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INVESTIGATIONS ON THE ROLE OF HAEMOCYTES IN DROSOPHILA HOST DEFENCE



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ABSTRACT

Innate immunity in *Drosophila* is mediated via two major responses, humoral and cellular immunity. Induction of the former is characterised by the production of antimicrobial peptides (AMP) via the Toll and Imd pathway. The cellular response is mediated via the haemocytes (blood cells), which engage in phagocytosis and the melanisation cascade. In parallel to the mammalian system, the possibility of haemocytes bridging the gap between pathogen recognition in the haemolymph and AMP gene induction in fat body cells was explored. To analyse their role, the haemocytes were ablated and various parameters for AMP gene expression were defined. The reduction in haemocytes was found to reduce AMP gene expression quantitatively as well causing a spatial restriction in the spreading of AMP expression in the fat body. To determine whether cytokine signals such as Spaetzle (Spz) or *unpaired3* (*upd3*) were involved in transducing the signal, they were knocked down in the haemocytes via RNA interference. The transcriptional reduction of Spz, the ligand for the Toll receptor, was shown to quantitatively reduce the expression of AMP while the reduction of *upd3*, the cytokine ligand for the JAK/STAT pathway, resulted in the same spatial restriction as larvae with reduced haemocyte counts. The reduction of *upd3* in the haemocytes also caused a reduction to signalling in the JNK pathway. The results here show that the haemocytes relay signal(s) to the fat body through the use of cytokines, a process surprisingly similar to the mammalian system. (240 words)

Supervisor: Dr Petros Ligoxygakis

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"Why has not man a microscopic eye? For this plain reason, man is not a fly"

Alexander Pope, An essay on Man, Epistle 1 (1733)

The research presented in this thesis
is my own original work unless otherwise
stated and has not been submitted
for another degree.

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LIST OF ABBREVIATIONS

Commonly used terms

AGM	aorta gonad mesonephros
AMP	Antimicrobial peptide
CFU	Colony forming unit
DAP	Diaminopimelic acid
DAPI	4-6-diamidino-2-phenylinodole
DNA	Deoxyribonucleic acid
FOG	Friend of GATA
GFP	Green fluorescent protein
GNBP	Glucan binding protein
HSC	haematopoietic stem cells
JAK/STAT	Janus kinase/Signal transducer and activator of transcription
JNK	Jun-N-terminal kinase
LB	Luria-Bertani
LPS	Lipopolysaccharide
MAPKKK	Mitogen activated protein kinase kinase kinase
NFκB	Nuclear factor kappa B
PAMP	Pathogen associated molecular patterns
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGN	Peptidoglycan
PGRP	Peptidoglycan recognition proteins
PPAE	Prophenoloxidase activating enzyme
proPO	Prophenoloxidase
PRR	Pathogen recognition receptor
PSC	posterior signalling center
RER	rough endoplasmic reticulum
RIP	Receptor interacting protein
RNA	Ribonucleic acid
RNA^i	RNA interference
ROS	Reactive oxygen species
Serpin	Serine protease inhibitor
SP	Serine protease
TEP	Thiolester protein
TNF	Tumour necrosis factor
UAS-GAL4	Upstream activating sequence-Regulatory protein GAL4

Commonly used gene names

Ced3	*C elegans cell death gene 3*
Coll	*Collier*
Crq	*Croquemort*
DD_1	*Drosomycin-GFP, Diptericin-LacZ*
Dipt	*Diptericin*
Dom	*Domino*
Drs	*Drosomycin*
Gcm	*Glial cells missing*
Hid	*Head involution defective*
Hml	*Hemolectin*
Ice	*Interleukin-1β-converting enzyme*
IL-1	*Interleukin-1*
Lz	*Lozenge*
Odd	*Odd-skipped*
Psh	*Persephone*
Puc	*Puckered*
PVR	*PDGF/VEGF receptor*
Pxn	*Peroxidasin*
Rp49	*Ribosomal protein 49*
Rpr	*Reaper*
Ser	*Serrate*
SPE	*Spaetzle processing enzyme*
Spn27A	*Serpin27A*
Spz	*Spaetzle*
Srp	*Serpent*
Tot	*Turandot*
Upd3	*Unpaired3*
Ush	*U-shaped*

1. INTRODUCTION

1.1 The model organism *Drosophila melanogaster*

The genus *Drosophila* of the family *Drosophilidae* consists of over nine hundred species, inhabiting all ecological regions of the Earth, except the driest deserts and the coldest tundras. *Drosophilae* only require moisture, a temperate climate and yeast as a food source to feed on. There are various species that are rare and seldom seen but the standard laboratory fruit fly, *Drosophila melanogaster* is a hardy and seasoned world traveller, probably introduced to the New World in cargoes of bananas. *Drosophila* began life in science as a model for experiments in evolution and variations in physiological characters that were crucial to survival in the wild such as vitality, fecundity, sex ratio and growth rates. T H Morgan was one of the few pioneers to employ *Drosophila* for experimental work in the early 1900s with those objectives. However, in January 1910 the discovery of the *white* mutant started a breeder reactor effect where his group soon found a flood of mutants and on 5 March 1912 they began to map chromosomes two and three (CARNEGIE INSTITUTION OF WASHINGTON. *et al.* 1919; MORGAN 1910). With the mapping, they began to physically reconstruct *Drosophila* for laboratory usage by creating standard mutant stocks and balancer chromosomes (chromosomes in *Drosophila* that have multiple aberrations preventing recombination, carrying either single or multiple markers, with at least one dominant visible marker) for specific laboratory purposes (KOHLER 1994).

The model animal chosen was well suited for an experimental life having a short generation time of approximately 10 days at room temperature. They are fertile all

year round with each female producing about 1,000 eggs and receptive to fertilisation a few hours after eclosion. They are tolerant to changes in their environment, such as weather and epidemics of mites or neglect. Their prominent characteristics include having easily distinguishable sexes where the males have outer genitalia that can be easily viewed and only four chromosomes distinguishable in a polytene chromosome preparation. A useful feature is that *Drosophila* males do not recombine their chromosomes. Their hardy ability to withstand mutation from various mutagens and crossbreeding experiments; as well as having a variety of genetic tools such as inversions, translocations, duplications, deletions and balancer chromosomes constructed from earlier years that allow genes or genetic mutations to be tracked allows them to be very versatile in the laboratory (ASHBURNER *et al.* 2005; GREENSPAN 2004). Finally, its genome has been fully sequenced and annotated increasing the appeal of using *Drosophila* as an insect model of choice (ADAMS *et al.* 2000).

1.2 *Drosophila* development

The generation time of *Drosophila melanogaster* at 25°C is approximately 10 days (Figure 1-1). The sperm enters the egg through the micropyle, a teat shaped structure at the anterior end. After oviposition, the embryo, which has an average length of 500μm and a diameter of about 180μm undergoes a series of nuclear multiplication without cell division, resulting in a syncytium (a mass of nuclei with a common cytoplasm) 70 minutes after fertilisation. Approximately 10 minutes later, the nuclei begin to move to the periphery of the cytoplasm and 2 hours after fertilisation, a syncytial blastoderm is formed. Three hours after fertilisation, the cellular blastoderm (where all future tissue is derived from as a single epithelial layer)

becomes evident, followed shortly by gastrulation. The movement of the endodermal and mesodermal tissues to their future positions inside the embryo leaves the ectodermal tissue as the outer layer driving the extension of the ventral blastoderm or germ band. This moves the posterior trunks regions round the posterior end onto what was the dorsal side. The end of embryonic development is signified by the retraction of the germ band and at this point in time, the first signs of external segmentation can be seen. There are 14 demarcated parasegments, 3 contributing to the mouthparts of the head, 3 thoracic segments followed by 8 abdominal parasegments. Twenty-four hours after fertilisation, the embryo hatches into a feeding first instar larva. Structures associated with the anterior region are called acron while at the posterior they are called telson. Between these two regions, the 3 thoracic segments and 8 abdominal segments can be distinguished via cuticle specialisations secreted by the epidermis. On the ventral side of each segment denticle belts and other cuticular structures characteristic of each segment are present. As the larva grows, it molts, shedding a cuticle every 24 hours, with each stage called an instar. Larvae are developmentally chimeric in that they contain tissue specific for their developmental stage, as well as imaginal discs (clusters of cells that give rise to structures of the adult fly). These imaginal discs are small sheets of prospective epidermal cells derived from the cellular blastoderm and usually contain about 40 cells each. They grow throughout larval life and become folded sacs of epithelia to accommodate their increase in size. There are discs for each of the six legs, two wings, two halteres, the genital apparatus, eyes, antennae and other adult head structures. In the segments of the abdomen there are groups of about ten histoblast cells that do not divide but will eventually contribute to the epidermis of the adult that ecloses 5 days after pupariation. At the end of the third

instar lasting approximately 2-3 days, a surge of ecdysone hormone is released that induces pupariation, triggering metamorphosis that lasts for approximately 5 days. The adult fly will begin mating 6-7 hours after eclosion (GILBERT 1994; WOLPERT 1998).

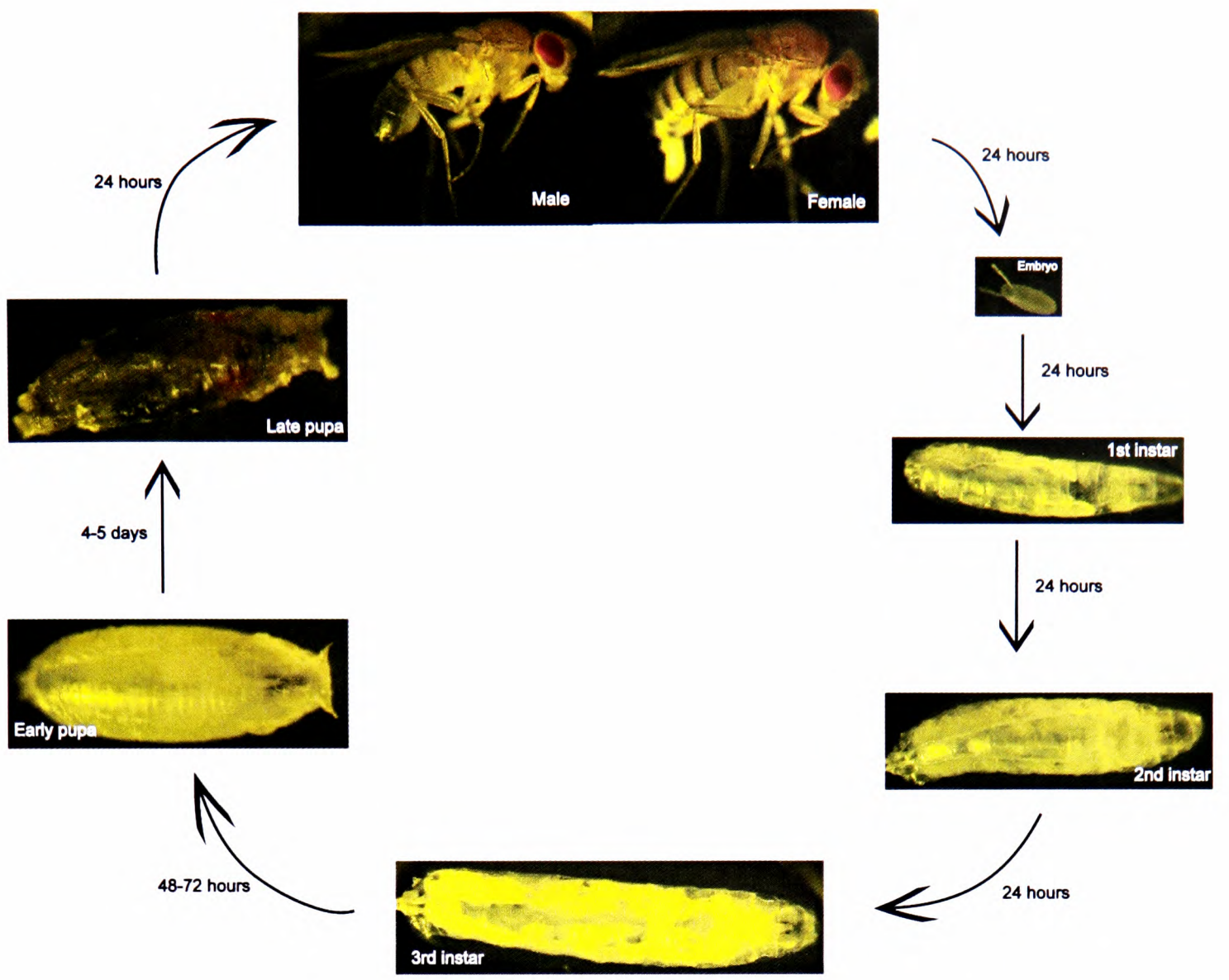


Figure 1-1 Illustrated life cycle of *Drosophila*. The time-line of each stage is indicated next to arrow bars. Figures are not to scale but are relative to each stage of the life cycle in the top figure. Adult female in the act oviposition, embryo (with outer chorion) and micropyle hatches into a feeding first instar and progressively increases in size through each instar until pupariation. In the late pupa, structures for the adult fly are visible through the outer cuticle. Staging of all stages as previously described (DEMEREC 1950).

1.3 Immunity in *Drosophila*

All multi-cellular organisms have developed various defence mechanisms that have the capacity to be triggered by a wounding event or an infection to protect the host organism that is commonly referred to as an immune response. Immunity can be divided into adaptive and innate with adaptive immunity a recent adaptation appearing only 500 million years ago in the ancestors of cartilaginous fish and independently in lamprey fishes (PANCER *et al.* 2004). Innate immunity is a phylogenetically ancient form of host defence that is encoded by the germline and in mammals it is co-opted to activate the adaptive response (HOFFMANN and REICHHART 2002). The innate immune system works by destroying the invading microbes and neutralising their virulence factors and has evolved under selective pressure imposed by infectious microorganisms. Induction of the immune system is only appropriate if the antigen recognised is derived from a pathogen. Structures selected for immune induction must have been absolutely distinct from self-antigens to avoid damage to self-cells and tissues. The innate immune response is believed to be modulated by pattern-recognition receptors (PRRs). These are specific molecules encoded by the host genome that recognise pathogen associated molecular patterns (PAMPs); invariant patterns that are normally present on the surfaces of pathogens and not present in the host (JANEWAY 1989). PAMPs are molecular structures that are essential for the survival of the microbes and thus are not subject to much variability as mutations in the structures would undermine the survival of the invading pathogen. Invariant structures that exist include the conserved lipid A of polysaccharides present in all Gram-negative bacteria, the cell wall minimal structure that is the peptidoglycan (PGN) and the β(1-3)-glucans that are present on the surfaces of fungal spores. PGN is an essential glycopeptide consisting of long glycan

chains of alternating N-acetylglucosamine and O-N-acetylmuramic acid residue that are cross-linked to each other by short peptide bridges. PGN from Gram-negative bacteria has a meso-diamenopimelic acid (DAP) instead of the lysine residue at the third position of the peptide chain and consists of a single layer, hidden in the periplasmic space underneath the outer membrane and lipopolysaccharides. PGN in Gram-positive bacteria is multilayered and exposed at the bacterial surface (FERRANDON *et al.* 2004; WANG and LIGOXYGAKIS 2006).

Invertebrates, although lacking the 'classical' mammalian adaptive defences are still able to fight an infection effectively, proving that an innate immune system with the ability to distinguish pathogen and discriminate against non-self is sufficient for the protection of the host. Although presumed not to have immunological memory, recent studies have shown that *Drosophila* may possess alternate means of adaptive response (PHAM *et al.* 2007). Innate immune responses in *Drosophila* can be subjectively divided into humoral and cellular responses, although it is expected that cross talk exists between them.

1.3.1 Humoral innate immunity

The humoral innate immune response has recently been under intense scrutiny, revealing the molecular mechanisms of both the Toll and Imd pathway (NAITZA and LIGOXYGAKIS 2004). Both pathways activate a family of nuclear factor-κB (NF-κB)/reticuloendotheliosis (Rel) transcription factors, initiating transcription of immune-responsive genes including antimicrobial peptides (AMPs, see page 16). The major site of AMP synthesis and secretion is considered to be the fat body, the insect equivalent of mammalian liver. The Toll signalling cascade regulates the

immune reaction against Gram-positive bacteria and fungal infections and is similar to mammalian Toll-like receptor/interleukin-1 receptor pathway (Figure 1-2) (LEMAITRE B *et al.* 1996). The pathway also plays key roles in development especially in establishing the dorsal-ventral gradient in the embryo (BELVIN and ANDERSON 1996). The immune cascade is initiated by the minimal subunit of Lys-type peptidoglycan, the muropeptide dimer (FILIPE *et al.* 2005). This trigger initiates a series of serine proteases (SPs) that undergo zymogen activation followed by a cleavage of substrate for the downstream protease (IMLER and HOFFMANN 2000). This culminates in the activating of the Toll receptor ligand Spaetzle (Spz), a cytokine-like nerve growth factor homologue. Activation of Spz is preceded by SP cascades that are involved in shaping and amplification of extracellular signals, inhibited by SP inhibitors (serpins). The activation of SPs during immune response differs from the ones during embryonic development. The ultimate SP for the Toll pathway is Spaetzle processing enzyme (SPE), recently elucidated during a targeted RNA^{i} screen (JANG *et al.* 2006). SPE has been shown to directly cleave and activate Spaetzle and has 89% amino acid identity with Easter (the embryonic SP that processes Spz for the dorso-ventral pathway). The proteolytic cleavage of Spz produces 2 fragments, of which the C-106 terminal fragment will dimerise and directly bind to the Toll receptor, activating the pathway (HU X *et al.* 2004; WEBER *et al.* 2003). Toll pathway activation is also mediated by Persephone (Psh) and the inhibitory serpin Necrotic (LIGOXYGAKIS *et al.* 2002a; ROBERTSON *et al.* 2003). Overexpression of Psh or the loss of function of Necrotic is sufficient to trigger Spz-dependant activation of Toll in the absence of an immune challenge. Once the Toll receptor has been activated, a hetero-trimeric receptor adaptor complex consisting of Myd88, Tube and Pelle is then formed; this activates an as yet unknown kinase that

phosphorylates Cactus, the *Drosophila* IκB homologue (BELVIN M P *et al.* 1995). Phosphorylation targets Cactus for ubiquitination and degradation through the proteosome pathway, leaving the NFκB homologue Dif free to translocate to the nucleus (RUTSCHMANN S *et al.* 2000). Additional components include the adaptor TRAF6, downstream of IRAK and the IκB kinases, IKK1 and IKK2. The *Drosophila* genome contains 3 TRAF-like proteins and 2 IKK-like kinases (SHEN *et al.* 2001). Dif and Dorsal, the NFκB-like *Drosophila* activators are encoded by two clustered genes, each containing an N-terminal Rel DNA binding domain and a C-terminal transactivator domain. It appears that Dif and Dorsal (both transactivators for the Toll pathway) play redundant roles at the larval stage (MANFRUELLI *et al.* 1999), while Dif is necessary and sufficient to mediate activation in adults (RUTSCHMANN *et al.* 2000a). The atypical protein kinase C (aPKC) and Ref(2)P which is the homologue of mammalian p62 are needed for Dif mediated transcriptional regulation downstream of Dif (AVILA A *et al.* 2002). Both aPKC and p62 has been shown to be required for mammalian NF-κB activation in a manner analogous to the Toll pathway. Flies deficient in Spz, Toll, Tube, Pelle are extremely sensitive to bacterial or fungal infection and fail to induce a subset of AMPs directed against those infections (LEMAITRE *et al.* 1996). Gain of function alleles for Toll or loss of function in Cactus induces nuclear localisation of Dorsal and Dif increasing the transcription of AMPs (NICOLAS *et al.* 1998).

Nine other Toll proteins are encoded in the *Drosophila* genome but none of which have any role in immune response. The Toll pathway also plays an additional role in haematopoiesis by controlling haemocyte density; constitutive activation of the Toll

pathway (gain of function mutants in Toll and Cactus) leads to hyper-proliferation of haemocytes and increased incidences of melanotic masses (QIU *et al.* 1998).

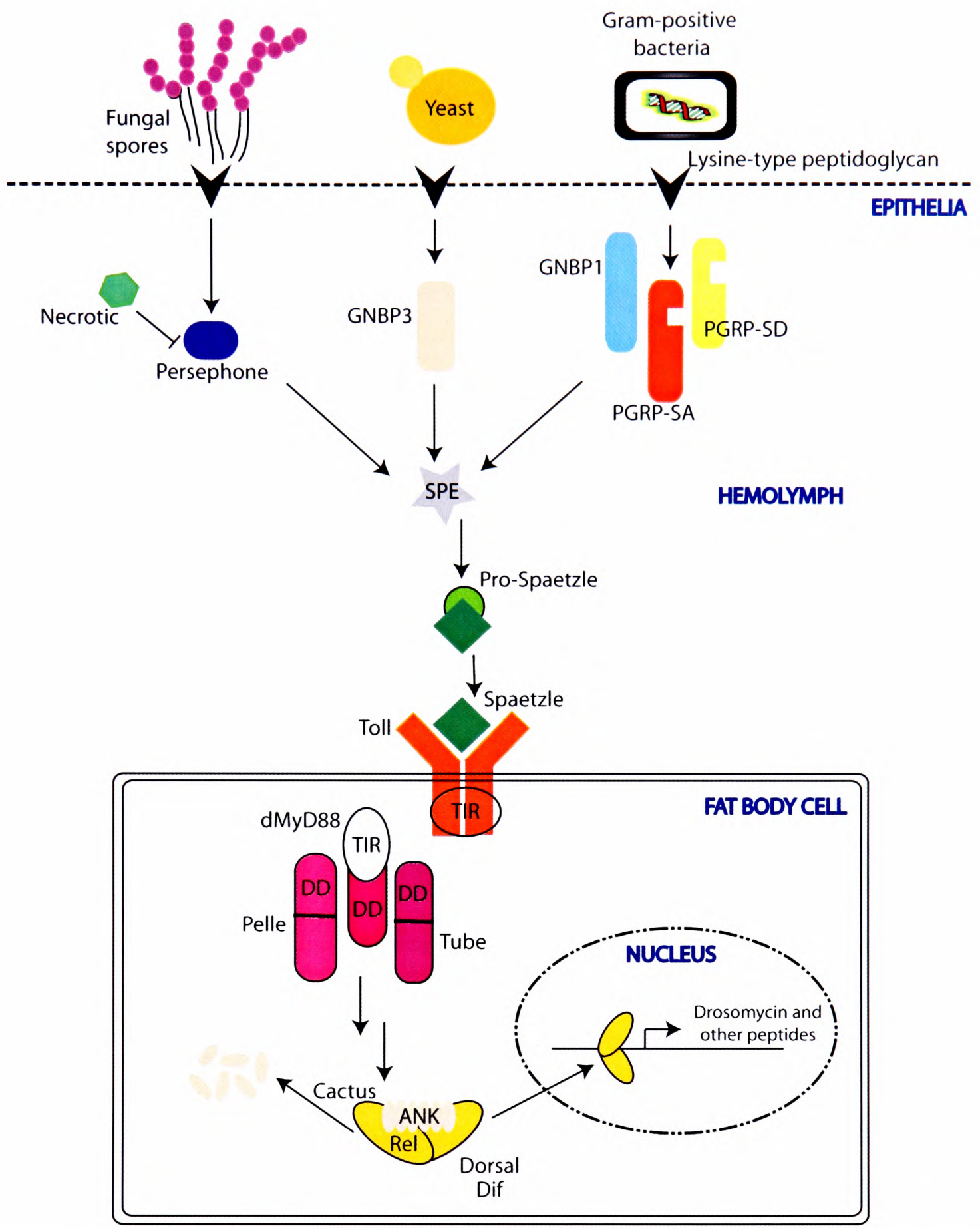


Figure 1-2 Schematic representation of the Toll pathway. Pathway explained in detail in the text. Protein domains abbreviated in the figures are TIR (Toll/Interleukin-1 receptor), DD (death domain) while common gene names are abbreviated are GNBP (Glucan binding protein), PGRP (peptidoglycan recognition protein) and SPE (Spaetzle processing enzyme). Infection sensing is discriminatory at the barrier epithelia triggering a cascade that will cleave the serine protease SPE to ensure a signalling cascade activating the Toll pathway that results in the translocation of either Dif or Dorsal to the nucleus to activate the necessary genes required for an immune response.

The immune deficiency (imd) gene is the most upstream member of the signalling cascade that leads to host activation following Gram-negative infection (Figure 1-3) (LEMAITRE *et al.* 1995). It is triggered mainly by DAP-type peptidoglycan. The Imd gene product has a Death domain similar to the one in human receptor-interacting protein (RIP) kinase (which mediates tumour necrosis factor receptor signalling) but lacks a kinase domain. Overexpression of Imd has been found to activate apoptosis (GEORGEL *et al.* 2001). The specific monomer GlcNAc-MurNAc(anhydro)-L-Ala-D-isoGlu-*meso*-DAP-D-Ala monomer (tracheal cytotoxin) has been identified as the minimal PGN motif capable of initiating the IMD cascade (LIM *et al.* 2006). The muropeptide is located at the end of the PGN strand and released during cell growth and division. The IMD pathway is remarkably similar to the mammalian tumour necrosis factor (TNF)-α pathway, where triggering of the pathway leads to the formation of an adaptor complex consisting of the Imd, dFADD and DREDD (LEULIER *et al.* 2002; NAITZA *et al.* 2002). dFADD induces the activation of dTAK1 (transforming growth factor activated kinase 1), the *Drosophila* homologue of mammalian mitogen-activated protein kinase kinase kinase (MAPKKK) which is involved in many mammalian signalling cascades (VIDAL S *et al.* 2001). As in mammals, dTAK1 mediates the phosphorylation and activation of an IKK complex comprising the *Drosophila* IKKβ and IKKγ (the kinase and accessory subunit of the enzyme) homologues *ird5* and *Kenny* (*key*), which leads to phosphorylation of Relish (LU Y *et al.* 2001; SILVERMAN *et al.* 2003). Epistatic analysis confirmed that TAK1 acts upstream of *ird5* and *key* but downstream of Imd (RUTSCHMANN *et al.* 2000b). The catalytically active *ird5* gene product is closely related to both α and β subunits of human IKK. Kenny is a protein distantly related to the γ subunit that is present in filamentous coiled-coil tail region of the myosin heavy chain. The region participates

in the formation of higher order complexes, linking IKK to the cytoskeleton. Relish, similar to mammalian p105, is then endoproteolytically cleaved in a proteosome independent manner and generates two stable fragments; Rel-68, the N-terminal Rel (DNA binding) homology domain that is translocated to the nucleus and the C-terminal inhibitory ankyrin repeat domain, Rel-49, which includes an IκB-like domain and remains cytoplasmic without inhibiting Rel-68 (STOVEN S *et al.* 2000). Relish mutants show no phenotypic effect on haemocytes or on cellular immune response but are extremely sensitive to bacterial infection (HEDENGREN *et al.* 1999). DREDD (a homologue of mammalian caspase-8) is presumed to recruit and activate a downstream caspase that cleaves and phosphorylates Relish but this has yet to be proven (LEULIER *et al.* 2002). The translocation of Relish to the nucleus initiates transcription of a variety of immune responsive genes (DE GREGORIO *et al.* 2002).

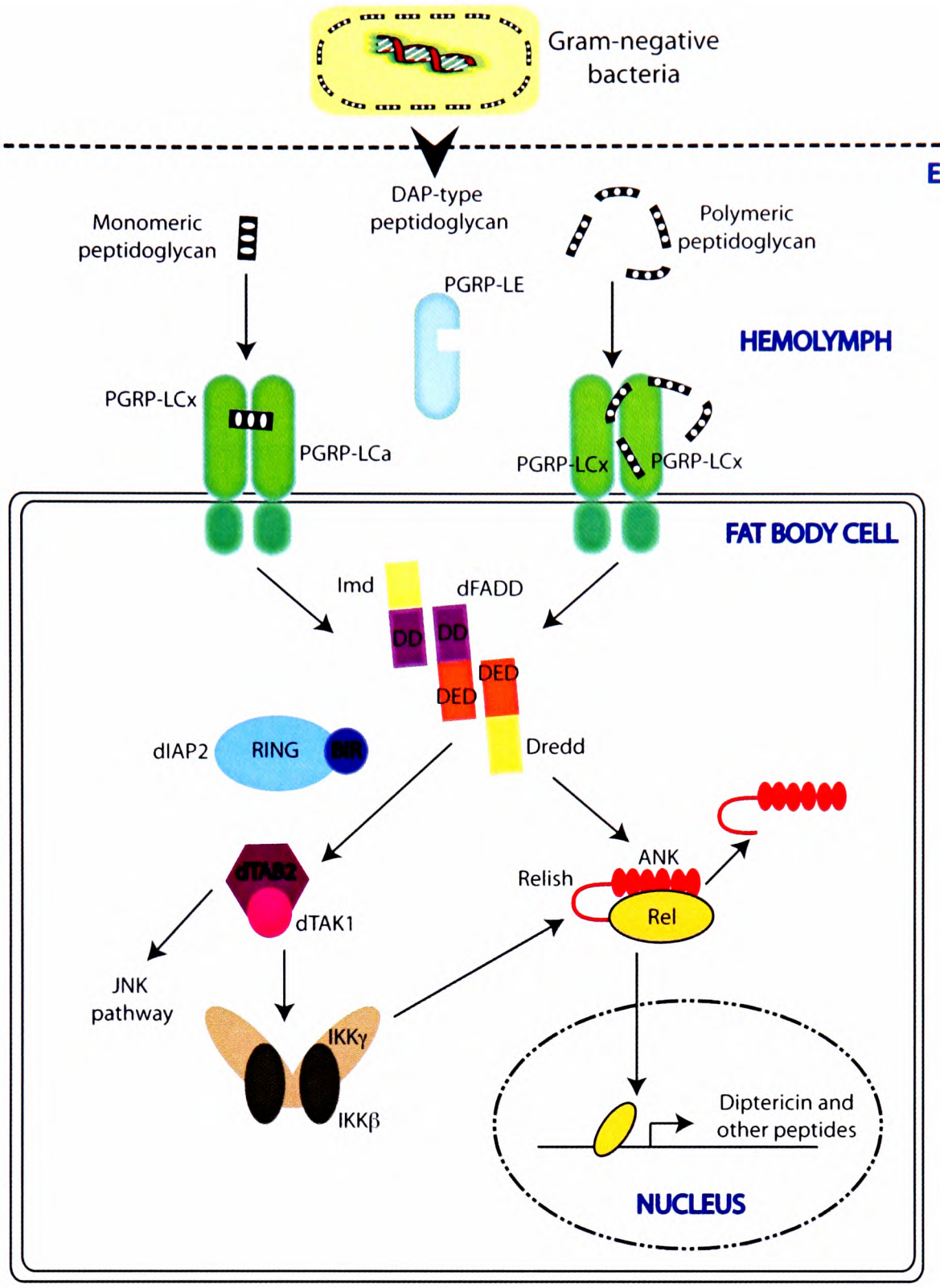


Figure 1-3 Schematic representation of the IMD pathway. Common protein domains abbreviated in the figure are DD (death domain), DED (death effector domain) and BIR (baculovirus IAP repeat) while gene names abbreviated are PGRP (peptidoglycan recognition protein), dIAP2 (*Drosophila* inhibitor of apoptosis 2), dTAK1 (*Drosophila* TGF-β activated kinase 1) and dTAB2 (*Drosophila* TAK1 binding protein). The pathway is triggered when the presence of lysed bacterial cell wall is detected by various PGRPs, triggering recruitment of an adaptor complex. The processes governing the interaction are mediated by various ubiquitination and proteosome processing pathways. Translocation of Relish to the nucleus initiates transcription of immune inducible proteins.

1.3.2 Receptors in immunity

In 1989, Janeway argued that innate immunity must operate by means of receptors that have been selected over evolutionary time to recognise highly conserved and widely distributed features of pathogens, especially features that are not found on the cells of multicellular organisms (JANEWAY 1989). Three different types of PRRs exist, humoral proteins circulating the haemolymph, endocytic receptors expressed on the cell surface and signalling receptors that can be expressed either on the cell surface or intra-cellularly (LEMAITRE and HOFFMANN 2007).

In *Drosophila*, PGN is the molecular pattern being recognised following bacterial infection (LEULIER *et al.* 2003). Peptidoglycan recognition proteins (PGRPs) are sensors that define the type of bacterial infection and trigger the proper signalling pathway (WERNER *et al.* 2000). PGRPs are highly conserved from insects to mammals and share a 160-amino acid domain (the PGRP domain) that share similarities to bacteriophage T7 lyzozyme, a zinc dependent N-acetylmuramyl-L-alanine amidase that cleaves peptidoglycans in their peptide side chains (KANG *et al.* 1998). Sequence analysis divided the 13 *Drosophila* PGRPs into 2 groups, the first one grouping PGRP-SA, SD, LA, LC, LD and LF as they lack the zinc binding residues required for amidase activity but still retain the ability to bind and recognise PGN. The second group is referred to as catalytic PGRPs (PGRP SC1A/B, LB, SB1/2) as they still retain the zinc dependent amidase activity that removes peptides from glycan chains, reducing or eliminating the biological activity of PGN (STEINER 2004). PGRP-LC deficient flies display a strongly reduced expression of AMP to Gram-negative bacteria, behaving very similarly to Imd deficient mutants, proving that this is the major receptor for the Imd pathway (TAKEHANA *et al.* 2004).

Alternative splicing produces three proteins (LCa, LCx and LCy) that share the same intracellular (signalling) domain but have differing extracellular (sensing) domains. Signalling is achieved through interaction of differing PGRP-LC molecules (see Figure 1-3). PGRP-LE is expressed both intra- and extra-cellular and is also involved in the Imd pathway. PGRP-LB and PGRP-SC are efficient amidases that remove peptides from the glycan chains and convert Gram-negative PGN into non-immunostimulatory fragments (ZAIDMAN-REMY *et al.* 2006). PGRP-LB is active only on DAP-type PGN and establishes a negative feedback loop thus turning off the Imd response whereas PGRP-SC digests both DAP and Lys-type PGN. From the second PGRP group, a mutation in PGRP-SA (secreted in the haemolymph) was found to block activation of Toll pathway by Gram-positive bacteria only (MICHEL *et al.* 2001). PGRP-SD, another secreted PGRP, was also found to mediate Toll activation by a subset of Gram-positive bacteria (BISCHOFF *et al.* 2004).

The members of the *Drosophila* glucan binding protein (GNBP) family include three canonical members, GNBP1-3 and a GNBP-like gene. They display a significant homology to bacterial glucanases, containing an N-terminal domain that can bind β(1,3-glucan) and a C-terminal domain, homologous to the catalytic domain of β-glucanases (KIM *et al.* 2000). The role of GNBP1 was postulated when mutants were found to be sensitive to Gram-positive bacterial infections (GOBERT *et al.* 2003) and recently it has been found to co-operate with PGRP-SA to hydrolyse PGN (WANG *et al.* 2006). The role of GNBP2 is currently under investigation but it also seems to be involved in Gram-positive bacteria sensing (LIGOXYGAKIS P., personal communications). GNBP3 mediates activation of the Toll pathway by detecting fungal spores. Experiments with yeast show that flies lacking GNBP3 are sensitive to

these fungi. However, entomopathogenic filamentous fungi seem to directly activate the protease cascade of the Toll pathway through fungal proteases that penetrate the host cuticle. When expressed in the fly, the fungal protease PR1 triggers the Toll pathway (GOTTAR *et al.* 2006). This activation depends on the host protease Psh (LIGOXYGAKIS *et al.* 2002a), a significant component of the branch, which leads to Toll upregulation through fungal infections (Figure 1-2).

1.3.3 Effectors of immune response pathways

The sequential activation of the signalling pathways induces transcription of a plethora of immune responsive genes, among which include the antimicrobial peptides (AMPs). The AMPs are small, cationic molecules exhibiting a wide range of activities, peptides that reach micromolar concentrations or higher (BULET *et al.* 2004). Twenty-immune inducible AMPs, grouped into 7 classes have been identified, exhibiting a wide range of bacteriocidal or fungicidal activities. They are listed in Table 1-1 with their properties and physiological concentrations. The precise mode of action for AMPs is unknown but they are presumed to lyse bacteria by forming pores in their membranes, as shown by studies involving *Phormia* (blowfly) recombinant defensin. The addition of *Phormia* defensin to the Gram-positive *M luteus*, disrupted the permeabilisation barrier of the cytoplasmic membrane and patch clamp experiments support the hypothesis of formation of channels through the membrane (COCIANCICH *et al.* 1993). Genes regulated by Imd generally show an acute phase profile while Toll target genes exhibit a late and sustained pattern of expression (BOUTROS *et al.* 2002; LEMAITRE *et al.* 1997).

The Turandot (Tot) family of genes are part of the humoral stress response being transcriptionally activated in response to bacterial challenge or other environmental insults (EKENGREN and HULTMARK 2001). The family of 8 genes are expected to be small peptides that are secreted into the haemolymph from the fat body. The expression of Tot proteins is modulated by the JNK and JAK/STAT pathways (AGAISSE *et al.* 2003). It is expected that Tot proteins interact with denatured proteins that arise from the stress applied to the animals in the extracellular environment (EKENGREN and HULTMARK 2001).

In vertebrates, thiolester containing proteins (TEPs) participate in the complement cascade and are commonly identified in as acute phase reactants (BLANDIN and LEVASHINA 2004). In *Drosophila*, 6 TEPs have been identified and 3 of them (TEP1, TEP2 and TEP4) have been shown to be upregulated after bacterial challenge in larvae (LAGUEUX *et al.* 2000). TEP1 is expressed in haemocytes of naïve and challenged larvae with inducible expression being controlled by either Toll or IMD. It is proposed that TEP function as opsonins to promote phagocytosis and/or as protease inhibitors (MEISTER and LAGUEUX 2003).

Antimicrobial peptide family	Main biological activities at physiological concentrations	Number of genes		Post translational modifications	Concentration in the blood (systemic response)	Epithelia expressing various antimicrobial peptides
		Per genome	Expressed			
Diptericin	Antibacterial, Gram-negative	2	2	Two O-glycosylations, COOH-terminal amidation	0.5μM	Midgut
Attacin	Antibacterial, Gram-negative	4	4			Midgut
Drosocin	Antibacterial, Gram-negative	1	1	O-glycosylation	40μM	Calyx, oviduct, tracheae
Cecropin	Antibacterial, Gram-negative	4	4	COOH-terminal amidation	50μM	Calyx, oviduct, seminal receptacle, spermathecae
Defensin	Antibacterial, Gram-positive	1	1		1μM	Seminal receptacle, spermathecae, labellar glands
Metchnikowin	Antifungal	1	1		40μM	Labellar glands
Drosomycin	Antifungal	7	2		100μM	Labellar glands, seminal receptacle,

Table 1-1 List of inducible antimicrobial peptides in *Drosophila*. Adapted from (HOFFMANN and REICHHART 2002)

1.4 Cellular immunity

1.4.1 Plasmatocytes

Cellular immunity in *Drosophila* is mediated by haemocytes (blood cells in insects and crustacea). They do not transport oxygen that is provided by the tracheal system but their main roles include seeking out and removing dead cells and debris, secreting and remodelling the extracellular matrix and secreting AMPs, as well as engulfing and encapsulating foreign invaders (MEISTER 2004; SAMAKOVLIS *et al.* 1992). Three types of haemocytes have been morphologically classified that share similarities with the mammalian myeloid linkage (MEISTER M 2004; MEISTER M and LAGUEUX M 2003; RIZKI T M and RIZKI R M 1984). The main part of the haemocyte population (approximately 95%) consists of plasmatocytes (Figure 1-4). They are small, rounded cells which function as phagocytes, clearing pathogens and removing apoptotic cells (MEISTER and LAGUEUX 2003). They are most similar to mammalian macrophage/monocyte cells and are involved in similar roles such as phagocytosis, development and signalling (TIROUVANZIAM R *et al.* 2004). Phagocytosis of non-self by plasmatocytes includes attachment of phagocyte to particle followed by cytoskeletal modification and internalisation of the target. The engulfed target (including bacteria, yeast, Sephadex beads, dsRNA or ink particles) is then destroyed by lysosomal enzymes, reactive oxygen species and nitric oxide within minutes (ADEREM and UNDERHILL 1999; ELROD-ERICKSON *et al.* 2000). They are derived from prohaemocytes; cells that are 4-6μm in diameter with little cytoplasmic volume, many free ribosomes and large lipid droplets (MEISTER and LAGUEUX 2003). Plasmatocytes contain more cytoplasmic volume and have abundant lysosomes, as well as large amounts of endoplasmic reticulum. Plasmatocytes are

crucial for morphogenetic processes as they secrete extracellular matrix proteins such as Peroxidasin (Pxn), Laminin, Tigrin, Glutactin, and MDP1, and the collagen proteins Dcg1 and Viking (FESSLER *et al.* 1994; HORTSCH *et al.* 1998; NELSON *et al.* 1994). Plasmatocytes mediate the recognition of apoptotic cells and pathogens by various receptors such as Croquemort (Crq), DSR-CI (*Drosophila* scavenger receptor), Eater, Dscam and PGRP-LC as well as secreting a small amount of AMPs (FRANC *et al.* 1996; KOCKS *et al.* 2005; PEARSON *et al.* 1995; RAMET *et al.* 2002b; SAMAKOVLIS *et al.* 1990; WATSON *et al.* 2005). The main role of plasmatocytes as professional phagocytes requires the expression of Crq (a CD36 homologue) (FRANC *et al.* 1996; FRANC *et al.* 1999). In mammals, CD36 is a class B scavenger receptor that acts in concert with the vitronectin receptor ($\alpha V\beta 3$) an integrin via the bridge of thrombospondin, an extracellular matrix protein to bind to the apoptotic corpse. Crq is expressed exclusively on embryonic macrophages where it is found at the surface of intracellular vesicles that contain apoptotic corpses. Mutants lacking Crq expression were inefficient at removing apoptotic corpses although they were able to carry out other macrophage functions (FRANC *et al.* 1999).

A corollary cell type, podocytes, are cells that contain extensive cytoplasmic projections or pseudopoedia (Figure 1-4) (RIZKI T M and RIZKI R M 1984). It is assumed that they are differentiated from plasmatocytes and will be further differentiated to lamellocytes, another cell type in *Drosophila*. It is possible that podocytes are just plasmatocytes with modified adhesion capacity. The hypothesis correlates with the increase in podocyte number to the decrease of plasmatocyte number at the onset of metamorphosis. The cell is not molecularly characterised as a

separate cell type and does not appear to have any specified role (LANOT R *et al.* 2001; RIZKI T M and RIZKI R M 1984).

1.4.2 Lamellocytes

Lamellocytes are an unusual cell type in *Drosophila*, being differentiated only after immune challenge in the larvae. They are large (15-40μm in diameter), flat adherent cells with sparse cytoplasmic constituents (Figure 1-4). The cells express L1 antigen and *misshapen* (part of the JNK signalling cascade) (CARTON and NAPPI 1997). Development of lamellocytes is induced when an object that is too large to be phagocytosed is detected in the larval cavity. They have not been detected in embryos and adults (LEMAITRE and HOFFMANN 2007). Within a few hours of parasitation, signals to the lymph gland induces an increase in proliferation and the massive differentiation of lamellocytes from prohaemocytes from the medullary zone and secondary zones of the posterior signalling centre (JUNG *et al.* 2005). Lamellocytes are then released from the lymph glands and form multilayered capsules around the pathogen. Typically, encapsulation is observed by parasitation of *Drosophila* embryos by the wasp *Leptopilina boulardi* (SORRENTINO *et al.* 2002). Wasp eggs laid in the embryo are encapsulated by lamellocytes and are blackened due to melanisation. The parasite is killed either by asphyxiation or by production of reactive oxygen species or cytotoxic free radicals that consist of the quinones or semiquinones from the intermediates of the melanisation cascade (SODERHALL and CERENIUS 1998). Encapsulation is believed to be triggered by the absence of a factor found on the 'foreign' basement membrane. Hemese, a transmembrane glycophorin-like protein whose expression is restricted to the cell surface of haemocytes and haematopoietic organs is believed to play a role in differentiating lamellocytes

(KURUCZ *et al.* 2003). Depletion of Hemese by RNAi suggests that it plays a modulatory role in the activation and recruitment of haemocytes for encapsulation.

1.4.3 Crystal cells

The final morphologically distinguishable cell type of the haemocyte population is the crystal cell, consisting approximately 5% of the total haemocytes (Figure 1-4) (MEISTER M and LAGUEUX M 2003; RIZKI T M and RIZKI R M 1984). They are 10-12μm in diameter, fragile and so named because they contain paracrystalline inclusions that contain a high concentration of enzymes and substrates for melanisation. They do not participate in phagocytosis and are instead involved in the process of melanisation. Crystal cells express Lozenge (Lz) and three different genes encoding prophenoloxidase (proPO) that have been found in the *Drosophila* genome including proPO-A (oxidoreductase related to hemocyanins and vertebrate tyrosinases that mediate the melanisation reaction) (BATAILLE *et al.* 2005). Physical breakage of the arthropod cuticle immediately induces haemolymph clotting and melanisation (BIDLA *et al.* 2005). Clotting is critical to limiting haemolymph loss and initiating the wound healing process. Melanisation seals the wound before the more elaborate wound-healing process and is also part of cuticle formation for adult animals (SODERHALL and CERENIUS 1998). The blackening results from the *de novo* synthesis and deposition of melanin. The phenoloxidase cascade consists of a series of proteases, which ultimately catalyse prophenoloxidase (proPO) to phenoloxidase (PO). ProPO is cleaved by a serine protease known as prophenoloxidase activating enzyme (PPAE) also activated through a stepwise cascade of other SPs (SODERHALL K and CERENIUS L 1998; THEOPOLD U *et al.* 2004). The PO catalyses the oxidation of mono- and diphenols to orthoquinones, resulting in a non-enzymatic

polymerisation to melanin. The intermediate products of the reaction are directly toxic to invading microbes. The melanisation cascade is regulated via Serpin27A through the specific inhibition of prophenoloxidase processing by the serine protease cascade (LIGOXYGAKIS *et al.* 2002b).

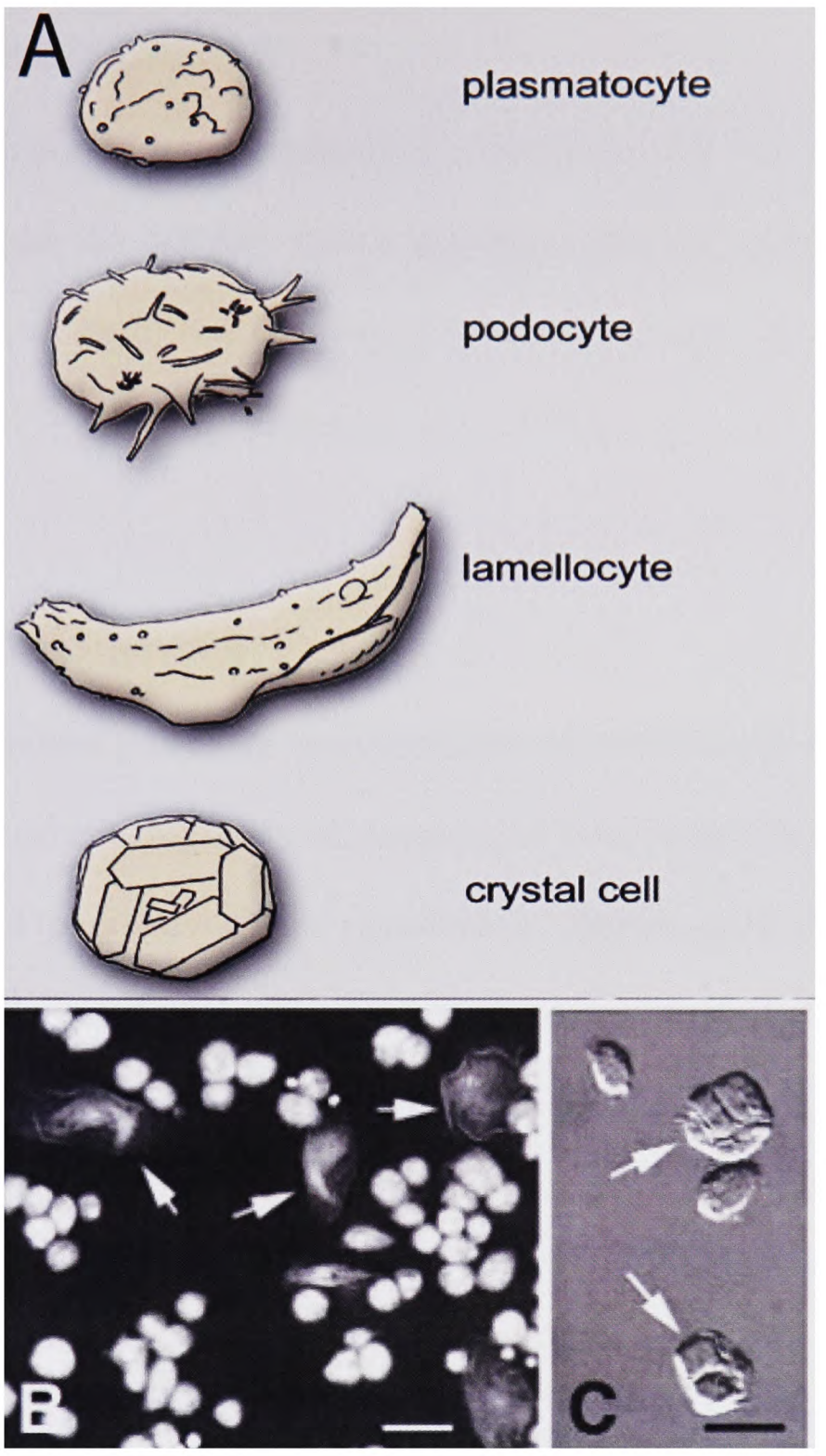


Figure 1-4 A) A cartoon representation of different cell types in *Drosophila*, adapted from (RIZKI T M and RIZKI R M 1984). All morphological characteristics have been described in the text. B) The brightly fluorescent, smaller rounded cells are plasmatocytes, while the larger flattened cells are the lamellocytes (highlighted by white arrows). C) A phase contrast microscopy image of circulating crystal cells (highlighted by the white arrows) showing crystalline inclusions. Latter images adapted from (MEISTER M and LAGUEUX M 2003)

1.5 Haematopoiesis in *Drosophila*

There are two phases of haematopoiesis in *Drosophila*, the first one during early embryogenesis from the cephalic (head) mesoderm and the second one at a later larval developmental stage from the mesodermal lymph glands (Figure 1-5) (HOLZ A *et al.* 2003; LANOT R *et al.* 2001; TEPASS U *et al.* 1994).

1.5.1 Embryonic haematopoiesis

Proliferation of haemocytes in the embryo follows an intrinsically fixed programme where by the end of embryogenesis, all haemocytes have been differentiated (TEPASS U *et al.* 1994). The prohaemocytes representing plasmatocytes move around the embryo undergoing 4 cell divisions at specified times and carry out sentinel duties while the crystal cell precursors remain concentrated around the proventriculus. A variety of genes are involved in specification of haemocytes in the embryo and will be discussed briefly.

Serpent (Srp, one of the five *Drosophila* GATA factor homologue) plays a central role in committing head mesodermal and proventriculus cells to haemocyte fate beginning in stage 5 and continues to be expressed in mature plasmatocytes (LEBESTKY *et al.* 2000). The GATA family of zinc transcription factors are highly conserved from yeast to vertebrates and have many diverse roles in developmental programmes including cell fate specification, differentiation and proliferation. Srp is transcriptionally controlled using 2 spliced isoforms containing either one (SrpC) or two (SrpNC) GATA zinc fingers (WALTZER *et al.* 2002). Members of the friend-of-GATA (FOG) proteins interact with the N-terminal zinc fingers and modulate their

activity. The FOG U-shaped (Ush) represses crystal cell fate and inhibits expression of the apoptotic body receptor Crq in crystal cells by interacting with SrpNC (FOSSETT *et al.* 2001). Ush is expressed in the head mesoderm by embryonic stage 8 and is maintained in plasmatocytes and downregulated in crystal cells. The interaction between Ush and Crq is not required for initial specification but required for subsequent steps in haematopoietic lineage commitment (Figure 1-6B).

Another gene involved in embryonic haematopoiesis is Lozenge (Lz). The gene is a member of the Runx family of transcription factor and has high homology (71% within the RUNT domain) to human AML/Runx1 (LEBESTKY *et al.* 2000). Expression of Lz appears to be triggered in a subset of Srp expressing cells in response to Notch signalling and is spatially restricted, remaining localised to their point of origin (LEBESTKY *et al.* 2003). Lz is required for differentiation of crystal cells; expression can be detected in the head mesoderm at approximately stage 10, in 2 bilateral clusters of 18 cells each within a subset of Srp-expressing prohaemocytes, termed the crystal cell precursors. Expression of Lz during stage 10-14 is necessary for proper crystal cell differentiation (WALTZER *et al.* 2003). By stage 17, they have matured into a single, loose cluster that remains in the head region. Lz is also expressed in mature crystal cells, both in circulation and in the lymph glands. Ectopic expression of Lz is not sufficient to repress plasmatocyte differentiation. Co-expression of Lz and Srp throughout the mesoderm causes large numbers of crystal cells to develop, while expression of either alone causes only a modest increase in crystal cell differentiation. Lz interacts directly, via the Runt domain, with Srp through its GATA zinc finger. Crystal cells are missing in Lz null mutants while plasmatocytes develop normally.

A set of novel transcription factors that have been conserved to vertebrates with no defined role are Gcm (glial cells missing) and its homologue Gcm2. They are required for expression by cells downstream of Srp entering the plasmatocyte lineage but are excluded from crystal cell precursors (Figure 1-6B) (ALFONSO and JONES 2002; BERNARDONI *et al.* 1997). Expression of both is restricted to plasmatocyte precursors soon after Srp expression in stage 5 embryos, where they co-localise with Pxn and Crq. Their expression diminishes after stage 11 and cannot be observed in fully differentiated plasmatocytes. Gcm and Gcm2 have been intensively studied during neurogenesis as they are required to promote glial cell fate at the expense of neuronal cell fate (BERNARDONI *et al.* 1999). Forced expression of Gcm in crystal cell precursors redirects them to plasmatocytes while mutant embryos for either one exhibit a limited reduction in plasmatocytes (25-40%) and simultaneous deletion has severe consequences in those mutants (ALFONSO and JONES 2002; BATAILLE *et al.* 2005). All Crq expression is lost and remaining presumptive plasmatocytes (expressing Pxn) are reduced in number and exhibit aberrant morphologies and migratory behaviour. Both Gcm and Gcm2 play critical overlapping roles in early expansion and definitive maturation of plasmatocyte lineage. They appear to function as genetic switches in the specification of plasmatocytes as all plasmatocytes expressing Srp also express Gcm/Gcm2.

As in mammals, haematopoiesis requires movement of proliferating and newly differentiated cells. The PDGF/VEGF receptor (PVR) is first expressed in prohaemocytes of the head mesoderm at stage 8 and is maintained in plasmatocytes (BRUCKNER *et al.* 2004; DUCHEK *et al.* 2001). The receptor tyrosine kinase PVR (homologous to vertebrate PDGF and VEGF receptor) functions in embryonic

haemocytes controlling cell migration and cell survival by providing migration cues for haemocytes in the early embryo. The cells migrate throughout the embryo along an invariant path.

To summarise, there exists a genetic hierarchy in embryonic haematopoiesis where Srp is the upstream factor of Lz and Gcm, both of which are mutually exclusive lineage specific transcription factors in haematopoiesis (Figure 1-6B). Crystal cells and plasmatocytes develop from a pool of bipotent haematopoietic progenitors and lineage segregation is a highly dynamic process of interplay between Gcm/Gcm2 and Lz.

1.5.2 Secondary haematopoiesis

The second wave of haematopoiesis is initiated during the larval stages by lymph glands, formed during embryogenesis (Figure 1-5) (JUNG *et al.* 2005; LANOT R *et al.* 2001). In the late embryo, the lymph gland is a single pair of lobes containing approximately 20 cells, expressing Srp and Odd-skipped (Odd). The lymph gland appears in the dorsal mesoderm in the thoracic segments during mid embryogenesis, migrates dorsally forming a tight cluster and attaches itself to the sides of the dorsal vessel after dorsal closure. The lymph gland consists of 4-6 paired lobes along the dorsal vessel, reduced in size from anterior to posterior, surrounded by non-cellular extra-cellular matrix. Notch signalling is required for the early specification of the lymph gland. Each lymph gland lobe is identified by its position on the dorsal vessel relative to the pericardial cells that separate the lobes. The number of lobe pairs varies between individual and depends on temperature, nutrition and/or immune challenge. Lobes are surrounded by thick, basement-like membrane and actively

participate in the synthesis of extracellular matrix at all stages of development. The first of 2 or 3 lymph gland lobes from 2nd instar larvae contain approximately 200 undifferentiated cells consisting of prohaemocytes that are grouped in clusters by a whorl of basement membrane. Another group of cells only observed in the primary lobes and slightly larger at 5μm are termed secretory cells. They have enlarged cistarnae of the RER, numerous Golgi vesicles, and are abundant at the periphery of the glands.

In the third instar, the size of the lymph glands increase by another 10 times, while continuing to express Srp and Odd. The primary lymph gland lobe has a strong branching network of extracellular matrix and is divided into 3 distinct regions: the periphery exhibiting a granular appearance with loosely arranged cells (cortical zone) with differentiating haemocytes, the medial region called the medullary zone is smooth with compactly arranged cells consisting differentiating haemocytes and finally the posterior signalling centre (PSC) that acts as an organiser (Figure 1-5A) (JUNG *et al.* 2005). PSC consists of a small cluster of cells at the posterior tip and is defined by the expression of the Notch ligand Serrate (Ser, clustering at the tip), the transcription factor Collier (Coll) and the absence of PVR (CROZATIER *et al.* 2004; KRZEMIEN *et al.* 2007; LEBESTKY *et al.* 2003). Ser signalling through Notch mediates the commitment of prohaemocytes to crystal cell lineage via the expression of Lz that is initiated in a small number of cells during the second instar. Coll is homologous to mammalian early B-cell factor and is expressed earlier than Ser and controls its expression. Coll mutant larvae fail to produce lamellocytes upon parasitisation alluding to the earlier statement that lamellocytes are produced massively from a lymph gland signal (CROZATIER *et al.* 2004). All lymph gland cells

express Srp but only a small subset express Lz. The number of Lz expressing cells increases until 50-100 per lobe, expression is scattered uniformly throughout the primary lobe but not the others. Ectopic expression of Notch signalling pathway dramatically increases the number of Lz-expressing cells within the lymph glands, especially in the secondary lobes (DUVIC *et al.* 2002; JUNG *et al.* 2005).

Circulating cells in larval stages express Pxn, P1 antigen and are largely derived from embryonic head mesoderm that arose through earlier embryonic proliferation and not from lymph glands unless immune challenged (HOLZ A *et al.* 2003; LANOT *et al.* 2001). There are approximately 200 cells in the first instar and this rises dramatically to approximately 5000 cells at the end of third larval instar. The crystal cell population reaches approximately 80 cells per animal (2%) and all the circulating crystal cells continue to express Lz (LANOT *et al.* 2001). A major blood cell pool until 3rd instar (approximately one-third) are anchored to the integument in direct contact with epithelia and are thought to be plasmatocytes and/or crystal cells. They have been arbitrarily called sessile cells and have not been morphologically and molecularly classified as a separate group. Sessile haemocytes are grouped in densely packed clusters in the neighbourhood of oenocytes or along the posterior region of the dorsal vessel that are in direct contact with the epidermal layer, linked through intricate interdigitations, with no desmosomes or septate junctions, contain no secretory cells and have rare undifferentiated blast cells (LANOT *et al.* 2001).

During the late 3rd instar wandering stage, phagocytes become more abundant. Phagocytosis within the first lymph gland lobe becomes more frequent as the larvae approach metamorphosis. The number of blood cells in the lobe drops sharply just

before pupariation (JUNG *et al.* 2005; LANOT *et al.* 2001). At the onset of metamorphosis, the lymph gland releases large numbers of active phagocytes, pupal macrophages that play a crucial role in tissue remodelling as they phagocytose cells of doomed larval structures, including lymph glands. This occurs under the control of the steroid hormone ecdysone that determines all tissue re-modelling related to metamorphosis (JUNG *et al.* 2005). Five hours after the release of ecdysone, the blood cell content of the first lobe has been significantly decreased and by 12 hours, they are almost empty and the secondary lobes release their haemocytes. The secondary lobes serve as a reservoir for prohaemocytes in a semi-quiescent phase, but have a less well-defined organisation than the primary lobes. A few hours after that, the lymph gland lobes are all empty and are not observable.

After pupariation and eclosion, no haematopoietic site has been described in the adult fly. Sessile haemocytes number approximately 1000-2000 cells/individual and are mostly concentrated on the halteres, legs and are near or within the dorsal vessel (MEISTER and LAGUEUX 2003). Haemocytes observed are mostly plasmatocytes, crystal cells are rare and lamellocytes are never seen. An elegant clonal analysis has shown that all adult haemocytes are composed of a mixture of embryonic and larval stages haemocytes (HOLZ *et al.* 2003).

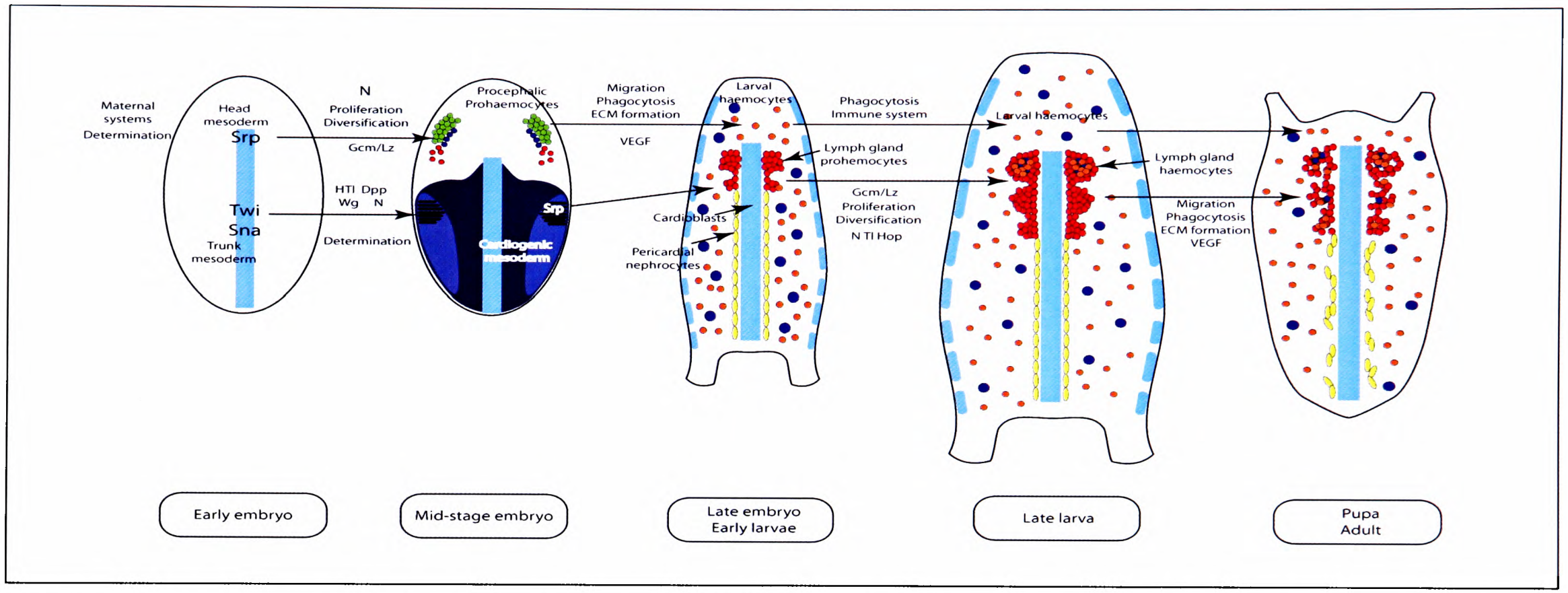


Figure 1-5 A summary of haematopoietic development in *Drosophila*. In the early embryo, expression of the GATA factor (Srp) is established by maternal components and the mesoderm specifying transcription factors Twist (Twi) and Snail (Sna). In the mid-stage embryos, haemocytes are differentiated through the expression of various signalling pathways such as Notch (N), Glial-cells-missing (Gcm) 1 and Gcm2 and the Runx factor Lozenge (Lz). The cardiogenic mesoderm is specified from the trunk mesoderm by Heartless (Htl), Decapentaplegic (Dpp), Wingless (Wg) and N. Continuing on to the late embryo, the vascular epithelial growth factor (VEGF) pathway plays a role in dispersing the haemocytes. Expansion of the lymph glands in the late larval stages involves a multitude of signalling pathways including N, Toll (Tl) and Hopscotch (Hop) while differentiation of the cells utilised the same N, Gcm and Lz as in embryonic haematopoiesis. At the onset of metamorphosis, characterised by ecdysone, the lymph gland will degenerate and release all the haemocytes into circulation.

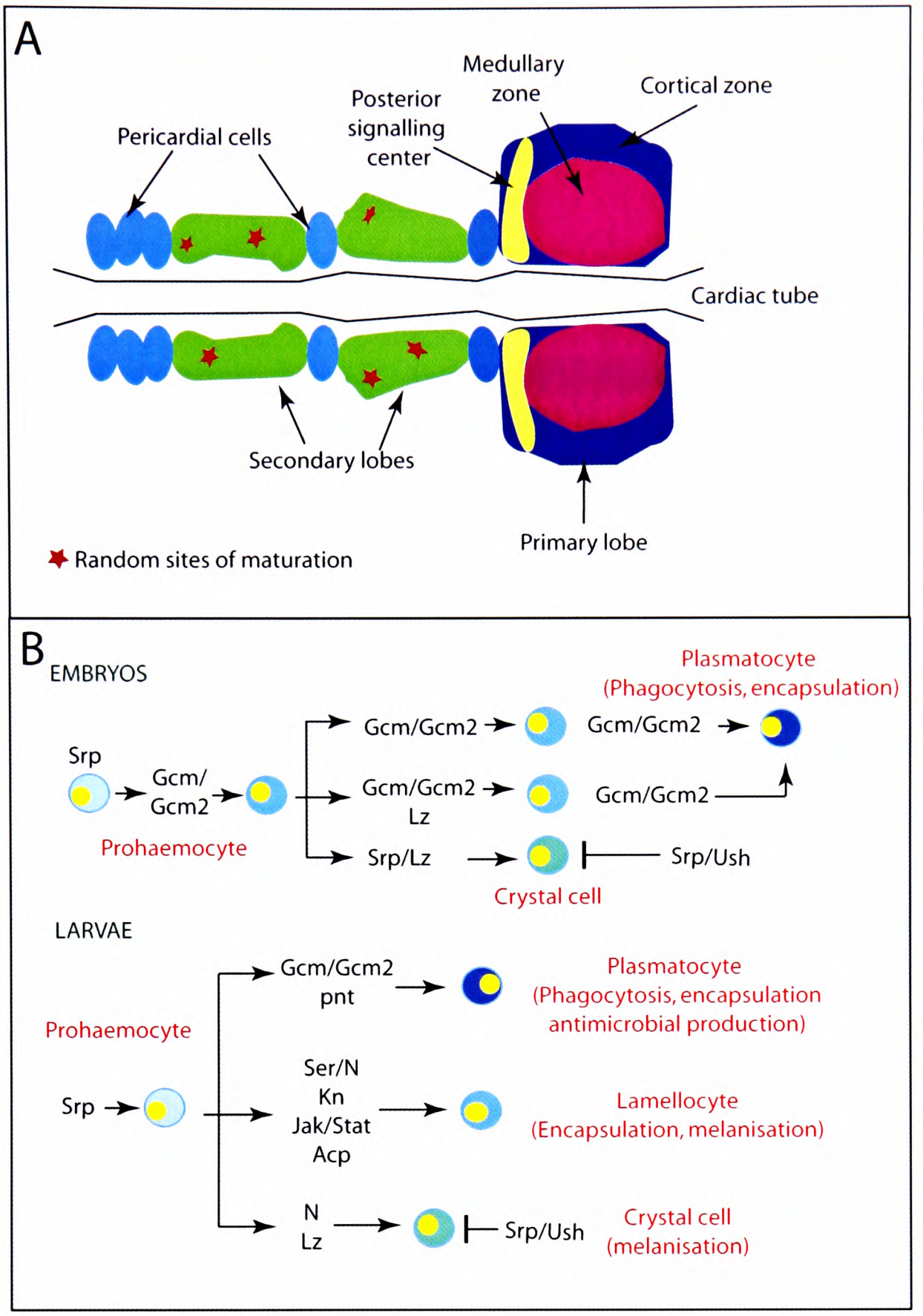


Figure 1-6 A) Schematic diagram of lymph gland organisation in third instar larva. The lymph gland flanks the dorsal vessel (cardiac tube) and pericardial cells separate the lobes. The primary lobe consists of three distinct zones; the cortical zone where maturing haemocytes are found, the medullary zone where haemocytes are yet to be differentiated and the posterior signalling center that has a unique population of specialised haemocytes. The secondary lobes usually contain immature haemocytes except in random sites of maturation. B) Schematic diagram for haemocyte differentiation in embryo and larvae towards different cell types. Abbreviations for genes as follows; srp (serpent), gcm (glial cells missing), Lz (lozenge), Ush (U-shaped), pnt (pointed), N (Notch), Kn (knot/collier), Acp (Anterior open),. The relevant pathways have been described in the text.

1.6 Perspectives of *Drosophila* haematopoiesis and immunity

1.6.1 Haematopoiesis

Each haematopoietic cell type arises through an ordered and regulated process of self-renewal and differentiation of stem cells. Both early haematopoiesis in the yolk sac and head mesoderm haematopoiesis provide developmental function by sculpting and remodelling tissue during development. When comparing lymph gland haematopoiesis to definitive haematopoiesis in the aorta-gonad-mesonephros (AGM) region of mammals, it can be noted that secondary development of both generates large populations of cells important for immunity (TRAVER and ZON 2002). *Drosophila* lymph gland progenitors develop from a subregion of the lateral mesoderm (cardiogenic mesoderm) and require input of various signalling pathways such as FGF (fibroblast growth pathway), Dpp (Decapentaplegic) and Wg (Wingless; Wnt1 of *Drosophila*) as well as being inhibited by Notch signalling. The differentiation of mesodermal AGM in vertebrates requires the input of the analogous FGF, BMP (bone morphogenetic pathway) and Wnt pathways. In arthropods, the cardiogenic mesoderm differentiates to blood, vascular and excretory cells that are separated from each other during embryogenesis but stay in contact during much of development. The situation is mirrored in vertebrates where the AGM gives rise to both endothelial and blood precursors that migrate to various sites with the embryo to assemble into blood vessels and diversify into separate haematopoietic lineages with the rest differentiating to the mesonephros (EVANS *et al.* 2003).

In vertebrates, VEGF (vascular endothelial growth factor) signalling is critical for the development of blood cells (vasculogenesis), the elaboration of vascular networks from existing cells (angiogenesis), the generation of blood cells and the homing of leucocytes to sites of inflammation. In *Drosophila*, VEGF signalling pathway is involved in haemocyte and border cell migration by recruiting blood and endothelial progenitors to correct sites of differentiation (TRAVER and ZON 2002). The GATA/Runx/FOG-like transcription factors in haematopoiesis are also conserved between *Drosophila* and mammals. In the murine model, GATA1 is involved in megakaryocyte development, GATA2 is required for definitive haematopoiesis, proliferation and/or survival of haematopoietic stem cells (HSC) as well as production of mast cells and GATA3 is necessary for T-cell development. This is conserved from the *Drosophila* GATA factor homologue Srp that is involved in directing cells to a haematopoietic fate. The essential role of Lz, a *Drosophila* homologue to AML1 in haematopoiesis correlates with the idea that AML1 is required for definitive haematopoiesis (EVANS *et al.* 2003).

Haematopoiesis occurs in *Drosophila* and vertebrates as two distinct temporal waves, in spatially distinct locations. Shifting sites of haematopoiesis is a common theme in embryogenesis amongst widely divergent organisms. In *Drosophila*, the primary site of haematopoiesis at the cephalic mesoderm in the embryos shifted towards the lymph glands in the larvae. In the murine model, the yolk sac and AGM region only transiently harbour HSCs. The foetal liver is populated by circulating HSCs and becomes predominant during mid-gestation, producing the first full complement of definitive, adult-type effector cells. Subsequent haematopoietic sites are seeded by migrating cells, explaining the transition of haematopoiesis to the bone

marrow. Different anatomical locations could specify distinct HSC populations or differentially support migrating HSCs. It is also possible that multiple sites may have evolved to meet the needs of the rapidly developing embryos (TRAVER and ZON 2002). From here, it can be summarised that both *Drosophila* and mammalian haematopoiesis share similar signalling pathways and developmental strategies.

1.6.2 Immunity

Insect and mammalian responses to infection are strikingly similar. The prominent immune reactions such as AMP production, iron sequestration, phagocytosis and ROS production are shared. The strong resemblance between the intracellular pathways activated upon infection between dipterans and mammals cannot be discounted. The *Drosophila* Toll pathway exhibit striking structural and functional homology to the IL-1 (interleukin-1) cascade. In mammals, binding of the IL-1 to its receptor IL-1R recruits the accessory proteins IRAK (IL-1R associated kinase) to the signalling complex. The accessory protein IRAK show considerable homology to *Drosophila* Pelle which is involved in the Toll pathway. In *Drosophila*, IMD and RIP have death domains present in all caspases which suggests similarity to the human tumour necrosis factor pathway (MATHEY-PREVOT and PERRIMON 1998).

In mammals, diverse sets of receptors are primarily found on monocyte/macrophage, granulocytic and dendritic cells involved in phagocytosis. Pathogens being phagocytosed are bound and killed through production and release of ROS. Similar types of receptors are present in flies and mammals, the Crq gene is functionally similar to CD36, both *psidin* and *eater* have a unique variant of multiple EGF

(epidermal growth factor)-like repeats (BRENNAN *et al.* 2007; FRANC *et al.* 1996; KOCKS *et al.* 2005; MEDZHITOV and JANEWAY 1997).

In addition, both insects and mammals are susceptible to disorders resulting from aberrant blood cell proliferation. Disruption of various NFκB/IκB components causes a variety of haematopoietic disorders including proliferation and differentiation defects in innate and adaptive immune responses. Human leukaemia are characterised by the uncontrolled proliferation of lineage specific blood cells blocked in their proliferation. In *Drosophila*, mutants with overproliferation of blood cells have a high incidence of melanotic masses that can either be benign or malignant and free floating or attached to internal organs (MATHEY-PREVOT and PERRIMON 1998).

The innate immune system in mammals limits the infection and provides signals required for suitable development of adaptive immune response. The innate immune system has to be present in a latent state at all times and rapidly activated upon infection to allow for destruction of the infectious agent (HOFFMANN and REICHHART 2002). The remarkable similarities between *Drosophila* host defence and essential facets of vertebrate innate immunity point to a common ancestry.

Currently, it is not known how microbial signals in the haemolymph are communicated to the fat body although the hypothesis is that the blood cells recognise and bind microorganisms, followed by the release of cytokine-like molecules. Previously published work concentrated on the mutant *domino*, discovered during a screen for genes involved in cellular immunity for *Drosophila*

(BRAUN et al. 1998). The recessive second-chromosome mutation results in the melanisation of the lymph glands, creating two black dots. The mutant larvae are devoid of all blood cells and any cells left were oversized and filled with melanotic masses. The mutants exhibit a prolonged third instar before pupation and subsequently die. Although the mutant maintains a wild type resistance to septic injury, when combined with other mutations for the humoral and melanisation cascade (Imd and Black cells), the immuno-compromised mutants suggested that a cross talk between the blood cells and the fat body (BRAUN *et al.* 1998). This theory was strengthened when a strong systemic response was detected in the fat body of third instar larvae after an oral infection with the plant pathogen, *Erwinia caratovora* (BASSET *et al.* 2000). However, the *domino* gene encodes members of the SWI2/SNF2 family of DNA-dependent ATPases, contributing to the silencing of homeotic genes (RUHF *et al.* 2001). The mutant therefore has abnormal diploid structures (ie neuroblast region, imaginal discs, germ cells and lymph glands) and is not well suited for attempts at elucidating role(s) of cellular immunity in signalling to the humoral immunity pathways in *Drosophila.*

Following on from this, the aim of this study was to elucidate the roles of *Drosophila* haemocytes in immune signalling during an infection using an alternative method. A viable working model of *Drosophila* was developed that had reduced haemocytes and the responses of larvae following immune challenge were studied in real-time. The AMP expression in the fat body was used as a read-out for signalling in a systemic response and the effect of this expression in animals without circulating haemocytes was observed. Based on this model, it was then possible to determine if

there were any signalling pathways that provide the cross-talk between cellular and humoral immunity.

2. MATERIALS AND METHODS

2.1 *Drosophila*

2.1.1 Stocks and crosses

Fly stocks were maintained at 25°C on standard fly food (Appendix A). Fly stocks used for construction and those constructed from this work are detailed in Table 2-1 and Table 2-2. All balancers used in this work have been previously described (LINDSLEY *et al.* 1992). The GAL4-UAS system used for ablating the haemocytes is a method of directed gene expression in *Drosophila* adapted from *Saccharomyces cerevisiae* (BRAND and PERRIMON 1993). A major advantage of using this system is that the gene under the control of the upstream activator sequences (UAS) is silent until crossed to the GAL4 driver; only the progeny will have activated gene enabling directed ectopic expression. The other advantage of using GAL4 is temperature sensitivity; the sequences are more active at 29°C, the optimal temperature for growth of yeast, than at 18°C, allowing fine-tuning of the expression of the gene. A generic mating scheme is shown in Figure 2-1. The standard haemocyte ablation crosses are shown in Figure 2-2. Where necessary, virgins were used to ensure that the progeny had correctly segregated chromosomes with appropriate balancers.

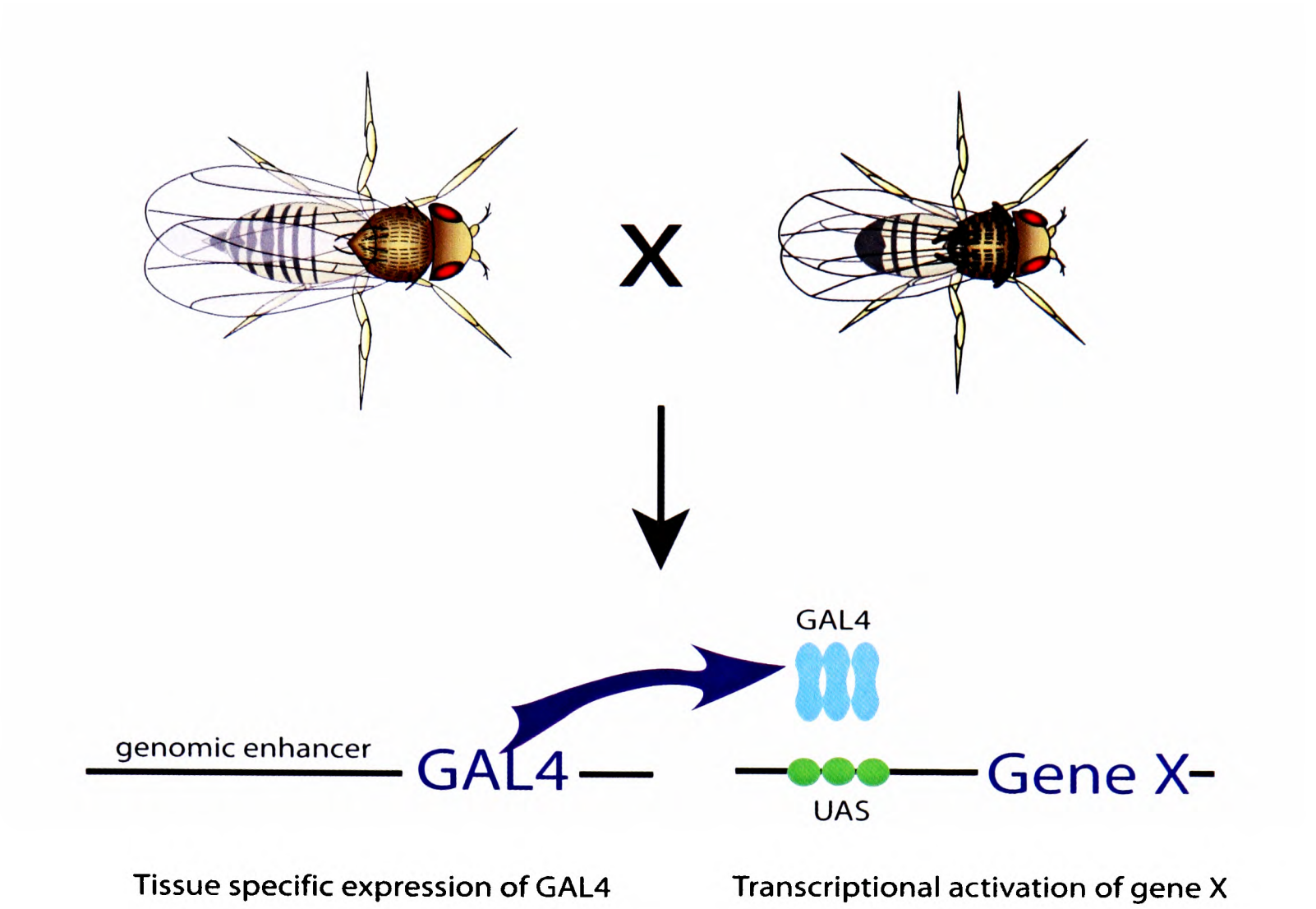


Figure 2-1 Directed gene expression in *Drosophila*. The genomic enhancer is silent until crossed to the GAL4 driver. The progeny of the cross will have Gene X expressed in tissues under the control of the GAL4 genomic enhancer, enabling control of ectopic gene expression.

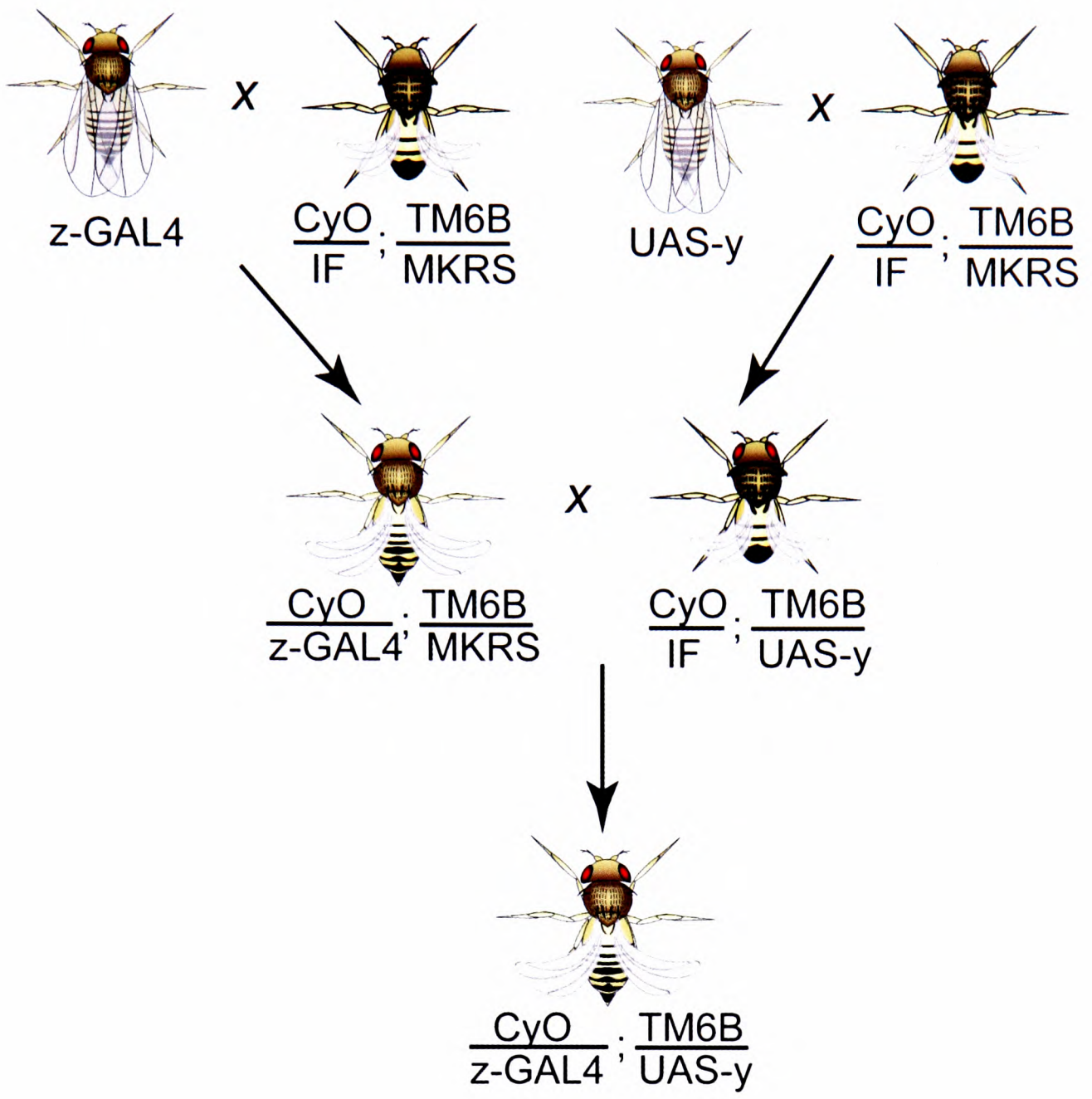


Figure 2-2 Generic mating scheme for haemocyte ablation. All stocks were crossed to wCam (CyO/IF; TM6B/MKRS) flies to balance them on the second and third chromosome. In this figure, z-GAL4 represents a haemocyte specific GAL4 while UAS-y represents a gene for activation of apoptosis.

Stock	Genotype	Chromosome	Source	Description
wCam	w; CyO/IF; TM6B/MKRS	I, II, III	Laboratory stock	Stock for balancing chromosomes
wCam^{GFP}	w; CyO^{GFP}/IF; TM6B/MKRS	I, II, III	Laboratory stock	Stock for balancing chromosomes
hml-GAL4	w; *hml*-GAL4, UAS-GFP	I, II	Laboratory stock	Haemocyte expressing line for ablation
pxn-GAL4	w; *pxn*-GAL4, UAS-GFP	I, II	M Galko[1]	Haemocyte expressing line for ablation
srp-GAL4	w; *srp*-GAL4	I, III	Laboratory stock	Haemocyte expressing line for ablation
RicinA	w; UAS-Ricin	I, III	C O'Kane[2]	Toxin for haemocyte ablation
Ice#2-2	w; UAS-ice#2-2	I, III	T Tanimura[3]	Apoptotic line for haemocyte ablation
Ice#7-1	w; UAS-ice#7-1	I, III	T Tanimura	Apoptotic line for haemocyte ablation
Ced3#12-5	w; UAS-ced3#12-5	I, III	T Tanimura	Apoptotic line for haemocyte ablation
Ced3#7-1	w; UAS-ced3#7-1	I, III	T Tanimura	Apoptotic line for haemocyte ablation
Hid,rpr	w; UAS-hid, rpr	I	Dr John Nambu[4]	Apoptotic line for haemocyte ablation
FbG4	w; *apt*-GAL4; TM6B/MKRS	II, III	Laboratory stock	Enhancer line for fat body expression
Drs-GFP	w; *drs*-GFP	I, III	Laboratory stock	GFP reporter for drosomycin gene
DD_1	w, DD_1; CyO/Bl	I, II	Laboratory stock	GFP reporter for drosomycin gene
Upd3^{RNAi}	w; UAS-*upd3*^{RNAi}	I, II	H Agaisse[5]	
Spz^{RNAi}	w; UAS-*spz*^{RNAi}	I, III	J M Reichhart[6]	
Puc	w; puc-lacZ	I, III	Laboratory stock	Enhancer trap line for puckered

Table 2-1 Complete list of fly stocks used in this work. All stocks used have been previously published except for Spz^{RNAi}, references for the stocks are listed numerically, 1-(GALKO and KRASNOW 2004), 2-(ALLEN *et al.* 2002) 3-(SHIGENAGA *et al.* 1997) 4-(ZHOU *et al.* 1997) 5-(AGAISSE *et al.* 2003) and 6-(gift from IBMC, Straubourg).

Genotype	Chromosome
w; *hml*-GAL4, UAS-GFP; UAS-Ricin	I, II, III
w; *hml*-GAL4, UAS-GFP; UAS-ced3#7-1	I, II, III
w; *hml*-GAL4, UAS-GFP; UAS-ced3#12-5	I, II, III
w; *hml*-GAL4, UAS-GFP; UAS-ice#7-1	I, II, III
w; *hml*-GAL4, UAS-GFP; UAS-ice#2-2	I, II, III
w, UAS-hid,rpr; *hml*-GAL4, UAS-GFP	I, II
w; *pxn*-GAL4, UAS-GFP; UAS-ice#2-2	I, II, III
w; *pxn*-GAL4, UAS-GFP; UAS-ice#7-1	I, II, III
DD1; *hml*-GAL4, UAS-GFP	I, II
w; *hml*-GAL4, UAS-GFP; drs-GFP	I, II, III
w; *pxn*-GAL4, UAS-GFP; drs-GFP	I, II, III
w; *hml*-GAL4, UAS-GFP; UAS-ice#2-2, *drs*-GFP	I, II, III
w; *pxn*-GAL4, UAS-GFP; UAS-ice#2-2, *drs*-GFP	I, II, III
w; UAS-upd3^{RNAi}; *drs*-GFP	I, II, III
w; *hml*-GAL4, UAS-GFP; UAS-spz^{RNAi}/*drs*-GFP	I, II, III
w; Fatbody-GAL4; UAS-spz^{RNAi}/*drs*-GFP	I, II, III

Table 2-2 Complete list of fly strains constructed from this work. For any genotype presented in the results not listed here, they were crossed for each experiment as a stable stock could not be obtained.

2.1.2 Recombination of alleles

Alleles to be recombined were crossed to each other to ensure heterozygosity. The heterozygous virgin females were crossed to male wCam *en masse* in bottles. The resulting progeny were crossed to female wCam to create stable lines. All stable lines were screened by selecting for the balancer on one chromosome and the presence of both constructs on the other.

2.1.3 Haemocyte counts

Wandering third instar larvae were randomly selected from cultures, washed twice with distilled water and dried on a paper towel. Ten larvae were bled on a glass slide by pulling them apart with fine forceps under a dissecting microscope (Nikon SMZ645). Haemolymph from 5 adults was extracted using a micro-injector (Nanoject, Drummond). Haemocytes were buffered with 50µl of *Drosophila* Schneider's medium (Invitrogen) and 10% fetal bovine serum (Sigma). When necessary, 2µl of 1µg/ml of 4,6-diamidino-2-phenylindole (DAPI) was added to the haemolymph to stain the nuclei of haemocytes before counting. The number of haemocytes in a 1mm grid was counted in an Improved Neubaur haemocytometer under a fluorescence microscope (Nikon Eclipse 801i). Five grids were counted for each experiment. Three separate experiments were performed for each genotype to determine mean values and standard deviation.

2.1.4 Larval infections

Wandering third instar larvae selected as previously described (Figure 2-3) were picked from cultures and rinsed in distilled water. Larvae were placed on a rubber

mat and pricked at the posterior end between the spiracles with a sharp needle dipped into a concentrated solution of freshly cultured bacteria. Larvae for microscopy were allowed to recover on apple juice agar in 24-well plates (Appendix A). Larvae for Northern blots were placed on Whatman filter paper pre-soaked with sterile phosphate buffered saline (PBS, Appendix A) in a vial and were collected 6 hours or 12 hours after Gram-negative or Gram-positive infection respectively and stored at -80°C until RNA extraction (described later).

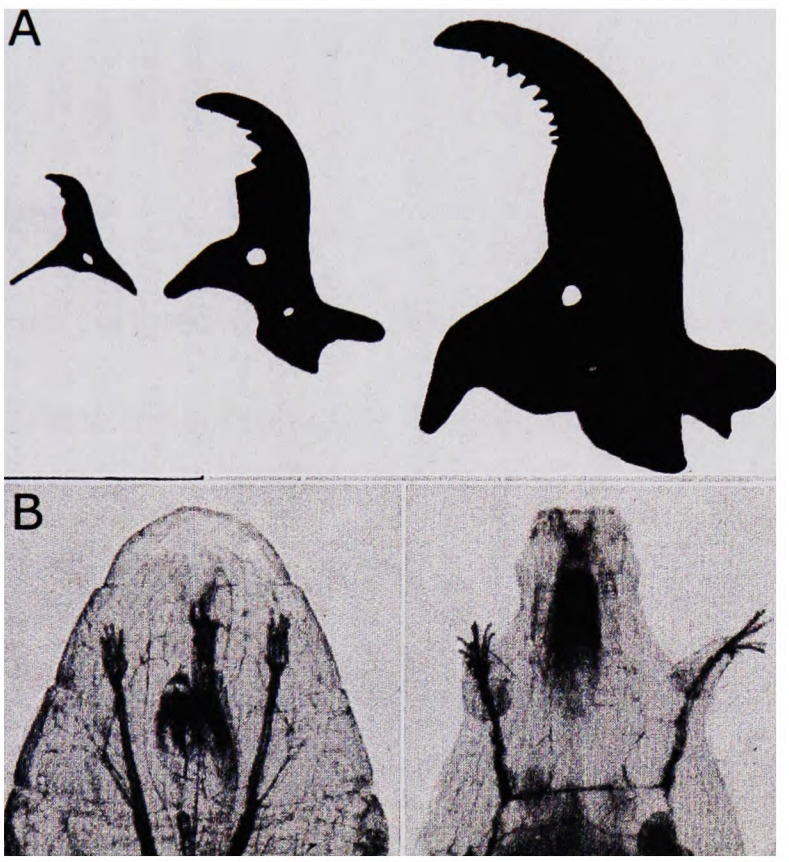


Figure 2-3 Morphological structures to select for third instar larvae (adapted from Biology of *Drosophila* Demerec, 1950) A) Mouth hooks of first, second and third instar larvae. B) Protracted anterior spiracles of third instar larvae and protruding anterior spiracle of third instar larvae. Either one or both of the characteristics were observed when selecting for third instar larvae for experiments.

2.1.5 Larval dissections

To determine the contribution for AMP production of different organs in larvae, 25 whole larvae were dissected into the constituent haemolymph, fat body and gut with outer cuticle in 50µl ice-cold PBS using a pair of fine forceps. The forceps were

cleaned after each dissection with a clean paper towel and water to avoid tissue cross- contamination. To prevent disintegration of the fat body, larvae were dissected in sets of 5 as quickly as possible and frozen at -80°C. All haemocytes with their haemolymph were pooled together as were the gut and carcass before RNA extraction using standard lab protocol. Any larvae infected with bacteria were left to recover for 6 hours before dissection.

2.2 Microbiology

2.2.1 Bacterial culture

All bacteria used were cultured in Luria-Bertani (LB) broth (Appendix A) at 37°C overnight using standard microbiological techniques. The Gram-negative bacteria used were *Escherichia coli* or *Erwinia carotovora* while Gram-positive bacterium used was *Micrococcus luteus*.

2.2.2 Preparation of electro-competent bacteria

To create an antibiotic resistant strain of *Erwinia carotovora* by transformation with pEYP (Clontech), electro-competent bacteria were prepared using a standard protocol (HINTON *et al.* 1985). Overnight cultures were grown at 30°C and used to seed cultures the next day. When optical density at A_{600} were approximately 0.6, bacteria were evenly cooled by swirling them in an ice bath. Cultures were then transferred to ice-cold centrifuge bottles and harvested by spinning them down at 1000g for 15 minutes at 4°C (Beckman J2-21). The supernatant was decanted and washed in 250ml ice-cold sterile milliQ water. Cells were again spun down at 1000g for 20 minutes at 4°C, following by decanting the supernatant and resuspending the

cells in 125ml ice cold 10% glycerol. The bottles were then centrifuged again as before and this time a Pasteur pipette was used to remove as much supernatant as possible. Pellets were now re-suspended in 0.5ml of ice-cold GYT medium (Appendix A) to approximately $2x10^{10}$ cells/ml. Bacteria were then tested by pipetting approximately 40µl into an ice-cold electroporation cuvette (2mm gap, Geneflow). If the bacteria arced during electroporation, the cells were ready and were washed down again with ice-cold GYT medium as described before. All bacteria were then aliquoted into sterile tubes and stored at -80°C until use.

2.2.3 Transformation

To transform bacteria, 40µl of thawed electrocompetent bacteria were pipetted into electroporation cuvettes. 1ng of pEYP or sterile milliQ water (1-2µl) were gently added to the bacteria and mixed using a pipette tip. Bacteria were electroporated at 25µF capacitance, 2.5kV and 200ohm resistance (BioRad MicroPulser). After electroporation, 1ml of SOC buffer (Appendix A) was added to the cuvette and the entire mix was transferred to a sterile microcentrifuge tube to incubate in a shaking incubator (200rpm) at 30°C for 1 hour. After the hour, bacteria were plated at appropriate dilutions on LB-ampicillin plates (100µg/ml) and incubated overnight at 30°C. Single colony transformants were selected and stored for use at -80°C in 15% glycerol.

2.2.4 Measurement of colony forming units (CFU)

To measure efficiency of bacterial phagocytosis, approximately 50 wandering third instar larvae were pricked with a sterile needle dipped in a freshly cultured

concentrated solution of bacteria and left to recover on apple juice agar plates. For colony counting, 5 larvae were randomly selected at each time point, washed twice in distilled water, dipped into ethanol to sterilise the outer cuticle, dried on a piece of Whatman filter paper and crushed in 100µl of sterile PBS using a sterile disposable microcentrifuge pestle (Sigma). Two sets of serial dilutions were prepared and each dilution was plated twice on LB-ampicillin plates. Colony forming units were calculated per ml of sterile PBS for 5 larvae.

2.3 Molecular biology

2.3.1 RNA extraction

Total RNA was prepared from 15 adult flies or 20 wandering third instar larvae using Trizol (Invitrogen) following manufacturer's instruction. 500µl of Trizol were added to the microcentrifuge tube and samples were crushed using disposable blue polypropylene pestles (Sigma). After the addition of 150µl of chloroform, each tube was vortexed vigorously then spun at 14000rpm for 10 minutes in a cold room. The supernatant RNA in each tube was precipitated with 250µl of iso-propanol for 15 minutes at room temperature. Samples were then spun down at 14000rpm for 15 minutes in a cold room. Pellets were washed again with ice-cold absolute alcohol. Total RNA was quantified for Northern blots. To quantify total RNA, pellets were re-suspended in 50µl RNAse free water (Qiagen) and heated at 65°C for 10 minutes in a heating block. 1:50 dilutions of the each sample were read at A_{260} using disposable UV-cuvettes (Brand).

2.3.2 Northern blots

Northern blots were carried out as a standard procedure in the lab as previously described (RUTSCHMANN *et al.* 2000a). Briefly, 20μg of total RNA after quantification was precipitated overnight at -20°C using 2.5X volume ice-cold absolute ethanol and 0.1X volume 3M NaAc. The next day, RNA pellets were obtained by centrifuging each tube at 14000rpm for 15 minutes in a cold room. The absolute alcohol was removed by pipetting and the pellet was left to dry at room temperature for approximately 5 minutes followed by resuspending the pellet in 20μl formaldehyde loading buffer (Ambion). The samples were then placed at 65°C for 5 minutes and then placed straight on ice. All Northern blots apparatus; gel tank, gel tray and combs were soaked in a 10% solution of H_2O_2 for 15 minutes, washed with sterile milliQ water and dried with paper towels. Gels were cast according to the recipe (Appendix A) and run in 1xMOPS buffer at 70V for 2 hours. After resolving the RNA, gels were washed twice in 10XSSC for 20 minutes. The blotting apparatus is detailed as below.

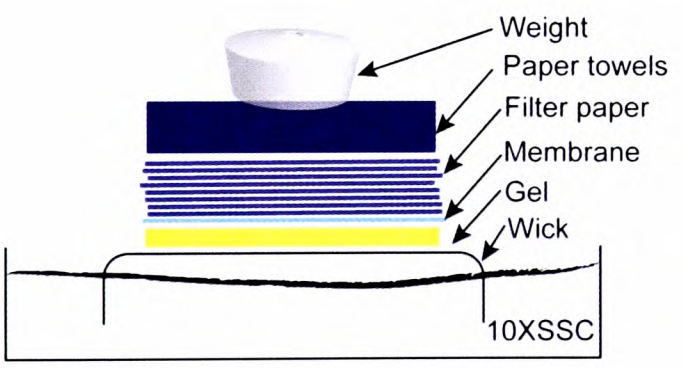


Figure 2-4 Apparatus for RNA transfer. The membrane was left overnight over the gel to allow the nucleic acids to move across.

The following day, the blot was auto-crosslinked in a UV-crosslinker (UV stratalinker, Stratagene) before blotting. The blocking solution was prepared as detailed in Appendix A. The blots and blocking solution were left rotating in a hybridisation oven (Techne) at 45°C for 45 minutes. Radioactive probes were

prepared by diluting 50μg of DNA to a total volume of 46μl. The DNA was then denatured at 95°C in a heating block for 5 minutes, left on ice for 5 minutes and then used to resuspend the Rediprime II random labelling system (Amersham Biosciences). After this, 4μl of α^{32}P-dCTP (Amersham Biosciences) were added and this was then incubated at 37°C for 10 minutes. After this, the pre-equilibrated ProbeQuant G-50 micro column (Amersham Biosciences) was spun down at 3000rpm for 2 minutes. The radioactive probe was pipetted directly into the center to purify the labelled probe from unincorporated labelled nucleotides by centrifuging at 3000rpm for 4 minutes. The labelled probe was denatured at 95°C for 5 minutes and immediately cooled on ice for 5 minutes then pipetted into the hybridisation tube to probe at 45°C for 16 hours.

The following day, the radioactive solution was decanted into appropriate waste and the blots were rinsed with 2xSSC/0.1% SDS wash solution. The blots were then washed twice in the same solution for 15 minutes at 45°C followed by 2 washes with 0.2xSSC/0.1% SDS; one at 45°C and the other at 65°C. The blots were then covered with Saran wrap and exposed in a phosporimager cassette for 8-16 hours depending on the strength of the blot. Blots were quantified using a Fujifilm FLA-3000 reader using proprietary software (Image Gauge V3.3, Image Reader V1.8E, Fuji, Japan). After reading, blots were stripped by heating in a solution of 0.1% SDS at 95°C for 5 minutes. Blots were then stored at -20°C until needed.

Data for *drosomycin* and *diptericin* transcripts were normalised to *ribosomal protein* 49 (*Rp49*) readings. Induction of antimicrobial peptides was determined by fold-activation of the peptide to an uninfected control.

2.3.3 Single fly DNA preparation

Anaesthetised flies were placed in a 0.2ml PCR tube on ice. The fly was mashed with a pipette tip containing 50µl of freshly prepared squish buffer with Proteinase K (Appendix A) without expelling any of the liquid. Each tube was incubated at 37°C for 30 minutes followed by 95°C for 2 minutes to inactivate the Proteinase K. Tubes were stored at 4°C until use.

2.3.4 Polymerase chain reaction

Probes for Northern blots were prepared by amplifying fragments of DNA from single fly preparations. Primers used are detailed as below. The standard reaction mixture is detailed in Appendix A. The cycling parameters were the same for all primers, after denaturing the DNA at 94°C for 2 minutes, 30 cycles of denaturing at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 1 minute. The reaction was completed by a final extension at 72°C for 7 minutes then rapidly cooled to 4°C in the reaction block (MJ Cycler). The products were then resolved on a 1% TBE-agarose gel with 5µl of a 10mg/ml ethidium bromide stock solution.

Probe	Primer	Size (bp)
Rp49- Forward	ATACAGGCCCAAGATCGTGA	430
Rp49- Reverse	GTGTATTCCGACCACGTTACA	
Drosomycin- Forward	AGCTCCGTGAGAACCTTTTCC	232
Drosomycin- Reverse	CATCCTTCGCACCAGCACTTC	
Diptericin- Forward	AGTTCACCATTGCCGTCGCC	298
Diptericin- Reverse	GTAGGTGTAGGTGCTTCCCA	

Table 2-3 Primers used for Northern probes

2.3.5 Purification of PCR products

PCR products were purified using the QIAquick Gel extraction kit (Qiagen). DNA fragments separated on agarose gel were visualised under UV light and excised using a sterile scalpel. The gel size was weighed and 3X volumes of Buffer QG were added to 1X volume of the gel. The microcentrifuge tube was then incubated at 50°C for 10 minutes or until the slice was melted. The sample was then applied to the centre of a QIAquick spin column placed in a microcentrifuge tube and spun down at 13000rpm for 1 minute. The flow-through was discarded and 750µl of the wash buffer (Buffer PE) was applied to the column and the spin was repeated again. After discarding the flow-through, the column was spun again for an additional minute to ensure the removal of excess ethanol. The spin column was then replaced into a clean microcentrifuge tube, adding 30µl of elution buffer (TE) to the centre of the membrane, letting the column stand for 1 minute, then centrifuging for 1 minute at full speed. Purified DNA was stored at -20°C.

2.4 Live imaging and microscopy

Wandering third instar larvae were randomly selected from cultures and infected using a sharp needle dipped into a freshly cultured concentrated solution of *E coli* or *M luteus*. The larvae were left to recover on apple juice agar and images were captured at hourly intervals. Each larva was cooled for as briefly as possible on a Peltier Stage (Linkam PE 94, UK) before photomicrography and replaced on apple juice agar in a 24-well plate at room temperature every hour until 6 hours post-infection. Larvae were visualised on a GFP stereo dissecting microscope (Leica MZFIII, UK) and images were captured using KyLink software (v2.0, Japan) and JVC KY-F75U camera (JVC, Japan). Images were colour corrected and composited

using Adobe CS2 (Adobe, USA Mac OSX). Intensity of GFP was measured using MetaMorph v6.3r7 (Molecular Devices, USA) using the Region Measurement function. All results presented were in arbitrary units as determined by the software calculating the pixel intensity for the region. As much as possible, all images were controlled to be taken in the same conditions, including shutter speed and exposure time. Three equivalent regions of interest were selected in a single image of a genotype at 6 hours post infection. Regions of interest were selected at approximate distances from each other across the length of the larva. Ten images were selected for each genotype and for each image, the intensities were averaged for the regions of interest. Standard deviation was calculated for all genotypes. Supplementary movies (Appendix C) were compiled in Final Cut Express (Adobe, MacOSX) as close to real time and exported as uncompressed QuickTime format movies (Apple, USA).

2.5 Staining of enhancer trap larvae

Larvae with *puckered*-lacZ enhancer trap were infected with *E. coli* for 24 hours then dissected using a fine pair of forceps in PBS+2mM $MgCl_2$ (this and all solutions preceding are detailed in Appendix A). Larvae were then fixed in LacZ fix for 6 minutes, and then washed 3 times for 5 minutes each in LacZ wash buffer. The LacZ staining solution was prepared fresh each time and larvae were placed in a dark chamber for 48 hours at 37°C. After staining, larvae were washed 3 times for 5 minutes each in LacZ wash buffer and then fixed in 4% formaldehyde (methanol free) overnight. After fixation, larvae were dehydrated in a series of increasing concentrations of glycerol. Fat bodies were dissected and mounted in 100% glycerol. Cover-slips were sealed with nail polish and images of stained fat body were

captured using a Nikon Eclipse 801i with a Photometrics 3CCD camera and Metamorph (v6.3r7, Molecular Devices, USA). Blue stained nuclei for a single plane were processed using the same software based on provided algorithms.

2.6 Statistical analyses

Any statistical analysis were performed using SPSS v 13.0 (SPSS, USA). To confirm if there was a correlation and the strength of any correlation between 2 variables, the Pearson product moment correlation test was used. Where presented, the p value dictates the significance level and the r value shows the strength of the relationship between the two variables. To assess whether the means of two groups are statistically significant from each other, an independent sample t-test was performed. Where presented, the p value dictates the significance level and the t-value indicates the ratio between the two means. A P value of <0.10 is deemed to be marginally significant while a P value of <0.05 is statistically significant.

3. RESULTS

3.1 Haemocyte ablation

To study the role of cellular immunity in *Drosophila*, haemocytes were ablated using the GAL4-UAS system. The study required a viable model and thus, any ablation that was too successful and resulted in embryonic lethality could not be used to determine the role of haemocytes in larvae. The promotor fusions and GAL4 enhancer traps have the potential to drive genetic cell ablations by cell-autonomous expression of cytotoxic gene products (see Figure 2-1). Promoter regions of different genes shown to be expressed by haemocytes during development were fused to GAL4 enhancers were used to determine any role(s) that haemocytes play in AMP expression from the fat body. The different genes are detailed below.

3.1.1 Genes selected to drive GAL4 expression in haemocytes

Hemolectin (hml) was biologically characterised by Goto (GOTO *et al.* 2003). This gene has sequence similarities to von Willebrand factor known to be involved in the clotting cascade of mammals. The 3843-amino acid disulphide-linked dimeric protein was first found secreted from haemocyte-derived cell line Kc167 (GOTO *et al.* 2003). Analysis of the gene coding region revealed 26 exons, covering 14kb and the gene mapped to chromosome 3 on the left region at position 70C1-5. Larvae depleted of *hml* using a UAS-RNA^{i} construct showed clotting defects not due to the phenoloxidase cascade (SCHERFER *et al.* 2004). *Hml* is expressed as a pulse during larval development, coinciding with the appearance of phagocytic haemocytes. The expression of *hml* in third instar larvae was shown to be limited to a sub-population of mature plasmatocytes and crystal cells (GOTO *et al.* 2003). In adults, GFP

expression was restricted to the leg and abdominal regions, although expression was weak compared to larvae. *Hml* expression is not required for viability nor required for either Toll or Imd signalling cascades.

Peroxidasin (*pxn*) expression appears early in *Drosophila* development and is used as a marker for haemocytes (NELSON *et al.* 1994). The gene has been mapped to chromosome 3L at position 67E. Transcription of the gene begins at two plates of the procephalic mesoderm at stage 10 of embryogenesis and continues to larval development. The trimeric 500kDa heme protein with an enzyme domain of 606-residues for peroxidase activity is unique in that it appears to be secreted for cross-linking and stabilising the extracellular basement membrane as well as phagocytosis. Expression of the gene is observed in haemocytes up to third instar larvae as well as the lymph glands, fat body, gastric caeca and cells that adhere to larval imaginal discs when staining with an enhancer trap line. It is not possible to extract significant amount of Pxn from embryos or larvae as it is probably cross-linked to the extracellular basement membrane as individual molecules or as part of a network. GFP expression in embryos for the selected driver reveals the previously described dispersal from the procephalic mesoderm at the anterior region to the whole embryo by stage 16. This suggests that the *pxn*-promoter driven GAL4 marks all prohaemocytes and will subsequently mark all circulating haemocytes in larvae, although it has not been observed in previous publications (STRAMER *et al.* 2005).

In the embryo, all prohaemocytes express the GATA transcription factor *Serpent* (*Srp*) that is required for blood cell precursor specification and maintenance (REHORN *et al.* 1996). In vertebrates, GATA factors play fundamental roles in

various aspects of haematopoietic development. *Srp* participates in specification of haemocytes in the embryonic primordium and remains expressed in the differentiated blood cells, suggesting a further role in maturation (LEBESTKY *et al.* 2000).

3.1.2 UAS constructs selected for gene ablation

Different genes encoding various toxins or apoptosis-inducing genes were used to determine which would be most suitable to ablate haemocytes. All haemocytes driven expression by different gene promoters were visualised by a UAS-GFP transgene present in all configurations.

The Ricin A construct used encodes the conditional cold-sensitive Ricin-A chain, ectopic expression of which has been shown to successfully ablate cells in other systems (ALLEN *et al.* 2002). In this study, there was not any significant difference between control and ablated flies, or between wandering third instar larvae when they were visualised under the microscope. It is possible that the toxin is not expressed when driven by the promoter, thus unable to drive death in the correct cells.

A set of constructs with human interleukin-1β-converting enzyme (ICE) and *Caenorhabditis elegans* cell death gene 3 (CED-3) were also used. CED3 is a protein required for programmed cell death in the nematode, a recessive loss-of-function mutant blocks all programmed cell death that occurs during development of *C. elegans* (SHIGENAGA *et al.* 1997). ICE, a mammalian homologue of CED-3, is a cysteine protease that cleaves the pro-interleukin-1β into the biologically active 1β; over-expression of this protein causes rat fibroblasts to undergo apoptosis, showing

that it acts as cell death gene (MIURA *et al.* 1993). Both proteins share 28% amino acid identity and a conserved pentapeptide QACRG, suggesting that ICE participates in cell death. A total of four different lines under the control of UAS were used, 2 of each for each protein to eliminate possible positional variegation effects. The *Drosophila* genes; *reaper* (*rpr*), *head involution defective* (*hid*) and *grim* are mapped to the same region on the genome and were shown to be involved in the regulation of apoptosis. All the genes are expressed in dying cells and ectopic expression of these genes in cells destined to die results in stage specific lethality, inducing excessive cell death. Co-expression of *rpr* and *hid* has been shown to induce a rapid midline cell death in the central nervous system, indicating synergistic actions between these two cell death regulators. A transgenic line with both genes under the control of UAS was used in our experiments (ZHOU *et al.* 1997). The use of apoptotic machinery genes to induce directed cell ablation is preferable as necrosis caused by expression of toxins could result in non-autonomous effects on neighbouring cells, as well as interfere with immune functions to be observed in this study. Whether necrosis induces an immune response has not yet been studied in *Drosophila*.

3.1.3 Ablation results

All three haemocytes drivers were crossed to all available apoptotic constructs, however the use of *srp*-GAL4 proved to be problematic due to high lethality rates in early larval development. Although these larvae were devoid of haemocytes they rarely reached the third instar larval stage of development, negating their usefulness in this study as we required viable animals and were not used for any further work. It is possible that the *srp*-GAL4 lines available in the laboratory was not entirely

specific for haemocytes and ablation would interfere with the development of other organs such as fat body (HAYES *et al.* 2001).

Expression of a UAS-GFP transgene under the control of the *pxn*-GAL4 driver, showed a variable pattern of expression in third instar larvae. Expression was not limited to haemocytes; most larvae viewed also had expression in the fat body (Figure 3-1). Ablation of *pxn*-GAL4 expressing cells was only possible with the ICE constructs, use of other constructs resulted in embryonic lethality. However, due to their pattern of expression in third instar larvae, it was not possible to determine via visual inspection if ablation of *pxn*-GAL4 also affected the morphology or immune function of the fat body. By selecting *pxn* larvae that did not have any GFP expression in the fat body for both normal and ablated larvae, it was possible to visualise that a significant level of ablation had occurred (Figure 3-2). A line with reduced expression in the fat body was selected for all further experiments, with the stability of the line being continually checked. With the ablated lines, it is worth noting that most ablation occurred within haemocytes circulating the haemolymph. Sessile haemocytes resistant to ablation are highlighted in the figure. This level of ablation observed concurred with my casual observation that non-balanced ablated larvae were much weaker compared to wild type stock, with large amounts of larval lethality before pupariation.

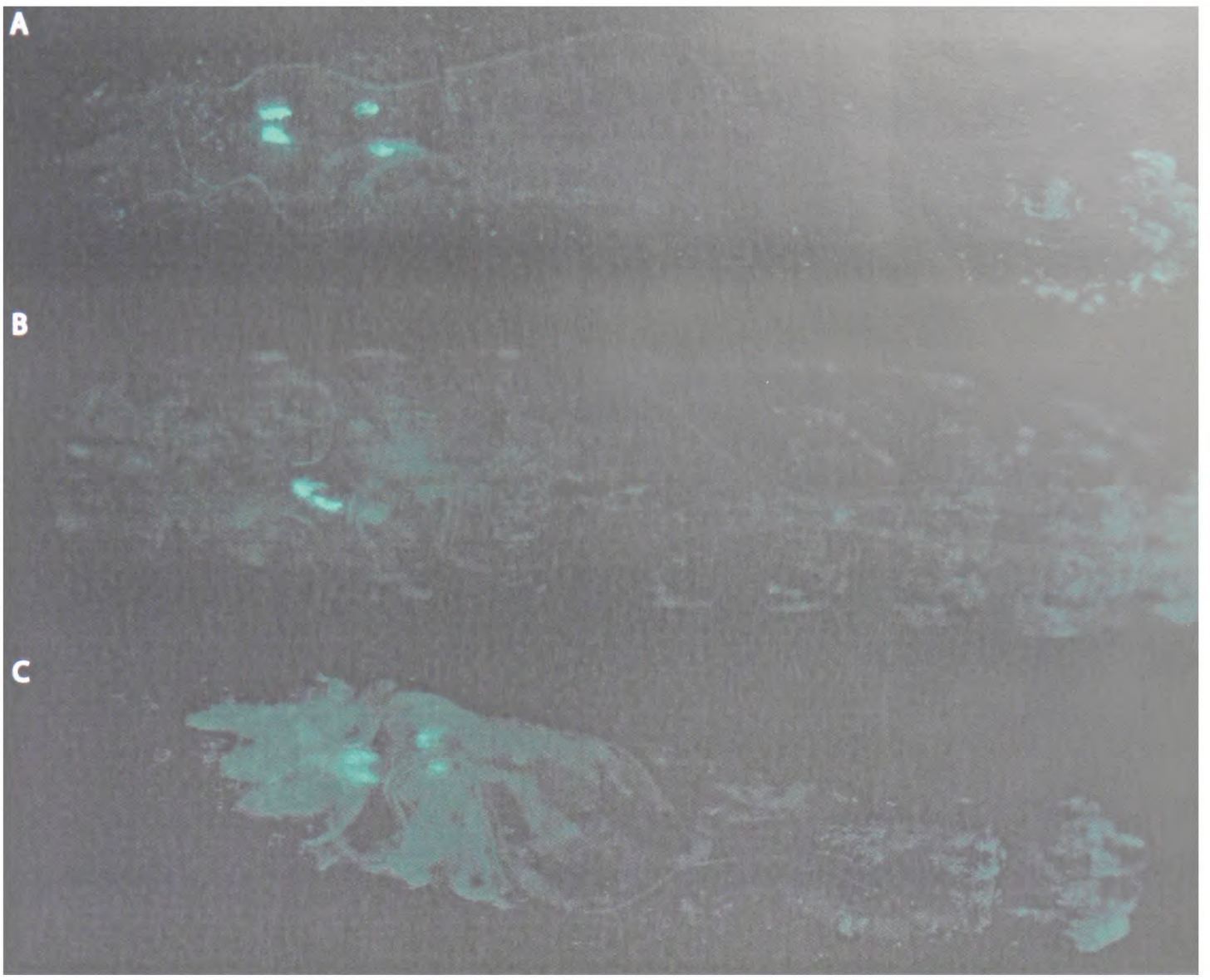


Figure 3-1 Variable expression in *pxn*-GAL4,UAS-GFP wandering third instar larva. A) Expression restricted to haemocytes located at the posterior and lymph glands. B) Gene expression in circulating and sessile haemocytes as well as lymph glands (compare expression to *hml*-GAL4,UAS-GFP in Figure 3-4A) C) Expression in the lymph glands, sessile haemocytes and anterior of the fat body. Images were captured at 40X magnification, the anterior of the larva at the left hand side of the image, viewing the ventral region. For all further experiments involving *pxn*, larvae were selected from a line that expressed GFP in the haemocytes only as seen in (A).

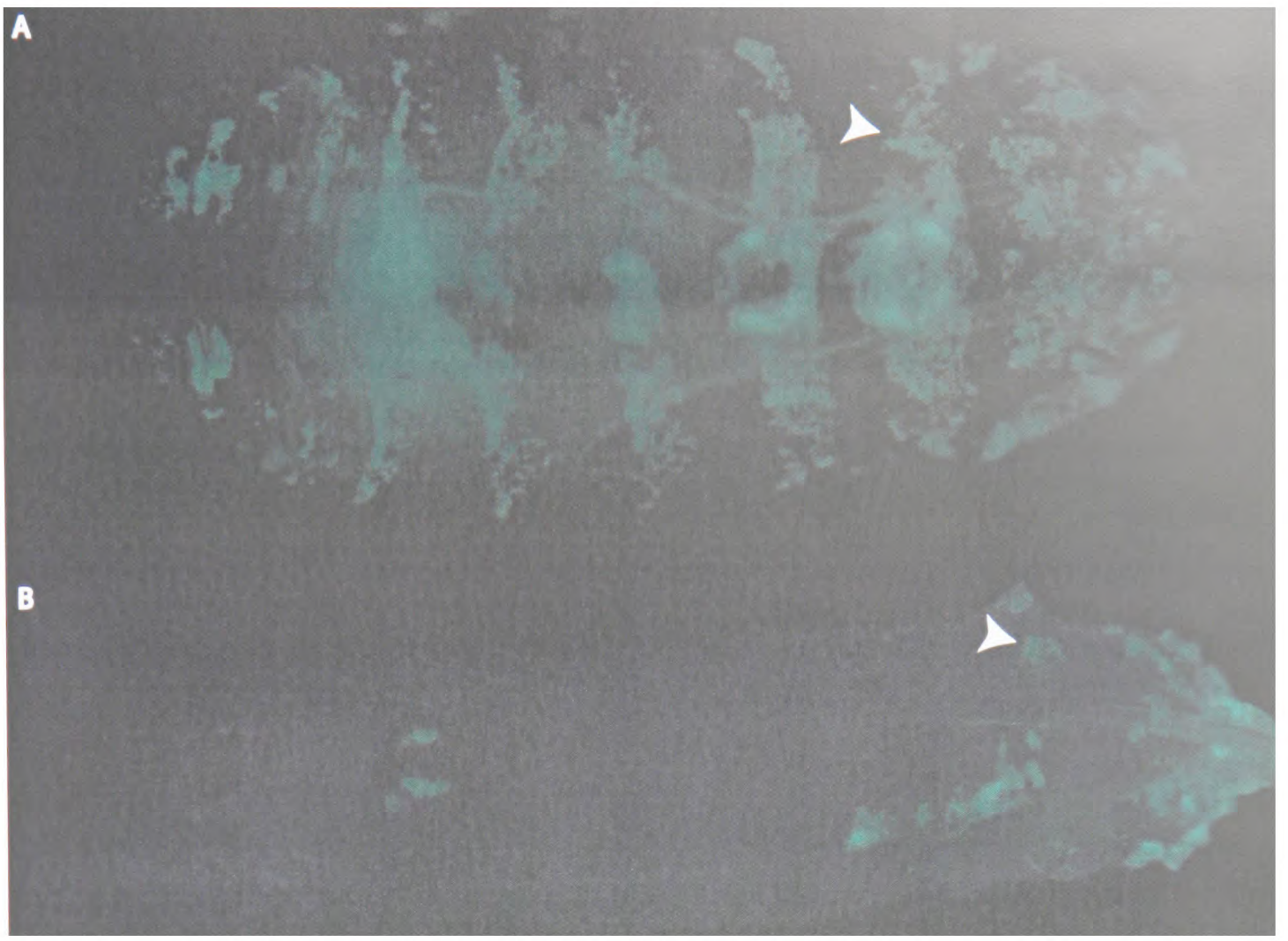


Figure 3-2 Ablation of *pxn*-GAL4 haemocytes A) *Pxn*-GAL4, UAS-GFP; TM6B/MKRS wandering third instar larvae, selected to exclude expression in fat body. B) *Pxn*-GAL4, UAS-GFP; UAS-ice#2-2. The white arrowhead highlights the presence of sessile haemocytes. Images were captured at 40X magnification, the anterior of the larva at the left hand side of the image, viewing the ventral region. The larva in the upper panel expresses the larval marker *tubby* (on the balancer TM6B) and thus appears shorter or smaller but both are of the same developmental stage.

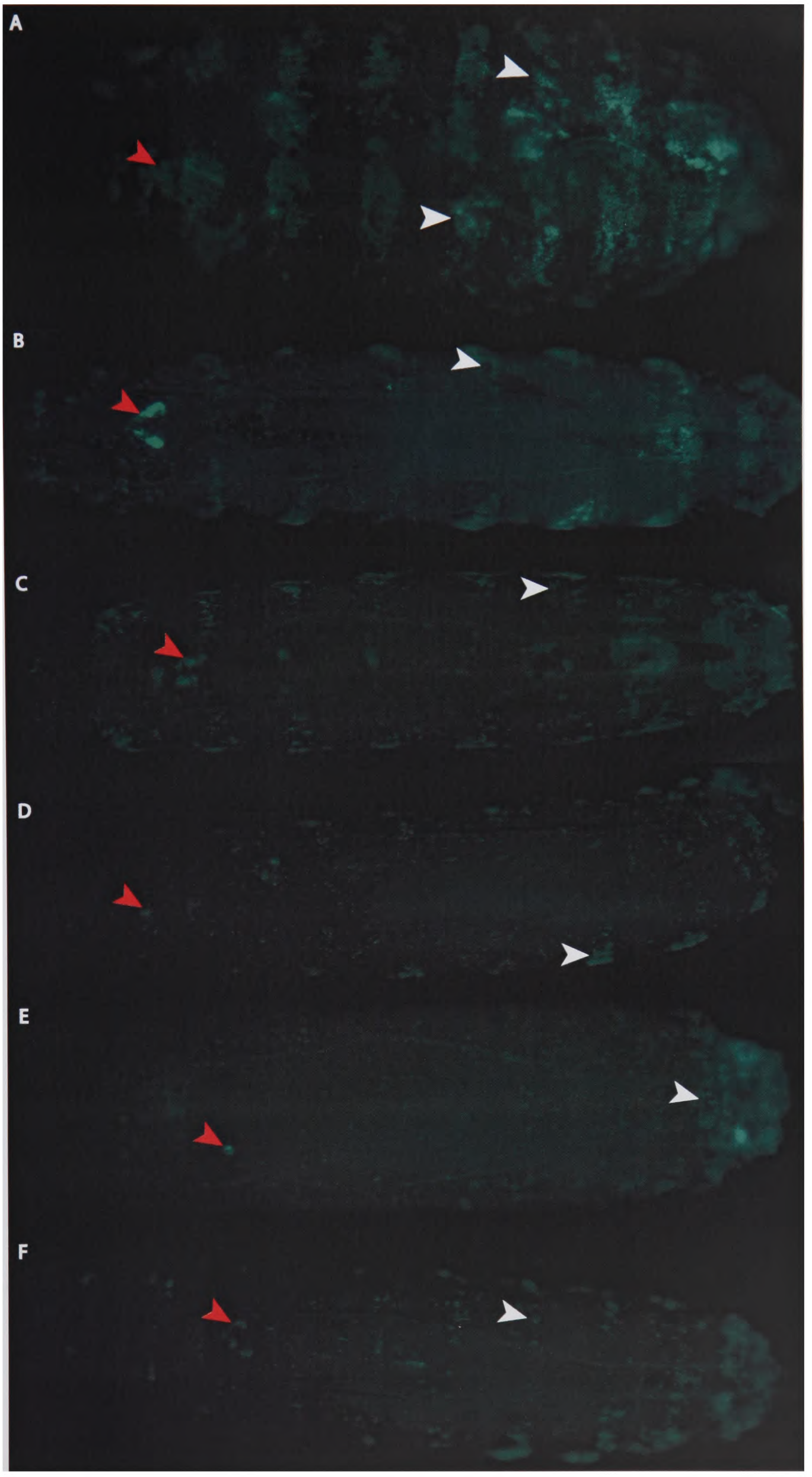
Due to the nature of the expression of the *hemolectin* (*hml*) gene, as a pulse that occurs in larval development, we were able to examine ablation in larval and adult progeny for this haemocyte driver. The ablated lines showed varying intensities of haemocyte ablation when wandering third instar larvae were visualised under UV light with a GFP filter (Figure 3-3, Supplementary movie 1 and 2). Here again, we see that most of the ablation occurred with circulating rather than with sessile haemocytes, the cells lining the integument in a segmented pattern (indicated by white arrow-heads in Figure 3-3). When viewing heterozygous larvae, the ablation for the same genotype was less evident if comparing to the homozygous strain (results not presented here). The dose-dependent effect could be explained by the fact that strains created contained multiple UAS constructs that had to be driven by a single GAL4 driver (see Figure 2-1).

In supplementary movie 1, note the rapid flow of haemocytes through the body cavity of the larva pushed by the pulse of the dorsal vessel for the normal larva while in the ablated larva, the lack of circulating haemocytes is evident. The movie is played as real-time. In supplementary movie 2, the higher magnification movie highlights the difference between the sessile haemocytes attached to the inner epithelia and circulating free-flowing haemocytes in the larvae. This difference between both types of cells is currently not understood.

Lymph glands, the paired lobes of larval haematopoietic organs were visualised and did not appear to be ablated compared to wild type although in the most ablated lines, they appeared to be reduced in size (annotated by red arrow heads in Figure 3-3). However, it has been previously noted that all larval haemocytes were derived

from the embryonic anlagen of haematopoiesis and therefore, ablation of lymph gland haemocytes were not necessary to study the immune function of third instar larvae. Lymph gland haemocytes are released at the onset of puparation, right after induction by ecdysone and are believed to be involved in tissue remodelling for eclosion of the adult fly. This concurred with the pupal lethality observed in two of the most highly ablated lines (Figure 3-4). Lethality that was observed in our most ablated lines when balanced stocks were kept (Table 3-1).

Figure 3-3 (following page) Variable ablation observed in *hml*-GAL4 ablated lines. A) *Hml*-GAL4, UAS-GFP; TM6B/MKRS B) *Hml*-GAL4, UAS-GFP; UAS-ced 3#7-1 C) *Hml*-GAL4, UAS-GFP; UAS-ice#7-1 D) *Hml*-GAL4, UAS-GFP; UAS-ced3#12-5 E) UAS-*hid,rpr*; *Hml*-GAL4, UAS-GFP F) *Hml*-GAL4, UAS-GFP; UAS-ice#2-2. Images were captured at 40X magnification, the anterior of the larva at the left hand side of the image, viewing the ventral region. Sessile haemocytes present highlighted by white arrow-heads, lymph glands (or remnants of lymph glands) resistant to ablation are indicated by red arrow-heads. The larva in (A) expresses the larval marker *tubby* (on the balancer TM6B) and thus appear shorter or smaller but it is of the same developmental stage as the others.


A
B
C
D
E
F

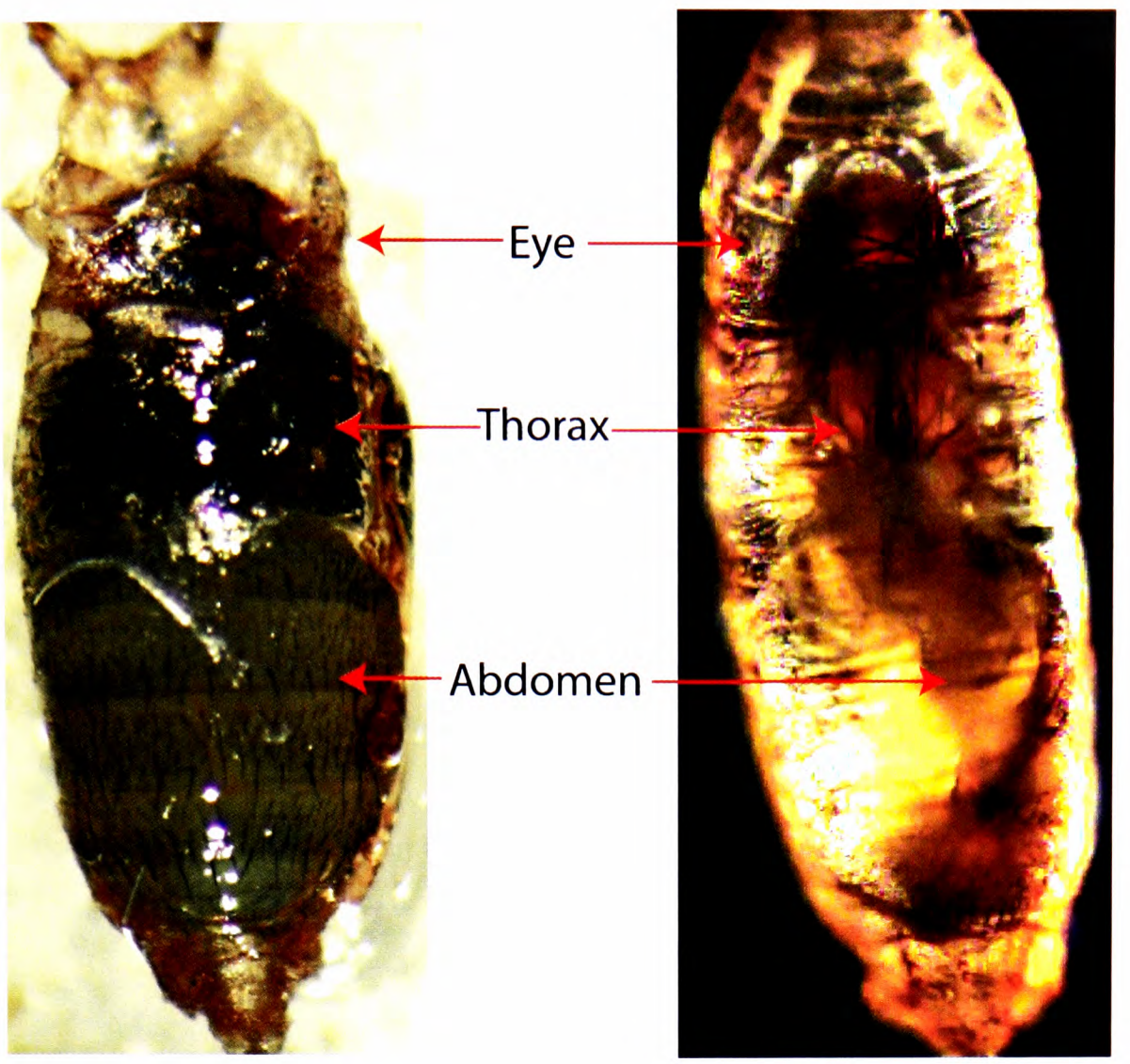


Figure 3-4 Comparison of doomed pupal structures observed in hml-GAL4, UAS-GFP; UAS-ice#2-2 on the right compared to a normal pupae on the left. Images were captured at 40X magnification, viewing ventrally. Note the tissue shrinking from the edge of the pupal case, indicating possible necrosis. Severely ablated larvae were unable to complete metamorphosis due to lack of haemocytes available for tissue remodelling.

Genotype	Homozygous	Balanced	Ratio
Hml-GAL4,UASGFP; UAS-ced#7-1	62	114	1:1.8
Hml-GAL4,UASGFP; UAS-ice#7-1	88	148	1:1.7
Hml-GAL4,UASGFP; UAS-ced3#12-5	92	195	1:2.0
Hml-GAL4,UASGFP; UAS-ice#2-2	16	131	**1:8.2**

Table 3-1 Lethality observed for the most severely ablated line by counting the number of progeny for ablated lines with and without the balancer TM6B, visualised by the marker *humoral*, characterised by the presence of extra bristles at the thorax for adults. Expected ratios are 1:2 as homozygous balancers are embryonic lethal.

The visual observations were confirmed by counting haemocytes driven by *hml*-GAL4 from randomly selected third instar larvae as well as adults to avoid artificial selection and ensure that biological variability was accounted for (Table 3-2, Figure 3-5). It was not possible to do haemocyte counts randomly for *pxn* due to variable expression of the GAL4 driver. In this experiment, haemocyte counts were restricted to comparison of *hml* expressing cells that have been previously shown to be expressed in plasmatocytes and a subpopulation of crystal cells (GOTO *et al.* 2003). Haemocytes were reduced to 15% of the normal complement of haemocytes in our most ablated stock.

In order to confirm that the haemocyte GFP counts were accurate for third instar larvae, haemocytes bled from the most severely ablated line were also stained with DAPI. Counting of both DAPI and GFP stained cells confirmed that the ablation was successful (Figure 3-6), with the total number of cells in the ablated line reduced to 36% of the total number of cells in the wild-type control. The GFP population in the normal control accounted for 34% of the total cells and in the ablated larvae accounted for 18% of the total cells (Table 3-3).

The number of haemocytes in adult flies was counted by extracting the haemolymph using a micro-injector. The totals indicate that the circulating haemocytes did not appear to be ablated (Table 3-4), although flies observed under a fluorescence microscope showed reduced numbers of sessile haemocytes (Figure 3-7). The morphological structure as well as the genetic profile has not been determined in adult haemocytes although it has been shown that they are composed of lymph gland haemocytes released just before metamorphosis as well as embryonic haemocytes

(HOLZ *et al.* 2003). The ablation in adults has worked on the sessile population but not the circulating haemocytes.

Genotype	Exp1	Exp 2	Exp 3	Average	% of haemocytes
Hml-GAL4,UAS-GFP	604	604	618	608.7	100
Hml-GAL4,UAS-GFP; UAS-ced3#7-1	496	476	469	480.3	79
Hml-GAL4,UAS-GFP; UAS-ice#7-1	348	426	441	405.0	67
Hml-GAL4,UAS-GFP; UAS-ced3#12-5	180	208	180	189.3	31
UAS-hid,rpr; *Hml*-GAL4,UAS-GFP	151	114	152	139.0	23
Hml-GAL4,UAS-GFP; UAS-ice#2-2	91	83	106	93.3	15

Table 3-2 Haemocyte counts of ten wandering third instar larvae. Counts are represented as GFP expressing haemocytes per μl. Three independent experiments were carried out for each genotype.

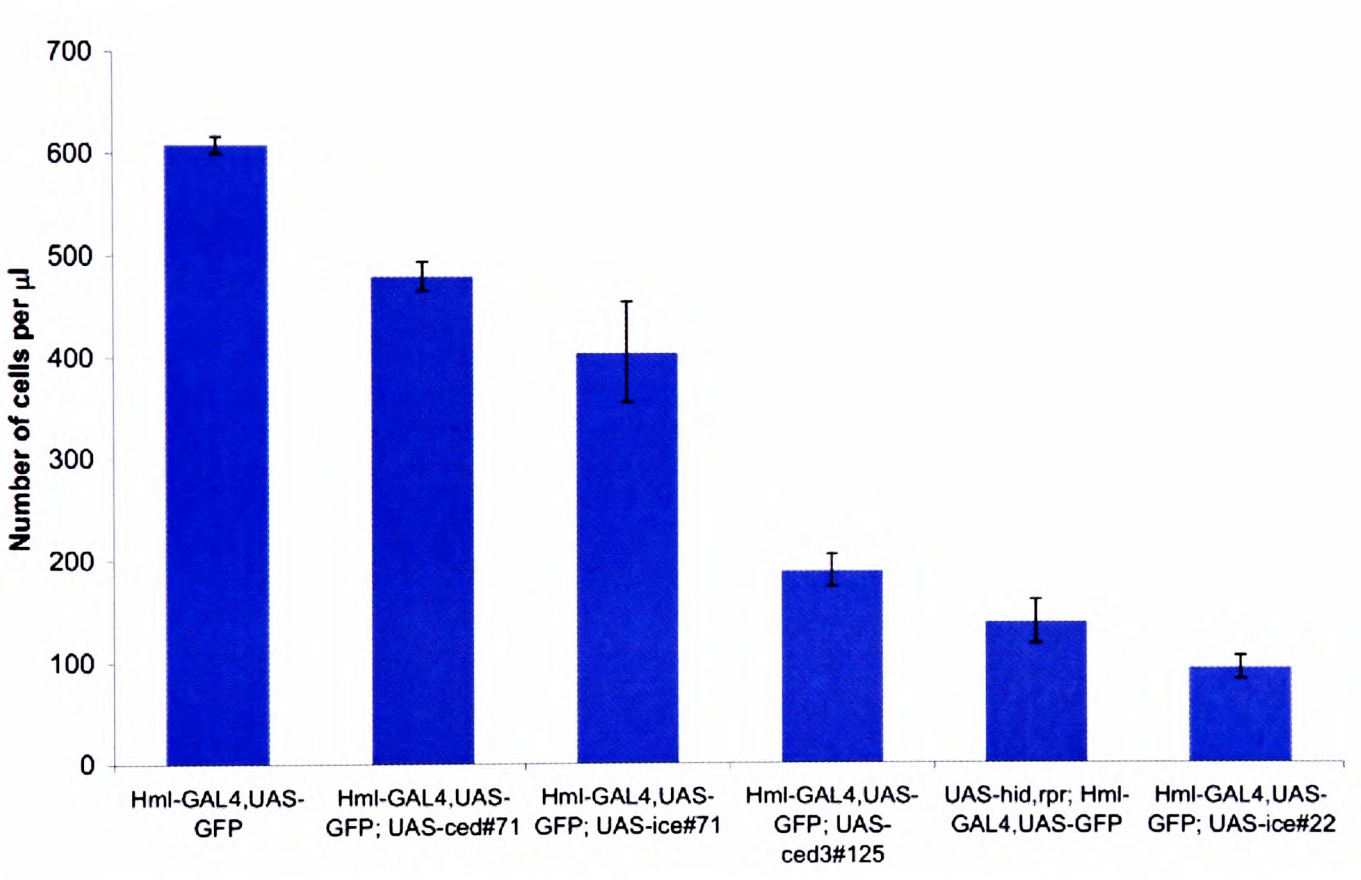


Figure 3-5 Reduction in haemocytes in wandering third instar larvae. Variations between the same gene was observed due to positional effects of UAS-constructs during preparation of transgenic flies. Data presented from the table above, with error bars representing standard deviation from the average.

Genotype	Haemocytes expressing		Total haemocytes	%GFP expression
	GFP	DAPI		
Hml-GAL4,UAS-GFP	542	1023	1565	34
Hml-GAL4,UAS-GFP; UAS-ice#2-2	99	463	562	18

Table 3-3 Haemocyte counts for normal and most ablated genotype using wandering third instar larvae. DAPI was added to the haemolymph to count haemocytes that were not expressing GFP. The counts are represented as haemocytes per μl. Averages are based on 5 independent experiments and percentage expression of haemocytes expressing GFP was based on the total number of haemocytes in the larvae, summed by the ones expressing GFP and DAPI.

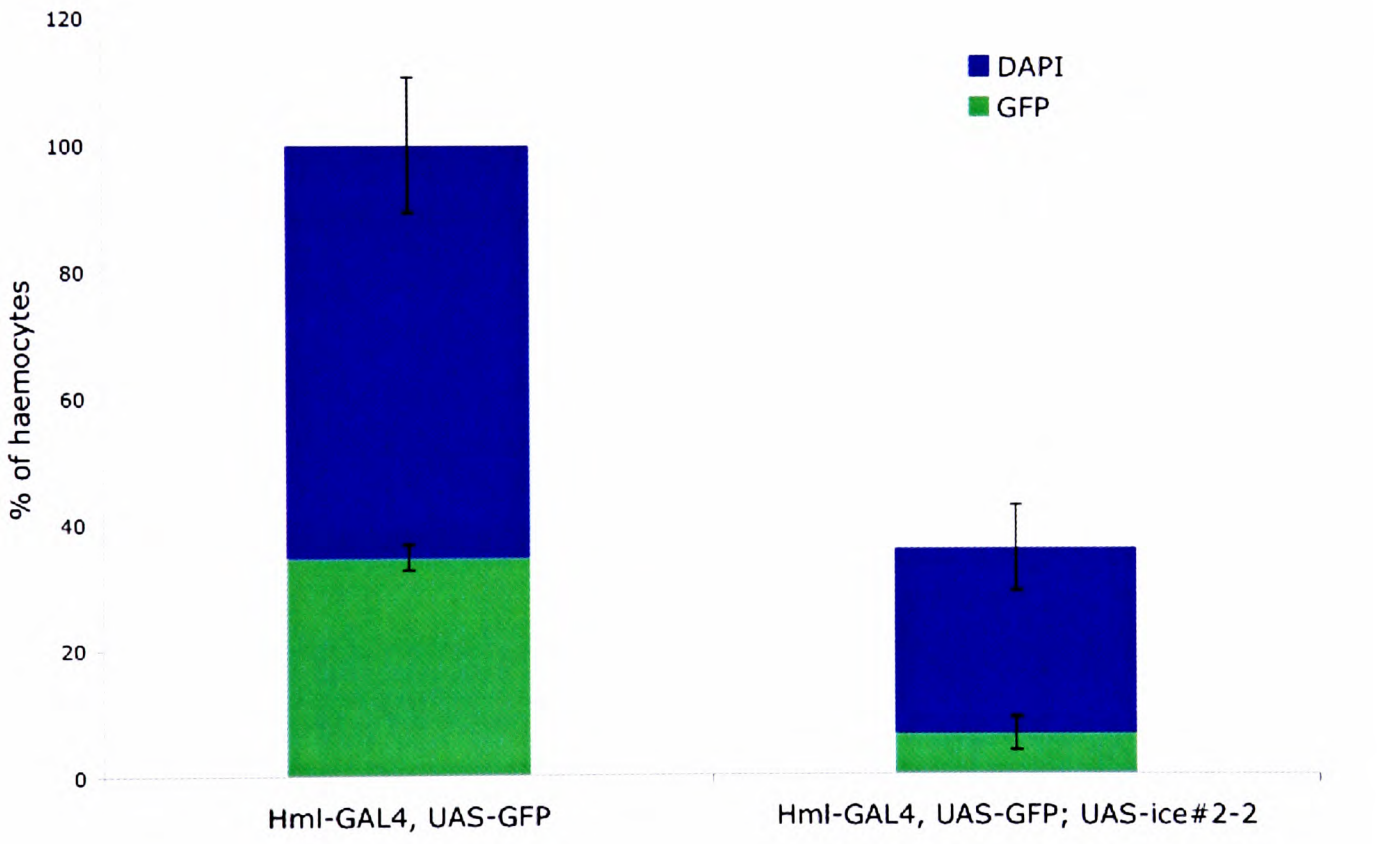


Figure 3-6 Chart representing the GFP population as compared to the total haemocyte population in wandering third instar larvae for normal and ablated larvae. The ablated larvae showed reduced numbers in the overall population as well as the GFP population, confirming earlier results. Error bars represent the standard deviation from the mean.

Genotype	Exp1	Exp 2	Average	% of haemocytes
Hml-GAL4,UAS-GFP	270	260	265	100
Hml-GAL4,UAS-GFP; UAS-ced3#71	210	210	210	79
Hml-GAL4,UAS-GFP; UAS-ice#71	180	210	195	74
Hml-GAL4,UAS-GFP; UAS-ced3#125	210	260	235	89
UAS-hid,rpr; *Hml*-GAL4,UAS-GFP	160	160	160	60
Hml-GAL4,UAS-GFP; UAS-ice#22	210	220	215	81

Table 3-4 Haemocyte counts of 5 adults of different haemocyte ablated lines. Counts are represented as GFP expressing haemocytes per μl. Two independent experiments were carried out.

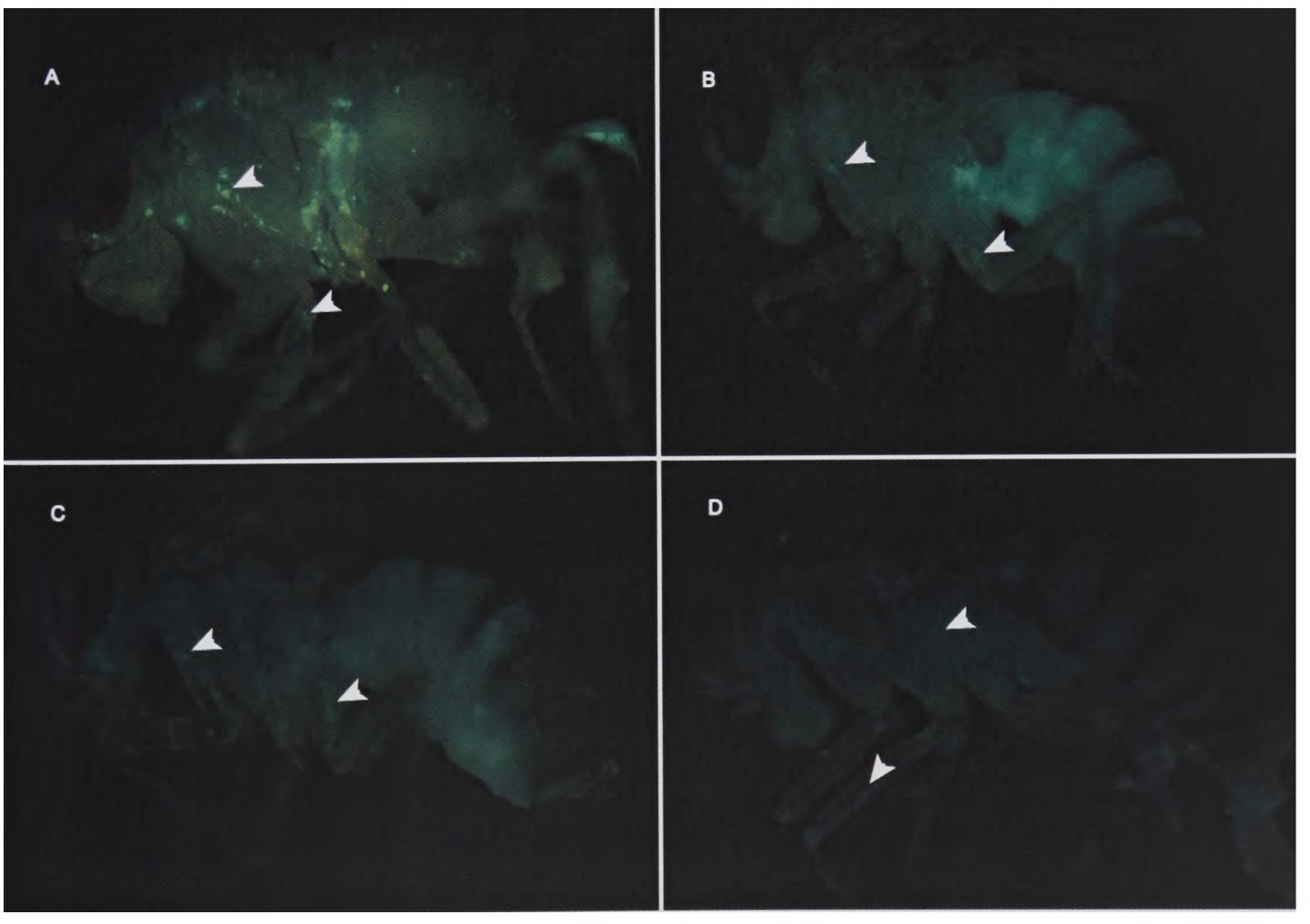


Figure 3-7 Expression of haemocytes in adult flies A) *hml*-GAL4, UAS-GFP; TM6B/MKRS B) *pxn*-GAL4, UAS-GFP; TM6B/MKRS C) *hml*-GAL4, UAS-GFP ; UAS-ice#2-2 D) *pxn*-GAL4, UAS-GFP ; UAS-ice#2-2. Images were taken at 20X magnification. Sessile haemocytes were observed with both lines in the thorax and legs, as indicated by white arrow-heads.

3.2 Antimicrobial peptide (AMP) expression of haemocyte ablated larvae

Once it was ascertained that cell death for haemocytes was evident in wandering third instar larvae, the antimicrobial peptide expression was analysed for all genotypes of ablated larvae to determine the effects. Transcriptional activation of the *drosomycin* (*drs*) gene was used as a readout for the Toll pathway after infection with Gram-positive bacteria (*M. luteus*) while *diptericin* (*dipt*) was used as a readout for the Imd pathway after infection with a Gram-negative bacterium (*E. coli*) (LEMAITRE *et al.* 1995; LEMAITRE *et al.* 1996). The larvae were allowed to recover for 6 hours after infection with *E. coli* and for 16-20 hours after infection with *M. luteus* for maximal AMP expression.

Readings for AMP gene transcription were all normalised to *Rp49*, a ubiquitously expressed gene. The induction of AMP expression was calculated by dividing the AMP result with a non-challenged larva of the same genotype. Maximal possible expression was ascribed to infected controls (non-ablated animals). A representative Northern blot is presented in Figure 3-8.

AMP expression was reduced by 85% in our most severely ablated line. It is possible to note a trend between the level of haemocyte ablation to the observed reduction in AMP gene activation after challenge (Figure 3-9). Statistical analysis using the Pearson correlation test showed that there is a strong association between the ablation of the haemocytes to the reduction of AMP, with $r=0.87$, $p<0.05$ and $r=0.88$, $p<0.05$ for *drs* and *dipt* respectively. To confirm that the reduction seen was not specific to the *hml* gene, expression of AMPs was also shown to be reduced to approximately 50% for a *pxn*-GAL4 ablated line (Figure 3-10). The reduction was

shown to be statistically significant when analysed by an independent sample t-test with $t=17.17$, $p<0.05$ and $t=3.44$, $p<0.05$ for *drs* and *dipt* respectively. The reduction in the *pxn*-GAL4 was more severe when compared to the equivalent, *hml*-GAL4, UAS-GFP; UAS-*ice*#7-1.

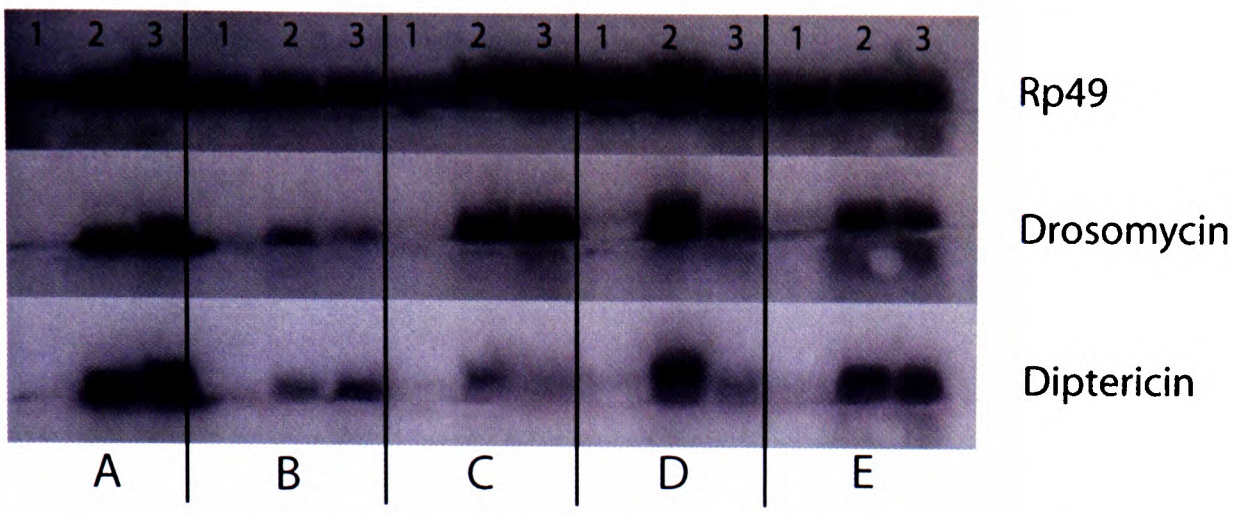


Figure 3-8 Representative Northern blot for all data presented. For each genotype, lane 1 represents a basal non-infected control, lane 2 is *E. coli* infected animals and lane 3 is *M. luteus* infected animals. The five genotypes tested on this blots are A) *hml*-GAL4, UAS-GFP B) *hml*-GAL4, UAS-GFP; UAS-*ice*#2-2 C) *hml*-GAL4, UAS-GFP; UAS-*ice*#7-1 D) *hml*-GAL4, UAS-GFP; UAS-*ced3*#7-1 E) *hml*-GAL4, UAS-GFP; UAS-*ced3*#12-5.

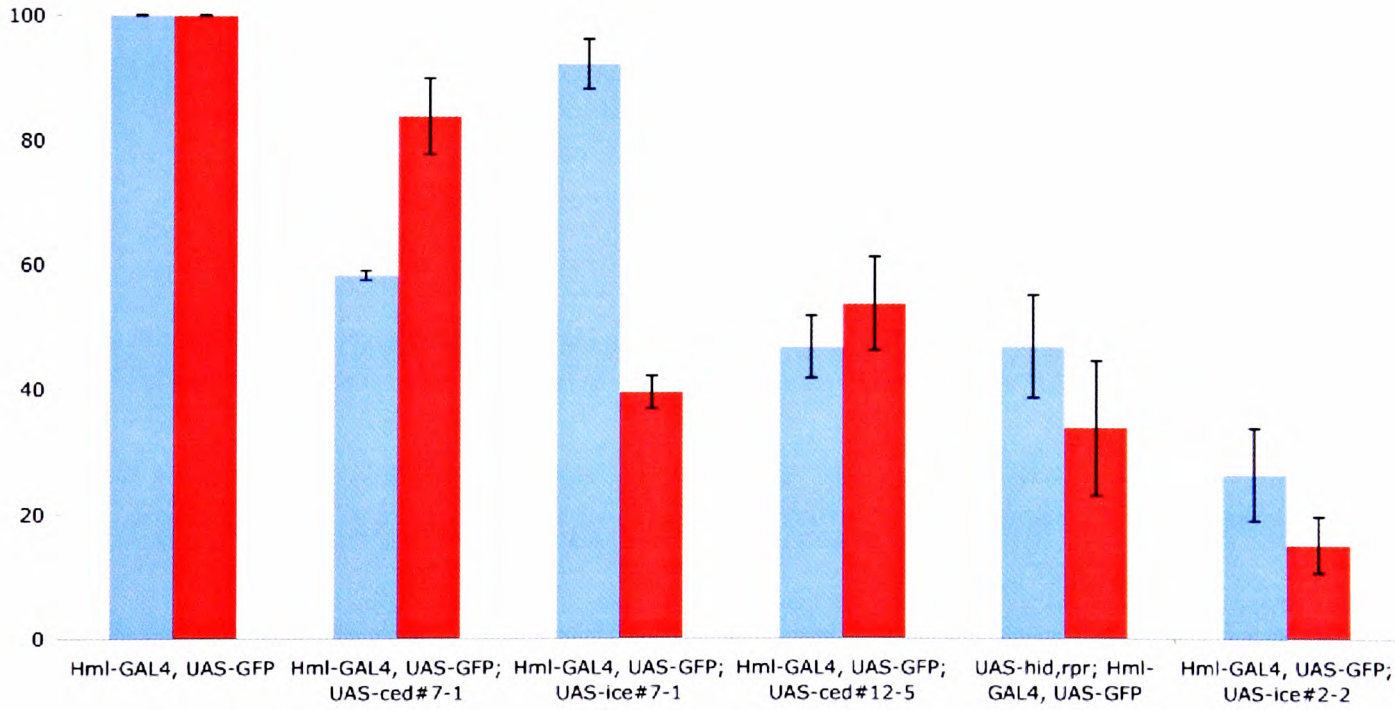


Figure 3-9 AMP expression of haemocyte ablated lines, samples collected 12 hours after infection with Gram-positive bacteria for drosomycin expression and 6 hours after infection with Gram-negative bacteria for diptericin expression. Genotypes are arranged in order of the extent of ablation, statistical analysis showing a strong correlation between the degree of ablation to the reduction of AMP expression. Data presented are averages of 3 independent experiments; error bars indicate the standard deviation from the mean.

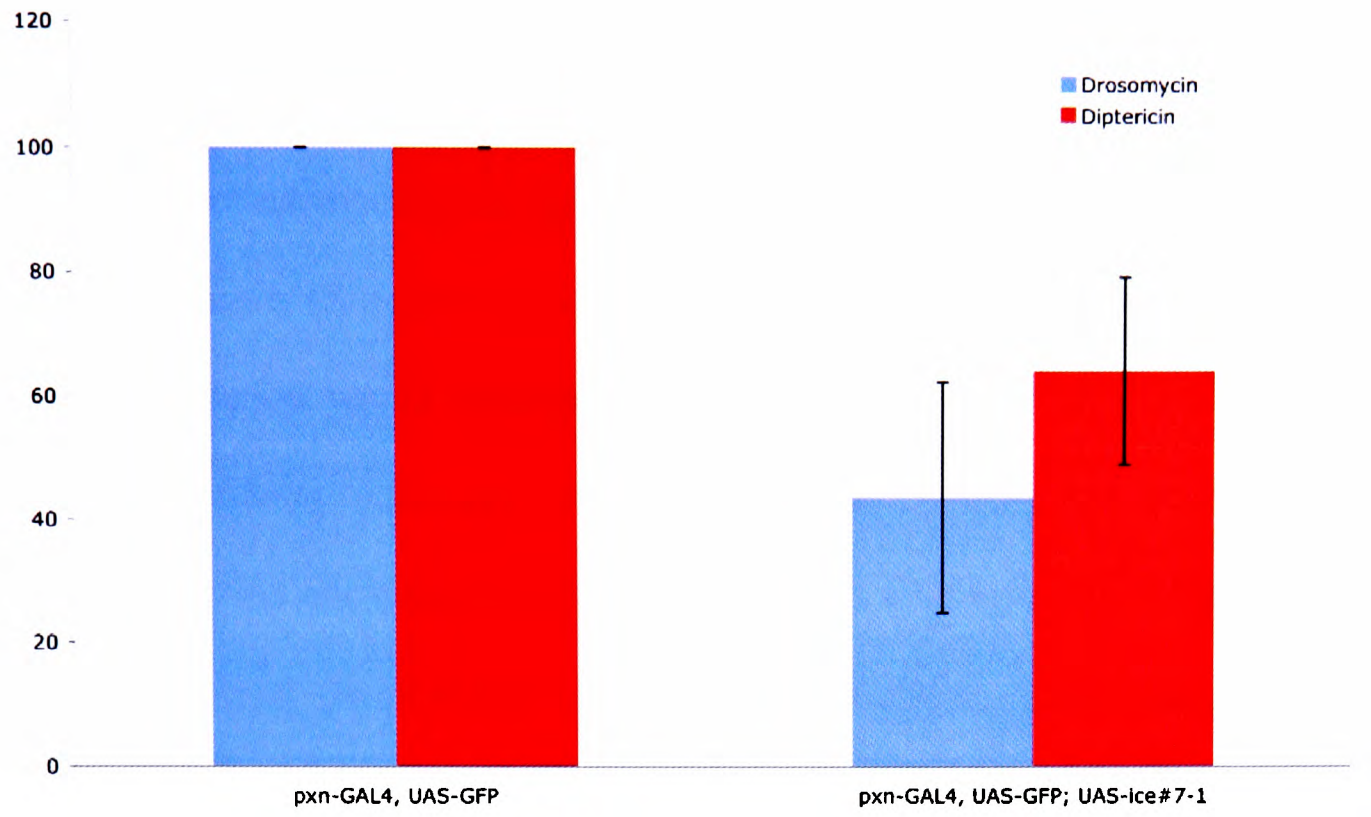


Figure 3-10 AMP expression for haemocyte ablated lines driven by *pxn*, samples collected 12 hours after infection with Gram-positive bacteria for drosomycin expression and 6 hours after infection with Gram-negative bacteria for diptericin expression. The reduction of AMP expression more severe when compared to *hml*-GAL4, UAS-GFP; UAS-*ice#7-1*. Data presented as averages of 3 independent experiments; error bars indicate standard deviation from the mean. The reduction of AMP production is statistically significant.

3.3 Constituent expression of antimicrobial peptide expression in larvae

However, it is possible that the reduction in AMP observed (Figure 3-9) could be due to reduction of haemocytes as they have been shown to secrete AMP as part of their roles in immunity (SAMAKOVLIS *et al.* 1992). Therefore, it could be assumed that the extent of ablation would correlate with reduction in AMP production since both humoral immunity pathways were affected. To confirm the amount of AMP expressed by the constituent parts of each larvae, wild type and the most severely ablated genotype (*hml*-GAL4; UAS-*ice*#2-2) wandering third instar larvae were dissected 6 hours post-infection and all their tissues were separated as carefully as possible (see materials and methods) to assay for AMP expression as before (Table 3-5). Tissues were separated into 3 main constituents; the fat body (with a small amount of salivary gland contamination), haemocytes and the surrounding haemolymph (including circulating proteins expressed by the haemocytes and/or secreted by the fat body) and the carcass (remainder of the larva dissected that included gut, trachea, outer cuticle, brain and mouth parts).

When normal larvae were infected with bacteria, the expression profile showed a small increase for AMP in the fat body and a marked increase for AMP in the haemolymph with the proportional reduction for AMP in the carcass. Ablated larvae showed a slightly different expression profile when infected with bacteria, with a decrease in the expression of AMP in the haemolymph. The expression of AMP in the fat body showed a similar increase to normal larvae and the expression of AMP in the carcass showed the proportional reduction.

It is of noteworthy that the remaining haemocytes in a non-immune challenged ablated larvae contributed significantly to overall AMP expression, with 24% of *drs* and 28% of *dipt* while in the normal larvae they only contributed 2.6% of *drs* and 4.2% of *dipt* expression. The reduction in the numbers of haemocytes increased the contribution of AMP expression.

The most significant contribution of AMP for haemocytes (a maximum of 28%) does not account for the extensive reduction seen in our most ablated lines where AMP expression in whole larvae was reduced by 85%.

A)

Genotype/Drosomycin	Fat body		Haemolymph		Carcass	
	%	±	%	±	%	±
hml-GAL4	40.1	12.3	2.6	3.1	57.3	15.1
Infected *hml*-GAL4	68.0	10.0	24.0	5.6	8.0	4.3
Ablated	37.9	20.7	24.0	12.8	38.1	11.2
Infected ablated	81.5	6.7	14.6	6.7	3.9	1.0

B)

Genotype/Diptericin	Fat body		Haemolymph		Carcass	
	%	±	%	±	%	±
hml-GAL4	19.2	14.4	4.2	7.3	76.5	21.3
Infected *hml*-GAL4	76.9	6.4	15.9	4.9	7.2	2.7
Ablated	20.8	5.3	27.6	24.2	51.5	28.9
Infected ablated	73.1	11.3	16.3	5.8	10.5	5.5

Table 3-5 Comparison of the contribution of immuno-competent tissue between dissected normal *(hml*-GAL4) and ablated larvae for (A) *drosomycin* and (B) *diptericin*. Percentage of expression for each constituent body part was determined by comparing it to the sum total of all body parts assumed to be maximal possible expression for AMP as no body parts were discarded. Results are an average of 3 experiments; errors represent the standard deviation from the average.

3.4 Phagocytic ability of haemocytes

With the reduction of haemocytes in the ablated larvae, it could be assumed that they would have a reduction in phagocytic ability. The reduction in phagocytosis would allow bacteria to proliferate in the haemolymph and it is entirely possible that the excess bacteria circulating would trigger AMP expression in the fat body. If this were true, it would compromise the hypothesis that haemocytes were responsible for modulating the signalling cascades that trigger AMP expression in the fat body.

To examine whether haemocytes regulate antimicrobial inducibility in the fat body, the proliferation of bacteria in normal and ablated larvae were calculated as colony forming units per ml (CFU/ml) after infection with a transformed strain of *E. carotovora* (Figure 3-11). The increase of CFU calculated at various time points indicated that bacteria were able to proliferate in the haemocel freely. In normal larvae, CFU/ml was rapidly reduced following challenge, from the initial load of $2.8x10^5$ to $1.8x10^5$ in one hour and to near extinction in 3 hours. In contrast, for haemocyte-ablated larvae, at the point of infection the lack of haemocytes in ablated larvae enabled 4 times the number of bacteria to enter the haemocel. The initial CFU per ml at $1.1x10^6$ increased to $1.5x10^6$ after an hour and was only reduced to $5.3x10^5$ at 6 hours post-infection confirming that the reduction in haemocytes affected phagocytosis in ablated larvae.

For normal larvae, expression of *dipt* peaked at 144-fold above the basal level, approximately 5 hours post-infection and tailed off slightly to 84-fold the basal level by the next hour. In ablated larvae, expression of *dipt* was only induced to 6-fold above the basal level 6 hours post-infection. The expression of *dipt* in haemocyte

ablated larvae was poorly induced regardless of the presence of excess bacteria and an intact normal fat body.

In accordance with previously published work, this reinforces the hypothesis that haemocytes are required for an integral part of the signalling network for AMP expression (BRENNAN *et al.* 2007).

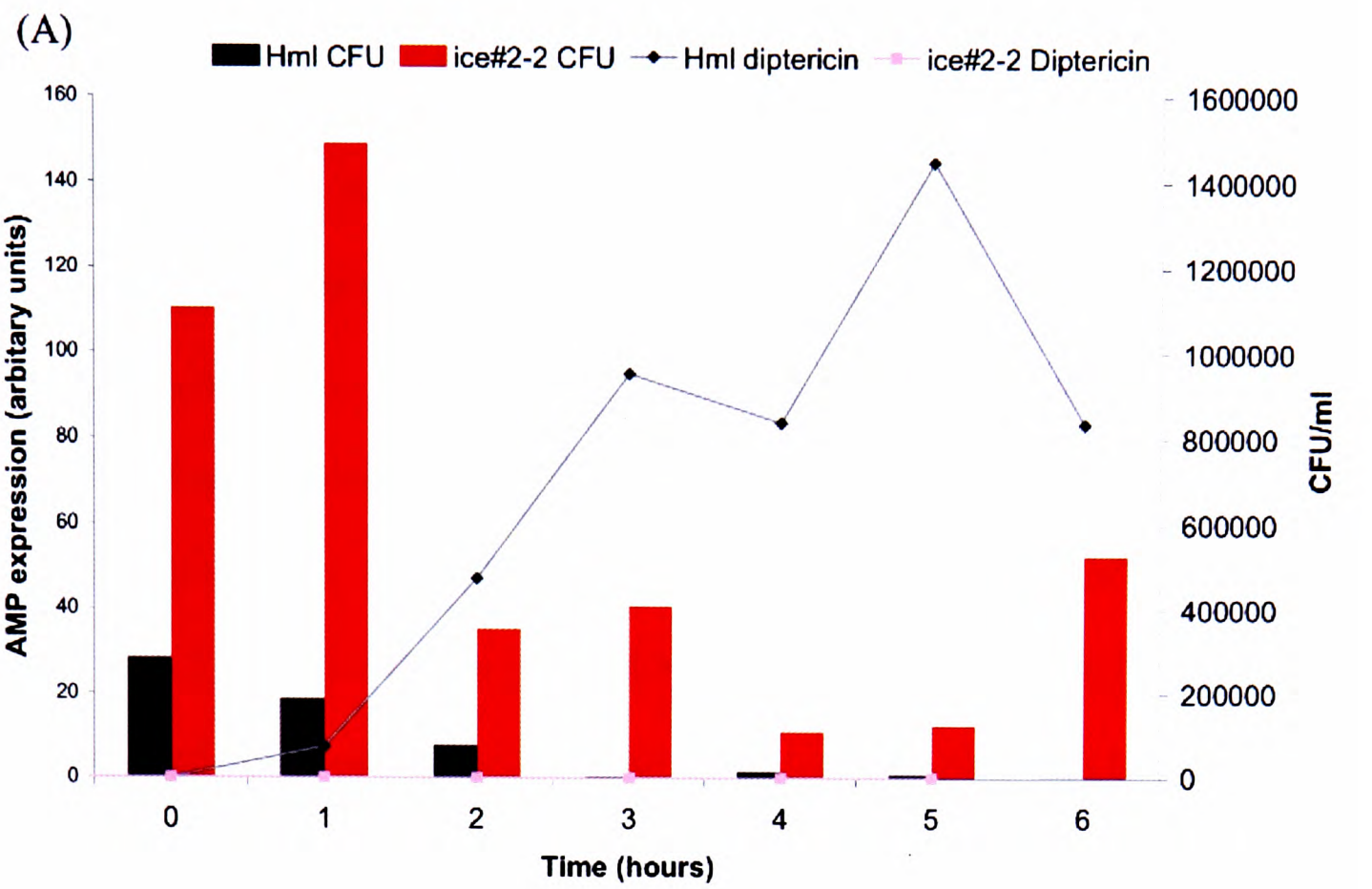


Figure 3-11 A) *Diptericin* expression and CFU per ml in normal and ablated larvae. Results shown are for a single experiment as it was not possible to calculate average CFUs. The experiment was reproducible with similar trends (B) A second CFU experiment to show a similar trend. Note that CFU in normal larvae were detectable 6 hours post infection but the amount was not noticeable on the scale. Expression of diptericin in ablated larvae was at such a low level that it appeared to be undetectable, it was only induced 6-fold at 6 hours post infection compared to 83-fold for normal larvae.

3.5 Detachment of sessile haemocytes in ablated larvae after infection

Following on from the previous results that ablated larvae were able to phagocytose bacteria after a delay and from previous work (HOLZ *et al.* 2003; LANOT *et al.* 2001; ZETTERVALL *et al.* 2004), it was hypothesised that the lymph glands and the sessile haemocytes present in the larvae acted as reservoirs to be recruited upon infection or at pupariation. Sessile haemocytes expressed markers for mature haemocytes and have not been molecularly characterised to be any different to circulating plasmatocytes in the larvae. It is presented here that it is possible for sessile haemocytes to be released into circulation after infection. The reduction of bacteria in the ablated larvae (discussed previously) was possibly due to the release of some sessile haemocytes that could not be ablated. When using TexasRed labelled *E. coli* strain to infect *hml*-GAL4, UAS-GFP; UAS-ice#2-2 (haemocyte-ablated) larvae it was observed that bacteria through the haemolymph flow were found associated with the sessile haemocytes. Detachment of sessile haemocytes away from the site of infection was possibly induced by the signals from the first group of haemocytes that were detached during infection and began circulating around the haemolymph (Figure 3-13). The infection was inflicted in the middle of the larvae on the ventral region (marked by a white box in the figure) where the sessile haemocytes were attached to the integument.

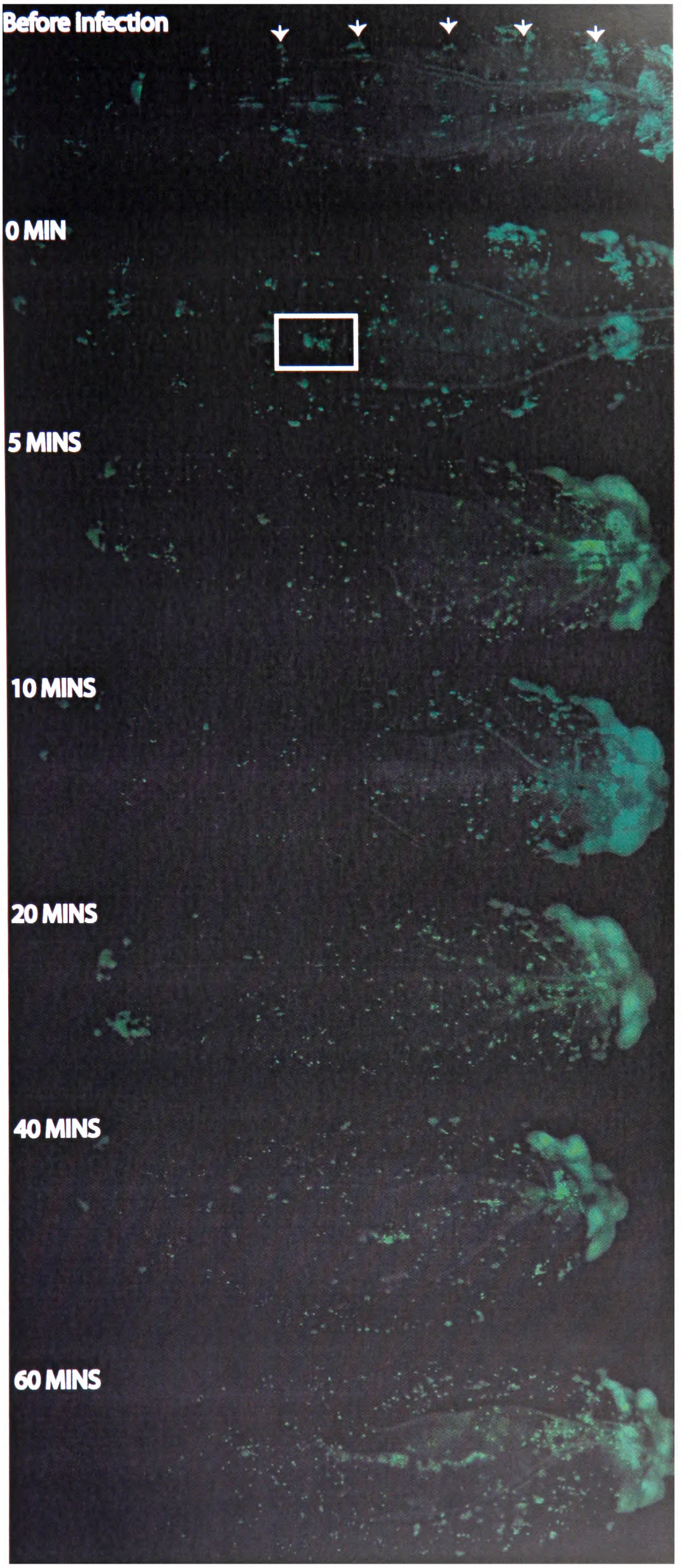

Before Infection
0 MIN
5 MINS
10 MINS
20 MINS
40 MINS
60 MINS

Figure 3-12 (previous page) Detachment of sessile haemocytes on the integument into circulation after infection. Arrowheads at the top figure indicate location of the bands of sessile haemocytes. Point of infection highlighted by box at 0 minute. Detachment of haemocytes from the interior of the epithelial was evident with more haemocytes appearing to be circulating in the haemocel. The disappearance of segments (highlighted by the white arrows before infection) correlated with the dispersal of the haemocytes into the haemocel. Images were captured at 40X magnification, the anterior of the larva at the left hand side of the image, viewing the ventral region.

3.6 Expression of *drosomycin* in fat body

Following on from the hypothesis that haemocytes contributed to the triggering of AMP expression in the fat body, it was proposed to investigate if patterns of AMP expression were influenced by the absence of circulating haemocytes. For this purpose, transgenic larvae with a *drs*-GFP were placed in a wild type or haemocyte ablated background and observed hourly up to 6 hours after infection by *M. luteus* under a fluorescence stereoscope. All expression data were obtained by infecting wandering third instar larvae with a freshly cultured concentrated solution of bacteria, pricking with a sharp needle to the posterior end between the posterior spiracles. To obtain accurate expression data, both lines of *drs*-GFP available as stocks in the lab were examined and both showed the same temporal and spatial expression patterns confirming there were no positional effect for the transgene (Figure 3-13, Figure 3-14A, Figure 3-14C). Both *drs*-GFP lines were on different chromosomes, DD_1 (consisting of *drs*-GFP and *dipt*-LacZ) on the X and *drs*-GFP on the 3rd chromosome. DD_1 showed a slight tendency to be expressed in the tracheal system, indicating a localised response and interfered with observations for the fat body, thus *drs*-GFP was preferred and used for further experiments. Both lines had equal expression in fat body lobes and temporally. To confirm that the GFP fluorescence detected was indeed expressed in the fat body, random samples were dissected. The expression was observed to be restricted to the specific organ, confirming the expression pattern observed (Figure 3-15).

The *drs*-GFP transgene was then examined together with different haemocyte drivers *in trans* to determine if there would be any non-autonomous cell expression but spatial and temporal expression patterns did not differ from wild type observations

(Figure 3-13 and Figure 3-14B, D and E). The standard expression pattern was then used to compare to the ablated genotype. To confirm that the data observed was consistent, the intensity of GFP expression was measured and was found to be similar in all control lines (Figure 3-19). The intensity of expression in *pxn* driven lines was stronger than *hml* driven lines but this was possibly due to the fact that it is possible for *pxn* to be expressed in the fat body.

In wild type larvae, the initiation of fat body GFP expression appears to initiate approximately 3-4 hours post infection (Figure 3-13). Sites of initiation for expression were random throughout the fat body but mostly concentrated to the anterior of the larvae, near the lymph glands. Expression of *drs*-GFP did not follow any linear pattern and started to appear at various points throughout the fat body that continued to spread throughout the tissue within 1 hour (Figure 3-14). Approximately 50 larvae were observed for each genotype and in each of them; over two-thirds initiated expression of *drs*-GFP at 3 or 4 hours post-infection. The haemocytes of both haemocyte driver lines used were not attracted to the site of infection at the posterior and it is presumed that they encounter bacteria through the rapid flow of the haemolymph.

In ablated larvae the temporal initiation of the transgene did not differ from the controls (Figure 3-17), however expression was spatially restricted to the initial site of activity that was concentrated at the anterior portion near the lymph gland (Figure 3-18). These results indicate that triggering and the continuing response of AMP expression for the whole fat body requires the contribution of the haemocytes.

Concurring with results obtained by Northern blots, the intensity of *drs*-GFP observed in ablated larvae was found to be reduced to 49% (Figure 3-19).

With these observations it was proposed that the haemocytes send inductive signals to the fat body cells to ensure a concerted response of antimicrobial expression in the whole tissue. These results indicate how cellular and humoral response can be integrated during host defence.

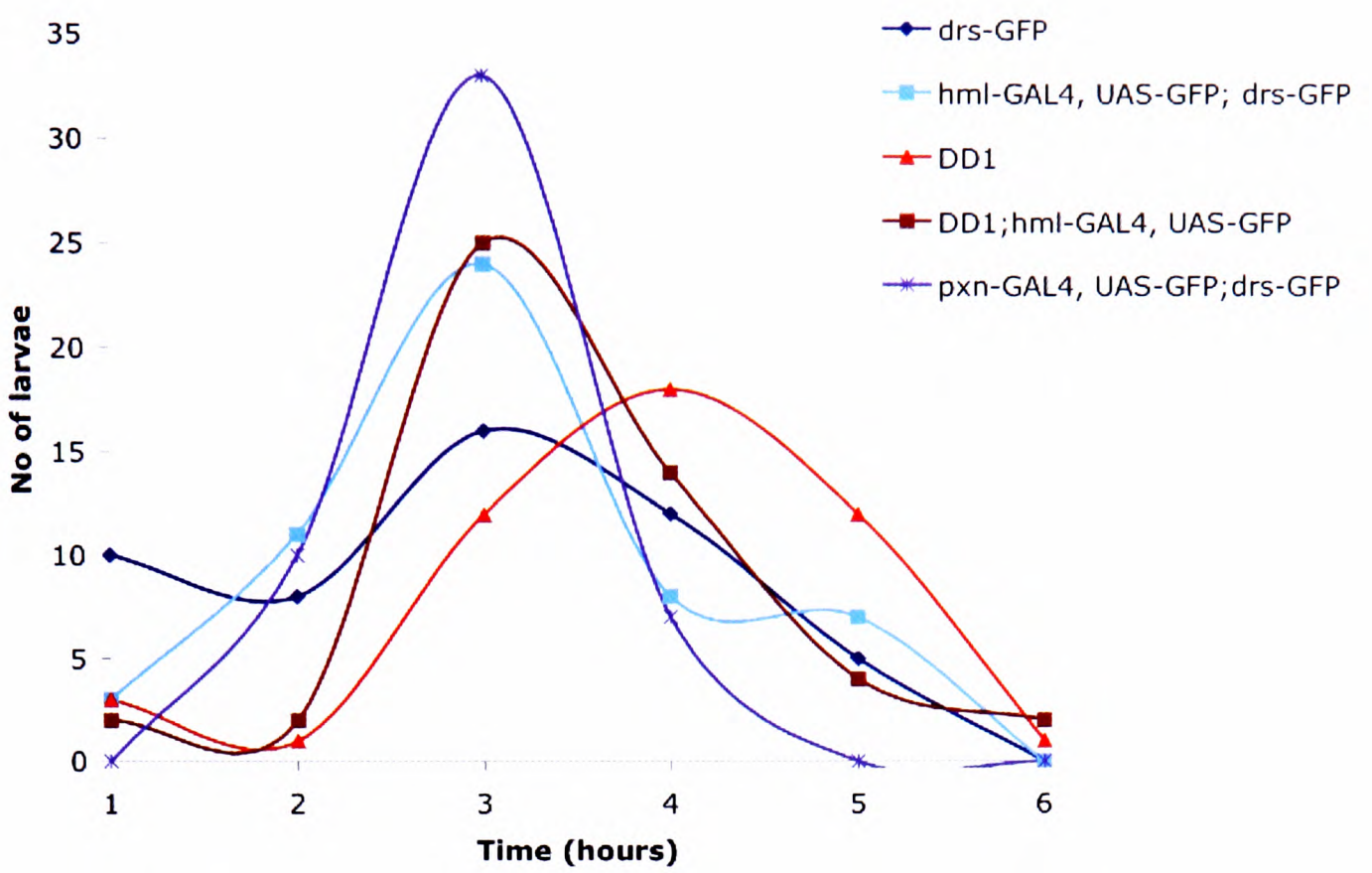


Figure 3-13 Temporal activation of *drs*-GFP expression in fat body of different genotypes. Approximately 50 larvae were observed for each genotype to allow for biological variance and to confirm the initiation of expression. Once initiation of expression was confirmed, larvae were observed until the end of the experiment. In a small number of larvae, *drs*-GFP was not observable and was excluded from analysis.

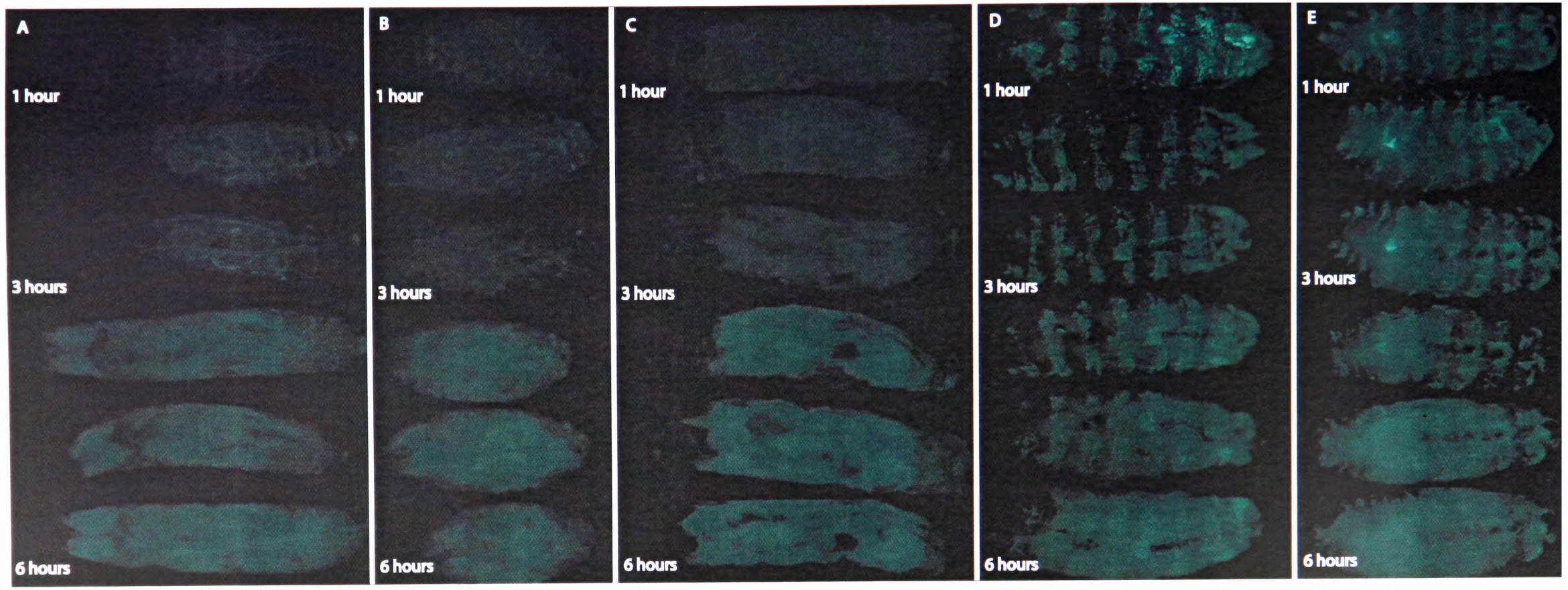


Figure 3-14 Initiation of *drs*-GFP expression in fat body of various genotypes up to 6 hours post infection A) DD_1 B) DD_1; *hml*-GAL4 , UAS-GFP; TM6B/MKRS C) *drs*-GFP D) *hml*-GAL4, UAS-GFP ; *drs*-GFP E) *pxn*-GAL4, UAS-GFP ; *drs*-GFP; TM6B.MKRS. Images were captured at 40X magnification, the anterior of the larva at the left hand side of the image, viewing the ventral region. Haemocytes expressing GFP in (B) had weaker expression as they only had a single copy of *hml*-GAL4, UAS-GFP. The initial expression of the antimicrobial peptide was random and was not concentrated at any foci, the antimicrobial peptide was expressed throughout the fat body fairly rapidly without any set pattern. The larva in (B) and (E) expresses the larval marker *tubby* (on the balancer TM6B) and thus appear shorter or smaller but it is of the same developmental stage as the others.

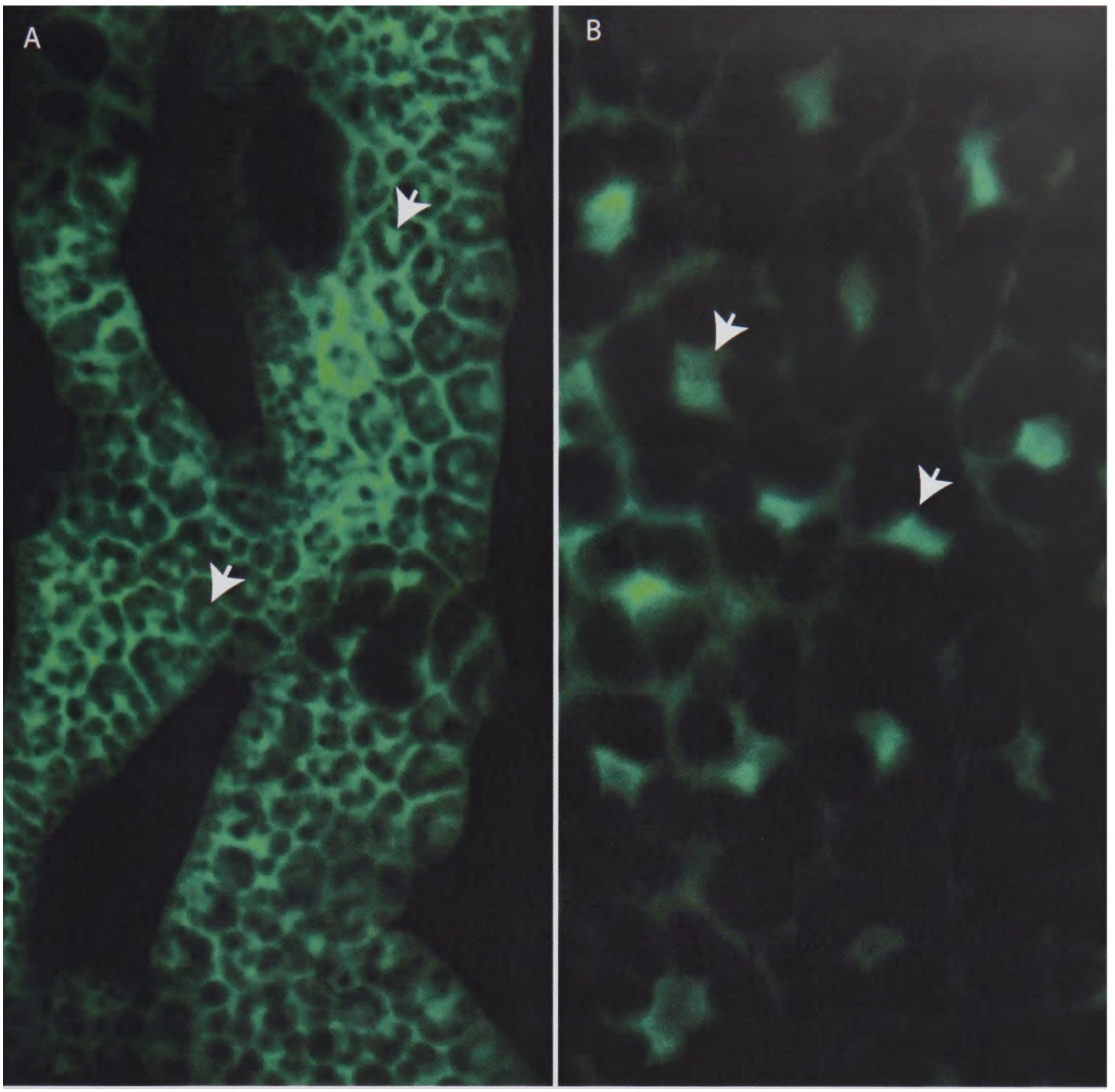


Figure 3-15 Fat body dissected from larvae 6 hours after infection. Fluorescence expression was clearly evident in the fat body observed with a *drs*-GFP transgene. A) GFP expression at 100X magnification, part of a fat body lobe. Expression of *drs*-GFP was detected at the nuclei B) GFP expression concentrated at the nucleus of fat body cells at 200X magnification of the same lobe, showing concentrated expression in the nuclei of cells, as highlighted by the white arrows.

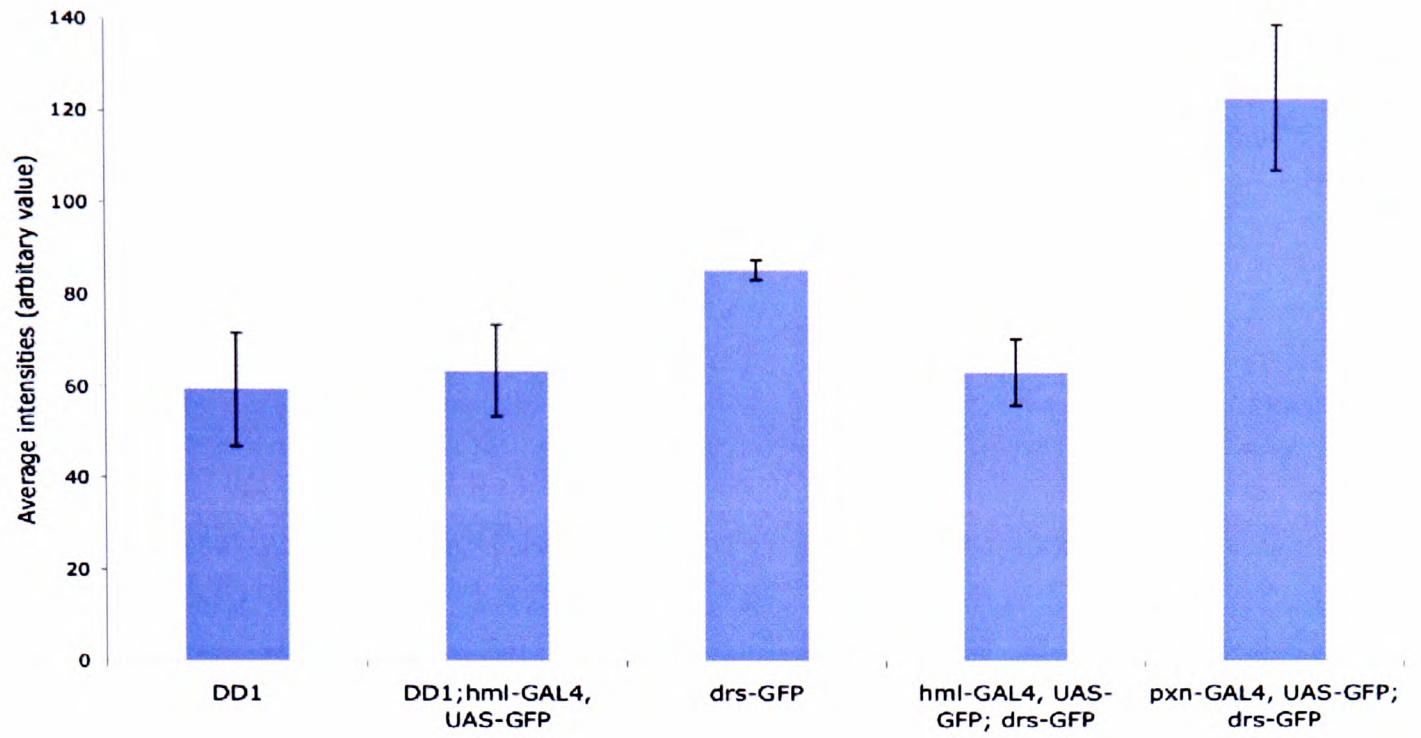


Figure 3-16 Comparison of the average intensities measured for the standard control animals. Data presented are averages of 10 independent measurements of the same genotype, error bars indicate the standard deviation from the mean.

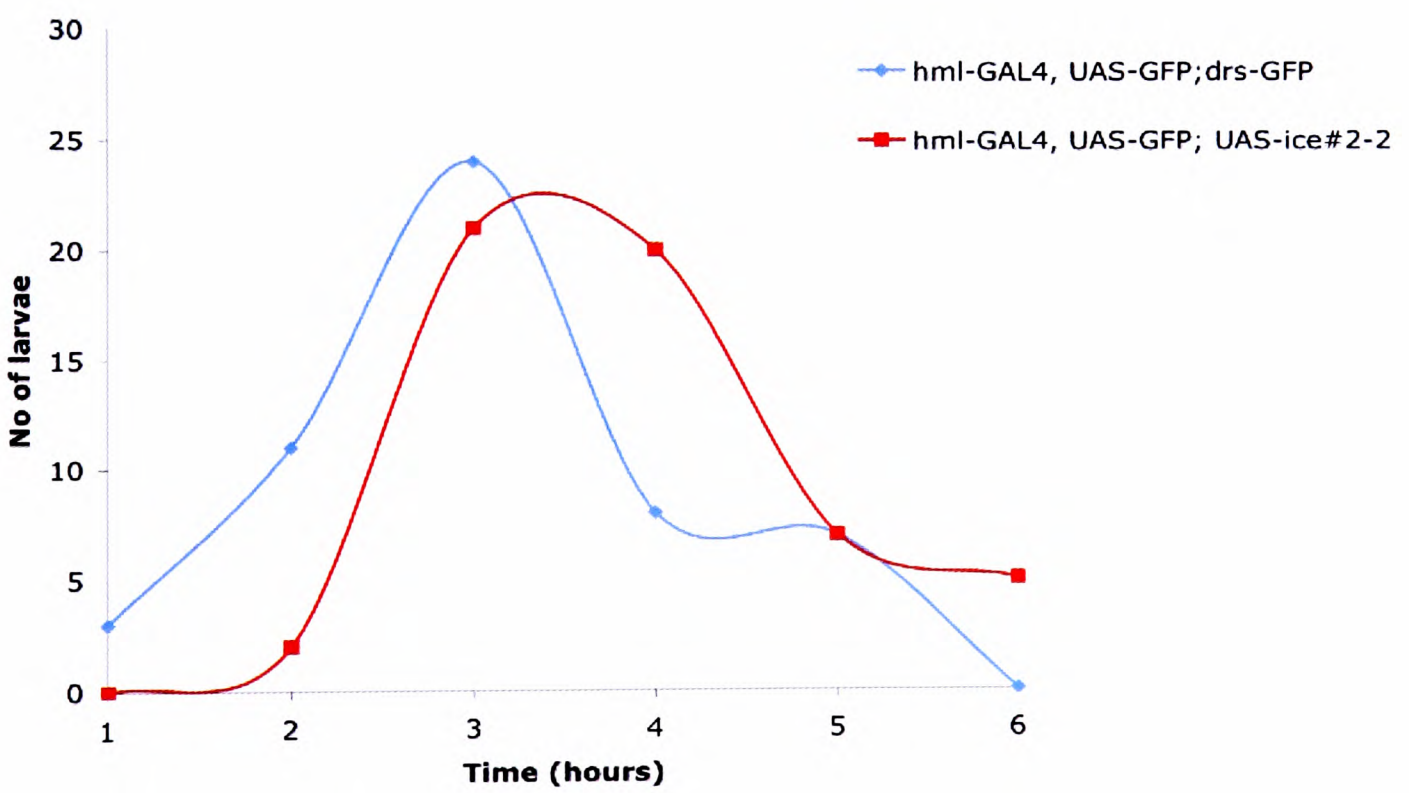


Figure 3-17 Comparison of temporal initiation between a normal and ablated genotype As before, approximately 50 larvae were observed for each genotype. Once expression of the AMP was detected, larvae was observed until the end of the experiment to confirm the pattern of expression of *drosomycin*. Both strains showed initiation of expression at approximately the same time, with over half of them starting at approximately 3 hours post infection.

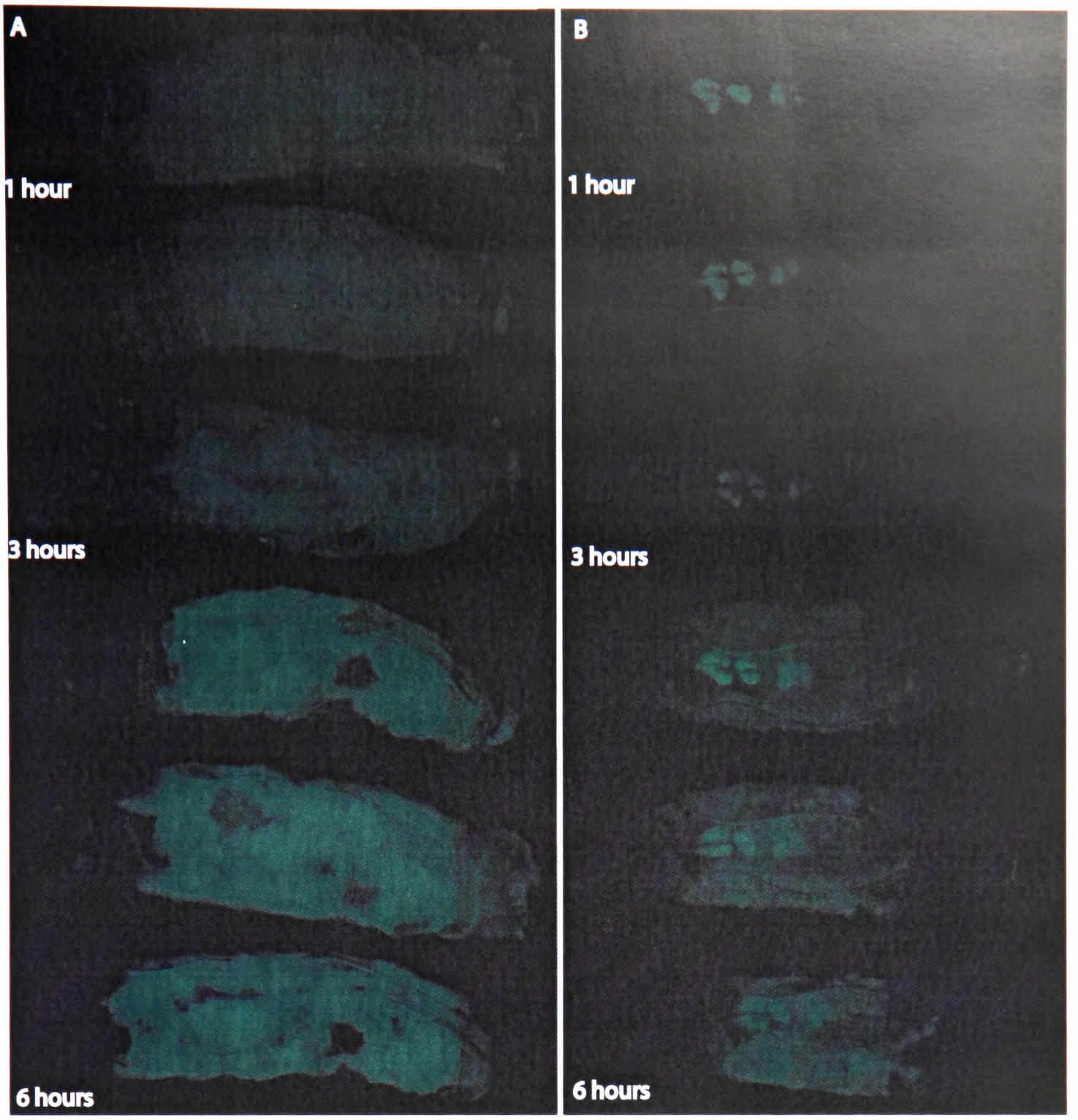


Figure 3-18 Comparison of *drs*-GFP pattern of expression in normal and ablated wandering third instar larva A) CyO/IF; *drs*-GFP B) *hml*-GAL4, UAS-GFP; UAS-ice#2-2. Images were captured at 40X magnification, the anterior of the larva at the left hand side of the image, viewing the ventral region. Initiation of *drs*-GFP is random and does not follow an invariant route, the propagation for ablated larva appears to be restricted to the anterior of the fat body (surrounding the lymph glands that were not ablated in this larva).

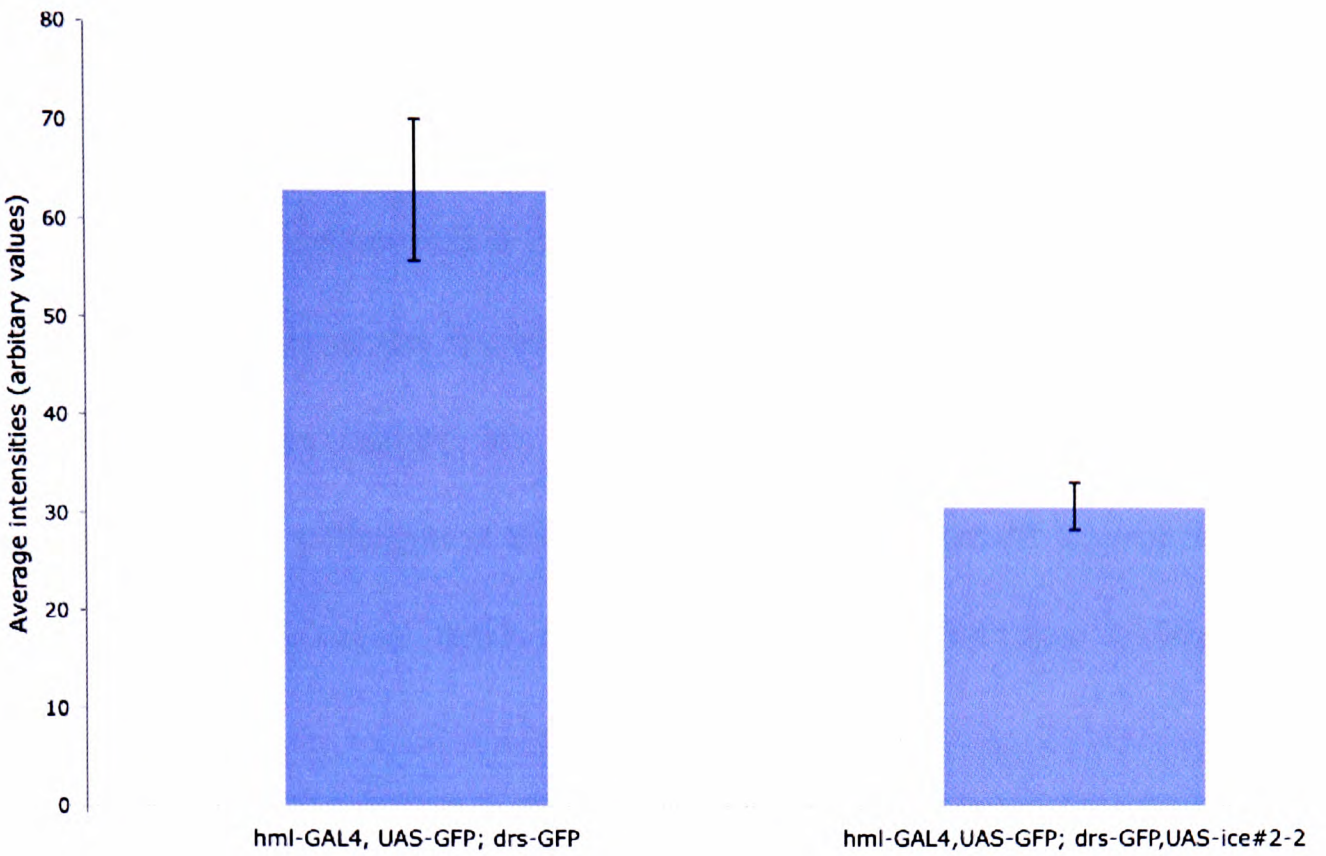


Figure 3-19 Comparison of the average intensities for a standard control to the most severely ablated phenotype. Data presented are averages of 10 independent measurements of the same genotype; error bars indicate the standard deviation from the mean. The ablated larvae showed a marked decrease in intensity of GFP expression

3.7 Inductive signals sent by the haemocytes to the fat body after infection

So far it has been implied that the regulation of antimicrobial responses in the fat body depends on haemocytes at both the level of expression and the activation of this expression throughout the tissue. The working hypothesis was that they integrate signalling cascades caused by the challenge (including wounding) as well as recognition of the pathogen in the haemolymph to channel the appropriate signals to the fat body (IRVING *et al.* 2005; MATHEY-PREVOT and PERRIMON 1998).

3.7.1 The cytokine ligand for the JAK/STAT pathway, upd3

Various signalling pathways have been implied to be involved in conducting the inductive signals required by the fat body from the haemocytes. *Upd3* is one of the three *upd* genes contained in the *upd/os* locus and is activated in adult haemocytes following septic injury and regulates the activation of the general stress response peptide TotA (AGAISSE *et al.* 2003). It is also the cytokine ligand for the JAK/STAT pathway (AGAISSE *et al.* 2003). To determine if *upd3* could function as a regulatory signal for AMP expression in the fat body, the GAL4-UAS technique was employed, this time utilising a UAS-RNA^{i} construct (AGAISSE *et al.* 2003) for tissue specific silencing in the haemocytes and the fat body.

When transcription of *upd3* was silenced in *hml*-expressing haemocytes, expression of AMPs was knocked down by 20% with a similar response when it was reduced in *pxn*-expressing cells, indicating that *upd3* is not specific to a driver and affects both immune pathways. In contrast, silencing of *upd3* in the fat body did not affect AMP expression (Figure 3-20).

To identify if *upd3* plays a role in *drs*-GFP expression in the fat body, the transgene was first introduced to UAS-*upd3*^{RNAi} without any GAL4 drivers to obtain accurate expression data. As before, 50 larvae were observed and they did not show any spatial or temporal differences to the standard *drs*-GFP larvae (Figure 3-21, Figure 3-22). When the hairpin construct was introduced to silence the gene in *hml*-expressing haemocytes, the expression of AMP was reminiscent to that of the ablated genotype (compare Figure 3-22C to Figure 3-18B). This observation was confirmed when knockdown of *upd3* in *pxn*-expressing haemocytes also showed similar spatial restriction for *drs*-GFP (Figure 3-22D). Silencing of the gene in the fat body did not affect the temporal nor spatial expression of *drs*-GFP.

To correlate the Northern blot data with the *drs*-GFP expression data, the intensity of expression was measured and found to be reduced to approximately 56% in *hml*-GAL4 and 41% in *pxn*-GAL4 silenced lines, with no reduction in the intensity for the ones expressed in the fat body (Figure 3-23).

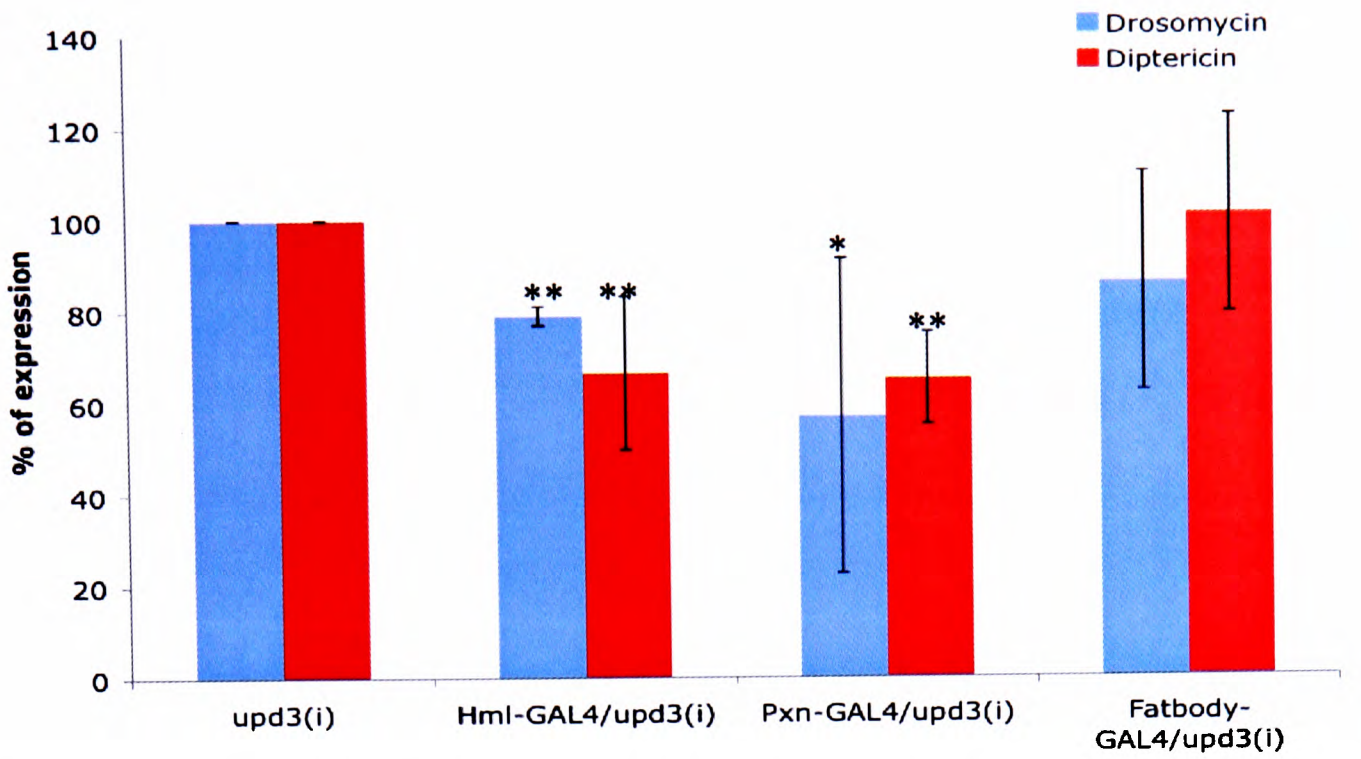


Figure 3-20 Comparison of antimicrobial peptide expression when *upd3* was silenced in fat body and haemocytes. Data presented are averages of three independent experiments and error bars indicate standard deviation from the mean. A double asterisk above the column indicates that the difference in the means of the sample was statistically significant compared to the control upd3(i), while a single asterisk indicates that it is marginally significant after an a t-test. For *hml*-GAL4/UAS-*upd3*(i), *drs* has values of $t=17.17$, $p<0.05$ while *dipt* has values of $t=3.44$, $p<0.05$. For *pxn*-GAL4/UAS-*upd3*(i), *drs* has values of $t=2.15$, $p<0.10$ while *dipt* has values of $t=5.92$, $p<0.05$. Finally, for fatbody-GAL4/UAS-*upd3*(i), *drs* has values of $t=0.97$, $p>0.10$ while *dipt* has values $t=0.11$, $p>0.10$

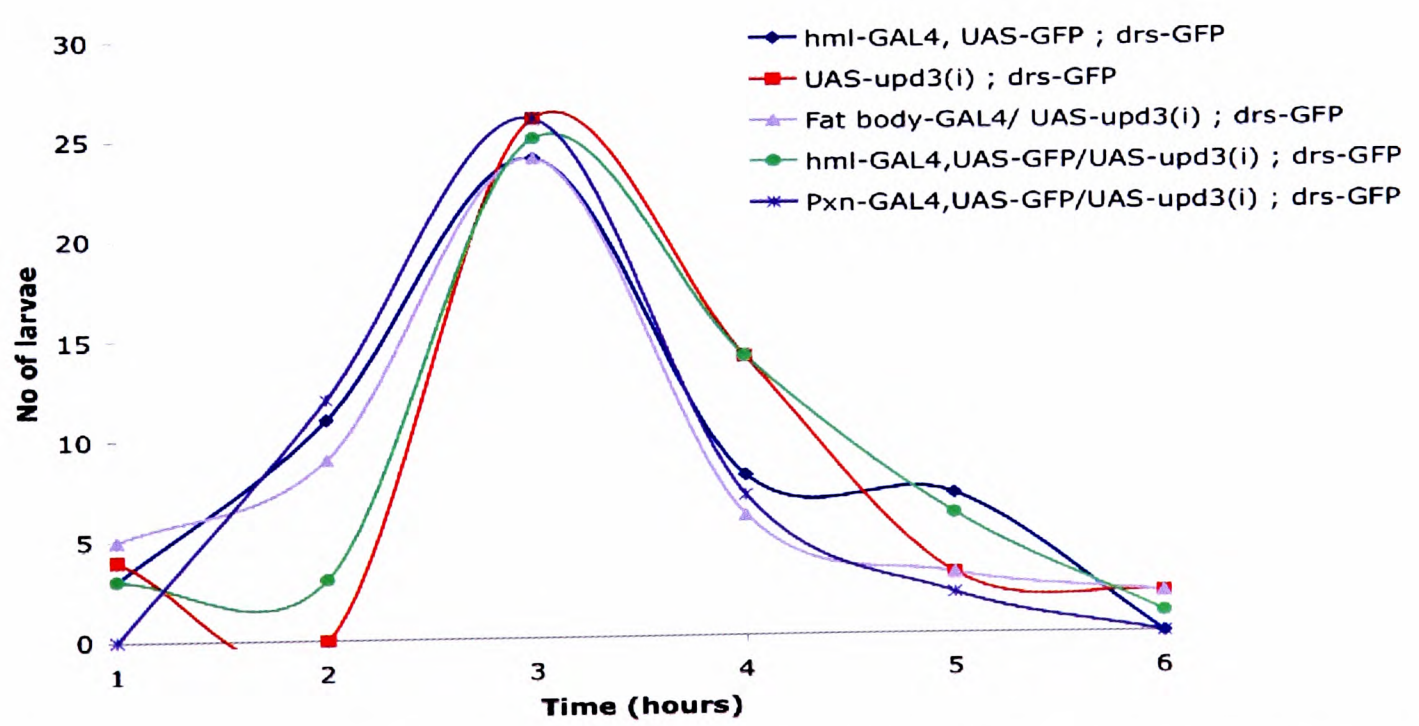


Figure 3-21 Temporal initiation of *drs*-GFP expression in different genotypes where *upd3* was knocked down by RNA^{i}. As before, 50 larvae were observed for each genotype and once initiation of the transgene was confirmed, larvae were observed until the end of the experiment. Over half of the larvae initiated expression at 3 hours post infection for all genotypes.

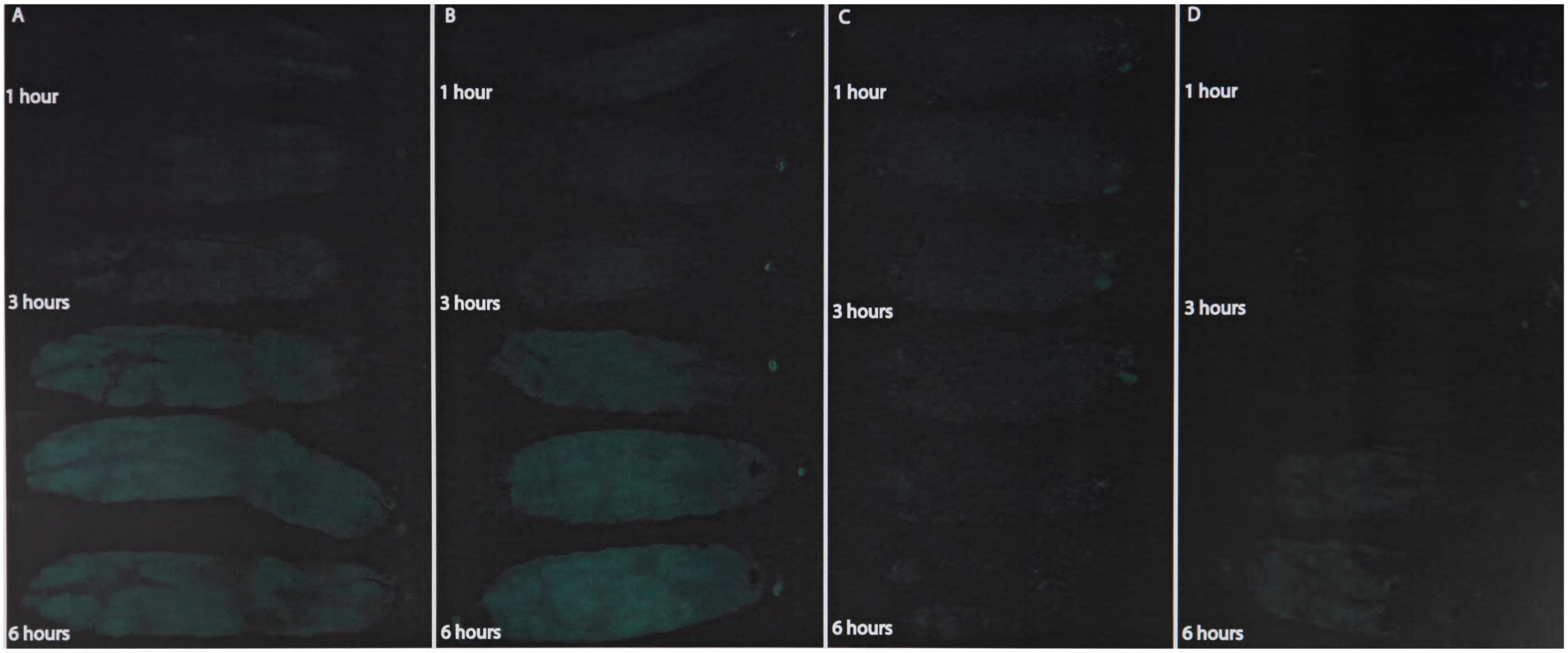


Figure 3-22 Initiation of *drs*-GFP expression in the fat body of various genotypes. A) UAS-*upd3*^{RNAi}; *drs*-GFP B) Fat body-GAL4/UAS-*upd3*^{RNAi}; *drs*-GFP C) *hml*-GAL4/UAS-*upd3*^{RNAi}; *drs*-GFP; TM6B D) *pxn*-GAL4/UAS-*upd3*^{RNAi}; *drs*-GFP. Images were captured at 40X magnification, the anterior of the larva at the left hand side of the image, viewing the ventral region. The initial point of expression was random for A) and B) without following an invariant route while spatial restriction was obvious in C) and D). The larva in (C) expresses the larval marker *tubby* (on the balancer TM6B) and thus appear shorter or smaller but it is of the same developmental stage as the others. Larva in (C) also had points of GFP expression, marking the haemocytes, not to be confused with fat body lobe expression.

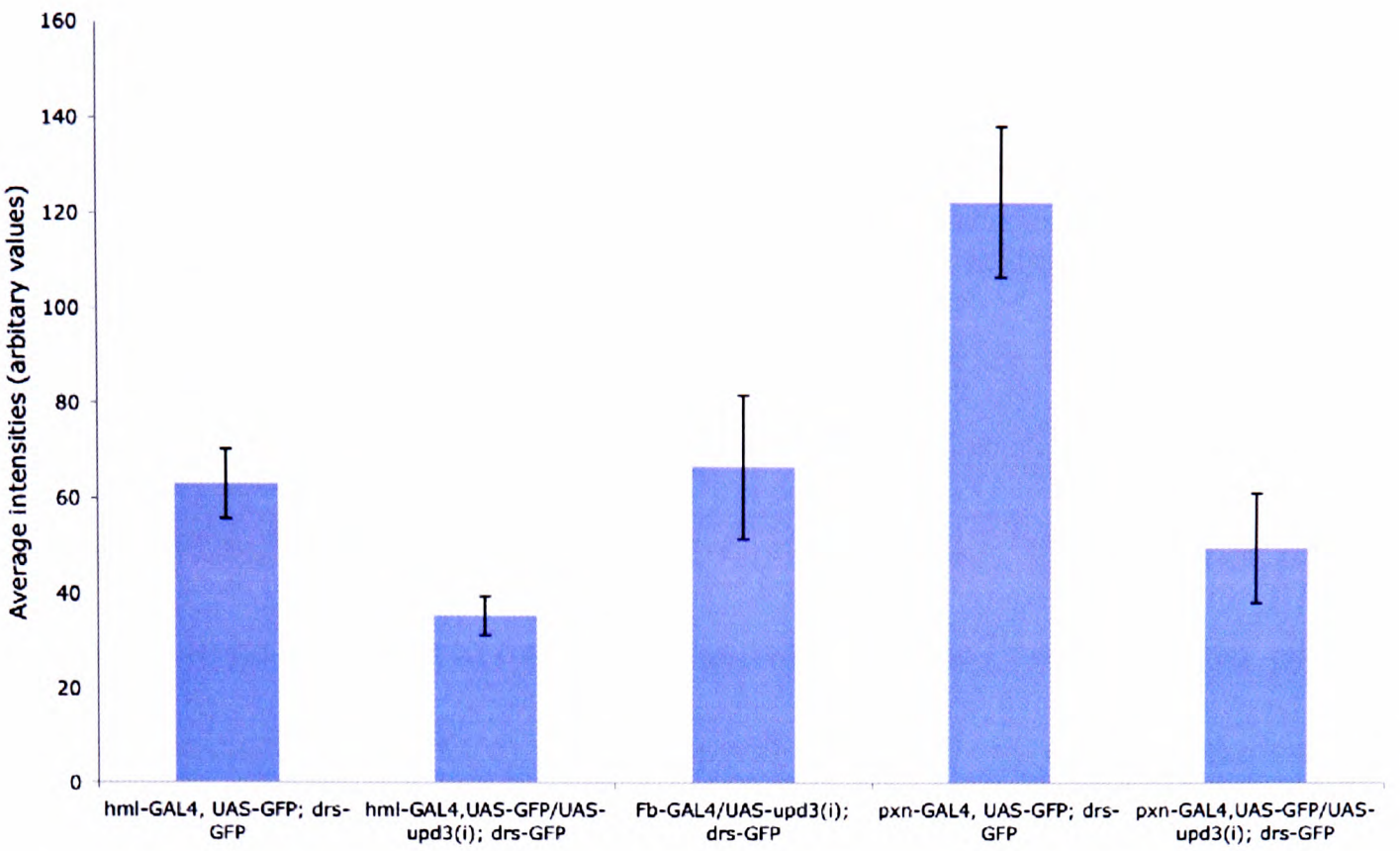


Figure 3-23 Comparison of the average intensities for control genotypes to various genotypes that had tissue specific reduction of *upd3* expression. Data presented are averages of 10 independent measurements of the same genotype; error bars indicate the standard deviation from the mean. A reduction in intensity was observed in two lines that had reduced expression of *upd3* in the haemocytes (*hml*-GAL4, UAS-GFP/UAS-$upd3^{RNAi}$; *drs*-GFP and *pxn*-GAL4, UAS-GFP/UAS-$upd3^{RNAi}$; *drs*-GFP). Reduced expression of the cytokine in the fat body did not affect the intensity of drs-GFP observed in the fat body.

3.7.2 The JNK phosphatase puc

The JNK pathway has been linked to various pathways, including stress response, cell migration, apoptosis and immune response in both mammals and *Drosophila* (ARBOUZOVA and ZEIDLER 2006). The pathway consists of canonical MAPK signalling proteins of which Puc is a dual-specificity phosphatase that dephosphorylates and inactivates the JNK family members. Expression of *puc* has been shown to be sufficient to monitor the activation of the Drosophila JNK pathway signalling (RAMET *et al.* 2002a). The temporal pattern of expression in the fat body of a *puc*-lacZ transgene suggests local signalling, with adjacent cells appearing to activate expression of the protein in their neighbouring cells (Figure 3-24). The intensity of expression appeared to be at its peak 8 hours after infection although the signal could still be detected 24 hours after infection.

In *Drosophila*, JNK signalling influences AMP expression (DELANEY *et al.* 2006). As has been previously presented, the JAK/STAT pathway also plays a role in signalling to the fat body. To observe how JAK/STAT signalling could influence JNK signalling, the expression of *puc* was observed in fat body cells when *upd3* was reduced in the haemocytes. Reduction of *upd3* in haemocytes appeared to alter the morphological appearance of the fat body cells, such that they became less tensile and unable to resist any physical stress. The number of cells that expressed *puc*-lacZ was also significantly reduced, indicating that the haemocytes were capable of tranducing signals from one pathway to another (Figure 3-25).

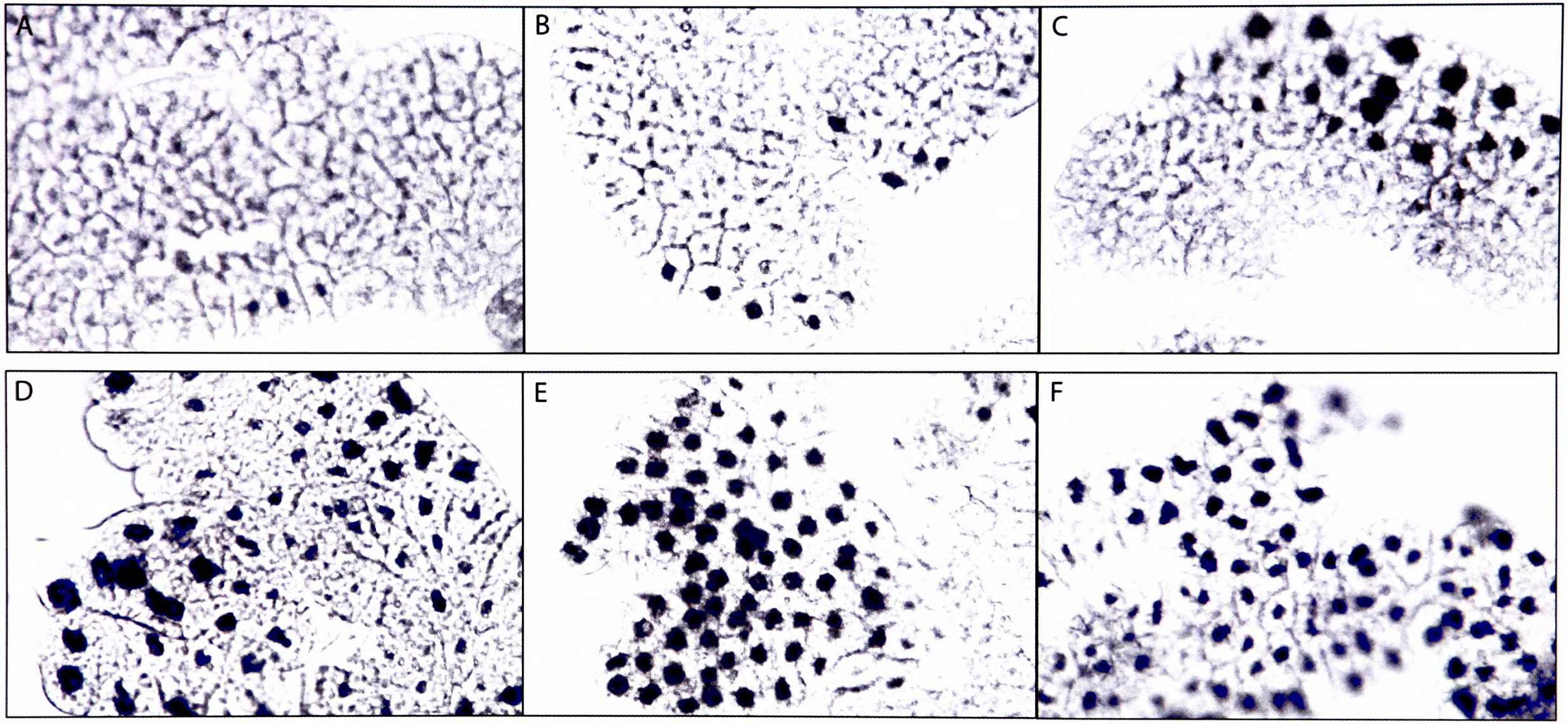


Figure 3-24 Temporal expression of *puc*-lacZ in the fat body of wandering third instar larvae after infection with *E. coli*. Fat body dissected at different time points A) 2 hours B) 4 hours C) 6 hours D) 8 hours E) 12 hours F) 24 hours. Images captured at 100X magnification.

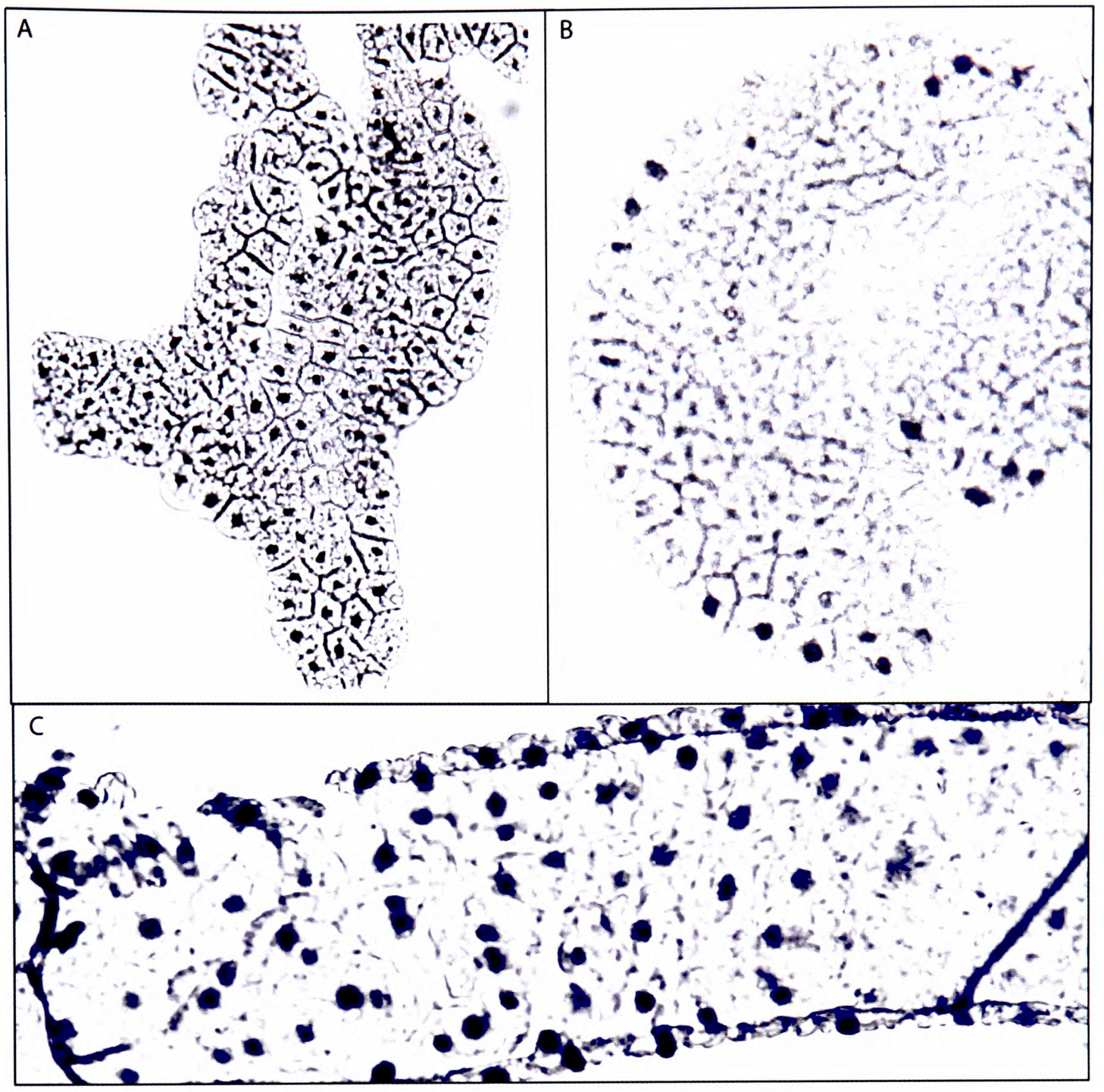


Figure 3-25 *puc*-lacZ expression in sections of A) fat body of *hml*-GAL4; *puc*-lacZ B) fat body of *hml*-GAL4/UAS-*upd3*^{RNAi}; *puc*-lacZ C) gut of *hml*-GAL3/UAS-*upd3*^{RNAi}; *puc*-lacZ. Images captured at 100X magnification. The changed morphology of the fat body cells in (B) appeared only with the knockdown of *upd3* in the haemocytes. The gut was used as a positive control for the staining procedure.

3.7.3 The Toll receptor ligand, Spz

The Spz protein is a ligand for the Toll pathway that initiates an intracellular signalling pathway culminating in the activation of NF-κB dependant transcription in fat body cells. A recent DNA microarray study has shown that the cytokine-line polypeptide is upregulated in larval haemocytes and not the fat body following infection (IRVING *et al.* 2005). Transcriptional silencing of *spz* in haemocytes through an RNA^{i} construct (a kind gift from Prof J-M Reichhart, IBMC Strasbourg) resulted in a 30% reduction of the AMP *drs* following infection. The expression was reduced further when the reduction of *spz* was enhanced by introducing a transheterozygous allele (Spz^{RM7}). Silencing of *spz* in the fat body did not alter the expression of *drs* (Figure 3-26).

To study the effects of *spz* on AMP expression in the fat body, the *drs*-GFP transgene was introduced to larvae that silenced *spz* in haemocytes and fat body. Although reduction in AMP expression levels was previously observed, no spatial or temporal differences for *drs*-GFP expression was observed in *spz* silenced larvae. Both lines showed most of the larvae initiated expression of *drs*-GFP at 3 or 4 hours post infection and confirmed previous observations that the initial point(s) of initiation for the *drs*-GFP transgene was variable (Figure 3-27 and Figure 3-28). The propagation of *drs*-GFP was as rapid as previously observed and did not follow any linear pattern. To correlate the reduction of *drosomycin* observed by Northern blots, the intensity of the expression in observed larvae was measured with a 25% reduction in intensity when *spz* was silenced in haemocytes and no reduction in intensity when silenced in the fat body (Figure 3-29).

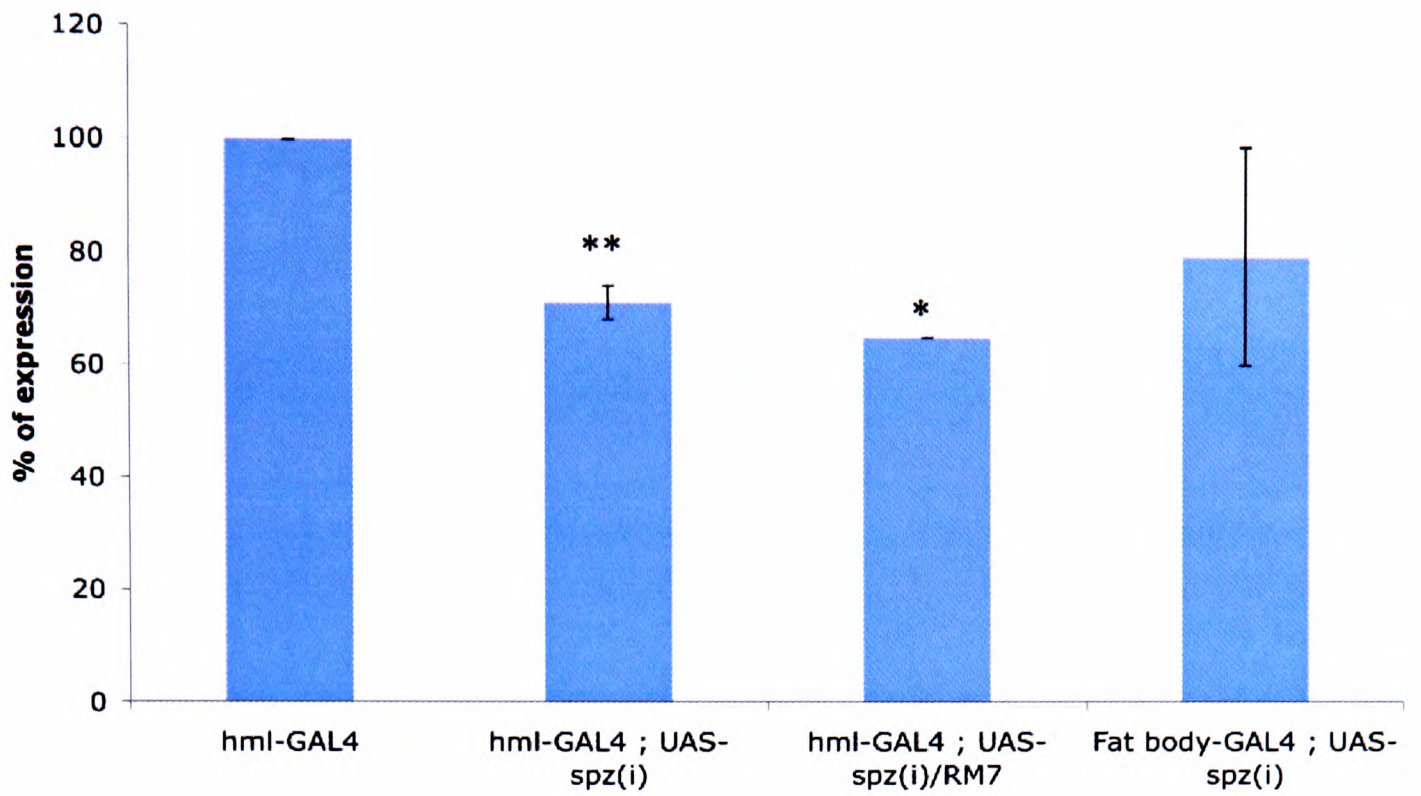


Figure 3-26 Expression of *drosomycin* (*drs*) expression in different genotypes where *spz* was silenced via RNA[i]. *Drs* is routinely used as a readout for the Toll pathway. Data presented is the average of three independent experiments with error bars indicating the standard deviation from the mean. A double asterisk above the column indicates that the difference in the means of the sample was statistically significant compared to the control *hml*-GAL4, while a single asterisk indicates that it is marginally significant, after a t-test. For *hml*-GAL4; UAS-*spz*(i), $t=16.72$, $p<0.05$, while for *hml*-GAL4; UAS-*spz*(i)/RM7, $t=1.66$, $p<0.10$ and for fatbody-GAL4; UAS-spz(i) $t=1.66$, $p>0.10$.

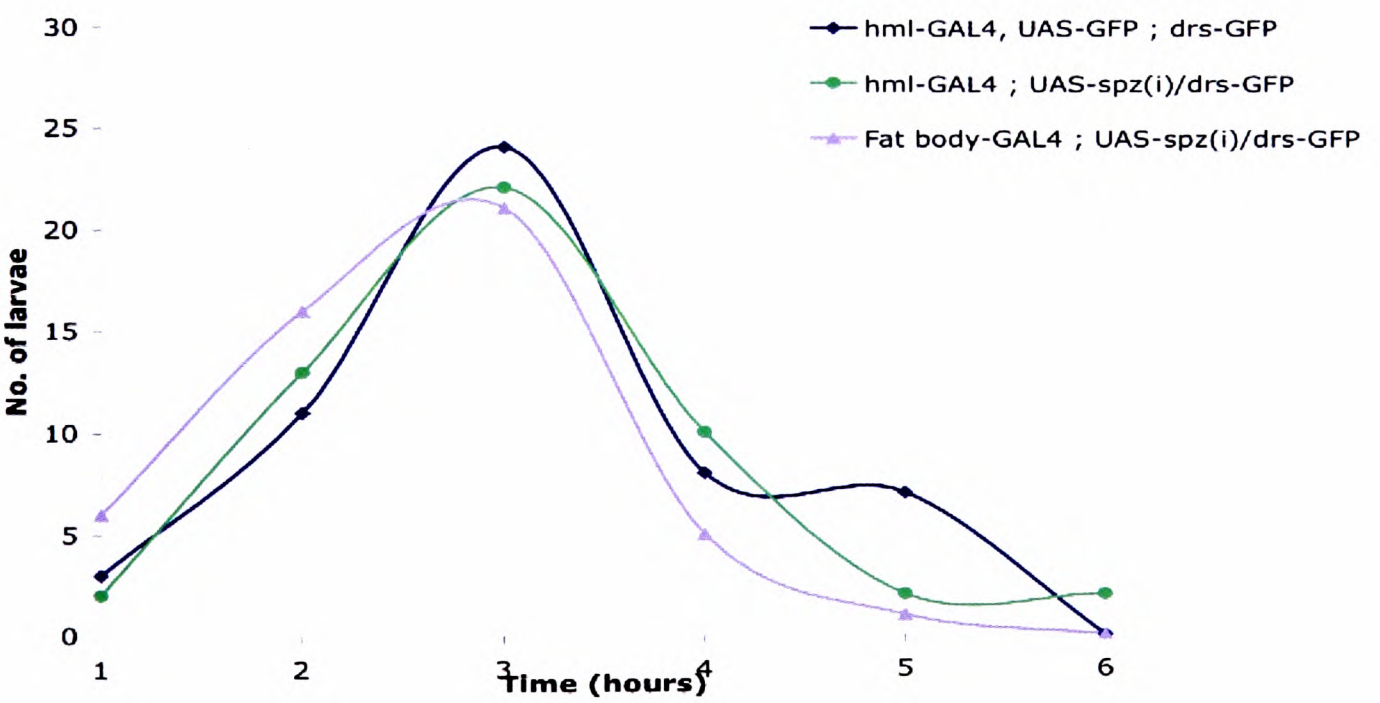


Figure 3-27 Temporal initiation of *drs*-GFP expression in different genotypes where *spz* was knocked down by RNA[i]. As before, 50 larvae were observed for each genotype and once initiation was confirmed, larvae were observed until the end of the experiment. Almost half of the larvae initiated expression at 3 hours post infection for all genotypes.

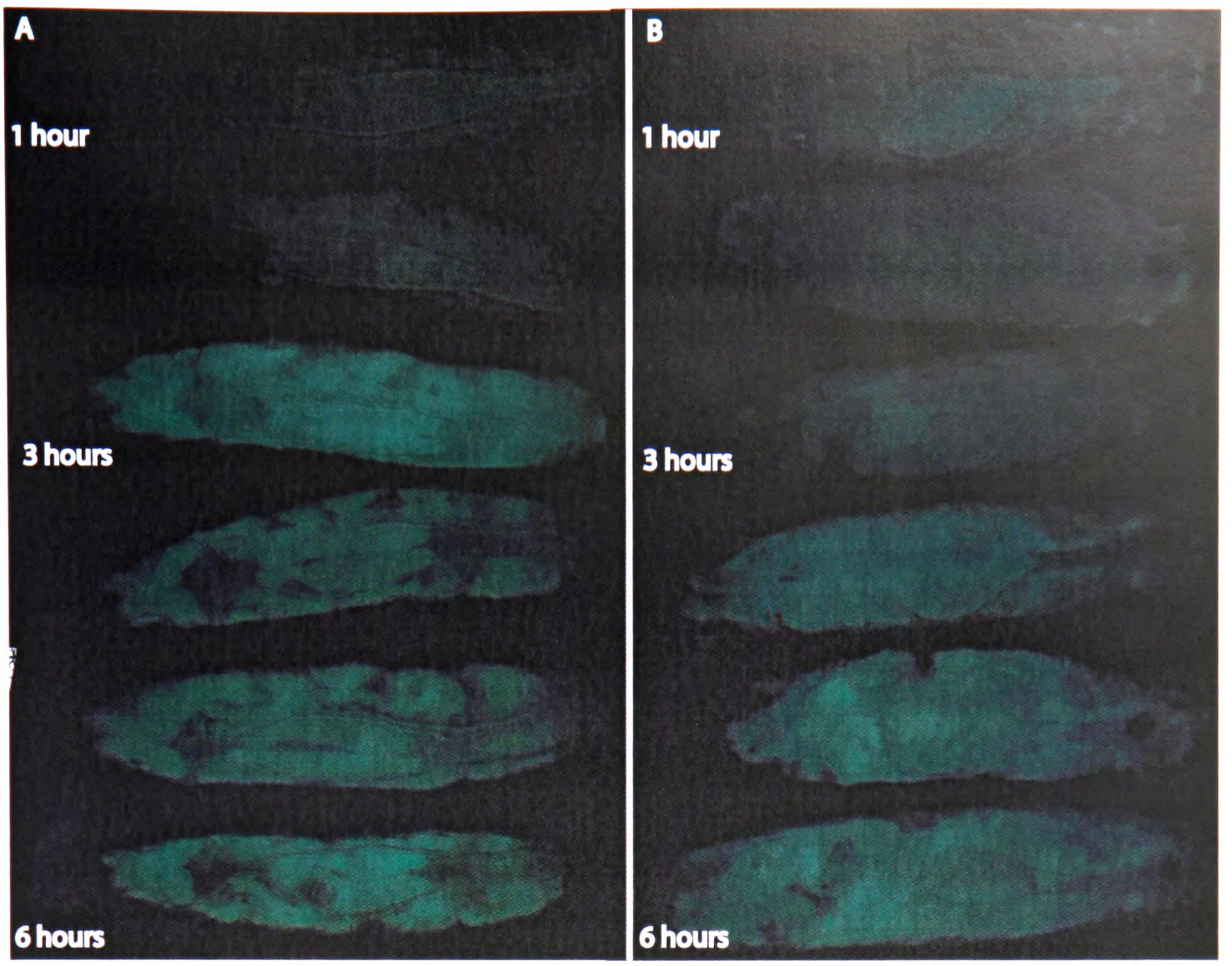


Figure 3-28 Initiation of *drs*-GFP expression in the fat body of various genotypes. (A) Fat body-GAL4; UAS-*spz*^{RNAi}/*drs*-GFP (B) *hml*-GAL4,UAS-GFP; UAS-*spz*^{RNAi}/*drs*-GFP. Images were captured at 40X magnification, the anterior of the larva at the left hand side of the image, viewing the ventral region. Initial point of expression was random and there were no noticeable spatial differences in the expression pattern. Expression in (B) was slightly weaker due to reduction in AMP expression.

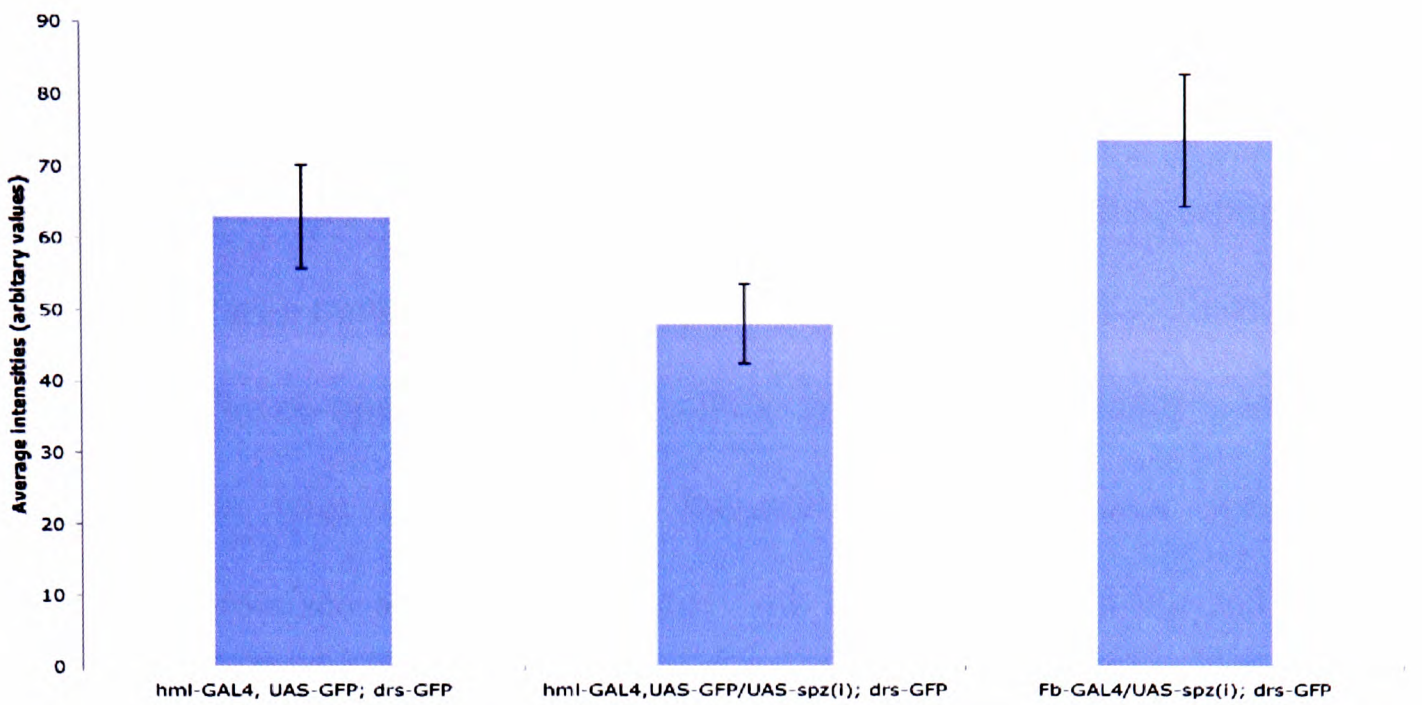


Figure 3-29 Comparison of the average intensities between control animals and reduced expression of *spz* in haemocytes or fat body. Data presented are averages of 10 independent measurements of the same genotype; error bars indicate the standard deviation from the mean. A slight reduction in intensity was observed when *spz* was silenced in the haemocytes that was not evident when *spz* was silenced in the fat body.

4. DISCUSSION

The role of haemocytes in integrating cellular and humoral immunity during the systemic response has not been dissected to date. In previous studies, septic injury to the larvae begins a multitude of signalling cascades, all of which have been observed in isolation. The co-operation between cellular and humoral immunity was observed when mutants with defects in the humoral immune response were further immunocompromised after being injected with beads. The bead injected flies had their cellular responses suppressed also had higher death rates after subsequent injection by *E. coli* (ELROD-ERICKSON *et al.* 2000). Although the haematopoietic mutant *domino* (*dom*) has significantly reduced numbers of haemocytes, it maintains a wild type resistance to septic injury (BRAUN *et al.* 1998). However, when combined with other mutations for the humoral and melanisation cascade (Imd and Black Cells), the immuno-compromised mutant suggested a synergy between them to fight infection (BRAUN *et al.* 1998). This theory was strengthened when a strong systemic response was detected in the fat body of third instar larvae after an oral infection with the plant pathogen *Erwinia carotovora*, indicating the existence of signalling from the gut to the fat body (BASSET *et al.* 2000). The results presented in this study amalgamate the idea that haemocytes are involved in signalling via various pathways to the fat body to induce transcription of AMPs to the fat body.

Current genetic models for studying the roles of haemocytes in immunity includes previously described mutants and cell lines that present disadvantages. Previously described haematopoietic mutants includes the *dom* and *l(3)hem* (BRAUN *et al.* 1998; GATEFF 1994). In both mutants, all diploid structures such as the neuroblast region of the brain, imaginal discs, germ cells and lymph glands are abnormal. The *dom* gene

encodes a member of the SWI1/SNF2 family of DNA-dependent ATPases that are also involved in the development of the animal (RUHF *et al.* 2001). *Dom* mutants exhibit a prolonged third instar and die as prepupae while any remaining haemocytes observed are also abnormal (BRAUN *et al.* 1998). The use of a cell line such as S2, although derived from a haematopoietic lineage, would not allow for an *in vivo* study. The use of cell cultures may also introduce exogenous growth factors present in the growth media (SAMAKOVLIS *et al.* 1992).

The various molecular tools available for *Drosophila* make it easy to create a transgenic model suitable for observing the roles of specific organs. The GAL4/UAS system is often used in *Drosophila* to drive tissue-specific ablation (BRAND and PERRIMON 1993). The successful ablation of a significant number of haemocytes using various GAL4 drivers described in this work (Figure 3-2, 3-3 and 3-5) allowed for further investigation into the role of haemocytes. The selection of appropriate GAL4 lines presented an important problem as *peroxidasin* (*pxn*) was expressed in other organs (Figure 3-1) and *serpent* (*srp*) plays a role in development (LEBESTKY *et al.* 2000). The ablation using *srp*, required for initial specification of cells to the haemocyte lineage would have been ideal however, a viable line using the GAL4 lines available for this work, could not be constructed. *Hemolectin* (*hml*) is involved in blood clotting and its expression as a pulse during larval development presented it as the choice for a post-embryonic GAL4 enhancer (GOTO *et al.* 2001). Although *hml* is only expressed in a subset of plasmatocytes and crystal cells, the total number of haemocytes ablated in the larval model was significant enough to observe their roles in signalling (Table 3-2 and Table 3-3).

As presented in this work, sessile haemocytes were resistant to ablation although they do not appear to be morphologically and molecularly different to circulating plasmatocytes (JUNG *et al.* 2005; LANOT *et al.* 2001). Their origins are unknown but it is possible that they are derived from embryonic haemocytes circulating around the haemolymph and attach themselves to the inner integument via different syndecans once a critical mass of haemocytes have proliferated (NARITA *et al.* 2004). It is also possible that they are under the control of a circulating signal and 'home-in' to the target. The sessile haemocytes are postulated to act as reservoirs for larvae (LANOT *et al.* 2001). This was shown to be the case (Figure 3-12) when their release into the haemolymph was observed after pathogen entry into ablated larvae that lack circulating haemocytes. Concurring with previous data that they were still capable of phagocytosis (LANOT *et al.* 2001), it appeared as if the released sessile haemocytes were able to partly contain the infection (Figure 3-11). It was also observed that the lymph glands did not appear to be ablated at all. However, this was not surprising as they are considered to be the stem cell reservoir for the animal, releasing lamellocytes upon parasitasation if necessary (SORRENTINO *et al.* 2002). It is possible that proliferative mechanisms suppress apoptosis in a cellular background that will abort any attempt for cell suicide (MINAKHINA *et al.* 2007). It is also possible that expression of *hml* in the small area of the cortical zone (where cells mature and differentiate) of the primary lobe of the lymph gland is potentially not sufficient to casually observe any reduction in the lymph gland size (JUNG *et al.* 2005).

In adult flies, the ablation pattern was reversed to that of larvae, there was a reduction in the number of sessile haemocytes but not in circulating haemocytes. If we consider sessile haemocytes as the pool of reserve haemocytes and that all

haemocytes in the adults were present in the larvae (HOLZ *et al.* 2003; LANOT *et al.* 2001), therefore, the reduction in sessile haemocytes could be to fewer reserves available for ablated flies when compared to wild-type flies. It could also be that the sessile haemocytes released during larval metamorphosis continue to be circulating in the adults when they have pupariated.

Upon successful ablation of haemocytes, it would be necessary to ascertain how they could affect the humoral immune system. It is known that directly introducing microorganisms into the haemocel of larvae triggers a response that can be monitored using known readouts of the immune pathways (LEMAITRE *et al.* 1995; LEMAITRE *et al.* 1996). The expression of AMPs is usually proportional to the number of bacteria injected. AMP expression reaches a plateau when flies are injected with massive doses of bacteria (LEULIER *et al.* 2003). The observation that a significant reduction in the number of haemocytes reduced the expression of AMP (Figure 3-9), concurred with the original hypothesis that they signal to the fat body in the event of an infection. However, it is certain that the reduction in the numbers of haemocytes reduces the phagocytic capability of the animal. Consistent with published results that it is possible for free floating bacteria in the haemolymph to directly activate the fat body (BETTENCOURT *et al.* 2004; BRAUN *et al.* 1998), the proliferation of bacteria in the haemocel and its effect on AMP expression was observed. In contrast to previous data, the reduction of phagocytosis in the ablated larvae left a significant number of bacteria in the body cavity but increasing numbers of bacteria did not significantly increase the induction of AMP (Figure 3-11). This is in contrast to previous results, where the *dom* mutant showed an almost wild-type response to a septic injury. However, further work done with the mutant not

presented in the thesis found that third instar *dom* larvae died significantly faster compared to the wild type counterparts, with the survivors 6 hours post infection showing an almost normal AMP expression.

It was also possible that the reduction of AMP detected in the whole animal could be due to a reduction in the haemocytes as a small number of them have been shown to express AMP (FERRANDON *et al.* 1998; SAMAKOVLIS *et al.* 1990). However, this was not the case as the contribution of the haemocytes in AMP gene expression (28%) was insufficient to explain the reduction of AMP expression observed (85%). It is interesting to note that while observing the contribution of AMP expression by different organs the levels of AMP expression in the haemolymph is increased in non-infected ablated larvae suggesting that a disruption in homeostasis is enough to induce expression of AMP (Table 3-5). The reduction of circulating sentinel cells capable of phagocytosis triggers the fat body to prepare a compensatory mechanism by secreting an amount of AMPs to the haemocel to prepare for an attack. It is interesting to speculate that the immune system not only recognises non-self but also danger signals that are emitted when there is a disruption (MATZINGER 2002). The idea that the immune system could be triggered by danger signals from disrupted tissues is highlighted by this set of data and previously hypothesised by Galko and co-workers when examining the post-embryonic healing responses (GALKO and KRASNOW 2004).

Following an infection, AMP gene expression is mainly concentrated in the fat body. This is in correlation with previous reports (FERRANDON *et al.* 1998) and data presented in this study (Figure 3-14 and 3-16). The fat body is the main site for the

secretion of AMP in *Drosophila* and plays an analogous role to the mammalian liver during the acute phase response (LEMAITRE and HOFFMANN 2007). The large size and the position of this tissue in the body cavity ensure that peptides reach their effective concentrations and are able to be circulated. This was confirmed when observing the contributions of different body parts after an infection (Table 3-5). The fat body of the larvae contributed at least 70% of the total AMP expression after an infection. When observing infected non-ablated third instar larvae, the increased contribution from the haemolymph is expected to be a combination of AMPs secreted by the fat body as well as a minor contribution from the haemocytes. The observed decrease of AMP expression in the carcass is potentially due to the fact that the concentrations of AMP expressed by the fat body are increased by such a large factor that although the rest of the tissues still had the same concentration, they appeared reduced in comparison.

The majority of AMP expression in a normal non-infected larva is in the carcass (basically, the larva without haemolymph and fat body) as expected. The majority of tissues in this sample represent the first line of host defence for *Drosophila*. The surface epithelia that are in contact with external environment have the ability to express antimicrobial peptides (AMPs) (FERRANDON *et al.* 1998; TZOU *et al.* 2000). Surfaces associated with expression include epidermis, the reproductive system, the respiratory tract and the digestive tract. The gut lumen is a hostile environment for microbes due to its physical and physiological properties, because it secretes lysozymes. It is possible for the surfaces to express the AMPs constitutively or for expression to be induced upon an infection. The induction of a local innate immune expression has been shown by natural infection of larvae using the Gram-negative

pathogen, *Erwinia carotovora* (BASSET *et al.* 2000). What is interesting from these data is how the host differentiates between the levels of commensal bacteria present in the gut to a pathogenic infection.

The *drs*-GFP reporter gene used in this study has been shown to behave as endogenous *drosomycin*. It appears to reproduce faithfully the transcription pattern of the endogenous gene, both qualitatively and quantitatively. As previously reported and confirmed in this study, GFP was detected uniformly in all lobes of the fat body of animals that were infected 4-6 hours after injury (Figure 3-15) (FERRANDON *et al.* 1998). However, time-lapse analysis for the spatial localisation for AMP expression has not been observed in *Drosophila* previously. The localisation of the AMP in an infection would suggest the signalling processes that were necessary for the induction of the AMP. There were no temporal differences in the activation of the *drs*-GFP reporter gene for all genotypes observed (Figure 3-13, 3-18, 3-22 and 3-28). The random foci of initiation followed by what appeared to be random diffusion of the reporter gene (Figure 3-14) and the restriction of the reporter gene in ablated larvae (Figure 3-18) suggested that fat body cells were being relayed a signal from the circulating haemolymph. The reduction in the quantitative expression of AMPs and the restriction on the spatial diffusion in haemocytes ablated larvae indicate the complex interplay that exists in the integrated systemic response.

Thus, haemocytes may control both the spatial expression as well as the intensity of AMP induction. Following on with the idea that *Drosophila* and vertebrates have similar immune responses and phagocytic defence mechanisms, the idea that cytokines could behave in a manner similar to vertebrates in *Drosophila* was brought

forward (AGAISSE *et al.* 2003). In vertebrates, released cytokines activate cognate receptors and mobilise two major signalling pathways, NFκB and JAK/STAT. Although both pathways are recruited via different signals, their co-operativity is essential for the integrity of this response and they converge at the level of transcription to induce expression of target genes. The JAK/STAT pathway was originally discovered as a cytokine induced signalling pathway required for myeloid and lymphoid cell lineages as well as being widely implicated in mammalian immunity (ARBOUZOVA and ZEIDLER 2006). In mammals, a major early step in acute phase response is the local release of signalling molecules, such as cytokines by activated macrophages, fibroblasts or endothelial cells at the site of damage. The cytokine IL6 has been identified as a major inducer, produced at the site of damage in response to primary cytokine such as IL1 or TNF in response to bacterial cell wall compounds such as LPS. IL6 triggers JAK/STAT through activation of cytokine class 1 receptor gp130 and JAK kinase. The process leads to the translocation of STAT dimers to the nucleus where they activate transcription of target genes encoding acute phase proteins such as C-reactive protein and LPS binding protein. The expression of mammalian acute phase proteins is modulated by the activation of specific signalling pathways (*e.g.* JAK/STAT) in response to systemic release of cytokines (*e.g.* IL6) by activated macrophages (BAUMANN and GAULDIE 1994). In *Drosophila*, the canonical pathway was discovered through its role in embryonic segmentation and consists of the ligand (*unpaired*), the receptor (*domeless*), the JAK (*hopscotch*) and the STAT (*marelle/STAT92E*). The role of this pathway in insect immunity was confirmed when *Anopheles* STAT was found translocated to the nucleus after an infection and the same observation confirmed an identical response in *Drosophila* (AGAISSE *et al.* 2003; BARILLAS-MURY *et al.* 1999). Gain of function

of STAT in *Drosophila* has been implicated in the transcriptional control of TEP (a target gene with putative immune function) (LAGUEUX *et al.* 2000). The signalling involves the cytokine *upd*, a secreted protein with no vertebrate homologues, a signal sequence and several potential N-linked glycosylation sites. *Upd3* is part of the same locus and was found to be upregulated in haemocytes after an infection and required for the production of TotA (a peptide related to stress response) (AGAISSE *et al.* 2003). The observation that transcriptional silencing of *upd3* in haemocytes quantitatively decreases the AMP response (Figure 3-20) and inhibits the diffusion of *drs*-GFP reporter gene (Figure 3-22), confirms a role for this cytokine as one of the signals relayed by the haemocytes to the fat body.

The JNK pathway is another signalling cascade which is induced following immune stimulation (BOUTROS *et al.* 2002; SLUSS *et al.* 1996). The JNK and NFκB pathways share a close relationship in *Drosophila*. *Puckered* (*puc*) is induced by JNK signalling and in turn terminates the cascade by a negative feedback loop. Overexpression of *puc* in the fat body reduced production of AMP by both pathways to about 10% of the wild type response. This indicated that JNK was required in the fat body for normal expression of AMP (DELANEY *et al.* 2006). The results presented here show that transcriptional reduction of the JAK/STAT cytokine (*upd3*) in the haemocytes directly impaired expression of the JNK signalling pathway (Figure 3-25).

In addition to immune challenge, wounding also activates the JNK pathway. It stimulates movement of haemocytes towards the wound, beginning cytoskeletal rearrangements and the formation of a melanotic plug (RAMET *et al.* 2002a).

Cytoskeletal gene expression during innate immune responses is controlled by JNK through the same upstream signalling cascade that activates the NFκB pathway hinting that the same pathway could potentially serve a dual purpose (GALKO and KRASNOW 2004; RAMET *et al.* 2002a). Temporal activation of *puc* observed (Figure 3-24) in the fat body of infected third instar larva corresponds to previous observation of protein accumulation in the fat body of adults via local cell to cell signalling seen in wound healing. However, caution must be exercised when analysing the activation with the lacZ line used here since β-galactosidase persists in the nuclei for an extended period of time (REICHHART *et al.* 1992).

The *Drosophila* JAK/STAT pathway also affects the MAPK pathway. D-Raf, the *Drosophila* homologue of human Raf protein, contains canonical STAT binding sites at its promoter that are absolutely required for activation by Hop (the kinase for the JAK/STAT pathway) after an immune challenge. The expression of D-Raf is localised in the larval fat body after an infection (KWON *et al.* 2000).

The morphological changes observed in the fat body (Figure 3-25) when *upd3* was knocked down in haemocytes in combination with the reduction in the spatial expression of AMPs and the role in JNK activation underlie the complex and multifunctional role of *upd3* in fat body physiology. It appears that the *upd3* plays a multitude of roles in the immune response of *Drosophila.*

The cytokine Spz is the ligand for the Toll pathway, involved in mediating the Gram-positive and anti-fungal responses. The ligand is cleaved by SPE (Spz processing enzyme) from the pro-protein to an active carboxyl terminal domain containing 106

amino acid residues (C106) that dimerises and proceeds to bind to the Toll receptor (JANG *et al.* 2006; WANG *et al.* 2006; WEBER *et al.* 2003). The cytokine has been found to be enriched in transcripts of *Drosophila* haemocytes after an infection, proposing it to be the signal mediated by haemocytes to the fat body for the Toll pathway (IRVING *et al.* 2005). The transcriptional reduction in haemocytes resulted in a reduction of AMP expression, confirming involvement in the Toll pathway however a spatial aberrance was not detected. It is entirely possible that control for this pathway lies with SPE instead of Spz. The use of SPE instead of Easter as the terminal SP for immune induction shows that different mechanisms are required for immune induction and development (MULINARI *et al.* 2006). At larval stages, SPE is highly expressed in the fat body and mature haemocytes. After an infection, the cleavage of SPE in the haemolymph activates Toll and upregulates transcription of itself to double about 3.5 hours after infection. The amplification response by a proteolytic cascade using the same pathway may represent energy conservation in the insect or unique divergence from its original role.

In higher vertebrates, innate immunity is an integral part of the host defence system that triggers the expression of co-stimulatory molecules initiating adaptive immunity. The activation of macrophages initiate an acute phase response, characterised by the release of inflammatory cytokines, IL1, IL8 IL6, TNFα, chemotactic factors and other cytokines such as CSF-1, G-CSF, GM-CSF that help recruit and activate immune cells (BAUMANN and GAULDIE 1994). The possibility that phagocytic degradation of microbes is required for the production of immunostimulatory signal(s) from the blood cells to the fat body, recently described by phagocytic mutants, *psidin* and *eater* (BRENNAN *et al.* 2007; KOCKS *et al.* 2005) is new to

Drosophila innate immunity. Their results indicate that defects in phagocytosis play a central role in activating the systemic immune response. This correlates with our results where absence of the blood cells and therefore reduced phagocytosis leads to an overall reduction in the systemic response mounted by the fat body (Figure 3-11). From here, it is possible to present a model that is remarkably similar to the mammalian system (Figure 4-1). In the event of an injury of using a sharp needle dipped with pathogen, the wounding triggers clot formation and epithelial regeneration (GALKO and KRASNOW 2004). The bacteria enter the open body cavity and are circulated in the free flowing system by the dorsal vessel where it is possible that the activity of an as yet unknown opsonin will begin the phagocytic process (HULTMARK and BORGE-RENBERG 2007). The phagocytic by-products produce minimal sub units that are required for pathogen recognition via PRGPs and/or GNBPs. The receptors involved in the specific sensing of the minimal subunits for a Gram-positive infection are found circulating (PGRP-SA, GNBP1, PGRP-SD) and will activate the Toll pathway. Haemocytes are an important source of at least one circulating pattern recognition receptor namely, PGRP-SA (IRVING *et al.* 2005). Sensing by a complex of PGRP-SA/GNBP1 or PGRP-SA/GNBP1/PGRP-SD (WANG *et al.* 2006) could be signalled to haemocytes and this event may in turn release Spz in the haemolymph. Production of circulating Spz would then trigger Toll activation in the fat body. Signalling via these receptors could be in parallel with or upstream of the cytokine components. The recognition of an invasive molecule will trigger other signalling pathways already known to be involved. The results indicate a cross talk between the cellular and humoral signalling pathways, in concordance with other results that show the immune response not as a single linear pathway but a concerted effort from the whole animal. During this time, the bacteria will continue to

proliferate in the body cavity. The AMP secreted from the fat body to the haemolymph will lyse the bacteria to complement the effort of phagocytosing haemocytes trying to clear the infection. The multifaceted roles of the haemocytes as professional macrophages bearing sentinel molecules are highlighted in this elegant system.

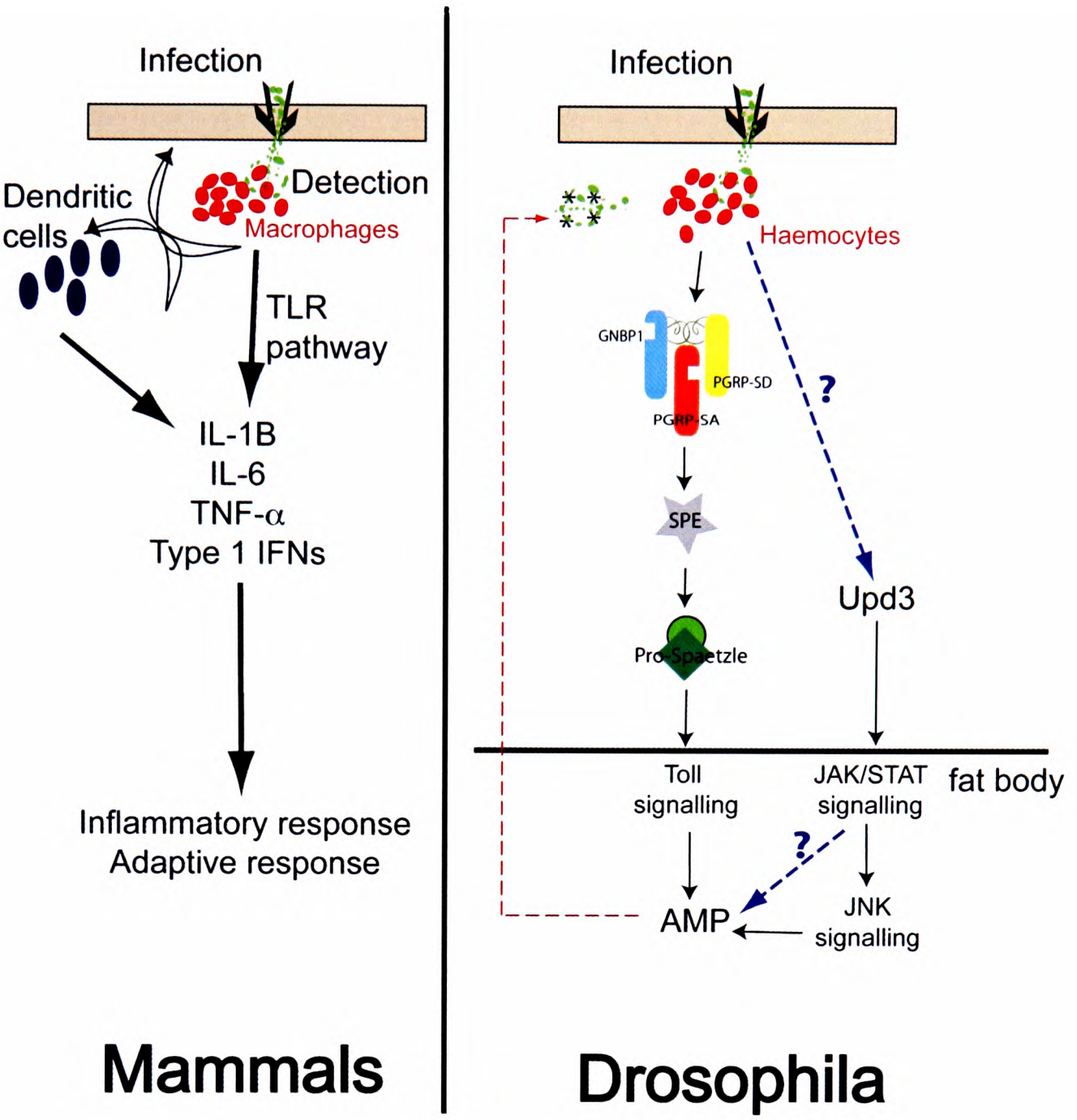


Figure 4-1 Comparison between mammalian and insect systems of cytokine release following detection of pathogens through macrophages (mammals) or haemocytes (*Drosophila*). The *Drosophila* model is surmised from the results of this work and current publications, elaborated in the text. The role of JAK/STAT signalling in diffusion of the AMP is currently unknown. AMPs that are secreted from the fat body to the haemocel complement phagocytosing haemocytes by lysing bacteria.

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APPENDIX A

Standard recipes

Drosophila

Fly food

A 20g agar

750ml double distilled water

125ml golden syrup

B 275ml double distilled water

125g wholemeal flour

C 250ml double distilled water

12ml nipagin (Stock solution 25g dissolved in 250ml alcohol)

7ml propionic acid

Stir all A ingredients into a saucepan and bring to boil. Mix B ingredients to a fine paste, add to the saucepan and bring it to boil. Simmer the mixture for 5 minutes. Mix the C ingredients away from the flame. Add it to the saucepan, bring it to boil and pour into sterile vials or bottles. Add live baker's yeast to the top of the food before use.

Apple juice agar

6.6g agar

325ml double distilled water

125ml apple juice

6.25g sucrose

10ml nipagin stock solution

Autoclave agar with 250ml double distilled water. Make up the sucrose solution with 125ml on a hotplate and add apple juice. When the autoclaved agar is cool enough, add the sucrose-apple juice solution and nipagin solution. Pour into sterile plates or 24-well tissue culture dishes.

Phosphate buffered saline (PBS)

10X stock solution

14.5g $NaH_2PO_{4.2}H2O$

57.5g Na_2HPO_4

80g NaCl

Dissolve all chemicals in 800ml of double distilled water, adjust to pH 7.4 and make up to 1litre using double distilled water.

Microbiology

Luria-Bertani broth (LB)

10g bacto-tryptone

5g bacto-yeast extract

10g NaCl

Dissolve all chemicals in 800ml of double distilled water, adjust to pH 7.0 and make up to 1litre using double distilled water. Add 1.5g agar if making medium. Autoclave before use.

GYT media

10% v/v glycerol

0.125% w/v yeast extract

0.25% w/v tryptone extract

Mix and filter sterilise. Store at 4°C until use.

Ampicillin stock solution

10mg ampicillin was dissolved in 10ml of double distilled water. Stock solution was filter sterilised and stored at -20°C until use.

Northern blot solutions

3M NaAc

Dissolve 24.61g of NaAc in 100ml double distilled water. Autoclave to sterilise.

20X SSC

175.3g NaCl

88.2g $Na_3C_6H_5O_7$ (sodium citrate)

Dissolve into 1litre of double distilled water

10X MOPS

0.2M MOPS (pH7.0)

20mM NaAc

10mM EDTA

41.8g MOPS in 700ml DEPC treated water, adjust to pH 7.0. Add 20ml 1M NaAc and 20ml 0.5M EDTA

Make up to 1 litre with DEPC treated water.

Formaldehyde gel

3g agarose (RNAse free)

30ml 10X MOPS

216ml sterile milliQ water

54ml paraformaldehyde

Dissolve the agarose with MOPS and water in a microwave and cool the gel down to 50°C in a water bath. Add the paraformaldehyde and cast.

Blocking solution

6ml 20X SSC

2ml 50X Denhardt solution

0.2ml 10% SDS

10ml formamide

0.2ml salmon sperm DNA

Denature the salmon sperm DNA solution at 95°C in a heating block for 5 minutes and cool in ice for 5 minutes. Mix all the solutions together into a hybridisation tube.

10% SDS stock solution

Dissolve 10.0g SDS in 90ml of water with gentle stirring.

Adjust to 100ml with deionised water.

Molecular biology

Squish buffer

10mM Tris-Cl (pH 8.2)

1mM EDTA

25mM NaCl

10µl of Proteinase K stock solution (14mg/ml) for every 500ml of squish buffer.

Polymerase chain reaction

36.75µl sterile double distilled water

5µl 10X PCR buffer

1µl 10mM dNTP stock solution

1µl 50mM forward and reverse primer

0.25µl 5U/µl Taq polymerase

Recipe for a standard 50µl reaction

Tris buffer (TE)

10mM Tris-HCl (pH 8.0)

1mM EDTA (pH 8.0)

10X TBE

108g Tris

55g boric acid

4ml 0.5M EDTA (pH 8.0)

Make up to 1 litre using double distilled water.

LacZ staining solutions

Stock solutions

1M $NaHPO_4$, pH 7.4, autoclaved and stored at room temperature

10X PBS, autoclaved and stored at room temperature

1M $MgCl_2$, sterile filtered, store at 4°C

2% NP-40, sterile filtered, store at 4°C

1% sodium deoxycholate, sterile filtered, store at 4°C

250mM EGTA, sterile filtered, store at room 4°C

500mM potassium ferricyanide, sterile filtered, store at room temperature in the dark

500mM potassium ferrocyanide, sterile filtered, store at room temperature in the dark

40mg/ml X-Gal in dimethylformamide, store at -20°C in the dark

4% formaldehyde (methanol free), store at room temperature

Staining solutions

PBS+2mM $MgCl_2$

449ml milliQ H_2O

50ml 10X PBS

1ml 1M $MgCl_2$

Mix and store at room temperature

LacZ fix buffer

175ml milliQ H_2O

20ml 1M $NaHPO_4$, pH7.4

4ml 250mM EGTA

1.6ml 25% glutaraldehyde

0.4ml 1M $MgCl_2$

Mix well and store at 4°C for 2 months

LacZ wash buffer

440ml milliQ H_2O

50ml 1M $NaHPO_4$, pH 7.4

5ml 2% NP-40

5ml 1% sodium deoxycholate

1ml 1M $MgCl_2$

Mix and store at room temperature

LacZ staining solution (made fresh on the day)

8.2ml milliQ H_2O

1ml 1M $NaHPO_4$

0.1ml 500mM potassium ferrocyanide

0.1ml 500mM potassium ferricyanide

0.1ml 2% NP-40

0.1ml 1% sodium deoxycholate

0.02ml 1M $MgCl_2$

0.4ml 40mg/ml X-Gal

Mix well and keep away from direct light.

APPENDIX B

Insert containing CD for supplementary movie 1 and 2. Movies are in QuickTime (Apple) format. To play the movie, the free movie player can be downloaded from www.apple.com/quicktime/download. Movies are shown in real-time as viewed for live animals.

Accompanying material
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