemocytometer. Dead cells took up the dye and appeared blue while live cells did not and, therefore, remained clear. The number of live cells per milliliter of media was obtained by multiplying the average number of live cells (per haemocytometer square) by 10^5.

2.2.2.4 Subculture of cell line

All cell linewere subcultured as per normal tissue culture practices (Phelan, 2003). The cells were pelleted at 2000 × *g* for 5 minutes and re-seeded in the supplemented culture medium at the required cell density. Cells were counted regularly and subcultured to maintain logarithmic expansion of cultures.

2.2.2.5 Freezing of cell line

After every passage, cells were frozen to build a stock of cells. Cultures in the logarithmic phase of growth were centrifuged and resuspended in 46.25% Schneider medium, and 15% glycerol at a density of 1×10^6-10^7 cells/ml. These cells were quickly aliquoted into 2 ml cryogenic vials (Corning, USA), frozen gradually (-1 °C per minute) and stored at -80 °C. To thaw the cells, the vial was immediately transfer to 37C water bath. The cell pellet was resuspended in Schneider medium and seeded as required in a tissue culture flask.

2.2.3 Karyological analysis of the dung beetle *E. intermedius* cell line

The reagents used were fixative (3 parts methanol: 1 part glacial acetic acid), Giemsa stain, DPX mountant, ethanol, and Colcemid (Sigma-Aldrich, Steinheim, Germany, 0.05 µg/ml). The selected cell culture was from log phase of the growth phase. Five millilitres (5 ml) of growth medium was removed from the culture for the use later. The cells were resuspended in 5 ml Schneider medium. Colcemid (final concentration of 0.04 µg/ml) was added and incubated at 37 ºC for 4 hours. Cell suspension was transferred to 10 ml and centrifuged at 1500 rpm for 5 minutes.

Cells were aspirated in a volume of 1.5 ml of medium and 6 ml of distilled water. The procedure was followed by incubation at 37 ºC for 15 minutes, and centrifugation at 1200 rpm for 5 minutes. The entire was medium removed; cells were resuspended in 2 ml fixative and incubated for 15 minutes at room temperature. The suspension was centrifuged at 1200 rpm for 10 minutes.

Note: The length of chromosomes is dependent on the concentration of colcemid and the time of mitotic arrest. The higher the concentration of colcemid and the longer the time of mitotic arrest, the shorter the chromosomes will be. Therefore, it is best to keep the concentration of colcemid and the time of exposure to a minimum. Colcemid will continue to be active until the addition of fixative.

The cell suspension a was taken up in a Pasteur pipette and dropped onto the slides and placed at an angle. Slides were dried and placed into 5 % Giemsa stain for 20 minutes. The slides were dehydrated by rinsing in distilled water mixed with 50 % ethanol and 100 % ethanol. The slides were air dried, ladled. A drop of DPX was mounted onto each slide, and covered with a dry cover slip. Metaphase nuclei were analyzed under visible light microscopy (Carl Zeiss) supplemented with AxioCam (MRm/MRc) camera and AxioVision software package (Carl Zeiss AG, Oberkochen, Germany).at 40 X and 100 X objective.

2.2.4 Microscopic examination of cell morphology

Cells were viewed and images captured using the AxioCam (MRm/MRc) camera and AxioVision software package (Carl Zeiss AG, Oberkochen, Germany). Brigth field optics and phase contrast was used at the magnification X640. This is the simplest and most direct method used to identify the state of cells. Obtaining information about morphology from comparative observations, both at high and low densities of cultures, depends on knowledge of several factors. Morphology varies between lines depending on the health of the cells.

2.2.5 Growth curve analysis

Using an absorbance spectrophotometer to monitor light scattered by non-absorbing suspended cells is common practice in life science laboratories. Such applications, more than any other, accentuate the differences amongst the optical systems of the numerous spectrophotometer designs. To monitor cell growth, NanoDrop, ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA) was used. The 'Cell Cultures' module displays the sample spectrum from 250 nm to 700 nm. One cursor used wavelength for monitoring cell suspensions (600nm) while the second cursor can be set to the wavelength of interest, 280 nm in this project.

2.2.6 Isoenzyme analysis

Isoenzyme analysis was used to characterize the Fylab *E. intermedius* embryonic cell line. Isoenzyme specimens may differentiate in molecular structures based on the electrophoretic mobility of certain enzymes. The *Drosophila* embryonic cell line, S2, was used as the insect cell line control. Isoenzyme electrophoresis analysis seven isoenzymes using the Authentikit (Innovative Chemistry, USA) gel electrophoresis system. The isoenzyme procedure was carried out according to the instructions in the AuthentiKit manual. Ten million cells were pelleted by centrifugation and suspended in 100 µl of cell extraction buffer. The mixture was incubated on ice for 30 minute and then sheared by repeatedly ejecting the suspension through a Pasteur pipette tip. The cell debris was removed by centrifugation. The debris contained the enzymes required for catalyses, RNA, DNA, organelles and some lipids.The supernatant was removed and transferred to another tube. One hundred µl of enzyme was added into tube (100 µl) stabilizer. The enzymes were unstable when removed from their normal environment. To prevent the enzymes from losing activity, the enzyme solutions were kept cold during processing and enzyme stabilizer was added to enzyme solutions at final concentration of 200 mg/mL and stored in the freezer. Prior to electrophoresis, the extract was checked for enzymatic activity present in order to obtain clear and consist measurements (Table 2).

Table 2: Maximum and minimum enzymatic activity levels required for electrophoretic analysis, using 1 µL of sample per well (Ziegenmeyer, 1988).

Enzyme	Maximum Activity at A565 nm	Activity IU/L	Minimum Activity at A565 nm	Activity IU/L
				IU/L
NP	0.5	500	0.1	100
G6PD	0.7	700	0.1	100
MD	0.9	900	0.1	100
MPI	0.6	600	0.1	100
PEP B	0.6	600	0.1	100
AST	0.6	600	0.1	100
LD	1.2	1200	0.1	100

2.2.7 *E. intermedius* RNA isolation from adult, SAIEI08 cell line, pupae, larvae, and embryos

Total RNA was extracted according to the Tri-Reagent manufacturer's protocol based on the Chomcynski (1993) method (Sigma-Aldrich Inc, USA). This reliable technique performs well with samples larger than ~5 mg tissue or 5 x 10^8 cultured cells. TRI Reagent solution combines phenol and guanidine thiocyanate in a monophasic solution to rapidly inhibit RNase activity. A biological sample is homogenized or lyzed in TRI Reagent solution, and the homogenate separated into aqueous and organic phases by addition of chloroform. RNA partitions to the aqueous phase, DNA to the interphase, and proteins to the organic phase. Next, the RNA was precipitated from the aqueous phase with isopropanol, washed with ethanol, and solubilized. The concentration of the RNA solution was determined by measuring the absorbance at 260 nm using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA). The A260/A280 ratio of the RNA is an indication of purity.

2.2.8 cDNA Synthesis

cDNA synthesis was carried out by using the ImProm-II™ Reverse Transcription System (Promega, USA). The first step in the 2 step RT PCR start by combining 1 µl of experimental RNA, 1 µl oligo (dT) (20 mM) and 3 µl RNase-free water were heated at 70 °C for five minutes and incubated on ice for the same amount of time. After the sample was spun down for 10 seconds and put on ice, the second step was carried out by adding to the previously obtained 5µl experimental reaction: 4µl 5x Reaction buffer, 1µl dNTP mix (0.5 mM each; Takara), 4.8 µl $MgCl_2$ (6 mM; Takara), 1 µl Reverse Transcriptase, 4.2 µl RNase free water. The 20 µl RT PCR reaction mix was placed in a controlled heat block at 25 °C for 5 minutes, followed by 42°C for one hour. To inactivate the reverse transcriptase samples were incubated at 72 °C for 15 minutes. The 20 µl sample was used as the DNA template for conventional PCR.

2.2.9 Polymerase Chain Reaction (PCR)

The Perkin Elmer (GEneAmp PCR System 2400, USA) and Bio Rad (MJ Mini, USA) were utilized. Primers, Hop27me Rps9designed and delivered by Inqaba Biotech (Pretoria, SA) were diluted to 10 µM/µl working solution (see Table 4).

The reaction consisted of 1x PCR buffer (10 mM Tris-HCl, 50 mM KCl; pH 8.3), 1.5 mM $MgCl_2$, 3 µg template DNA, 200 µM dNTPs, 0.2 µM of each primer, and 0.5 units *Taq* DNA polymerase. The final volume was made upto 50 µl with sterile distilled water. The following protocol was used: 40 cycles at 94 ^{o}C for 30 seconds (denaturation), 55 ^{o}C for 1 minute (annealing) and 72^{o}C for 45 seconds (extension). The amplification cycles were preceded by 95^{o}C PCR denaturation for 5 minutes and followed by 10 minutes of a final extension step at 72 ^{o}C. Following the reaction, the PCR product was then qualitatively analyzed on a 1% agarose gel.

2.2.10 PCR Fragments Purification:

PCR fragments purified using GenElute™ Agarose Spin Columns (Sigma-Aldrich) by centrifugation/filtration. A slice of agarose gel containing the PCR fragments placed into a spin column, and spun at maximum speed (13000 rpm) in a microcentrifuge. Agarose is retained on a filter in the spin column and soluble molecules, including DNA, pass through the filter and collect in a microcentrifuge tube.

2.2.11 Ligation

Purified PCR products were ligated into the pGEM®-T Easy or pJET1.2 cloning vectors using the pGEM®-T Easy vector pJET1.2 system cloning kit. Both systems use T4 DNA ligase to ligate the PCR product (containing TA overhangs) into the TA cloning vector. Ligation of the *hopscotch* and Rps9, with 380 bp and 380 bp, respectively was carried out for the *E. intermedius* adult and embryonic cell lines.

2.2.12 Transformation

The ligation reaction mixture of 10 µl contained approximately 50 ng of vector with insert added to 90 µl of competent *E. coli* cells. The mixture was incubated on ice for 20 minutes and heat shocked for 10 minutes at 37 ºC, which caused the cells to take up of the recombinant plasmids. One ml of the transformed cells was mixed with 900 µl LB and was shaken gently for 1 hour at 37 ºC to allow for regeneration. Seventy µl of transformed cells were then spread on LB-appropriate antibiotic plates to select for those cells that took up the resistant recombinant plasmid. The plates were then incubated overnight at 37 ºC.

2.2.12.1 Preparation of Competent cell

Chemically competent *E. coli* XL1-Blue cells were prepared by following a slightly altered procedure designed by the Fred Hutchinson Cancer Research Centre. A single bacterial colony was used to inoculate 5 ml of LB and incubated overnight at 37 °C with vigorous shaking. Following incubation, the starter culture was diluted 100 times with 250 ml LB and supplemented with Ampicillin as the antibiotic. The culture was shaken at 37 ºC until an OD600 of between 0.5 and 0.6 was reached, at which point they were transferred to GSA bottles and centrifuged at 5000 x g for 10 minutes at 4 ºC. The supernatant was removed and the pellet resuspended in 100 ml of ice cold 100 mM $MgCl_2$. This was followed by 30 minute incubation on ice after which the cells were again pelleted by centrifugation at 4000 x g for 10 minutes at 4 °C. The supernatant was discarded and the cells were resuspended in 10 ml ice-cold 100 mM $CaCl_2$ containing 15% glycerol. The now competent cells were aliquoted into small pre-cooled eppendorf tubes and stored for a time period not exceeding 6 months, at -70°C.

2.2.13 Selection of transformed cells and positive clones

E. coli XL1-blue bacterial cells transformed with recombinant pGEM-T Easy plasmid were selected on LB agar plates supplemented with 100 μg/ml ampicillin, 12 μg/ml tetracycline, 25 μg/ml X-gal and 100 mM IPTG. Cells containing recombinant clones were white in color, due to the disruption of the β-galactosidase coding region. The β-galactosidase gene produces an enzyme that catalyzes X-gal breakdown and cells carrying the non-disrupted gene turn blue when grown on a plate containing X-gal.

2.2.14 Small scale preparation of plasmid DNA (Miniprep)

The procedure for small scale preparation of plasmid DNA was derived from that published by Birnboim (1983). A single bacterial colony containing the desired plasmid was used to inoculate 2 ml of LB, supplemented with the appropriate antibiotic, and incubated at 37 ºC overnight with vigorous shaking. The next day, cells were harvested by centrifugation at 13 000 x g for one minute. The cells were resuspended in 100 ml of Miniprep solution I and then 200 ml Miniprep solution II, mixed, and incubated for 3 minutes at room temperature. One hundred and fifty millilitre (150 ml) of Miniprep solution III was then added, mixed, and incubated on ice for 20 minutes. Following incubation, the mixture was microfuged at 13 000 x g for 5 minutes and the supernatant retained. RNA was removed through the addition of 50 μg RNaseA and incubation for 5 minutes at room temperature.

To remove protein contaminants an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the solution, mixed, and microfuged for 5 minutes at 13 000 x g. The aqueous layer was transferred to a fresh tube and an equal volume of chloroform was added and mixed, before microfuging at 13 000 x g for 3 minutes. Once again, the aqueous layer was transferred to a fresh tube and 2.5 X the volume of 25:1 ethanol acetate was added, mixed by

inversion, and placed at -70°C for 20 minutes. The mixture was then microfuged at 13 000 x g for 10 minutes, the supernatant aspirated, and the pellet resuspended in 500 ml of 70% ethanol. The DNA was once again pelleted by centrifugation for 10 minutes at 13 000 x g at 4 °C, and the supernatant aspirated. The pellet was allowed to dry at 55 °C for 10 minutes and then resuspended in 80-150 ml of distilled water, depending on the size of the pellet.

2.2.15 Restriction endonuclease digestion of plasmid DNA

The restriction analysis reaction was prepared according to the manufacturer's instructions. Digestion with restriction enzymes was carried out as described (Ausubel *et al.*, 1995). Restriction enzyme digestion was assembled by adding plasmid DNA 2 µg, restriction enzyme 1 µl, 1 µl 10x buffer and 6 µl nuclease-free water. Reaction was incubated on ice in 0.5 ml tubes for 10 min. The mixture incubated at 37°C for 16 hours then immediately heat-inactive at 65°C for 15 minutes. The digestions were analysed by agarose gel electrophoresis. *Eco*RI, *Xho*I and BglII were used throughout this project according to the pGEM®-T Easy or pJET1.2 cloning vectors (see Appendix 1, page 100 and Appendix 2 page 106). Restriction enzyms cute sites are shown in the Figure 16. *Eco*RI can cut the pGEM®-T Easy in both sides and the *Xho*I and BglII can cut the pJET1.2 from both sides.



Figure 16: Restriction enzymes recognition site used in the project

Chapter 3

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3 Results

The dung beetle, *E. intermedius*, used in this project was bred in plastic containers filled with compact, moist, and sandy soil. In order to study cell characterisation, an embryonic cell line was established from early embryonic *E. intermedius* cells, the EISA08 cell line, as is named in this study. The cell line represents the South African *E. intermedius* which was established in 2008 in the Flylab at University of the Witwatersrand. The cells were grown in 25 cm^3 tissue culture flasks under lab conditions.

In this study, the cell line was characterized and investigated at the molecular level for the first time. The study shows that the embryonic cell line represents neural stem cells. The cells divide dramatically and asymmetrically due to the early expression of UD-3 which activates the JAK/JAK/STAT pathway mediated by the *hopscotch* gene expression in all the dung beetle *E. intermedius* developing stages. The *hopscotch* gene is over expressed in the early stages of *E. intermedius*, in the embryo, larvae, and the pupae. The JAK/STAT transduction pathway could play an important role in *E. intermedius* immunity cell division and embryonic development.

Although many insect cell linehave been created over the past three decades, few of these have been from the order Coleoptera (Lynn, 2007). In fact, a large numbers of the over 600 insect cell lineare derived from lepidopteran and diptera (Lynn, 2007). Twenty nine cell linehave been established from eight species of Coleoptera insects belonging to the families *Scarabedidae* (Mitsuhashi, 2003), *Chrysomelidae* (Long *et al.*, 2002), *Curculionidae* and *Cerambycidae* (Iwabuchi, 1999). More Coleoptera cell lineshould be established to enable the study of insects in this order. Therefore, the generation of an additional established cell line from the dung beetle, *E. intermedius* embryo, would contribute to both insect physiology and biology.

Insect cell linehave contributed to the progress in physiological studies on the species from which they are derived (Hoshino *et al.*, 2009). They can be used in numerous areas of biological research, including physiology, toxicology, and pathology. They have proven useful for the production of recombinant proteins and for propagation of specific pathogens of interest (Hoshino *et al.*, 2009).

Complete characterization of the *E. intermedius* cell line is essential for the cell banks and for the regulatory requirements in biopharmaceutical production. Characterization of this cell line provides information regarding the lineage or speciation of the cell line, genetic stability of cells, and cross-contamination of cell lines. This cell line was characterized using morphology, karyotype, cell growth, and isenozyme analyses. In addition, the project used information and comparison of the *hopscotch* and ribosomal protein 9 (Rps9) genes in *E. intermedius* and its cell line (SAEI8). Furthermore, expression of the *hopscotch* gene in the embryo, larvae, pupae, and adult and cell line was performed.

The evolutionary conserved Janus kinase (JAK)/Stat signal transducer and activator of transcription (STAT) cascade plays a key role in a wide variety of biological processes (Arbouzova and Zeidler, 2006). In addition to its function in embryonic segmentation, the HOP/STAT92E pathway is also involved in several other developmental events, including sex determination, polarity determination in the eye, and imaginal tissue formation (Hou and Perrimon, 1997; Dearolf, 1999; Zeidler et al., 2000b). The JAK-STAT pathway was shown to partly contribute to the antiviral response in *Drosophila* (Dostert *et al.*, 2005).

Based on sequence similarity, it was predicted that the conserved signalling pathway in the beetle is composed of the orthologs of *Dm*-Domeless, *hopscotch* and STAT92 (Iwanaga and Lee, 2005). This pathway, triggered by a cytokine-like molecule, is able to activate multiple noncytolytic intracellular antiviral pathways that can interfere with many steps in the life cycles of virus, thereby

limiting the amplification and speed of the virus and attenuating the infection (Guidotti and Chisari, 2001). After the cells are activated, signal transduction through the system will induce the expression of hundreds of genes (de Veer *et al.*, 2001; Ehrt *et al.*, 2001). These self-amplifying systems can also be triggered directly by viral components (Liu *et al.*, 2009). This pathway was first identified in interferon systems and responses to a wide range of cytokines and growth factors (Campos-Ortega and Hartenstein, 1985). The JAK-STAT pathway is used to control cell proliferation, differentiation, survival, and apoptosis during diverse growth and developmental processes in multiple tissues (Williams *et al.*, 2000; Tsai and Sun, 2004; Cabral-de-Mello *et al.*, 2008; De Arcanjo *et al.*, 2009). This pathway also plays a crucial role in tumorigenesis (Aurelian, 2005; Mbayu and Ntwasa, 2010). The JAK-STAT signalling cascade is essential for several biological processes, including the control of haematopoiesis and immune responses, as well as for cellular homeostasis and embryonic development (Hombria and Brown, 2002).

The JAK-STAT pathway is highly conserved in the evolution from invertebrates to humans. The activation mechanism of the JAK-STAT pathway is shared by invertebrates and humans (Gronholm *et al.*, 2010). In general, activation of the JAK-STAT pathway entails the binding of an extracellular ligand to a transmembrane receptor, which causes the activation of the receptor-associated JAKs. These tyrosine kinases then phosphorylate themselves and their associated receptors to provide docking sites for the STAT transcription factors. After activation by the receptor-JAK complex, STATs translocate to the nucleus and regulate the expression of target genes.

The main components of the pathway are: the ligand Unpaired, *Unpaired (Upd)*3, which promotes phagocytosis and participates in an antiviral response, the receptor domeless (Dome), the non-receptor tyrosine kinase JAK *hopscotch* (one gene as opposed to four mammalian genes), and the normally cytosolic STAT (STAT92E/Marelle; one gene as opposed to seven in mammals (Royet *et al.*, 2005). Diapause in insects refers to developmental arrest (Tanigawa *et al.*, 2009). Many

insects remain in diapause to avoid unfavourable climatic conditions. Insects may enter diapause during any of the developmental stages, i.e., adult, pupa, larva, or egg, and each species enters diapause at fixed stages. Different stages of entry into diapause have been reported in insect eggs (Danks, 1987). It would be interesting to know how development is arrested at an early stage before cell differentiation. *E. intermedius* offspring activates from its emergence until September, staying underground without feeding, from December (see Figure 17). *E. intermedius* diapause or the developmental arrest reduces the availability of the adult dung beetle throughout the year.

3.1 The embryonic development of *E. intermedius*

Insect embryos share a conserved embryonic stage called the segmented germ-band in which the elongated embryo shows segmentation of both the ectoderm and the mesoderm (Sander, 1983). Most insects develop as short germ-band embryos, where only the anterior most segments are specified at the blastoderm stage (usually head and thorax). While long germ-band development is seen as evolutionary highly derived (Sander, 1976). The short germ-band mode of embryogenesis is regarded as basal and is observed in most arthropods. As short germ-band is found throughout the insects, whereas the long- germ type is restricted to the higher insects, it is likely that a form of short or intermediate germ-band segmentation is evolutionarily ancestral (Davis and Patel, 2002).

The more posterior segments are formed in an anterior to posterior succession from a posterior growth zone. This mode of segmentation is believed to be ancestral (Davis and Patel, 2002). By contrast, long germ insects specify all segments during the blastoderm stage. At the molecular level, segmentation is well-understood only in the long germ insect *Drosophila melanogaster* (Pankratz and Jackie, 1990). Beetles are the only holometabolous insect order with definite short germ-band development (Sander, 1976; Schwalm, 1988; Davis *et al.*, 2002). Embryogenesis in the dung beetle, *E. intermedius* (as suggested in this study), represents a more ancestral form of embryogenesis in insects.

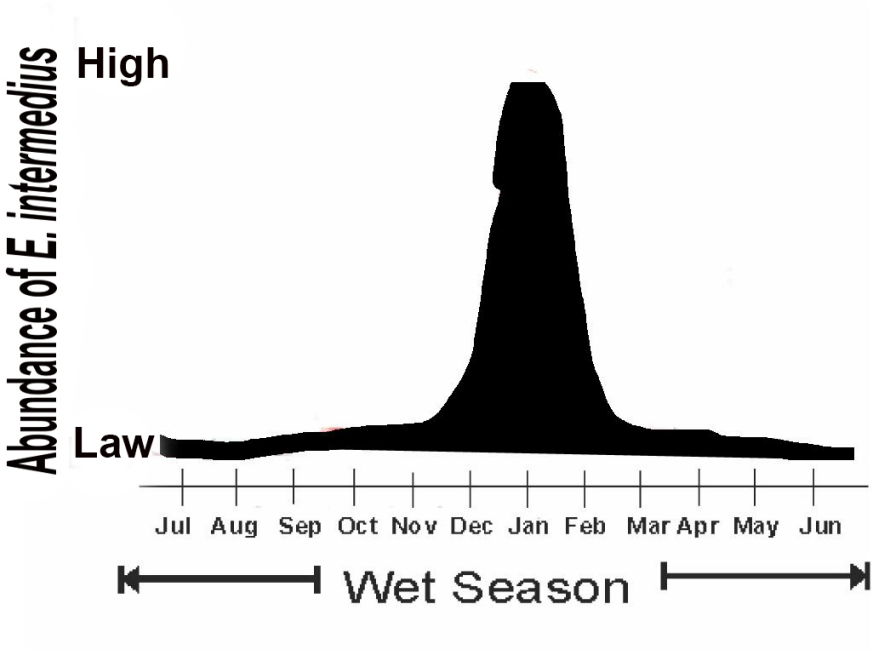


Figure 17: Typical activity pattern of dung beetle *E. intermedius* with a short development period in Johannesburg, South Africa.

3.1.1 *E. intermedius* embryonic development described as short germ-band

Adult *E. intermedius* were collected during the month of September from the south of Johannesburg, Grasmere farm. The dung beetles were maintained in plastic containers filled with compact, moist, and sandy soil, under laboratory conditions. The life cycle with 3 larval stages is complete within 6 weeks at 26 °C. The lifespan of the adult's lasts upto 2 months. *E. intermedius* behaviour and life cycle development was observed by carefully opening the brood ball, and monitored using a dissecting microscope. To observe the embryonic development, *E. intermedius* eggs were collected and dipped in halocarbon oil 700 from Sigma. Development was monitored and documented Development was monitored and documented the AxioCam (MRm/MRc) camera and AxioVision software package (Carl Zeiss AG, Oberkochen, Germany).

This study described the embryogenesis of *E. intermedius* (non-stained embryo, see Figure 18) in comparison with *Tribolium* embryogenesis (embryo whole mounts subjected to a variety of histochemical stains). *Tribolium* and *E. intermedius* (according to this study) develops as a short germ-band embryo (Schroder *et al.,* 2008). Unlike the long germ-band embryo of *Drosophila*, where all body segments form almost simultaneously at the blastoderm stage, segmentation in *Tribolium* occurs gradually. Only anterior segments are determined at the blastoderm stage in *Tribolium* as well as in the *E. intermedius*. After the initiation of gastrulation, more segments are added progressively from the anterior to the posterior dependent on the action of the posterior growth zone (Schroder *et al.,* 2008).

The beginning of insect embryogenesis is characterized by processes generally known as egg activation and maturation, syngamy and karyogamy (Zissler, 1992) . As karyogamy is being

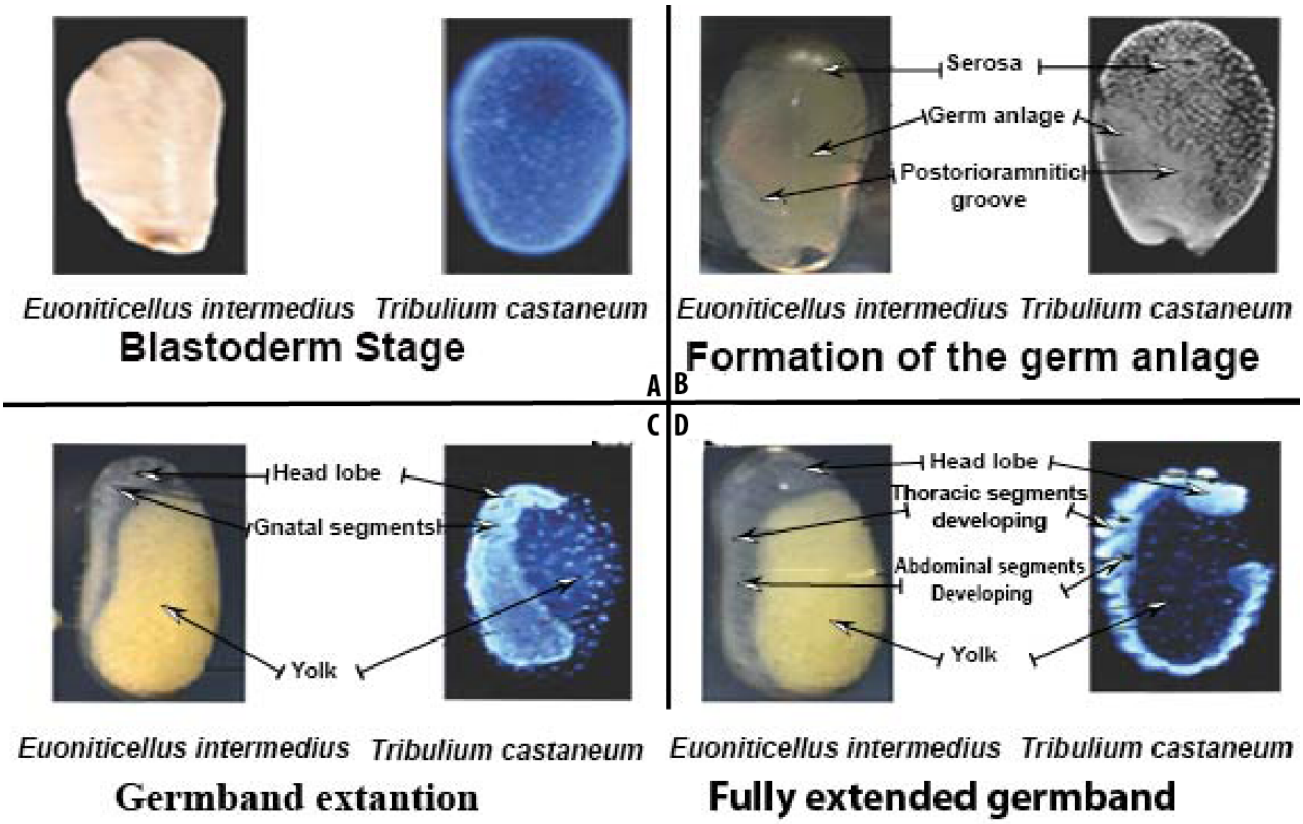


Figure 18: Early embryonic development comparison in non-stained *E. intermedius* and stained *Tribolium.*

completed, the zygotic nucleus undergoes a series of synchronous nuclear divisions (Figure 19) a process somewhat equivalent to cleavage in other animal phyla, although no cell membranes are formed in insects. Nuclei migrate to the periphery (Figure 19, 2A) to form the syncytial and then cellularized blastoderm.

In a dramatic departure from long germ behaviour, the *Tribolium* (as in the *E. intermedius* embryo) undergoes dynamic cellular reorganization to shape the embryonic rudiment. A posterio-lateral invagination, the primitive pit, forms as cells aggregate in the ventral, posterior region of the egg. This field of aggregating cells forms the embryo, whereas the remaining peripheral cells form the extra embryonic covering. The ventral aspect of the primitive pit becomes the caudal end of the embryo while the dorsal side, contributes to the amnioserosa which eventually envelopes the embryo ventrally. The condensing embryonic primordium slides along the ventral side of the egg and the primitive pit moves anterio-ventrally away from the posterior pole. As the caudal end of the primordium sinks into the yolk, the gastral furrow forms in a posterior-anterior progression to generate the mesoderm. At the same time, the amnioserosa encloses the ventral side of the embryo in a posterior to anterior direction. Finally, the head lobes form as the amnioserosal layer folds over from the lateral, anterior edges.

The resulting embryonic rudiment occupied greater than 80% of the egg length on the ventral side, with anteriorly placed head (protocephalic) lobes and a long protocorm sunk deeped in the yolk. During the subsequent phase of embryogenesis, this rudiment elongated and differentiated into a segmented germ-band. As the cephalic region developed, it extended around the anterior pole of the egg. Simultaneously, the protocorm elongated and eventually reached around the posterior pole. Similar to *Drosophila*, when the germ-band is fully extended the anterior-most portion of the embryo lied adjacent to the caudal end during germ-band elongation the embryo showed overt

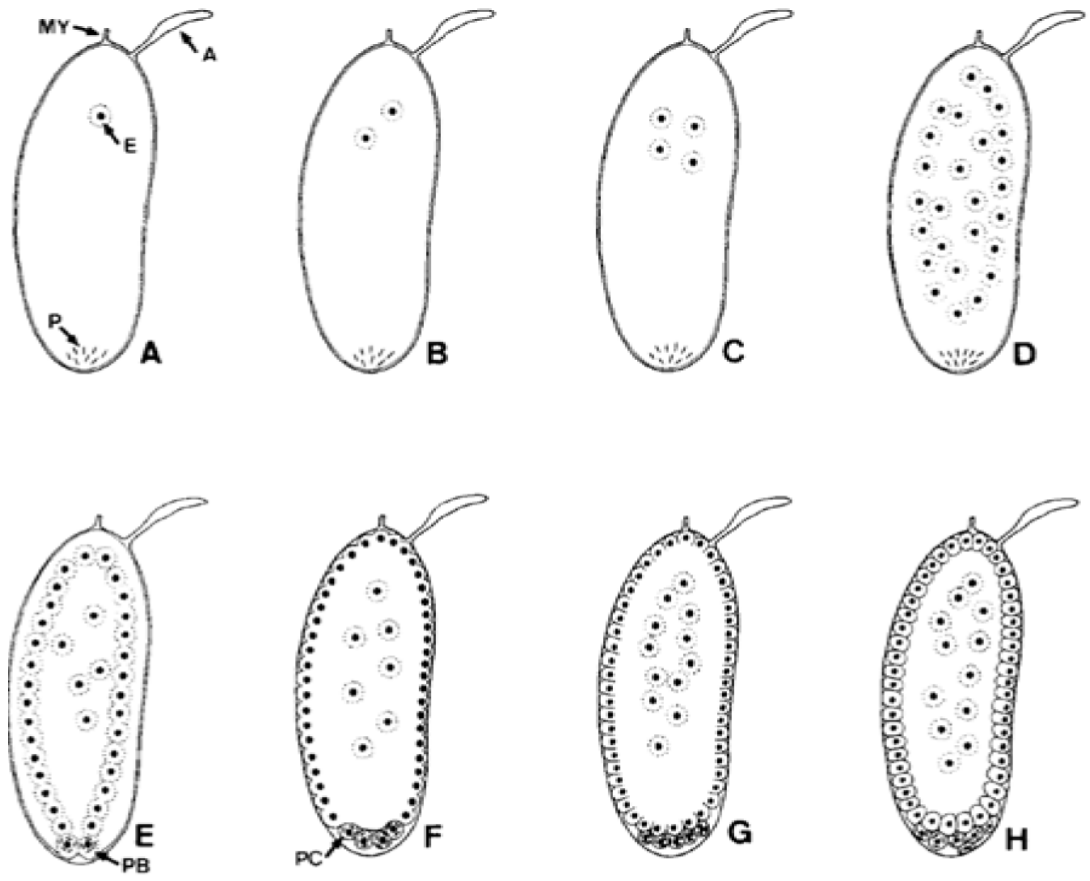


Figure 19: *Drosophila melanogaster* schematic drawing of early embryonic stages. Anterior, top. Modified from Zalokar (1976) and Foe and Alberts Alberts (Foe and Alberts, 1983) A Fertilized egg. D-E The first seven mitotic divisions (cycles); in D, illustrated are only 26 of the 128 energids present in the interior of the yolk system. E**:** Somatic cell nuclei undergo The 9th cycle. Energids migrate to the cell surface. In the posterior egg region, polar buds are formed. F Syncytial blastoderm. Pole cells are pinched off. G Interphase of cycle 14. H. Cycle 14. The blastoderm is formed and the cells of the pole are divided. PB, polar bud; PC, pole cell; E, energid; MY, micropylar cylinder; P, polar granules; A, respiratory appendage.

signs of segmentation. Soon after the germ anlagen formed, the mandibular and maxillary segments were discernible just posterior to the protocephalon. Segmentation occurred quite rapidly in an anterior-posterior progression through the thoracic region. Next the germ-band retracted until the embryo is the same length as the egg. During germ-band extension and retraction appendage buds form on particular segments. Thereafter, the embryo grew dorsally to enclose the remaining yolk. The dynamic process of germ anlagen formation and sequential segmentation exhibited by *Tribolium* and *E. intermedius* provided a context different from *Drosophila* within which to assess the function of homeotic and segmentation gene homologs.

The results obtained shown in Figure 18, indicated that the dung beetle *E. intermedius* is described as short germ type. Only segments of the head are specified in the blastoderm, whereas the remaining segments of the thorax and abdomen form progressively from a posterior growth zone following gastrulation.

3.1.2 *E. intermedius* larval development

The life cycle of *Euoniticellus intermedius* is shown in the Figure 20. Females can produce between 75 and 127 eggs during the two months life time (Tyndale-Biscoe, 1978). An embryo develops inside the egg and undergoes three larval instars once it emerges from the egg. The dorsal expansion ('hump') and caudal flattening of the *E. intermedius* larvae is functionally related to movement within a confined, spherical space (see Figure 21, Figure 22 and Figure 23). This enables the larvae to repair damage to the brood-mass wall with liquid faeces, manipulated with its legs and mandibles. The pupal stage begins when the larvae encase themselves in a cocoon-like structure. Newly moulted pupae are entirely white and lie head down in the pupal chamber. This stage begins when the larvae encase themselves in a cocoon-like structure. Newly moulted pupae are entirely white and lie head down in the pupal chamber. The pupal stage lasts 10-15 days. After which the young beetle

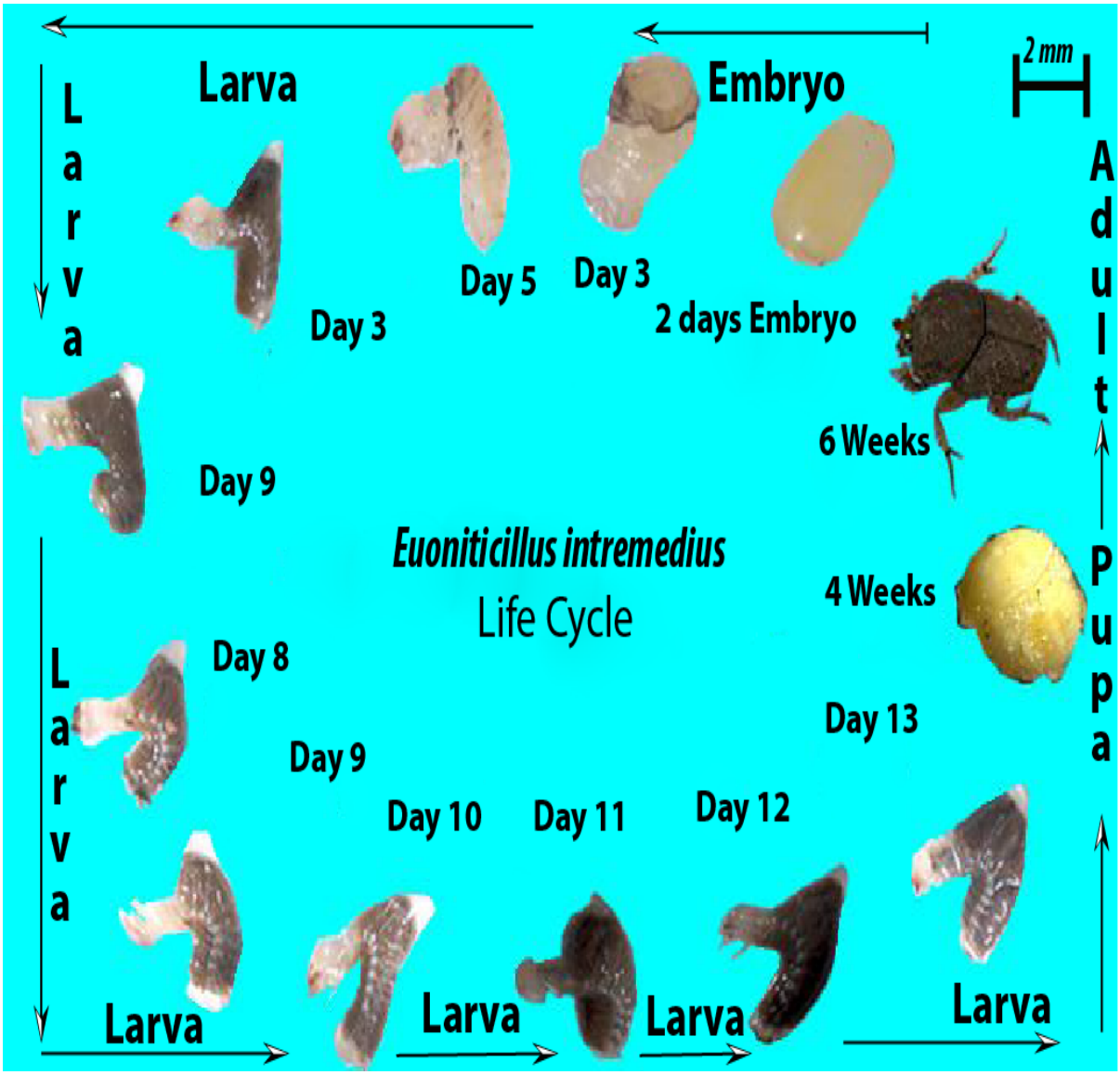


Figure 20: *E. intermedius* (Coleoptera: *Scarabaeidae*) life cycle stages.

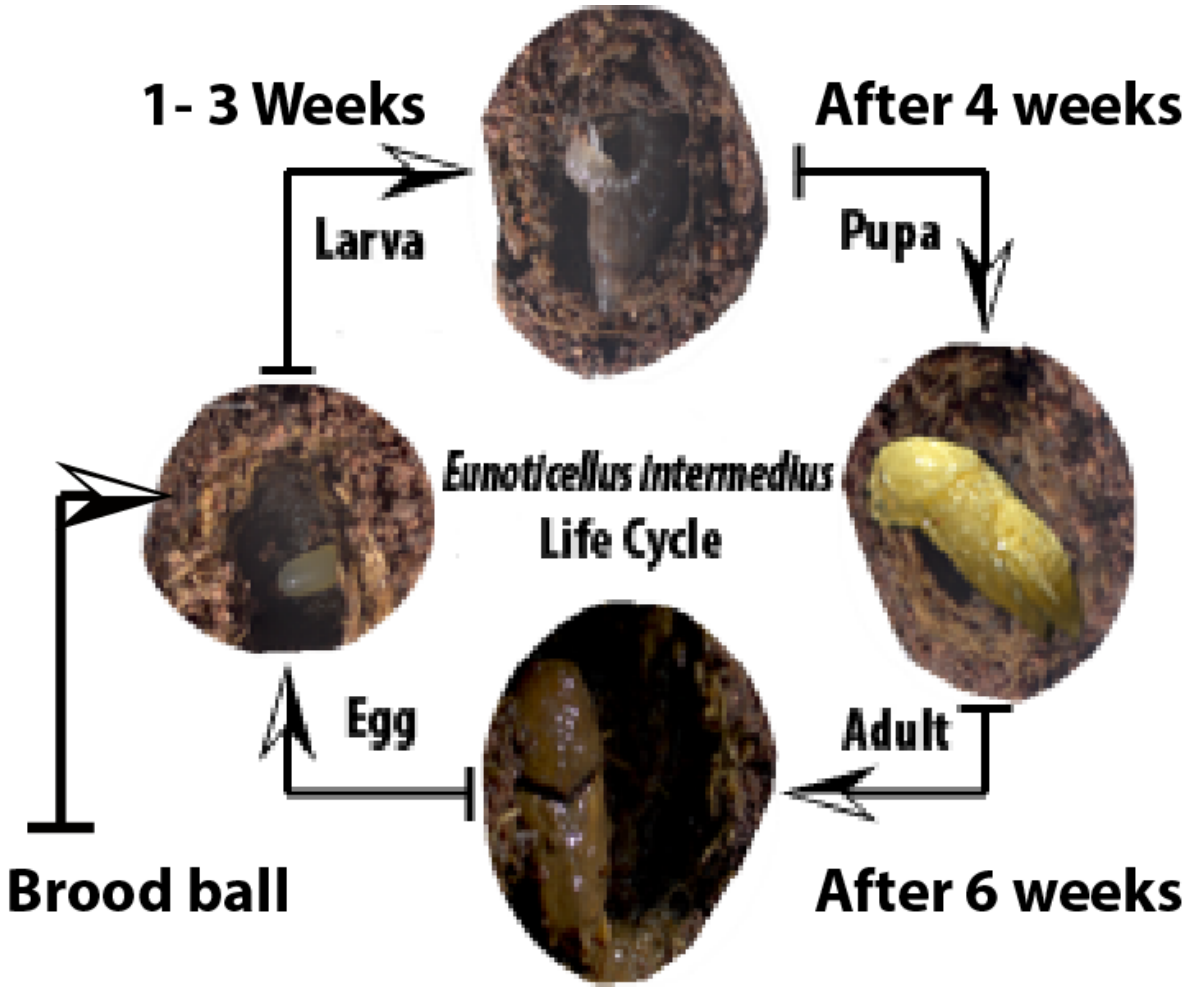


Figure 21: Life cycle of tunnelling dung beetle *E. intermedius*.

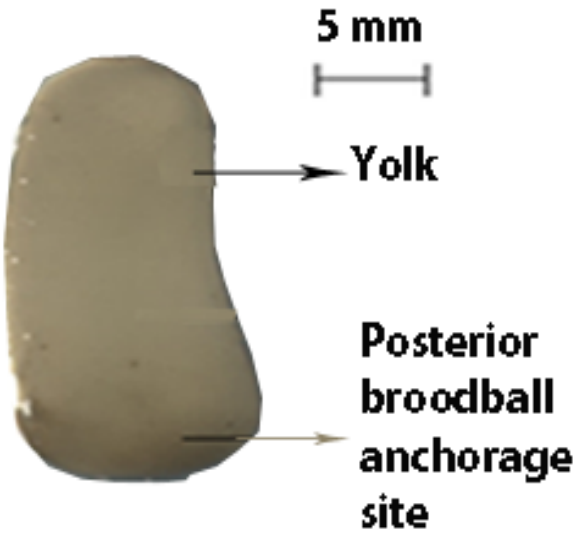


Figure 22: *E. intermedius* egg after 44 hours.

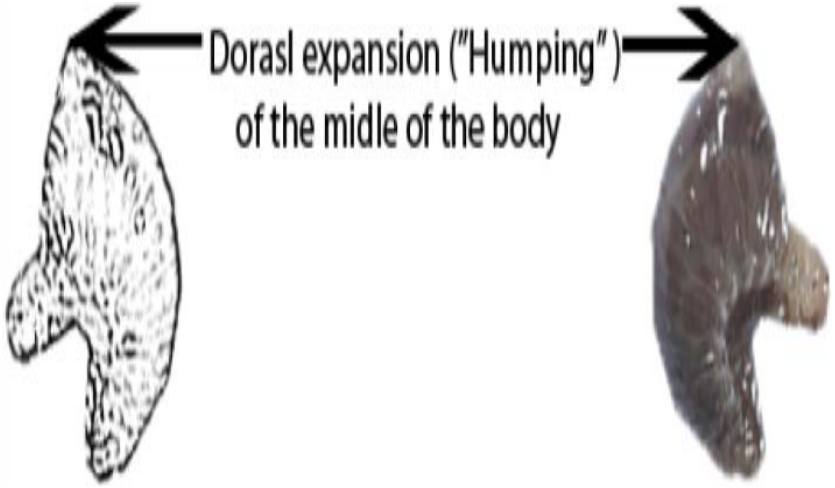


Figure 23: Lateral view mature larva *E. intermedius*.

emerges, eats its way out of the brood ball, forms a new tunnel to crawl out through, and goes in search of fresh manure.

3.2 *E. intermedius* embryonic cell line karyotype

In order to characterize the newly established Flylab cell line *E. intermedius* embryonic (EISA08), a karyotype analysis was carried out. These results have been obtained using conventional squash techniques as described in the Material and Methods section. The chromosomes were viewed and images captured using the AxioCam (MRm/MRc) camera and AxioVision software package (Carl Zeiss AG, Oberkochen, Germany) at 400 X magnification. The cell line chromosomes were obtained and compared with the adult karyotype in order to confirm the originality and the purity of this cell line. The results are illustrated in Figure 24 and Figure 25. The cell line chromosome number detected was 24 with sex chromosomal mechanisms described by Xy. The results showed that the Flylab cell line (EISA08) was derived from a male embryo. This result corresponded to the adult *E intermedius* gonad tissue which gave a chromosome number of n = 12 and sex mechanism Xy as shown in Figure 25 and Table 3.

3.3 *E. intermedius* cell line morphological and growth characteristics

3.3.1 The *E. intermedius* cell line (EISA08) divides asymmetrically

To identify and understand the functionality of the newly established cell line; a cell morphology examination was carried out. Phase-contrast and bright field optics was used to monitor the morphology of the *E. intermedius* (EISA08) cells at the passage 50. The *E. intermedius* cell line, EISA08, exhibits a spherical appearance and does not adhere to surfaces but grows in suspension. The cells present an unusual characteristic in that they seem to be multiplying in

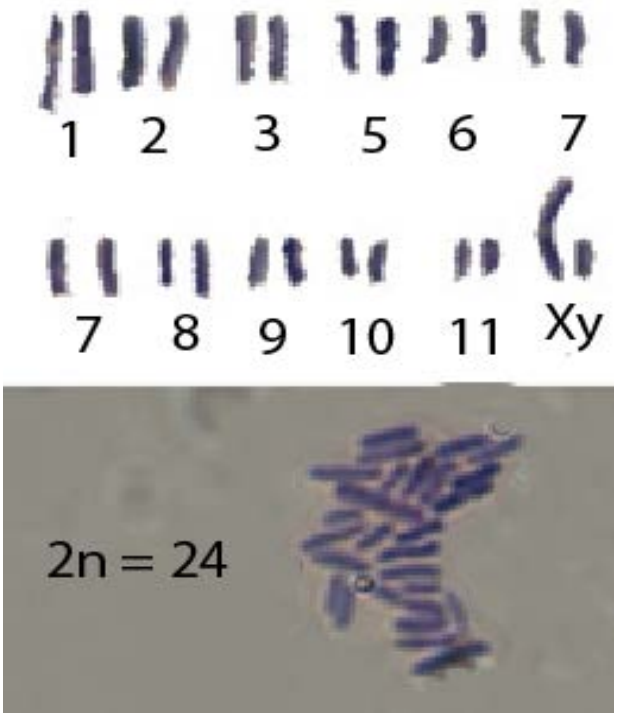


Figure 24: Chromosome Number for *E. intermedius* cell line EISA08: Analysis of cell line by Colcemid Treatment to final concentration of 0.04 µg/ml and staining with 5% Giemsa gave a karyotype number of 2n= 22+XY. The result indicates that the cells were derived from males.

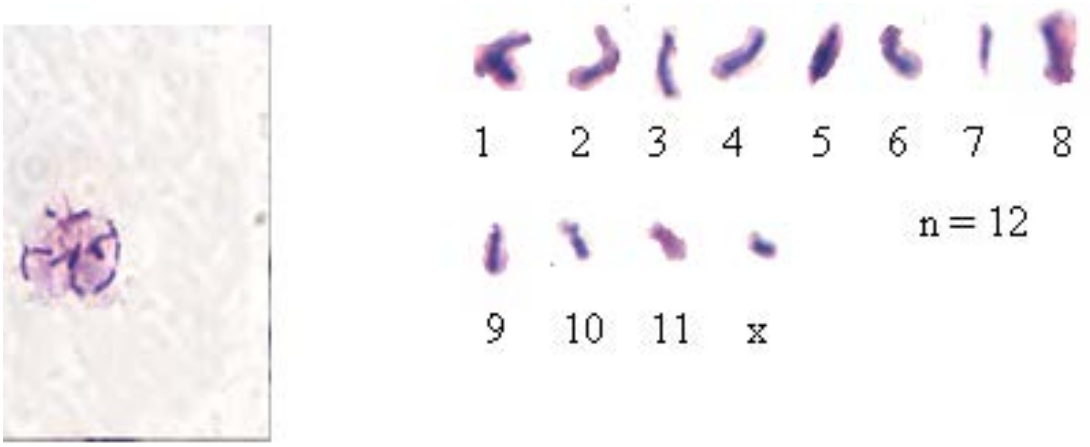


Figure 25: Chromosome number for adult *E. intermedius*: Analysis of gonad tissue through maceration and staining with 2 % Giemsa gave a karyotype number of n=12.

Table 3: Cytogenetic data of Oniticellini species.

Species	Chromosomal formula (2n males)	References
Euonticellus fulvus	20	(Smith and Virkki, 1978)
Euonticellus pallipes	20 = 9 + Xy	(Yadav *et al.*, 1979)
Euonticellus intermedius	24 = 11 + Xy	This work

chains with some daughter cells still attached to the parent cells. The cells divide asymmetrically producing two different daughter cells which differ in size (Figure 26)

3.3.2 *E. intermidius* cell growth

The quantification of cellular growth is an essential tool to characterize cell lines. Such techniques enable not only the optimization of cell culture conditions, but also the determination of growth factor and cytokine activity. In this study, the growth curve and population doubling time was calculated. The growth curve was measured at the 40th passage and 26 °C (Figure 27). The curve demonstrates that the logarithmic growth phase occurred between 3 to 12 hours after subculturing (Figure 27). The cells growth plateaued at 25 hours, and thereafter cells begin to die.

The *E. intermedius* population doubling time during logarithmic growth was calculated at 4.5 hours according to Hayflick's formula (Hayflick, 1980)

$$T = t.\lg 2/\lg (N/N0)$$

Where, T=population doubling time; t= appointed time after subculture; N= number of cells at the appointed time; N_0= number of cells at the beginning of subculture. The result revealed that the embryonic *E. intermedius* cell line grows rapidly. Cells were inoculated in 25 cm^2 flasks at 27.10^4 cells/ml (flask cell suspension = 5 ml). Cell numbers were counted using a haemocytometer (Table 4, figure 27).

3.4 E. intermedius cell line isoenzyme analysis

This isoenzyme analysis uses the genetic polymorphism of enzymes (Steube *et al.*, 1995). Polymorphic enzymes are genetically controlled variants of an enzyme resulting from point mutation(s) of the structural gene. These mutations yield electrophoretically solvable phenotypes (Wright *et al.*, 1981; Halton *et al.*, 1983). The determination of the isoenzyme phenotype of a group of polymorphic enzymes may differentiate according to the specific cell line and can be applied in the discrimination between cells from the same species (O'Brien *et al.*, 1980; Halton *et al.*, 1983).

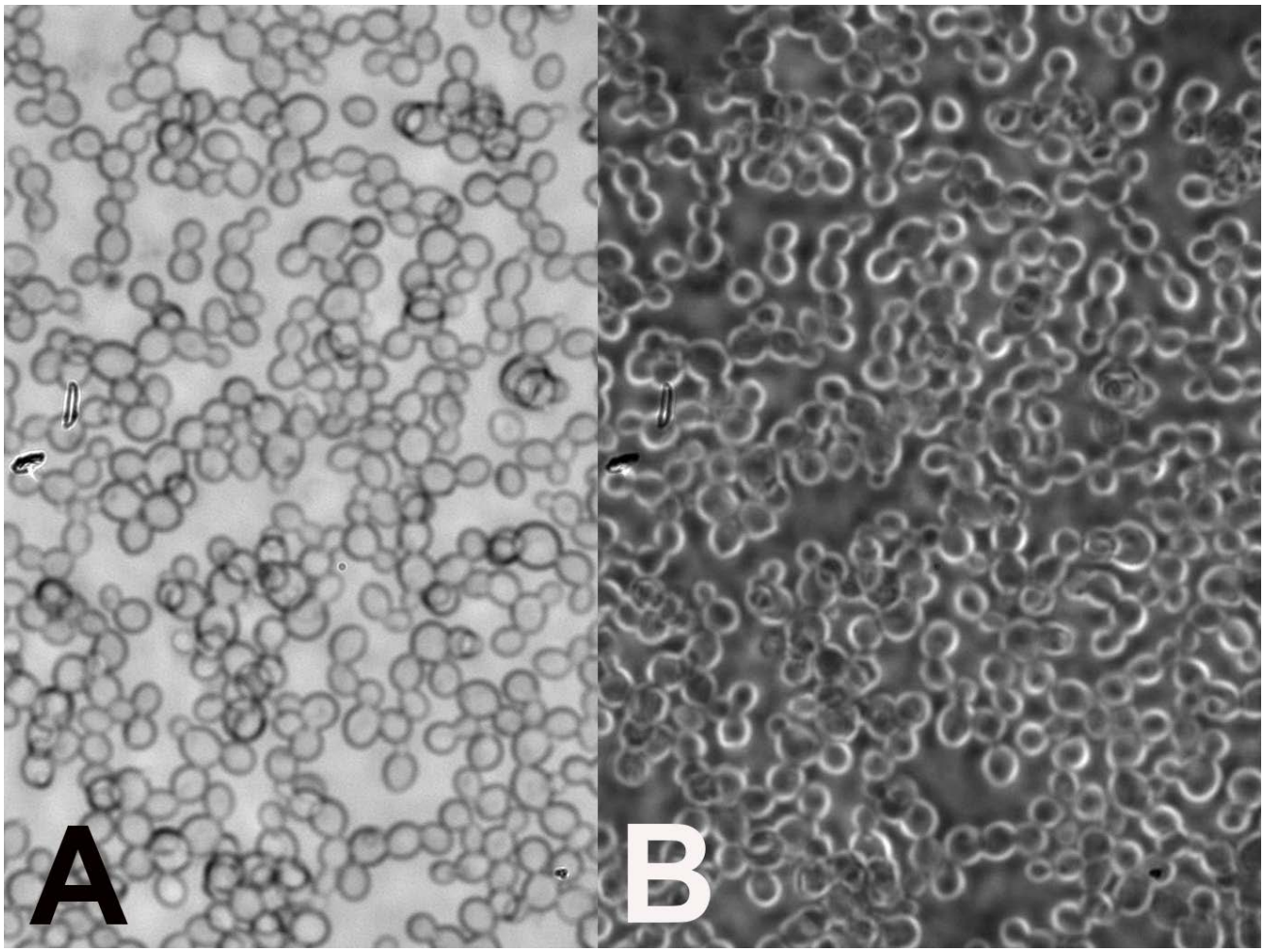


Figure 26: Microscopy image of South Africa dung beetle *E. intermedius* (SAIEI08) embryonic cells line at Passage 40, 2 days after subculture. The cells were cultured in Schneider media (Highveld #L22) supplemented with 10% fatal calf serum without antibiotic. A) Bright field optics, B) Phase contrast. X 640. The cells divide asymmetrically.

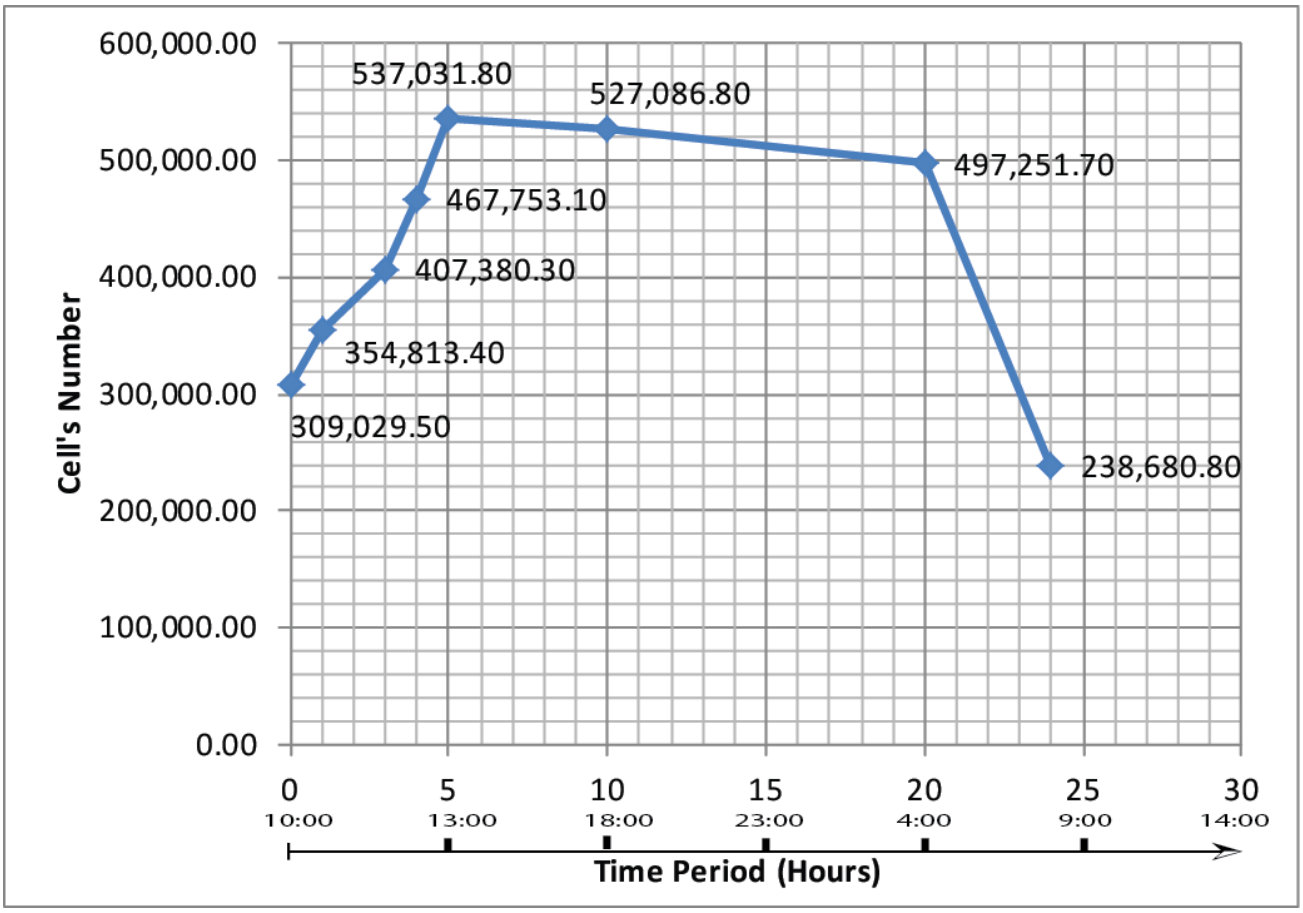


Figure 27: Analysis of the growth curve of *E. intermedius* cell line in Schneider medium containing 10% fetal bovine serum at 26 ^{o}C.

Table 4: *E. intermedius* cell growth during 24 hours period.

Time Point	Time Period (Hours)	Cells Number
10:00 AM	0	309,029.50
11:00 AM	1	354,813.40
1:00 PM	3	407,380.30
2:00 PM	4	467,753.10
3:00 PM	5	537,031.80
8:00 PM	10	527,086.80
6:00 AM	20	497,251.70
10:00 AM	24	238,680.80

The examination of the gel electrophoresis banding patterns and migration distances for particular isoenzymes of intracellular enzymes has been used as a tool for the confirmation of cell line species of origin since the 1970s (Peterson *et al.*, 1979; O'Brien *et al.*, 1980; Halton *et al.*, 1983; Peterson *et al.*, 1984).

In this research, isoenzyme patterns (homologous enzymes) for the *E. intermedius* embryonic cell line (EISA08) were investigated in order to provide profiles relating to cell expression. The isoenzyme mobility data obtained for the *E. intermedius* cell line (EISA09) are shown in Figure 28 and presented in Table 5. The procedure used is described in the Materials and Methods section. The isoenzyme results obtained from *E. intermedius* cell extracts show that the analyses were positive only for glucose-6-phosphate dehydrogenase (G6PD) and nucleoside phosphorylase (NP). The study used a quick and potent isoelectric focusing (IEF) technique. The method is based on the isoelectric separation of a specific set of isoenzymes used to differentiate between cell line origin (Steube *et al.*, 1995). Isoenzymes were analyzed using Innovative Chemistry's (Marshfield, MA) Authentikit system kit. Furthermore, the study used an embryonic *Drosophila* cell line as an insect cell control. Cell extracts from *E. intermedius* and *Drosophila* cell line were prepared and every single cell line was analyzed for the presence of different isoenzymes. These embryonic cell line were grown in Schneider medium with 10% (v/v) fetal bovine serum (FBS) incubated in 26 ^{o}C.

The Authentikit system uses precast agarose (1% in 65 mM barbital buffer; pH 8.6) electrophoresis films and a sodium barbital buffer system. The procedure was followed as described by the manufacturer. Seven enzymes were analysed: nucleoside phosphorylase (NP), malate dehydrogenase (MD), glucose-6-phosphate dehydrogenase (G6PD), lactate dehydrogenase (LD), peptidase B (PepB), aspartate amino transferase (AST), and mannose 6-phosphate isomerase (MPI).

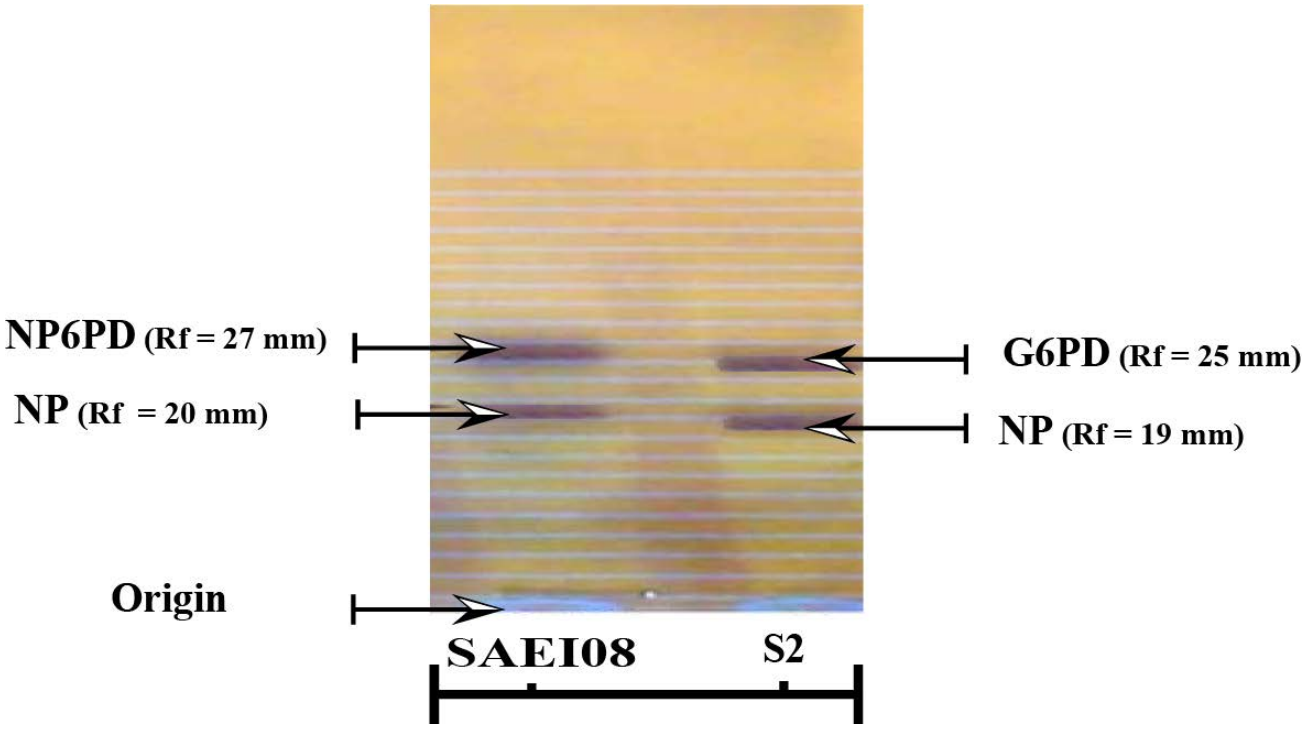


Figure 28: Electrophoresis of extracts from the dung beetle *E. intermedius* cell line (SAIEI08) and S2, *Drosophila melanogaster* embryo. The gels were analyzed for the enzymes NP and G6PD. The arrows indicate the approximate location of the gel origin and the G6PD and NP enzyme for the SAEI08, and S2 cell line.

Table 5: Relative Isoenzymes Mobility.

Insect species and cell line designation	G6PD migration distance (mm)	NP migration distance (mm)
E. intermedius(SAEI08)	27	20
Drosophila(S2)	25	19

The results obtained show that the *E. intermedius* cell line isoenzyme patterns differed from the controls (cells from mouse (L929), HeLa, and *Drosophila* (S2) cell linefor both G6PD and NP enzymes. The *E. intermedius* cell line had exhibited rapid isoenzyme migration profiles of the G6PD and NP isoenzymes. EISA08 and S2 cell extracts have a single G6PD band which migrates differently. Whereas the band for EISA08 cell line, the isoenzymes migrate at a more rapid rate during agarose gel electrophoresis. On the other hand, each of the insect cells formed a single nucleoside phosphorylase (NP) band. The EISA08 nucleoside phosphorylase migrated less rapidly when compared to the S2 cell extract (Figure 28).

3.5 *E. intermedius* cell line as a model to study JAK-STAT pathway

3.5.1 *E. intermedius* (EISA08) cell line *hopschotch* cloning

One of the objectives of this project was to study patterns of immune associated genes in the newly established *E. intermedius* embryonic cell line. Thus *hopscotch* gene expression in *E. intermedius* life cycle stages; adult, pupae, larvae, embryo and the cell line was determined. The houskeeping Ribosomal protein 9 gene was used as a positive control. The results were expected to lead to the confirmation of the cell line EISA08 origin that could participate in the JAK-STAT pathway of the dung beetle, *E. intermedius,* using the EISA08 cell line. The *hopscotch* gene was identified in *Drosophila* as the gene associated with the *hopscotch* pathway. *E. intemedius* adult and its embryonic cell line cDNA synthesis were visualized using a 1% agarose gel (w/v). As is shown in (Figure 29, A, B) *hopschotch* and Rps9 bands are 330 bp and 420 bp long, respectively.

3.5.1.1 Cloning into pGEM T-easy vector

The *hopscotch and Rps9* genes were cloned into the pGEM®-T Easy (Figure 39, Appendix 2, page 106) and vector pJET1 (Figure 40, Appendix 3, page 107) vectors. The primers used in this project were

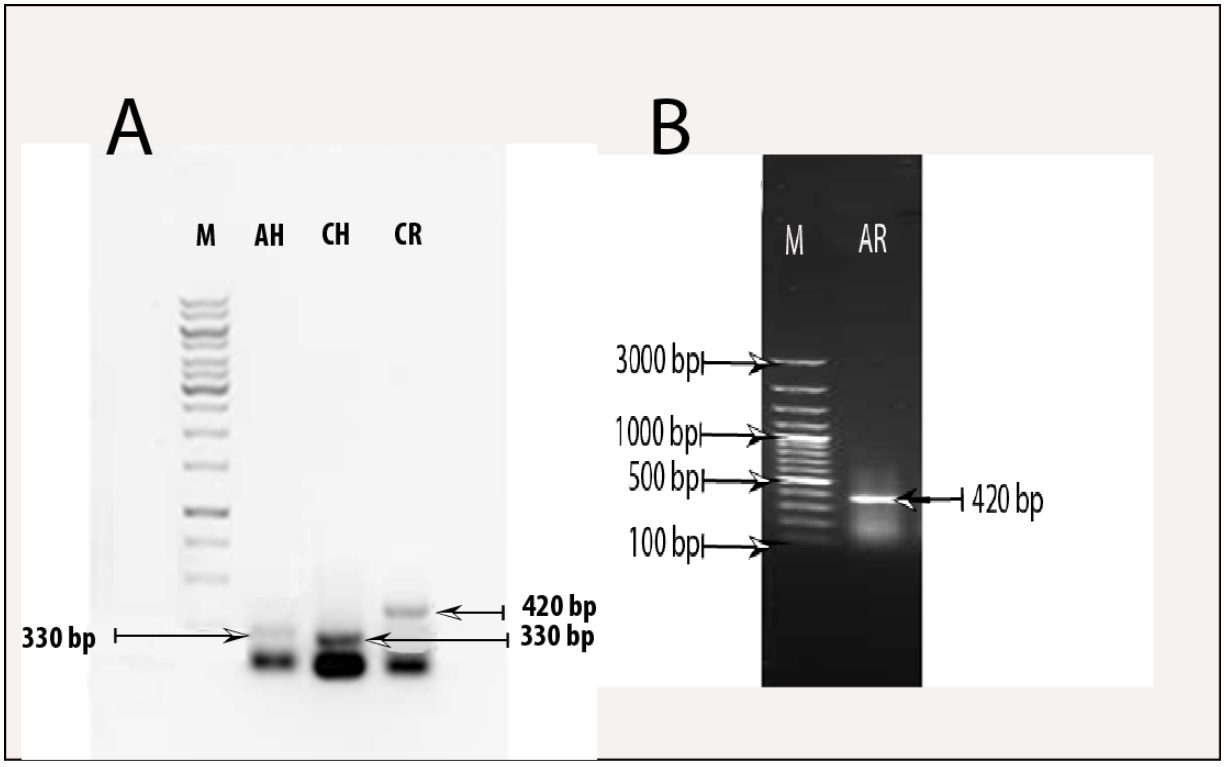


Figure 29: A- RT-PCR agarose gel (1%) analysis of *E. intermedius* adult and cell line EISA08 cDNA. RT-PCR was performed using *hopscotch* and Rps9 primers. Lane M: GeneRuler 1kb DNA marker, lane AH: Adult *hopscotch* 330 bp, lane CH: Cell line *hopscotch* 330 bp and lane CR: Cell line Rps9 420 bp. B- Agarose gel (1%) analysis of a RT-PCR *E. intermedius* adult cDNA. RT-PCR was performed using RPS9 primer. Lane M: Molecular base pair standard (100-bp ladder). Lane 1: Adult RPS9 (420 bp).

available in the Flylab. cDNA was synthesized from mRNA isolated from adult beetles and sequenced by GS (FLX) technology used by a commercial facility (Inqaba Biot.). The housekeeping gene, Rps9, was used as a control. Using a PCR reaction for amplifying specific DNA fragments, the PerkinElmer (GEneAmp PCR System 2400) and Bio-Rad (MJ Mini) thermal cycles were utilized. The cDNA synthesis was carried out by using the ImProm 2 step RT PCR kit and protocol (Promega, USA). Following PCR, the amplified DNA product was analyzed qualitatively by electrophoresis on 1% agarose gel and a fragment size of approximately 330 bp was obtained for both *E. intermedius* adult and cell line. A fragment of 420 bp was obtained for both *E. intermedius* adult and cell line. (Figure 29 A, B). A single clone was sent for DNA sequencing. The PCR product was then extracted and ligated into the pGEM®-T Easy pJET1.2 vector. The process was followed by transformation of competent *E. coli* XL1-Blue cells. White colonies were screened by colony PCR to verify the presence of PCR product in the recombinant plasmids.

3.6.1.2 Bioinformatics analysis

Using the acquired sequence mentioned in the previous paragraph, an alignment was done for bothadult of the *E. intermedius* and cell line and using *hopscotch and* Rps9 PCR products. The cloned sequences of *hopscotch* and Rps9 cell lineare shown in Figure 30 and Figure 31. The PCR base pairs are 330 bp and 420 bp, respectively.

To investigate the similarities between adult dung beetle and cell line sequences for both genes *hopscotch* and Rps9 an alignment was done using DNAman tool (Lynnon Corporation, Bioinformatics solution). The results obtained are shown respectively in Figure 32 and Figure 33. The results of this study revealed that the *hopscotch and* Rps9 cloned sequences were similar in both adult *E. intermedius* and the EISA08 cell line. These results confirm that the EISA08 embryonic cell line originated from the adult dung beetle, *E. intermedius.* The over expression of the *hopscotch* gene in the embryonic cell line, SAEI08, leads to the suggestion that the *hopscotch*

```
1         GCGGRATCTTCTAGAGATGTCGATGAAAATCACGTGAAATTTCCGATTTCGGTTTGGCTC
1          A  X  S  S  R  D  V  D  E  K  S  R  E  I  S  F  G  L  A  Q
1
61        AGTTCATTAATACCGGCGATTATTACGTTTTGAAAACTAACAGGGATTTACCTATTAAAT

21           F  I  N  T  G  D  Y  Y  V  L  K  T  N  R  D  L  P  I  K  W

121       GGTATGCCCCAGAAAGTTTAAGAGAAGGAAAATTTTCTACGGAATCGGACGTTTGGTCCT
41           Y  A  P  E  S  L  R  E  G  K  F  S  T  E  S  D  V  W  S  Y

181       ACGGCGTAACGTTATGGGAGATGTTCTCTTACGGGGAAGAACCAAAACTCGGAGACGGTA
61           G  V  T  L  W  E  M  F  S  Y  G  E  E  P  K  L  G  D  G  T

241       CGAAAGGGGTGAAGAACCCGAACATCTTGCTGAAAAACTCGAGCCATCCGGAAGATCTGG

81           K  G  E  E  P  E  I  L  L  K  N  S  S  H  P  E  D  L  A

301       CGGCCGCTCTCCCTTWACTGAGTCGAGAC
101          A  A  L  P  X  L  S  R  D
```

Figure 30: *E. intermedius* cell line *hopscotch* cloned sequence of 330 bp.

```
1         ACTCGATGCTCGAGTTTTTCAGCAGATTTTAGATCAAGAATTGAAAATCATCGGAGAGTA
1          T  R  C  S  S  F  S  A  D  F  R  S  R  I  E  N  H  R  R  V

61        TGGTCTCCGTAACAAACGTGAAGTATGGCAGGGTTAAATACACCCTAGCAAAAATTCGTAA

21          G  L  R  N  K  R  E  V  W  R  V  K  Y  T  L  A  K  I  R  K

121       AGCCGCTCGTGAGTTATTGACGCTTGAAGAAAAGGACCCTAAACGTCTATTTGAAGGTAA
41          A  A  R  E  L  L  T  L  E  E  K  D  P  K  R  L  F  E  G  N

181       TGCCCTTTTACGTCGTTTGGTGCGTATCGGCGTCTTGGACGAAGGCAGGATGAAGCTCGA
61          A  L  L  R  R  L  V  R  I  G  V  L  D  E  G  R  M  K  L  D

241       TTACGTGTTGGGTTTGAAAATCGAGGACTTCTTGGAGAGACGCTTGCAAACGCAAGTTTT
81          Y  V  L  G  L  K  I  E  D  F  L  E  R  R  L  Q  T  Q  V  F

301       CAAATTGGGATTGGCCAAGTCGATTCATCACGCGCGCGTTTTGATCCGTCAGCGACACAT
101         K  L  G  L  A  K  S  I  H  H  A  R  V  L  I  R  Q  R  H  I

361       TATCTTTCTAGAAGATCTCCTACAATATTCTCAGCTGCCATGGAAAACGATKKKTTCTTA
121         Y  L  S  R  R  S  P  T  I  F  S  A  A  M  E  N  D  X  F  L

421       A
```

Figure 31: *E. intermedius* cell line Rps9 cloned sequence of 420 bp.

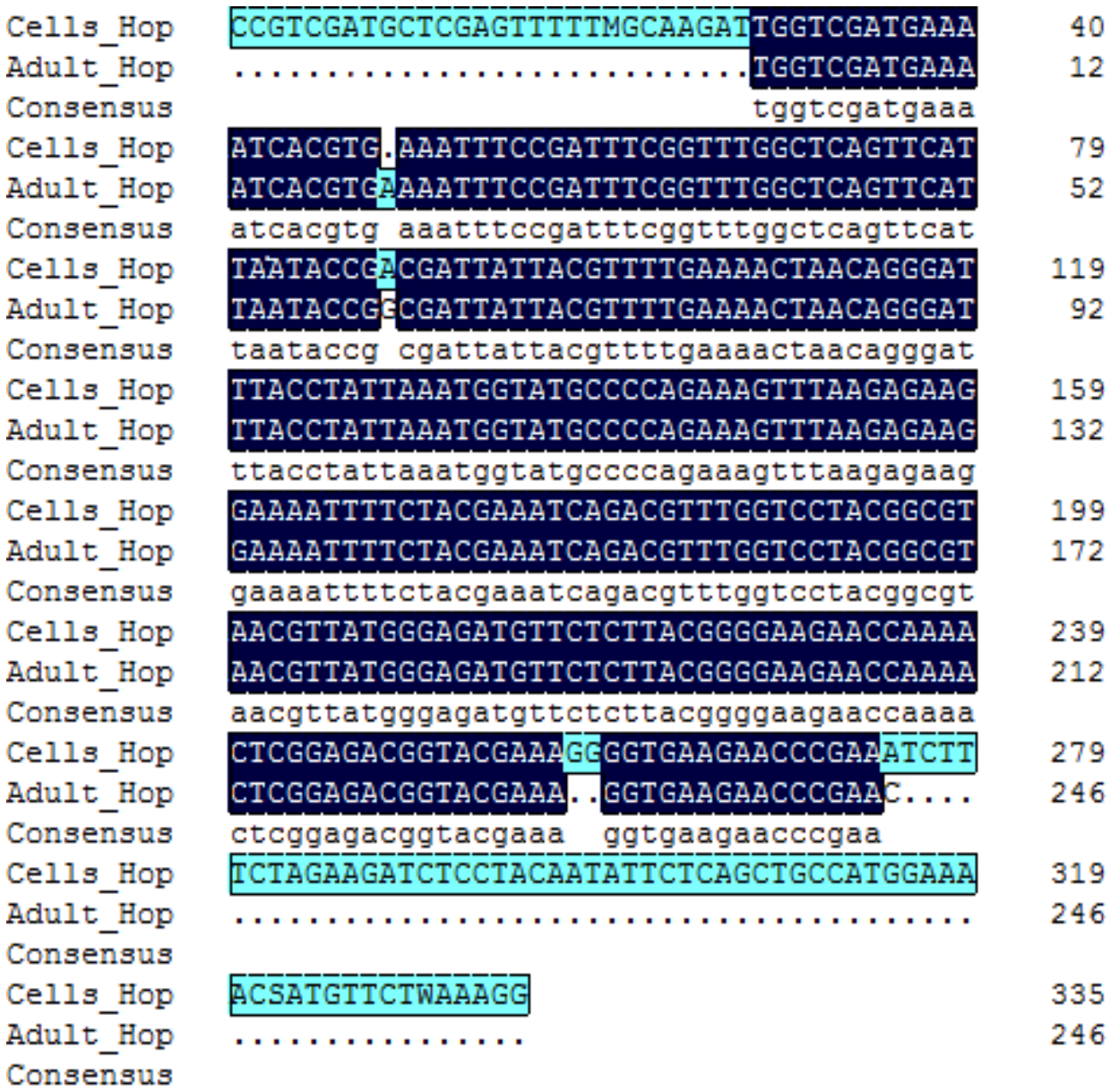


Figure 32: *E. intermedius* cell line *hopscotch* cloned sequence alignment vs. *E. intermedius* adult data sequence. The dark color indicates 99 % similarity between the two sequences.

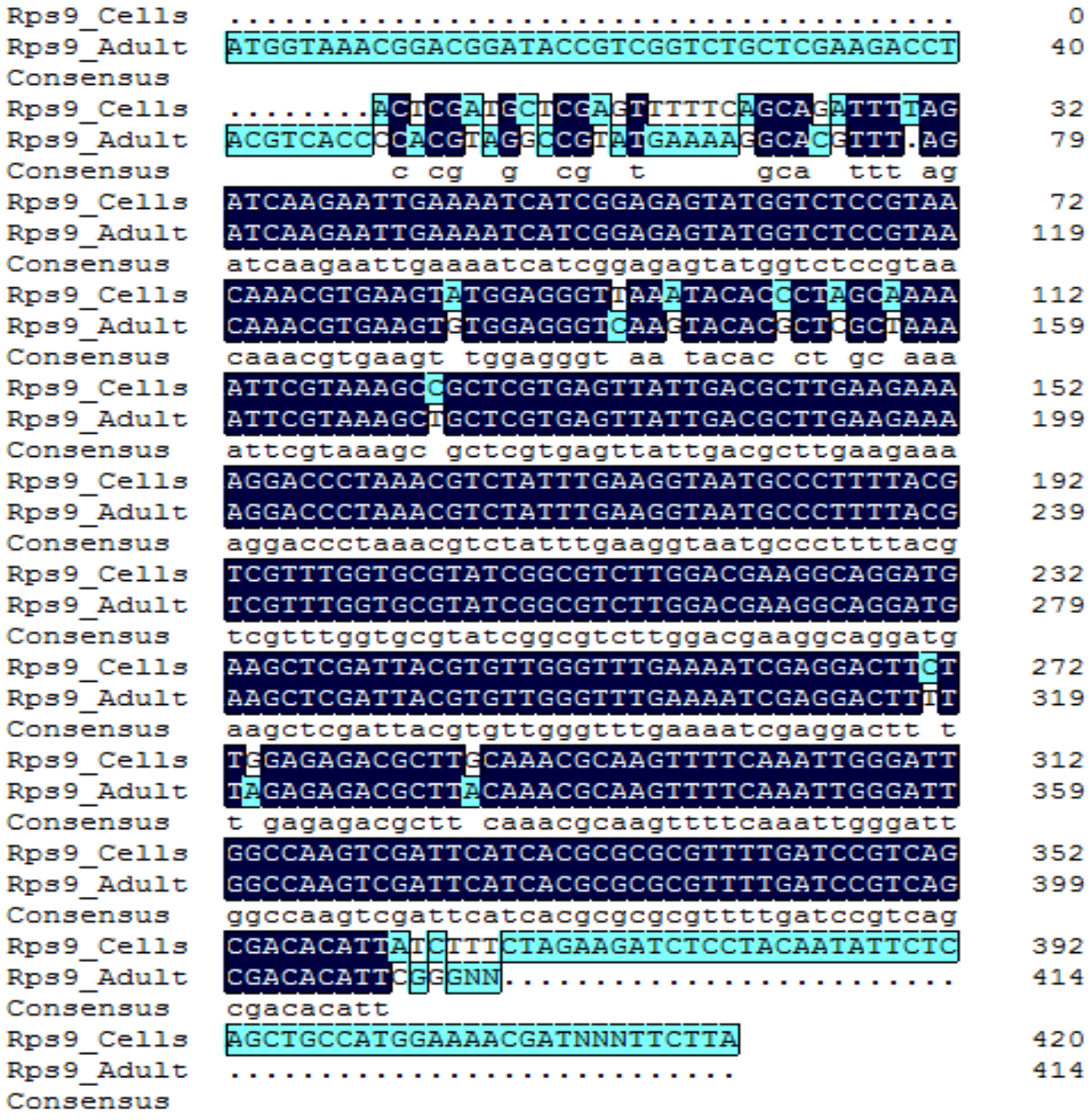


Figure 33: *E. intermedius* cell line Rps9 PCR cloned sequence alignment vs. *E. intermedius* flylab data sequence. The dark color indicates 99% similarity between the two sequences.

pathway plays an important role in the early embryonic development process and immunity defence of *E. intermedius*. The *E. intermedius hopscotch* cDNA cloned, sequenced and compared with adult genomic DNA. The Figure 34 and Figure 35 clearly show the existence of one intron separating two axons which confirm the 100% similarity with the newly established cell line.

3.5.2 Expression pattern gene *hopscotch* and Rps9

RT-PCR was used to detect the expression of *hopscotch* and Rps9 genes in all *E. intermedius* stages adult, cell line, pupa, larvae, embryo and the newly established cell line EISA08 (Figure 36). The Figure illustrates *hopscotch* and Rps9 bands. The results show over expression of the *hopscotch* gene in larvae, pupae, and the embryonic cell line. This results revealed that the JAK-STAT pathway is early activated in the *E. intermedius* life cycle. Thus the pathway may plays an important role in *E. intermedius* immunity defence and tissue development.

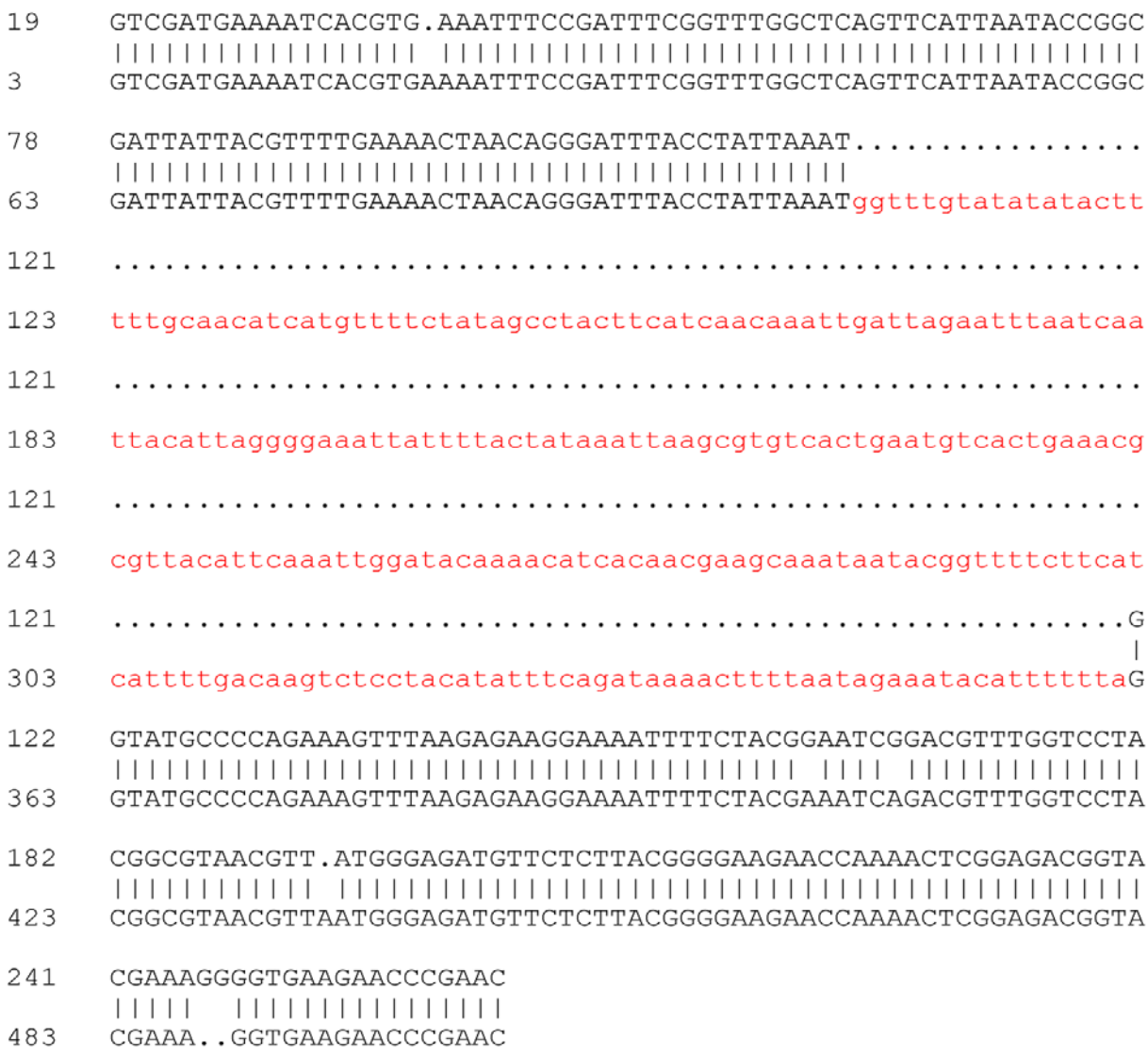


Figure 34: Adult genomic DNA *hopscotch* PCR product sequence for dung beetle *E. intermedius* (Mbayu, 2010).

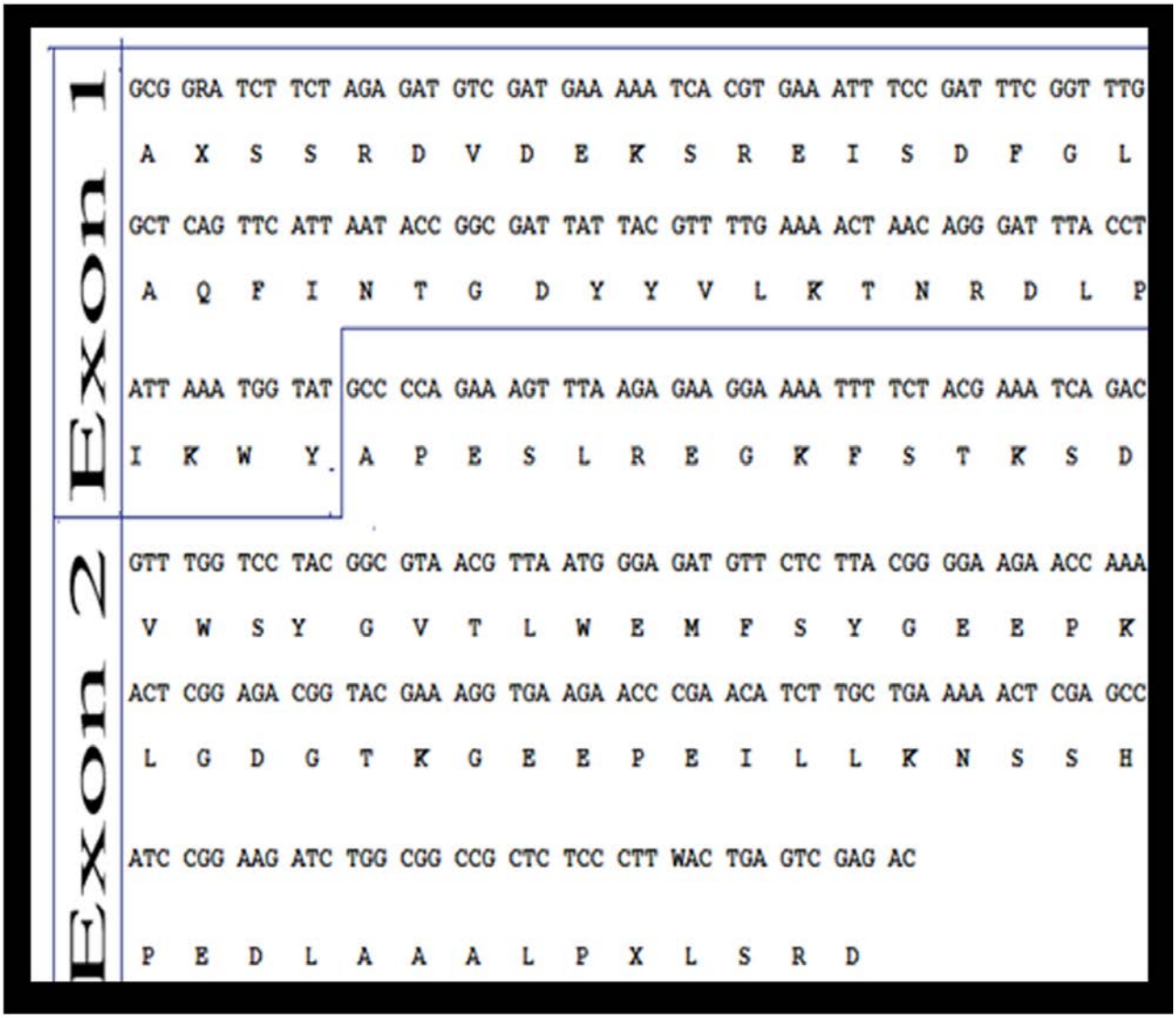


Figure 35: *E. intermedius* cell line (SAEI08) *hopscotch* cloned sequence.

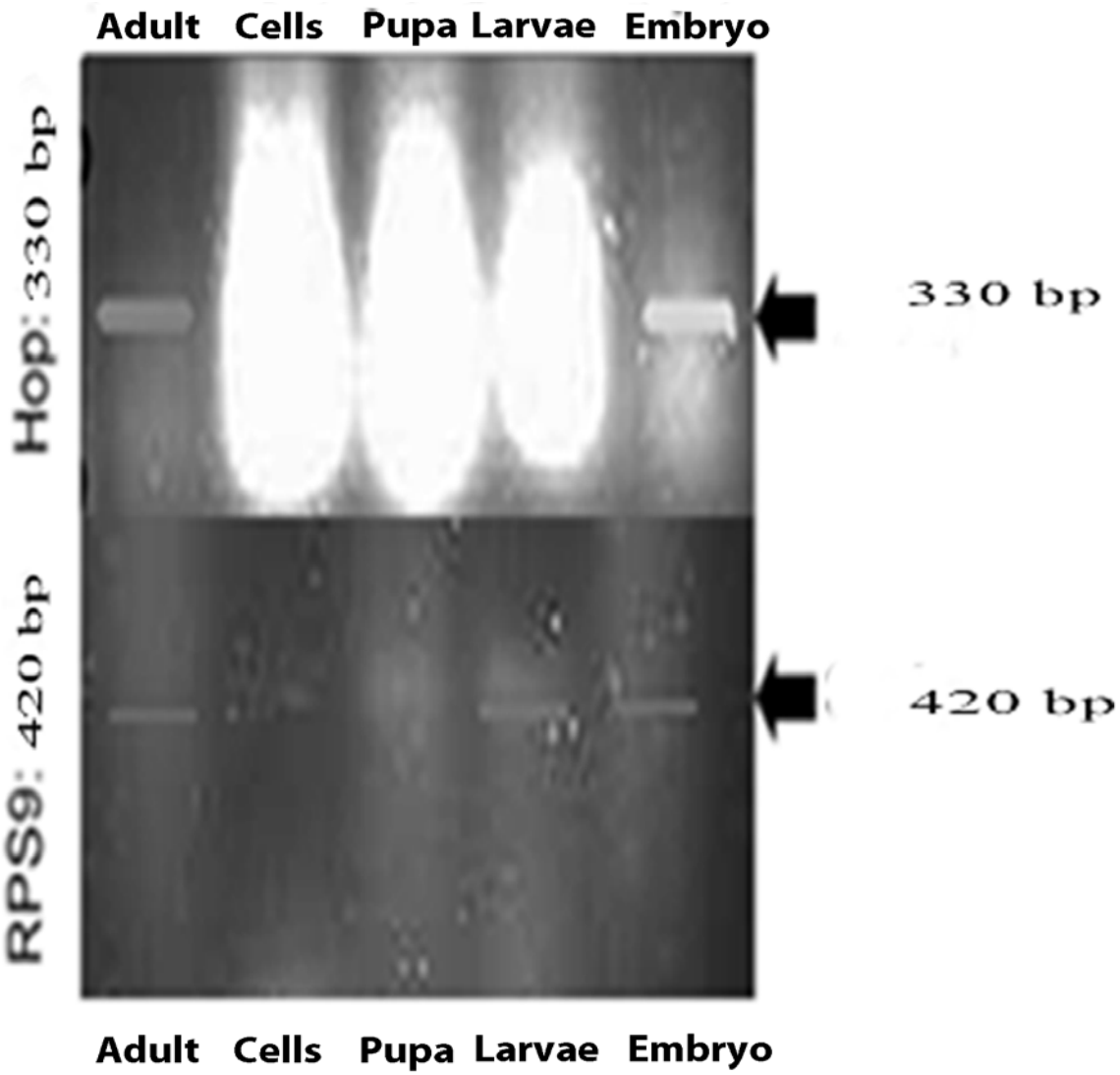


Figure 36: Agarose gel (1%) electrophoresis analysis of adult, cell line, larvae and embryo *E. intermedius* cDNA using specific primers for *hopscotch* and Rps9 gene regions. cDNA after 40 reaction cycles.

Chapter 4

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4 Discussion

4.1 *E. intermedius* chromosome number

The aim of this study was to describe the karyotype of *E. intermedius* (Coleoptera, *Scarabaeidae*) using conventional cytogenetic techniques. There is no published data for the chromosome number of *E. intermedius*. However, data are available for two other species within the genus of *Euoniticellus.* Both of these species, *Euonticellus fulvus* and *Euonticellus pallipes*, have a chromosome number of 20 and chromosomal sex mechanism Xy (Table 3) (Yadav *et al.*, 1979; Colomba *et al.*, 1996). The *E. intermedius* karyotype results obtained is illustrated in Figure 24 and Figure 25. The *E. intermedius* cell line of the chromosomes number detected was 24 chromosomes and chromosomal sex mechanisms Xy. The chromosomal formula 2n = 23 + X is similar to that verified in the majority of the Coleoptera species. The newly established *E. intermedius* embryonic cell line (EISA08) is pure and original based on the obtained results.

Only about 380 *Scarabaeidae* species have been studied by cytogenetic techniques, with the description of diploid number, sex determination mechanism and, to a lesser extent, chromosome morphology (Smith and Virkki, 1978; Angus *et al.*, 2007). The *Scarabaeidae* family presents a conserved karyotype, with a diploid number of 2n = 20 and sex determination mechanism Xyp (Cabral-de-Mello *et al.*, 2008). This karyotype is considered to be primitive and modal for the group as well as the Coleoptera (Cabral-de-Mello *et al.*, 2008). The ancestral karyotype modifications have been observed in representatives of this family with variations in the diploid number ranging from 2n = 8 to 2n = 30 and different sex determination mechanisms (Yadav *et al.*, 1979; Angus *et al.*, 2007; Cabral-de-Mello *et al.*, 2007).

Derived karyotypes have been revealed for several of the species studied. These species present diploid numbers of 2n = 8, 12, 14, 18, 19, 21, and 24 and sex mechanisms Xy, XY, Xyr,

neoXY and X0 (Smith and Virkki, 1978; Yadav *et al.*, 1979; Angus *et al.*, 2007; Cabral-de-Mello *et al.*, 2007). This condition is considered primitive to this group and also to the whole order of Coleoptera (Smith and Virkki, 1978; Moura *et al.*, 2003; Angus *et al.*, 2007; Cabral-de-Mello *et al.*, 2008). The family presents seven sex mechanisms (XY, Xy, XYp, Xyp, Xyr, XO and neo-XY) with variations in the chromosomal morphology in some species (Yadav *et al.*, 1979; Moura *et al.*, 2003; Cabral-de-Mello *et al.*, 2007). In some Coleopteran families, wide karyotypic structure variation has been reported, as described for *Buprestidae* (2n = 12 to 2n = 46) and *Elateridae* (2n = 4 to 2n = 23), whereas karyotypic conservation has been observed in others, such as *Lampyridae* (2n = 19, XO), and *Cantharidae* (2n = 13, XO) (Smith and Virkki, 1978; Schneider and Granato, 2007).

4.2 *E. intermedius* embryonic development

The results obtained in this project, revealed that *E. intermedius* develops a short germ embryo. Only the anterior most segments are specified at the blastoderm stage. The long-germ mode of development is linked to fast progression through embryogenesis. In higher dipterans, like *Drosophila*, embryogenesis is completed within one day. Gastrulation is a rapid process with mesoderm internalization requiring only 15 minutes (Sweeton *et al.*, 1991). This poses high demands on the precision and coordination of morphogenetic movements. *E. intermedius* and *Tribolium* embryogenesis, on the other hand, requires about 4 days and the gastrulation, movements are about four times slower than in *Drosophila* (Figure18) (Handel *et al.*, 2000). Although this is still rapid in comparison to hemimetabolous insects which frequently require several weeks to complete embryogenesis, some of the constraints on gastrulation unique to higher dipterans might be absent in *Tribolium* (Handel *et al.*, 2000) as could be considered for *E. intermedius* has suggested in this study.

Despite the differences in germ type and speed of development, many components of the regulatory network controlling DV patterning appear to be conserved between *E. intermedius*, *Tribolium* and *Drosophila* (Sommer and Tautz, 1994). The major target genes of *Drosophila* dorsal, like twist, snail, dpp and zerknullt are present in *Tribolium* (Sommer and Tautz, 1994). Several short germ-band beetles display patterns of segmentation and gene expression that correspond to those in *Drosophila* (Patel *et al.*, 1994; Tautz *et al.*, 1994). The main differences are the timing of the production of these patterns in relation to gastrulation (Handel *et al.*, 2005).

4.3 Features of the *E. intermedius* cell line

4.3.2 Enzymes

The aim of this study was to provide an enzyme profile relating to embryonic *E. intermedius* cell expression. The isoenzyme analysis based on the electrophoretic mobility of certain enzymes of the *E. intermedius* results revealed in this study using the Innovative Chemistry AuthentiKit, are illustrated in Figure 28. Isoenzyme cell extracts tested positive for glucose-6-phosphate dehydrogenase (G6PD) and nucleoside phosphorylase (NP) and were negative for malate dehydrogenase (MD), lactate dehydrogenase (LD), peptidase B (Pep B), aspartate amino transferase (AST) and mannose 6-phosphate isomerase (MPI). One single G6PD band was obtained for each of the two enzymes (Figure 28) with isoenzyme migration distance (Rf) values of 20 mm and 27 mm, respectively. The result differs from an embryonic *Drosophila* cell line isoenzyme migration distance (used as a control) obtained for the same enzymes. The Rf values were 19 mm and 25 mm respectively (Figure 28 and Table 5)

The AuthentiKit method uses the genetic polymorphism of enzymes, which is applied to discriminate between cells from the same speices. The enzymes are associated with particular cell types (Flexner, 1955; Ebert, 1959; Rutter *et al.*, 1968). In 1969, Wright and Shaw proved that the

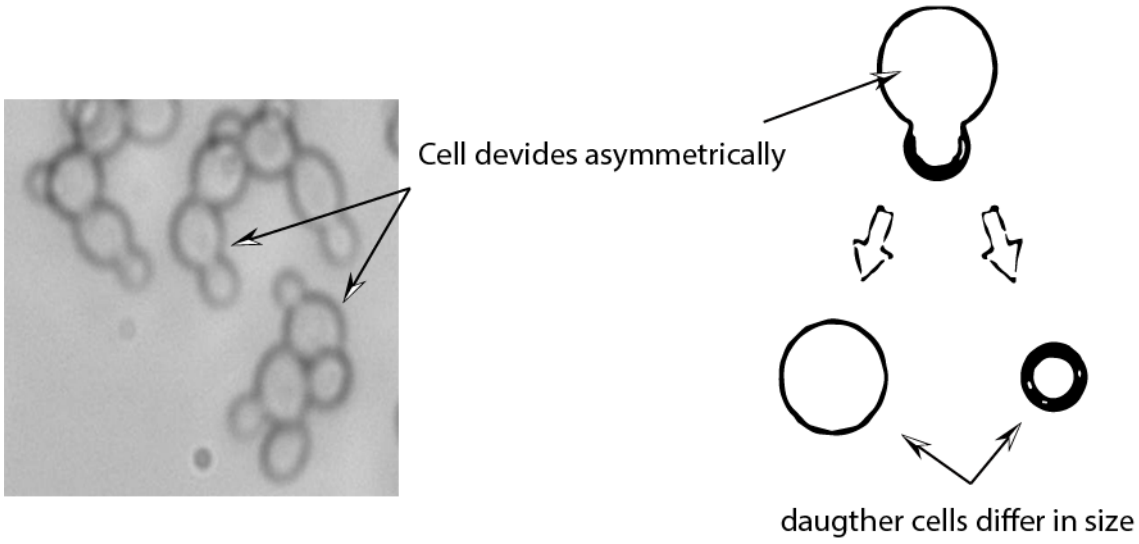


Figure 37: *E. intermedius* cell line (SAEI08) cells divide asymmetrically giving rise to two daughter cells differ in cell size.

egg and early embryo specialized enzymes may be lacking but relatively large amounts of the "ubiquitous" enzymes (enzymes found in most of the tissues of an organism) were present in the egg cytoplasm (Wright and Shaw, 1969).

As represented in the results in Figure 28 and Table 8, only two isoenzymes, G6PD and NP, were detected instead of seven enzymes. In this study, it is suggeted that an increase or deacrease in enzyme activity might reflect synthesis devolopment due to the cells that are derived from an early neural embryonic cells.

4.3.3 Cell Growth

E. intermedius cell growth results obtained in this project are shown in Figure 27. This study revealed that *E. intermedius* embryonic cell growth is very rapid with a population doubling time of 4.5 hours at 26 °C and 50 passage cultured in Schneider media (Figure 27)

The first continuous coleopteran cell line, designated DSIR-HA-1179, was derived from the scarab beetle *Heteronychus arator.* The population doubling time was 6 days, at 27 °C (Crawford, 1982). The slow cell growth rate doubling time of 6 days was similar to lines derived from homoptera but slower than most lepidopteranand dipteran lines, which take between 16 and 72 hours to double (Hink, 1976). The cell population doubling time for BCIRL-Lepd-SL1 cell line , established from pupal tissue of the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: *Chrysomelidae*). at 28 °C was estimated to be 3.4 days at passage 56 and 2.3 days at passage 111 (Long *et al.*, 2002). A continuous cell line from a culture of embryonic cells of the cupreous chafer, *Anomala cuprea* Hope (Insecta, Coleoptera, *Scarabaeidae*) doubling time was about 4.5 days (Mitsuhashi, 2003). *Anopheles stephensi var. Mysorensis* establishment of a larval cell estimated a population doubling time of 16 hours and a monolayer was usually formed in 2-4 days (Pudney and Varma, 1971).

The rapid growth of the EISA08 cell line with population doubling 4.5 hours is shown in Figure 27. In this study, it is suggested that the rapid growth could be due to the early expression of the cytokine, unpaird-3(*Unpaired (Upd)*-3) leads to the early activation of JAT-STAT pathway in *E. intermedius* embryonic cell line. The JAT-STAT transduction pathway stimulation activates transcription in the stem cells and maintains the self-renewal process (Kiger *et al.*, 2001). JAK-STAT signalling is required in the germ line for stem cell maintenance and for controlling morphology and proliferation. The loss of the *Unpaired (Upd)*, JAK or Stat results in disorders in stem cell maintenance, while excessive JAT-STAT pathway activation caused by Unpaired (Upd)ectopic expression leads to an unlimited stem cell growth (Valk-Lingbeek *et al.*, 2004). The study suggests that this pathway plays an important role in *E. intermedius* embryonic cell growth. The asymmetric cell division and embryonic development of this cell line could be derived from neural stem cells.

4.4 Dung beetle *E. intermedius* and EISA08 embryonic cell line *hopscotch* gene.

In this component of the project, the *hopscotch* gene was identified in the adult dung beetle *E. intermedius* and its embryonic cell line. The gene is a key factor for the *E. intermedius* JAK-STAT pathway. The early activation of the pathway in the embryonic cell line could cause a dramtic cell growth and unequal cell division (asymmetric division). It is suggested that the *E. intermedius hopscotch* pathway participates in regulation during embryonic development and cell proliferation regulation.

The JAK-STAT pathway has been extensively studied in *Drosophila* and has been demonstrated to be involved in stem-cell maintenance, ovarian-cell migration and sex determination (Jinks *et al.*, 2000). In development, this pathway is important for embryonic segmentation and larval hematopoiesis as well as for development of the eye, wing, trachea, hindgut and limb (Luo et

al., 1999; Chen et al., 2003). The results in Figure 29, Figure 32 and Figure 36 indicate the existance of *hopscotch* gene expression in both adult and the cell line of the *E. intermedius* dung beetle.

4.5 *hopscotch* gene Expression

The results obtained in this study reveal that the *hopscotch* gene is expressed in the dung beetle, *E. intermedius* life cycle development stages: Adult, Pupae, Larvae Embryo, and the cell line (Figure 36). The Figure demonstrates that the *hopscotch* gene is highly expressed in *E. intermedius* early stages; in the embryonic cell line, larvae and pupae stages. This result leads to suggession that the *E. intermedius* JAT-JAK/STAT pathway is involved in multiple developmental events and regulates the cellular immune response. The pathway could play an important role in cell proliferation and asymmetric division.

4.6 Conclusion and Recommendations

This study was undertaken to characterize the embryonic dung beetle *E. intermedius* cell line (EISA08) and to investigate expression patterns of immune associated genes. The chromosomal formula for *E. intermedius* was verified as 2n = 23 + XY (Figure 24 and Figure 25). This is similar to the majority of the Coleoptera species. The *E. intermedius* embryonic cell line (EISA08) was found to be neural stem cells (Figure 26). They are generated early in embryonic development in a process called neurogenesis. The isoenzyme cell extracts tested (using the authentikit) detected only for glucose-6-phosphate dehydrogenase (G6PD) and nucleoside phosphorylase (NP) (Figure 28) proved that the egg and early embryo and specialized enzymes may be lacking. The growth rate is rapid as the population doubling time was calculated to be closer to 5 hours (Figure 27). This rapid growth could lead to the suggestion that the Unpaired (Upd) in *E. intermedius* JAK-STAT pathway is expressed early in germ cells and leads to a dramatic increase in the number of stem cells.

The presence of the *hopscotch* gene was identified in all *E. intermedius* development stages, as well as in the embryonic cell line (Figure 26) and is highly expressed in the early *E. intermedius* life cycle (Figure 36). The cloned (cDNA) sequences for both *hopscotch* and Rps9 genes were sequenced and aligned to the adult based, in the Flylab data base (www.flylab.wits.ac.za) (see Figure 31, Figure 32, Figure 33, Figure 34). The study suggested that the *hopscotch* gene is involved in the early activation in the *E. intermedius* JAK-STAT pathway. From the results obtained from this study, dramatic cell devision (see Figure 27), assymmetric cell division (see Figure 26) and highly *hopscotch* gene expression in the *E. intermedius* early life cycle developing stages. Thus lead to the suggestion that the the JAK-STAT pathway may involved in stem-cell maintenance, cell proliferation, multiple developmental events and the regulation of the cellular immune response in this beetle .

It is recommended that more work should be performed to demonstrate the role of the *E. intermedius* JAK-STAT pathway in stem-cell maintenance, cell proliferation, asymmetric division, embryonic development and viral defence using the characterized EISA08 embryonic cell line. The results presented in this study could provide an understanding of this system in order to provide useful strategies for treatment of infectious diseases affecting humans and animals.

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Chapter 6

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8 Appendix 1

8.1 Media

Media were prepared as listed in Table 6.

Table 6: Growth media

Buffer	COMPOSITION
Schneider medium	See Table 12 (104)
LB medium	1% tryptone 0.5% yeast extract 1% NaCl.
LB agar	LB medium + 1.5% agar

8.2 Buffers and solutions

Buffers and solutions were prepared are listed in Table 9

8.3 Living organisms

The bacterial strains used for cloning is listed in Table 7

Table 7: Living organisms

Strain	Source
Euoniticellus intermedius	Flylab
Drosophila melanogaster	Invitrogen
Escherichia coli XL1-Blue	Flylab
E. coli BL 21	Flylab

Staphylococcus aureus	Flylab

8.4 Oligonucleotides

Table 8 lists the sequence of the oligonucleotides used in this study.

Table 8: Oligonucleotides used during PCR and RT-PCR

Primer name	Sequence	Tm Max (°C
Hop27me For (27bp)	5′ TGGTCGATGAAAATCACGTGAAATTTC 3′	61.57
HoP27me Rev (27bp)	5′ GTTCGGGTTCTTCACCCCTTTCGTACC 3′	69.16
Rps9 For (23bp)	5′ GTCTGCTCGAAGACCTACGTCAC 3′	66.33
Rps9 Rev (20bp)	5′ AATGTGTCGCTGACGGATCA 3′	60.4

8.5 Chemicals and kits

All chemicals used during the project were of analytic grade and if not mentioned were purchased from either Sigma-Aldrich (South Africa) or Merck Biosciences (South Africa). Agarose D1 LE (Low electro endosmosis) was obtained from Techcomp Ltd (Hong Kong). Agar, tryptone and yeast extracts were from Difco Laboratories (Detroit, Michigan, U.S.A.). Ampicillin was from Sigma-Aldrich (South Africa). Restriction endonucleases were from Promega, USA. Deoxynucleotide triphosphates (dNTPs) were from Roche Applied Science (South Africa). Chemicals, kits and their suppliersused in this study are listed in Table 5.

Table 9: Chemicals, Kits and names of suppliers

Chemical/Kit	Supplier
TRIzol® reagent	Invitrogen
pGEM®-T Easy vector system; ImProm-IITM reverse transcription system.	Promega, USA
AuthentiKit System	Innovative Chemistry
CloneJET™ PCR Cloning Kit	Invitrogen
GeneRulerTM 100bp, 1kb DNA ladder plus	GeneRulerTM 100bp DNA ladder plus

8.6 Addresses for the various bioinformatics tools used in this study

Web addresses for various bioinformatics tools are listed in Table 10

Table 10: General molecular biology buffers and solutions

Buffer	Components
50X Tris Acetate EDTA (TAE)	0.45 M Tris-base 0.45 M Boric Acid 0.01 M EDTA.
6X DNA loading buffer	0.25% Bromophenol blue 0.025% xylene cyanol 30% glycerol

DEPC treated water	DEPC 0.01% Dissolve DEPC in 50 % Ethanol water to 1% Mix 1:1 distilled water
Grinding buffer	5% sucrose 80 mM NaCl 100 mM Tris-HCl pH8.5 0.5% SDS 50 mM EDTA.
Miniprep solution I	5 mM sucrose 10 mM $Na_2EDTA\text{-}2H_20$ 25 mM tris pH to 8.0 with NaOH
Miniprep solution II	0.2 M NaOH 1% SDS; prepare fresh
Miniprep solution III	3 M sodium acetate, pH4.8 made up withacetic acid
Phosphate buffered saline (PBS),50X	1.4 M NaCl 27 mM KCl 101 mM Na_2HPO_4, 18 mM KH_2PO_4, pH to 7.3 with HCl
SDS sample buffer (5X)	1 M Tris-HCl, pH 6.8 10% glycerol 10% SDSβ-mercaptoethanol; 1% bromophenol blue; store at $4^{\circ}C$.

Table 11: Web addresses for various bioinformatics tools

Bioinformatics Tool	Web address
ALIGN Query	www2.igh.cnrs.fr/bin/align-guess.cgi
BLAST	http://www.ncbi.nlm.nih.gov/BLAST
FlyBase	http://flybase.bio.indiana.edu
Flylab	http://Flylab.wits.ac.za/EI/est2uni/home.php
GeneBank	http://www.ncbi.nlm.nih.gov

Table 12: Schneider's *Drosophila* Medium*(Schneider, 1964)*

Component	mg/litter
$CaCl_2$	600
KCl	1600
KH_2PO_4	450
$MgSO_4 \cdot 7H_2O$	3700
NaCl	2100
$NaHCO_3$	400
$Na_2HPO_4 \cdot 7H_2O$	1321
α-Ketoglutaric acid	200
Fumaric acid	100
D-Glucose	2000
Malic acid	100
Succinic acid	100
Trehalose	2000
Yeast	2000
ß-Alanine	500
L-Arginine	400
L-Aspartic acid	400
L-Cysteine	60.00
L-Cystine	100
L-Glutamic acid	800
L-Glutamine	1800
Glycine	250
L-Histidine	400

L-Isoleucine	150
L-Leucine	150
L-Lysine Hcl	1650
L-Methionine	800
L-Proline	1700
L-Serine	250
L-Threonine	350
L-Tryptophan	100
L-Tyrosine	500
L-Valine	300
L-Phenylalanine	150

9 Appendix 2

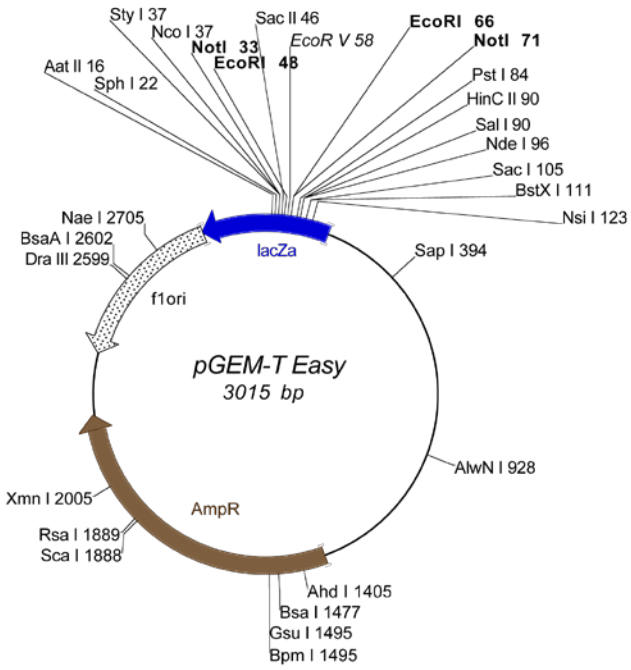


Figure 38: Map of the pGEM-T Easy Cloning Vector.

10 Appendix 3

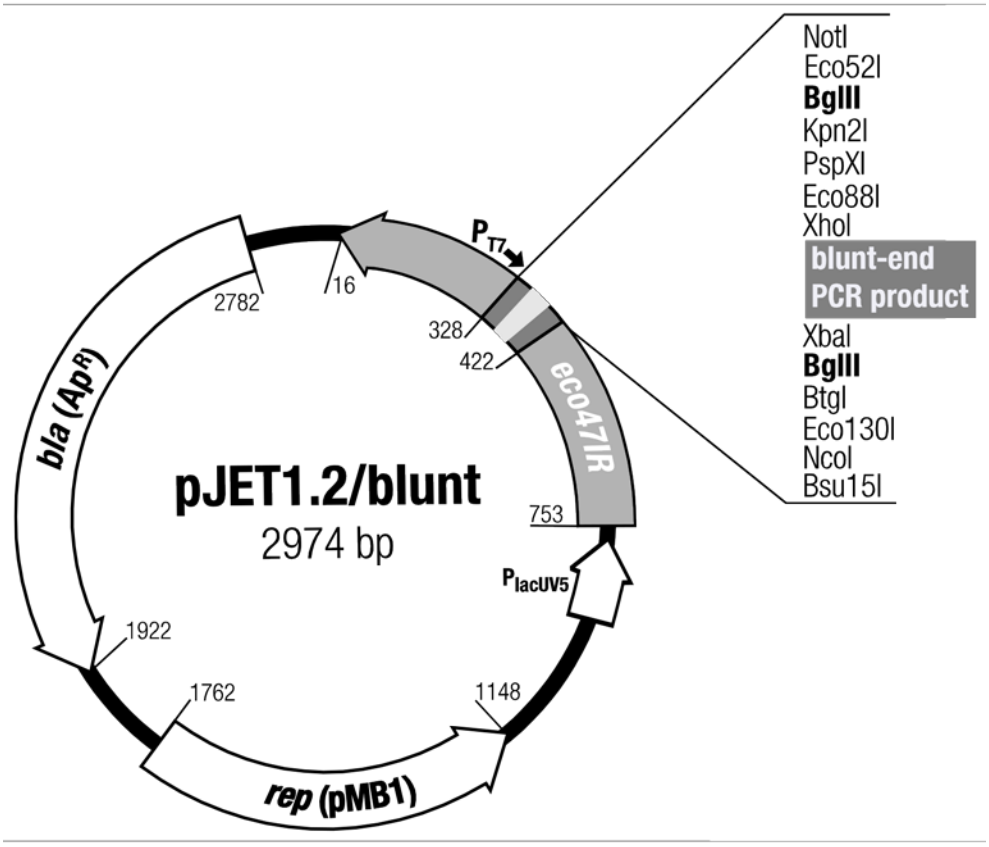


Figure 39: Map of the Pjet1.2/BUNT Cloning Vecor