

https://dspace.org/

Stress response to genotoxic agents and to infection

Hull, Rodney

2012-10-08
http://hdl.handle.net/10539/12067

Downloaded from a DSpace repository. https://dspace.org/

Stress response to genotoxic agents and to infection

By

Rodney Hull

9808976y

A thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.

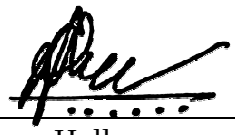
Johannesburg, 2012

A child of five would understand this. Send someone to fetch a child of five.

-Groucho Marx

Declaration

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



Rodney Hull

11th Day of May 2012.

Abstract

Insects have evolved various physiological responses to cope with stressors such as pathogens, toxins and environmental factors. It is known that the responses resulting from infection or DNA damage share some of the same pathways. Exposure of *Drosophila melanogaster* and the dung beetle *Euoniticellus intermedius* to stress led to changes in the expression of proteins involved in metabolism, development, protein degradation, mRNA processing and stress responses. Stress responses in *D. melanogaster* are well characterised. However, the role played by *Drosophila* p53 (Dmp53) and a member of the retinoblastoma binding protein 6 (RBBP6) family, Snama, are unknown. Snama has been proposed to play a role in Dmp53 regulation. Following DNA damage we investigated the role of Dmp53 and Snama. Flies recovering from camptothecin treatment display a glycolytic flux, involving a metabolic shift, different to that observed in cancer cells. Camptothecin treatment leads to an increase in the mortality of both sexes. Furthermore, females show a specific decrease in fecundity which is due to an increase in Dmp53 dependent apoptosis in the ovaries and is accompanied by a depletion of *Snama* and an increase in *Dmp53* transcripts. Expression data indicated that Dmp53 activity may be largely regulated at the protein level. Bypassing glycolysis through methyl pyruvate supplementation led to differential expression of Dmp53 and Snama and improved reproduction and embryonic development. These results highlight differences between the metabolic strategies used by cancerous and non-cancerous cells which may be exploited in future chemotherapies. While immune responses amongst insect orders are evolutionarily conserved, many remain uncharacterised. To investigate the immune system of an organism that lives in a microbe rich environment, *E. intermedius* was infected with the fungal pathogen *Beauveria bassiana.* This resulted in decreased lifespan and fecundity. Homologs of proteins involved in the immune response of insects were identified in *E. intermedius*, including a member of the Toll family of proteins, an insect defensin (present in the hemolymph) as well as a

homolog of the serine protease Persephone. These results show that immune signalling pathways are conserved in this dung beetle.

Acknowledgements

I would like to thank my supervisor, Dr Monde Ntwasa for all his support guidance, encouragement, and enthusiasm throughout my studies. This thesis would not have been possible without my parents. I would like to thank them for their support (both financially and otherwise) and encouragement. I would like to thank Miss Shüné Oliver for her companionship, support, academic advice, proofreading and late night suppers over the many years. I would also like to thank Dr Marcus Byrne for his help and advice regarding the dung beetles.

I would like to thank my lab mates past and present for their assistance. Special mention must be made of Dr Arshad Mather, Mr Mpho Rakgotho and Dr Tshepo Seekoe for "showing me the ropes"; to Brent Oosthuysen for the late night entertainment and to Umar Faruq Cajee and his wife for their assistance (the international bank of Umar).

A special thank you must be made to beer, coffee and my long suffering blue golf (FDH242 GP) without them this would have been a lot more difficult.

A final thank you must go to my friends, Kenneth Beecroft and Dean Higgins for their IT support; to Michael Shepperson for filling the passenger seat and to Roald Ellis for his amusing antics.

I would like to not thank The South African National Research Foundation.

Table of contents

Declaration			I
Abstract			II
Acknowledgements			IV
List of Figures			XI
List of Tables			XIII
List of Abbreviations			XIII
Chapter 1: Introduction			
1.1.1	Stress signalling pathways		3
	1.1.2	MAPK cascades	3
	1.1.3	Eiger	4
1.2	Cell cycle arrest: DNA Damage and Cell Cycle control		6
	1.2.1	Sensing DNA damage	6
	1.2.2	ATM/ATR/Chk2/Chk1	6
	1.2.3.	Cyclin dependent kinase (CDK) control	7
1.3	Apoptosis in *Drosophila*		7
	1.3.1	H99 deficiency genes	8
	1.3.2	IAPs	10
	1.3.3	The role of Caspases in apoptosis	11
	1.3.4	Apoptosomes	12
1.4.	Protein regulation and transcriptional response to stress: p53		14
	1.4.1	DNA binding domain	16
	1.4.2	Post-translational modifications of p53	16
	1.4.3	Regulation	17
	1.4.4	Mdm2.	23
	1.4.5	Functions of p53	24
		1.4.5.1 Cell death	24
		1.4.5.2 Transcriptional repression	26
		1.4.5.3 DNA repair	27
		1.4.5.4 Cell cycle arrest	29
		1.4.5.5 Metabolic role of p53	30
	1.4.6.	p53 ancestry	32
	1.4.7	Proteins that interact with p53	33
		1.4.7.1 E2F-1/Retinoblastoma protein	33
		1.4.7.2 Snama (RBBP6)	36
1.5	The transcriptional response to stress: Infection and immune response		38
	1.5.1	A brief summary of the insects immune system	40
		1.5.1.2 Cellular response	40
		1.5.1.3 Recognition	42
		1.5.1.3.1 PAMPS	42
	1.5.2	Signalling pathways	46
		1.5.2.1 Toll signalling pathway	46
		1.5.2.2. Imd pathway	50
		1.5.3 Anti-microbial peptides	53
		1.5.4 The cost of immunity	54
1.6	Integrated Stress response		55

1.7 *Euoniticellus intermedius* 56
1.8. *Drosophila melanogaster*. 65
1.9. Aims of this study 65
Chapter 2: Material and Methods 67
2.1 Materials 68
2.1.1 Media 68
2.1.2 Buffers and solutions 69
2.1.3 Stains 71
2.1.4 Living organisms 72
2.1.5 Primers 72
2.1.6 Antibodies 73
2.2 Methods 74
2.2.1 Organism maintenance and challenging 74
2.2.1.1 Beetle maintenance 74
2.2.1.2 Fly maintenance 74
2.2.1.3 Pathogens and Toxins 75
2.2.1.3.1 Bacterial pathogens 75
2.2.1.3.2 Fungal pathogens 75
2.2.1.3.3 Beetle infection 76
2.2.1.3.4 Camptothecin and methyl pyruvate exposure of flies 76
2.2.1.4 Mating of flies exposed to camptothecin 77
2.2.1.5 Validation of *dmp53* double mutants 78
2.2.1.6 Mortality assays 79
2.2.2 Genomic DNA preparation 79
2.2.3 Topoisomerase I assay 79
2.2.3.1 Preparation of cytoplasmic and nuclear protein extracts from *Drosophila* embryos. 79
2.2.3.2 Assay for topoisomerase I activity 80
2.2.4 Acridine orange staining 80
2.2.5 Protein extraction 81
2.2.5.2 Collection and preparation of hemolymph samples 82
2.2.6 Protein purification 82
2.2.6.1 High Salt Cation exchange column 82
2.2.6.2 C18 Sep pak vac column 83
2.2.6.3 Cleaning and concentration of samples 84
2.2.6.4 Molecular weight cut off purification 84
2.2.6.5 Reverse phase HPLC 84
2.2.6.6 Peptide concentration 86
2.2.7 SDS-PAGE analysis 86
2.2.7.1 Size estimation and banding pattern 86
2.2.7.2 Tris Tricine SDS-PAGE 86
2.2.8 Western blotting 88
2.2.9 TCA precipitation 89
2.2.10 Two-dimensional gel electrophoresis and Mass spectrophotometry 89
2.2.10.1 Two-dimensional gel electrophoresis 89
2.2.10.2 Image Analysis 90
2.2.10.3 MALDI-TOF Mass Spectrometry 91

		2.2.10.4 Statistical and Bioinformatic analysis of protein sequencing data	92
	2.2.11	GST assay	93
	2.2.12	Inhibition assays	94
		2.2.12.1 Solid and liquid media inhibition assays	94
		2.2.12.2 Radial diffusion assays	95
	2.2.13	Chemical characterization of inhibitory sample	95
		2.2.13.1. Proteinase K assay	96
		2.2.13.2. Heat stability of inhibitory peptide	97
	2.2.14	RNA isolation from flies and beetles	98
	2.2.15	Northern blots	98
		2.2.15.1 Preparation of DNA probes	99
		2.2.15.2 Labelling of DNA probes with [α 32P] dCTP	99
		2.2.15.3 Hybridisation, detection and stripping blots	100
	2.2.16	Design of degenerate primers and RT-PCR	100
	2.2.17	General Molecular Biology techniques	102
		2.2.17.1 Competent cells	102
		2.2.17.2 Ligations	102
		2.2.17.3 Transformation	102
		2.2.17.4 Colony Screening	103
		2.2.17.5 Colony PCR, sequencing of DNA and construction of phylogenetic trees	103
	2.2.18	Quantitative PCR	104
		2.2.18.1 RT –PCR to confirm RNA levels following DNA damage	104
		2.2.18.2 Internal standard	105
		2.2.18.3 qPCR	105
Chapter 3. Results: The response of *Drosophila melanogaster* to DNA damage.			107
3.1	Introduction		108
3.2	The physiological effects of camptothecin exposure		111
	3.2.1	Camptothecin exposure results in a decrease in the reproductive ability of female *Drosophila melanogaster*	112
	3.2.2	Staurosporine exposure has no effect on the female's fecundity	116
	3.2.3	Camptothecin decreases the survival probability of male and female flies	118
	3.2.4	Higher levels of apoptosis occur in embryos produced by flies that have been fed camptothecin	120
3.3	TopoisomeraseI assays were inconclusive		128
3.4	Proteomic analysis of *Drosophila melanogaster* following camptothecin exposure		130
	3.4.1	Camptothecin exposure results in a glycolytic flux	130
	3.4.2	Camptothecin exposure results in an increase in antioxidant and detoxification activity as well as cytoskeletal changes.	132

	3.4.3	Some proteins have multiple identities using Mascot	137
	3.4.4	Changes in optical densities mirror changes in the enzyme activity of Glutathione S transferase	137
3.5	Methyl pyruvate supplementation		141
	3.5.1	Methyl pyruvate supplementation failed to significantly improve the reproductive ability of the female flies when both sexes were exposed to camptothecin	141
	3.5.2	Methyl pyruvate is able to partially increase the viability of embryos produced by flies exposed to camptothecin	144
3.6	Effects of camptothecin exposure on *Snama, Dmp53* and *Reaper* mRNA levels.		146
	3.6.1	Significant changes in the transcription of *Snama* and *dmp53* following camptothecin exposure could not be determined using northern blots.	147
	3.6.2	The exponential production of *Rp49* from a cDNA template occurs at approximately 32 cycles	149
	3.6.3	*Reaper* transcription patterns do not match that of *Dmp53*	152
	3.6.4	Methyl pyruvate alters the transcription of *Dmp53* and *Snama*	155
	3.6.5	*Snama* and *Dmp53* demonstrate an inverse transcriptional relationship following stress exposure.	157
	3.6.6.	Levels of *Dmp53* transcription do not change in larvae treated with camptothecin, while *Snama* and *Reaper* transcription is highest in untreated larvae.	158
	3.6.7.	Real Time PCR results confirm those of the semi-quantitative RT-PCR	160
3.7.	Changes in the expression pattern of Dmp53, Snama following camptothecin exposure		162
	3.7.1	Anti-Dmp53 detects two band of the correct size	163
	3.7.2	Anti-Snama detects 3 different bands, none of which are the correct size.	167
3.8. Functional role of Dmp53 following camptothecin treatment.			167
	3.8.1	Camptothecin exposure has no adverse effects on the fecundity or development of p53 null mutant flies	168
3.9	Discussion		169
	3.9.1.	DNA damage has negative effects on fecundity, lifespan and the viability of *D. melanogaster* offspring.	169
	3.9.2	The Stress response of normal cells to DNA damage by camptothecin results in a glycolytic flux.	175
	3.9.3	The link between Dmp53, metabolism and	179

		apoptosis.	
	3.9.4	The effects of bypassing glycolysis.	184
	3.9.5	Other cellular changes that occur as a result of DNA damage.	185
Chapter 4: The identification and characterization of Antimicrobial peptides in *Euoniticellus intermedius*			189
4.1	Introduction		190
4.2	The effects of infection and the cost of the immune response		191
	4.2.1	HPLC elution profiles and SDS-PAGE gels show changes in protein expression patterns following bacterial infection	193
	4.2.2	Mortality assays show increased mortality rates and lower survival probabilities	199
	4.2.3	Fungal exposure lowers beetle fitness	201
4.3	Antimicrobial activity		201
	4.3.1	Solid media inhibitions indicated the presence of an antimicrobial substance in cationic hydrophobic samples	203
	4.3.2	The Antimicrobial agent is a heat stable protein	206
	4.3.3	Radial diffusion assays were adopted due to their increased sensitivity and reproducibility	209
	4.3.4	Wavelength absorption maxima were used to identify peaks of interest	216
	4.3.5	Only one of the isolated peaks shows any antimicrobial activity	219
4.4	Changes in overall protein expression following infection in *E. intermedius*		221
	4.4.1	Small peptides are present in samples that inhibit bacterial growth	221
	4.4.2	Common proteins are found in both the 40-60 and 60-80% acetonitrile fractions.	222
	4.4.3	2D gel electrophoresis analysis of differently infected and purified samples revealed changes in protein expression patterns.	225
	4.4.4	Tandem Mass spectrometry and bio-informatic analysis identified partial protein sequences of proteins that may be involved in immunity	227
	4.4.5	Peptide fragments may represent proteins that modulate protein stability	229
	4.4.6	Many of the peptide sequences share identity with sensory proteins	230
	4.4.7	Many of the peptide fragments share sequence similarity with proteins that play a role in insect development	230
	4.4.8	Fragments which share sequence identity with proteins that have an immune function are both up and down- regulated	231
	4.4.9	Only 5.5 percent of the peptide fragments share identity with Stress response proteins.	232
	4.4.10	Fragments were identified that were Cytoskeletal	232

		proteins or played a role in cytoskeletal modification	
	4.4.11	Treatment of beetles with fungus increased the expression of many proteins that share sequence identity with proteins involved in metabolic pathways.	233
4.5	Identification and characterization of Immune related genes		234
	4.5.1	The identification and cloning of a serine protease.	234
	4.5.2	The serine protease (5310) appears as two transcripts one of which is down-regulated following infection	236
	4.5.3	The Serpin probe detects only two of eleven expected transcripts, and transcript levels seem to be independent of sex or infection status	240
4.6	Discussion		240
	4.6.1	Antimicrobial activity is present in the hemolymph as a heat stable, positively charged, hydrophobic protein.	243
	4.6.2	Fungal infection of *E. intermedius* results in the induction of defence as well as stress response proteins.	244
	4.6.3	Other protein expression changes that accompany infection	247
Chapter 5: Discussion			253
5.1	DNA damage in *Drosophila melanogaster*		254
5.2	The immune response of *E. intermedius*		258
5.3	Stress responses common to both DNA damage and infection		259
5.4	Future Studies		262
Chapter 6: Appendix			264
6.1	Vectors		265
6.2	E. intermedius gene sequences		267
6.3	Drosophila melanogaster sequences		268
6.4	Markers		273
Chapter 7: References			279

List of figures

Figure 1.1	MAPK and Insulin signalling pathways in *Drosophila melanogaster*	5
Figure 1.2	Apoptosis pathways in *Drosophila*.	13
Figure 1.3	The different domain structures, regulation and functions of Dmp53 and human p53.	18
Figure 1.4	Human p53 structures	21
Figure 1.5	The signalling pathways of the *Drosophila melanogaster* orthologue of p53.	28
Figure 1.6	Homology amongst members of the p53 family.	34
Figure 1.7	Insect cellular pathogen recognition pathways.	47
Figure 1.8	Integration of the Stress Response pathways	58
Figure 1.9	Phylogenetic trees showing relative location of *E. intermedius* to other arthropod model organisms.	62
Figure 1.10	External Morphology of *E. intermedius*	63
Figure 1.11	Life cycle of *E. intermedius*.	64
Figure 2.1	Acetonitrile gradient for Reverse Phase HPLC.	85
Figure 2.2	Standard curve for protein concentration determination using the Bradford assay.	87
Figure 3.1	The influence of camptothecin on *Drosophila* fecundity and development times.	114
Figure 3.2:	The effect of the protein kinase C inhibitor, Staurosporine on the ability of female flies to produce embryos.	117
Figure 3.3:	The Mortality rate of untreated flies compared to those exposed to camptothecin	119
Figure 3.4	Acridine orange staining of 0-3 hour embryos to detect apoptosis	122
Figure 3.5	Acridine orange staining of 3-6 hour embryos to detect apoptosis	123
Figure 3.6	Acridine orange staining of 6-9 hour embryos to detect apoptosis.	125
Figure 3.7	Proportions of embryos displaying normal or abnormal patterns of apoptosis.	127
Figure 3.8	Topoisomerase relaxation assay.	129
Figure 3.9	2D gel electrophoresis of *D. melanogaster* crude protein extract before and after exposure to camptothecin.	131
Figure 3.10	The optical densities of the proteins isolated and identified using 2D PAGE and mass spectroscopy	134
Figure 3.11	GST enzyme assay for camptothecin and untreated fly extracts to confirm optical density changes do reflect changes in protein amount	140
Figure 3.12	The effect of methyl pyruvate on embryo numbers	143
Figure 3.13	The ability of methyl pyruvate to rescue the camptothecin phenotype	145
Figure 3.14	The effects of camptothecin and DMSO treatment on the transcription of *dmp53* and *snama* in *Drosophila*.	148
Figure 3.15	Determination of the exponential phase of RT-PCR amplification of the internal control *rp49*.	151
Figure 3.16	The semi-quantitative Reverse Transcriptase-PCR analysis of the level of RNA transcripts present for *reaper*, *dmp53*, *snama* and the control *rp49*.	153
Figure 3.17	The semi-quantitaive Reverse Transcriptase-PCR analysis of the level of RNA transcript levels of *snama*, *reaper* and *dmp53* in larvae following camptothecin and methyl pyruvate exposure of parental	159

flies.
Figure 3.18 Real time quantification of *snama* and *dmp53* transcripts in flies that have been left untreated, treated with camptothecin and after Camptothecin treatment. 161
Figure 3.19 Western blot analysis of *Drosophila* melanogaster treated with camptothecin, methyl pyruvate and both camptothecin and methyl pyruvate 164
Figure 3.20 The effect of camptothecin on *dmp53* null mutant flies. 170
Figure 3.21 Confirmation of the genotype of y1 w118 p53 5A-1-4 mutants through PCR. 171
Figure 4.1 HPLC elution profiles showing changes in peptide levels between challenged and unchallenged beetles 195
Figure 4.2 Inhibition assays: Photographs of solid media inhibition assays performed against Microoccus luteus. 196
Figure 4.3 Expression patterns on Tris Tricine PAGE gels as an indicator of peptide expression pattern change after immuno-challenge. 198
Figure 4.4 Mortality Assays. A Kaplan-Meier product limit estimate of the survival functions of each individual beetle grouped according to treatment was performed at a 95% confidence interval. 200
Figure 4.5 The effect of fungal treatment on broodball production 202
Figure 4.6 Activity of extractions against *M. luteus* recorded by solid media inhibition assays. 205
Figure 4.7 Identification of the nature of the inhibitory factor. 207
Figure 4.8 Liquid inhibition assays demonstrate that the antimicrobial agent is a heat stable protein. 208
Figure 4.9 Examples of radial diffusion assays 211
Figure 4.10 Radial diffusion assays with varying concentrations of an Ampicillin control. 212
Figure 4.11 The inhibitory effectiveness of different peptide samples was established based on, the diameters of the clearings they produced in radial diffusion assays. 214
Figure 4.12 Wavelength absorption maxima correlated with the percentage of certain amino acids in coleopteran antimicrobial peptides 217
Figure 4.13 Inhibition of M. luteus growth with HPLC purified samples. 220
Figure 4.14 Tris Tricine SDS Gel patterns of Inhibitory peptide samples 223
Figure 4.15 2D PAGE analysis of hydrophobic antibacterial samples. 224
Figure 4.16 2D gel electrophoresis of *E. intermedius* hemolymph proteins
Figure 4.17 Relative quantity of proteins in fungal infected and uninfected hemolymph samples as determined by 2D PAGE. 228
Figure 4.18 RT-PCR amplification of target mRNA using primers designed using peptide sequences: 235
Figure 4.19 Alignment of the 5310 translated amino acid sequence with serine proteases amino acid sequences from insects across different insect orders. 237
Figure 4.20 Northern blot analysis of the transcript levels of the serine protease 5310, following fungal challenge. 241
Figure 5.1 Comparison between the proteomic response of *D. melanogaster* to DNA damage and *E. intermedius* to infection. 260
Figure 6.1 pGEM-T-Easy Cloning vector (Promega cat# A1360) 265

Figure 6.2 The pJM 954 vector containing the rp49 gene 266
Figure 6.3 DNA and RNA markers 273
Figure 6.4 Protein markers 274

List of Tables

Table 2.1 Amplification parameters of the second step RT-PCR reaction for different primers 101
Table 3.1 The identification of up or downregulated proteins using MALDI-TOF MS 138
Table 4.1: List of coleopteran families, sub families and tribes where an analysis of the coleopteran immune system has been carried out 192
Table 4.2 The spectrum of antimicrobial activity of protein extracts from fungal challenged beetles 215
Table 4.3 Characteristics of antimicrobial peptides isolated form coleoptera 248

Table 6.1 Identification of peptide fragments based on sequence similarity with proteins in different databases 275

List of Abbreviations

(Gadd) 45	Growth Arrest DNA Damage
Apaf-1	apoptotic protease-activating factor 1
ARF-BP1/Mule	*ARF-b*inding *p*rotein *1*/*M*cl-1 *u*biquitin *l*igase *E*3
Ask1	apoptosis signal-regulating kinase 1
ATM	Ataxia-telangiectasia mutated
ATR	Ataxia-telangiectasia and RAD3 related
CARD	caspase recruitment domains
CDC25	Cell Division Cycle 25
CDKs	cyklin dependent kinases
CTLs	C Type lectins
dap	*dacapo*
DARK	*Drosophila* APaf-1 related killer
DEAF1	Deformed Epidermal autoregulatory factor
DED	death effector domains
dFADD	Fas (TNFRSF6)-associated via death domain
DID	death-inducing domains
Dif	Dorsal related immune factor
dpp	decapentaplegic
FBX33	F-box protein 33
FOXO	Forkhead Box containing protein
fzr	*fizzy-related*
GNBPB	gram negative binding protein
HAUSP	herpesvirus-associated ubiquitin-specific protease
hect	homologous to E6-APC-terminus
hep	Hemipterous

HSF	Heat shock transcription factors
Hsp	Heat Shock Proteins
HUS1	hemolytic-uremic syndrome
IAP	inhibitor of apoptosis
IKK	IkappaB kinase
ILPs	Insulin Like Peptides
Imd	Immune deficiency
INR	Insulin receptor
IP3	Inositol triphosphate
IPCs	Insulin Producing Cells
IRAK	IL-1 receptor-associated kinase
Jak	Janus kinases
JNK	c-Jun N-terminal kinase
LGR	Leucine Rich Repeat G protein
Mdm2	murine double minute 2
MDM4	murine double minute 4
MEKK4	mitogen-activated protein kinase kinase kinase 4
MKK7	mitogen-activated protein kinase kinase 7
Mnk	MAP kinase-interacting serine/threonine kinase
mre11	meiotic recombination 11
MTF	metal responsive transcription factors
MTN	Metalothioniens
Myt1	Myelin transcription factor 1
Nbs1	Nijmegen breakage syndrome 1
NES	nuclear export sequence
NLS	nuclear localization sequence
PAMPS	Pattern associated Molecular patterns
PCNA	Proliferative Cell Nuclear Antigen
PDGF	Platelet-derived growth factor
PDGF	Platelet-derived growth factor
PGRP	Peptidoglycan recognition protein
PIP3	phosphatidylinositol-3,4,5-triphosphate
Pirk	poor immune response upon knock in
PKC	Protein kinase C
PLC	phospholipase C
PRRs	Pattern recognition receptors
Rb	retinoblastoma protein
RBBP6	retinoblastoma binding protein 6
RING	Really Interesting New Gene
ROS	reactive oxygen species
RTK	Receptor Tyrosin Kinase
SCF	Skp-1/Cul1/F-box
SMG-1	SMG1 homolog, phosphatidylinositol 3-kinase-related kinase
SPE	Spaetzle processing Enzyme
STAT	Signal transducers and activators of transcription
TAK1	TGF-beta activated kinase 1
tefu	telomere fusion
TGF	Transforming growth factor
TGF	Transforming growth factor

THD	TNF homology domain
TIR	Toll/IL-1 receptor
TNF	tumour necrosis factor

Chapter 1

Introduction

1.1. Stress signalling pathways Stress signalling

1.2. Cell cycle arrest : DNA Damage and Cell Cycle control

1.3. Apoptosis in *Drosophila melanogaster*

1.4. Protein regulation and transcriptional response to stress: p53

1.5. The transcriptional response to stress :Infection and immune response

1.6. Integrated Stress response

1.7. *Euoniticellus intermedius*

1.8. *Drosophila melanogaster*

1.9. Aims of this study

1. Introduction.

Biological organisms can be influenced by external environmental factors that act upon them. These factors or stressors may cause changes in behaviour, development, aging, reproduction and survival and may act upon a cell, individual or populations of individuals. Once an organism has been exposed to these external factors the organism is under stress, which then leads to a stress response. Korsloot (Korsloot et al., 2004) defines stress as follows.

"Stress is a condition evoked in an organism by one or more environmental factors that bring the organism near to or over the edges of its ecological niche"

Stress is not permanent and results in a specific set of molecular responses that should counteract it and increase the chances of the individual's survival. Insects have evolved to occupy every biological niche other than those of the Polar Regions and deep marine environments. In terms of species number insects represent the largest class in the animal kingdom, with about 1-3 million species currently described and many as yet undiscovered species. Stress signalling in arthropods especially in model organisms such as *Drosophila* is of particular interest due to the relationship between stress and ageing. The lifespan of an organism is related to its ability to withstand extrinsic or intrinsic stresses (Vermeulen and Loeschcke, 2007).

Generally four distinct pathways can be used to deal with stress in a cell.

1) Cell cycle arrest (halting unnecessary cellular functions).

2) The safeguarding and repair of proteins, RNA and DNA.

3) Programmed cell death such as Apoptosis.

4) Responses to specific stressors resulting from transcriptional induction or the activation of currently inactive peptides, that will lead to increased survival (expression of survival genes).

Stress response pathways should therefore ensure the organisms survival, while reducing the age related genetic variation to a minimum (Song, 2005). However, before a stress response can be mounted, that stress must be recognized and the correct response must be initiated. The focus of this chapter is to review signalling pathways used by insects to respond to stress caused by pathogens and DNA damaging agents. It will discuss the major molecules used to detect these stressors and the subsequent pathways used to convey stress signals.

1.1.1 Stress signalling pathways.

1.1.2 MAPK cascades.

The Mitogen Associated Protein Kinase pathway conveys signals from stress and growth (mitogen) pathways that culminate in the activation of transcription factors. There are four main MAPK pathways but only two are important for stress.

1) c-Jun N-terminal kinases (JNKs)
2) p38 MAPKs

These two pathways respond to stressors such as, ultraviolet radiation, X-ray irradiation, heat shock, osmotic shock and cytokines such as tumour necrosis factor (TNF) and interleukin (IL)-1. These signalling components in MAPK pathways are

highly conserved between mammals and *Drosophila* (see figure 1)(Takeda et al., 2008).

1.1.3 Eiger.

Eiger is an invertebrate Tumour Necrosis Factor superfamily ligand that was identified due to its ability to induce apoptosis in the *Drosophila* eye (Igaki et al., 2002). Eiger induced apoptosis occurs independently of caspases, and relies on the JNK pathway, but it can be blocked by the over expression of *Drosophila* Inhibitor of Apoptosis 1 (DIAP1). The C-terminal TNF homology domain (THD) of Eiger is 20-25% homologous to the C termini of human TNFs (Moreno et al., 2002). Eiger is expressed in a similar pattern to JNK during embryonic morphogenesis and may also be the signal controlling the JNK signalling pathway in this process (Stronach, 2005).

The tumour necrosis factor Receptor required for Eiger signalling is Wengen. Unlike most tumour necrosis receptors Wengen contains no TRAF2, or death domains in its cytoplasmic region. Wengen over expression cannot stimulate the JNK pathway suggesting that Eiger/Wengen may use a novel type of TNF signalling mechanism (Kanada et al., 2002).

The signalling molecules linking Eiger and Wengen to the JNK pathway may include Tak1 which may be the JNKKK in the TNF pathway (Igaki et al., 2002), but *Ask1*, *Hemipterous* and *Basket* are active downstream. Traf1 is an inhibitor of Eiger induced apoptosis and appears to directly interact with Wengen and Eiger (Stronach, 2005).

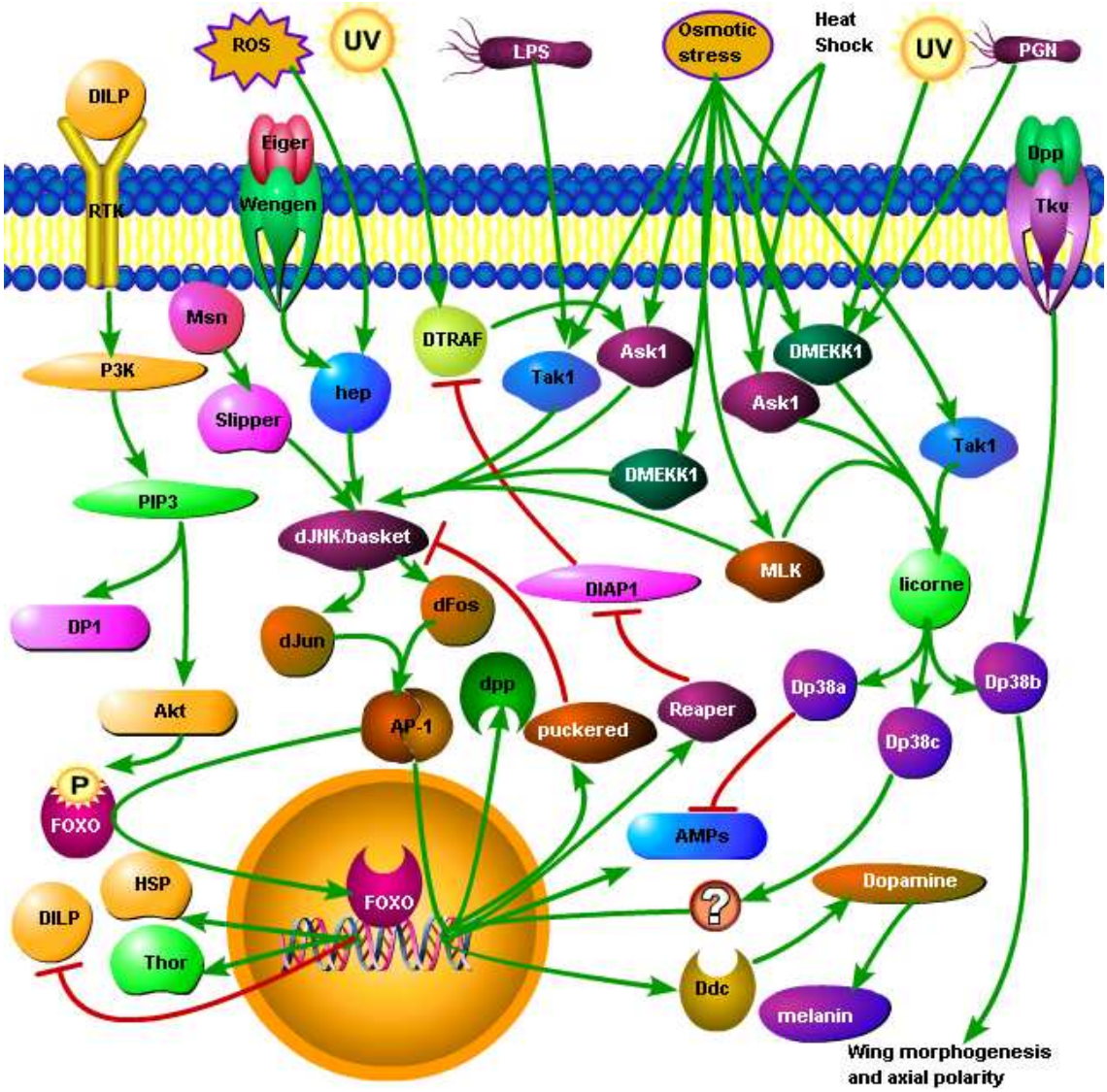


Figure 1.1 MAPK and Insulin signalling pathways in *Drosophila melanogaster*. The Mitogen Associated Protein Kinase, respond to stressors, such as ultraviolet radiation, X-irradiation, heat shock, osmotic shock, and cytokines such as tumour necrosis factor (TNF) - Eiger and Wengen and immune challenge (Moreno et al., 2002). In *Drosophila* Insulin like peptides bind to the Insulin receptor (INR) and initiate a signalling pathway that activates downstream kinases PDK1 and Akt. Akt acts against apoptosis by phosphorylating FOXO leading to its retention in the cytoplasm. The JNK pathway antagonises the activity of the Insulin Like signalling pathway by de-phosphorylating FOXO resulting in FOXO being translocated to the nucleus (Stronach, 2005).

1.2 Cell cycle arrest: DNA Damage and Cell Cycle control.

1.2.1 Sensing DNA damage.

DNA damage is detected by the *Drosophila* homologs of the human Rad17, Rad9, Rad1 and HUS1 proteins. These sensor proteins then relay the signal to the phophoinositide 3 kinase proteins which include tefu, mei-41 and SMG-1. In addition to this the MRN complex of proteins is conserved between flies and humans and is responsible for the detection and repair of double strand breaks. This complex is made up of the mre11, Rad50 and Nbs1 proteins (Song, 2005). The Ku protein is responsible for detecting double strand breaks. The *Drosophila* homolog was identified in a mutagen sensitive strain, and is involved in the repair of DNA following p element insertion (Sekelsky et al., 1998).

1.2.2. ATM/ATR/Chk2/Chk1.

Ataxia-telangiectasia mutated (ATM) is a kinase activated by ionizing radiation while Ataxia-telangiectasia and RAD3 related (ATR) is a kinase activate by incomplete DNA replication. These kinases can both activate p53 directly through phosphorylation on serine 15 and initiate a phosphorylation cascade by targeting the checkpoint kinases Chk1 or Chk2. These two checkpoint kinases phosphorylate p53, resulting in the breakdown of the Mdm2 and p53 complex (Sutcliffe and Brehm, 2004).

In *Drosophila* the Chk2 homolog is given the names Loki or Mnk. Following X-ray irradiation of embryos transcription of the TNF Eiger is induced via Dmp53 in a Loki dependent manner. Here the levels of Dmp53 do not increase and like the mammalian Chk2 the increase in the p53 activity is due to the phosphorylation and stabilization of Dmp53 (Brodsky et al., 2004). The *Drosophila* homolog of ATR (mei41) and the homolog of Chk1 (grapes) along with the product of the *mus304* gene are involved in a signalling pathway that co-ordinates cell cycle arrest delaying the cells entry into mitosis following DNA damage (Jaklevica and Su, 2004) (Song, 2005).

1.2.3. Cyclin dependent kinase (CDK) control.

Cyclin dependent kinases control cell cycle regulation. In *Drosophila* CDKs are controlled through the inhibitory proteins dWee and dMyt1. These inhibitory kinases phosphorylate the CDKs preventing their activation (Price et al., 2002). The CDC25 (string) protein in turn negatively regulates dWee allowing for CDKs to become active and initiate cell cycle progression. DMyt1 is the *Drosophila* homolog of Myt1 which is an inhibitor of CDK1 (Price et al., 2002). The expression of dWee is repressed by p53, but p53 activity is inhibited by dWee1 and dMyt1 activity (Price et al., 2002).

1.3 Apoptosis in *Drosophila melanogaster.*

Programmed cell death or apoptosis is essential for the continued existence of any multicellular organism, being required for development and homeostasis. Apoptosis is characterized by membrane blebbing, cytoplasmic condensation and the degradation of nuclear DNA. Apoptotic signals most commonly result in the activation of a set of

cysteine proteases known as caspases. These caspases are evolutionary conserved from the worm *C. elegans* to man, but show an increase in the complexity and number of proteins in the system with an increase in the complexity of the organism. For example there are 14 caspases in humans, 7 in flies and 1 in worms (Richardson and . 2002). Cells make use of two separate apoptotic pathways, the intrinsic and extrinsic pathways. The intrinsic pathway leads to the release of cytochrome C from the mitochondria, while the extrinsic pathway involves tumor necrosis factor (TNF) receptors. Although young freshly eclosed flies show higher levels of caspase activity in their neuronal tissue, older adults show the highest levels of apoptosis occurring in the reproductive system due to gametogenesis (Zheng et al., 2005).

1.3.1 H99 deficiency genes.

The first genes involved in apoptosis were identified due to a deletion Df (3L) H99 on chromosome 3. This region was found to contain three pro-apoptotic genes *Reaper*, *Grim* and *Head involution defective (hid)*. Two other pro-apoptotic genes also exist in *Drosophila*. The fourth, *Sickle* lies near to the H99 region, while the fifth *Jaffrac* 2 maps to a completely different location (Cashio et al., 2005). Deletions of these genes completely block apoptosis during embryogenesis. However, induction of these genes results in increased, aberrant apoptosis, which can be inhibited using the caspase inhibitor p35. This suggests that RHG proteins rely on caspases to induce apoptosis (see figure 1.2) (Bergmann et al., 2003). All five of these proteins show very little sequence similarity except for a common motif at their N terminal named the RHG motif. Truncation of this motif results in the partial or complete loss of the proteins pro-apoptotic function (Bergmann et al., 2003). Jaffrac2 is the only *Drosophila* RHG protein this motif is only revealed following proteolytic processing. This is similar to

the functional human homologs of the RHG proteins Smac/Diablo and Omi/HtrA2 (Bergmann et al., 2003).

Reaper is required for the proper development of the adult ganglion by removing excess neuroblasts. Reaper mutants display neural hyperplasia, an enlargement of the adult ganglion due to an increase in the number of neurons (Peterson et al., 2002) (Cashio et al., 2005). Reaper and Hid have similar expression patterns and Hid is probably able to substitute for the absence of Reaper (Peterson et al., 2002).

Hid may also be regulated by the expression of a 21 nucleotide miRNA Bantam. The over expression of bantam results in excessive growth (see figure 1.2). Following irradiation Bantam levels are increased to allow for the survival of some cells and it is the balance between the levels of Hid and Bantam which dictates weather apoptosis occurs or not. Apoptotic cells appear to signal to neighbouring cells to activate the expression of bantam and this may explain why some cells in irradiated tissues survive while others die (Jaklevic et al., 2008). Reaper and Hid overexpression result in the release of cytochrome C from the mitochondria (Abdelwahid et al., 2007).

1.3.2 IAPs.

Apoptosis is negatively regulated through the function of the inhibitor of apoptosis proteins (IAP). IAPs generally function by binding to caspases inactivating them (see figure 1.2) (Richardson and . 2002). IAPs share a common BIR (baculovirus IAP repeat) domain. This domain is about 80 residues in length and has a zinc binding fold (Cashio et al., 2005). The BIR domains mediate the binding of IAPs to caspases and differ slightly from each other, allowing them to inhibit different caspases (Bergmann

et al., 2003). A second motif that is commonly but not always found in IAPs is the RING finger domain. This implies that IAPs possess E3 ubiquitin ligase activity. In addition to interacting with caspases, IAPs also interact with the RHG proteins. This interaction leads to the inhibition of IAPs and apoptosis (Cashio et al., 2005).

The *Drosophila* genome encodes four IAPs of which diap1 appears to be the most prominent and contains two BIR domains and one RING domain (Cashio et al., 2005). In order to perform its anti-apoptotic function correctly Diap1 must be degraded implying that the caspases that are bound to Diap1 must be degraded as Diap1 is degraded. In contrast the RING finger of the IAPs appears to catalyses the IAPs autoubiquitination. This leads to the degradation of the IAP and this action is pro-apoptotic (Bergmann et al., 2003). Two E2 ligases were identified that are involved in this RING activity are morgue and ubcD1. Both of these proteins interact with Diap1 and both affect Reaper induced apoptosis and while UbcD1 is a classical E2 enzyme, morgue lacks some of the classical residues found in most E2's. However, it does show some structural similarity to E3,s (Bergmann et al., 2003). In *Drosophila* the TNF ligand Eiger is regulated by dIAP1 but is not caspase dependent. This means that dIAP is able to inhibit caspase dependent and independent apoptosis (Igaki et al., 2002).

1.3.3 The role of Caspases in apoptosis.

The caspases (a family of Cystine proteases) are the main effectors in most apoptotic pathways (see figure 1.2). They cleave their targets specifically after an aspartame residue (Kornbluth and White, 2005). Initially they exist as large inactive procaspases

of 20–30 kDa that consist of a variable prodomain, a large subunit (~20 kDa), and a small subunit (~10 kDa) (Cashio et al., 2005). Caspases are divided into two classes, initiator caspases and effector caspases. These differ in structure, the initiator caspases contain long prodomains comprised of two subunits, while the effector caspases lack a pro-domain or have a very short prodomain (Cashio et al., 2005).

Caspases are activated by cleavage at aspartic acid residues by either another caspase or by themselves through autocatalysis. The large and small subunits then associate to from heterodimers (Villa et al., 1997). The long N-terminal prodomains of initiator caspases commonly contain protein–protein interaction domains. The two most common domains are the caspase recruitment domains (CARD) or death effector domains (DED). DED domains associate with the adaptor protein FADD (Fas-associated death domain), while CARD domains associate with DARK. These domains allow the initiator caspase zymogens to associate in death complexes where they will be cleaved and activated (Cashio et al., 2005). This allows caspases to be activated in an amplifying proteolytic cascade, cleaving one another in sequence resulting in an exponential rate of activation (Kornbluth and White, 2005).

D. melanogaster has seven caspases *Dcp-1, Dredd, Drice, Dronc, Decay, Stric* and *Damn* (Cashio et al., 2005) (Cooper et al., 2009). Dredd is most similar to caspase 8 containing a death domain and two death-inducing domains (DID). Dredd seems mainly involved in immune signalling and is involved in the activation of the IMD pathway by cleaving Relish. Dredd may also be involved in activating the JNK pathway. Dronc is the only *Drosophila* caspase with a CARD domain, this domain interacts with DARK that results in Dronc activation. Dronc is the primary effector of

caspase dependent cell death in *Drosophila* and is essential for developmental toxic agent *x*- and *γ*-irradiation induced apoptosis (Cooper et al., 2009). Dronc is also involved in compensatory proliferation of cells, spermatid individualization, and cell migration (Cashio et al., 2005). Strica is a long prodomain containing caspase that contains no regular motifs but has a serine and threonine rich prodomain. Strica related apoptosis involves Hid and is and plays a role in *Drosophila* oogenesis (Cooper et al., 2009). Drice is the primary target of Dronc and consists of a short prodomain. Drice can also be induced by the hormone ecdysone and requires Reaper and Hid. Dcp1 is similar to Drice in structure and substrate specificity. It also requires Reaper and Hid. Decay is also similar to Drice and is involved in Hid-mediated cell death. Damm seems to be redundant in the regular apoptosis system and may perform as yet unknown functions (Cooper et al., 2009).

1.3.4 Apoptosomes.

Initiator caspases are activated on specific adaptor protein complexes or apoptosomes. In vertebrates this function is performed by the Apaf-1 (apoptotic protease-activating factor 1), the *Drosophila* homolog of this protein is DARK (Drosophila APaf-1 related killer) (see figure 1.2). The apoptosome consists of eight DARK molecules (octomer) (Bao and Shi, 2007).

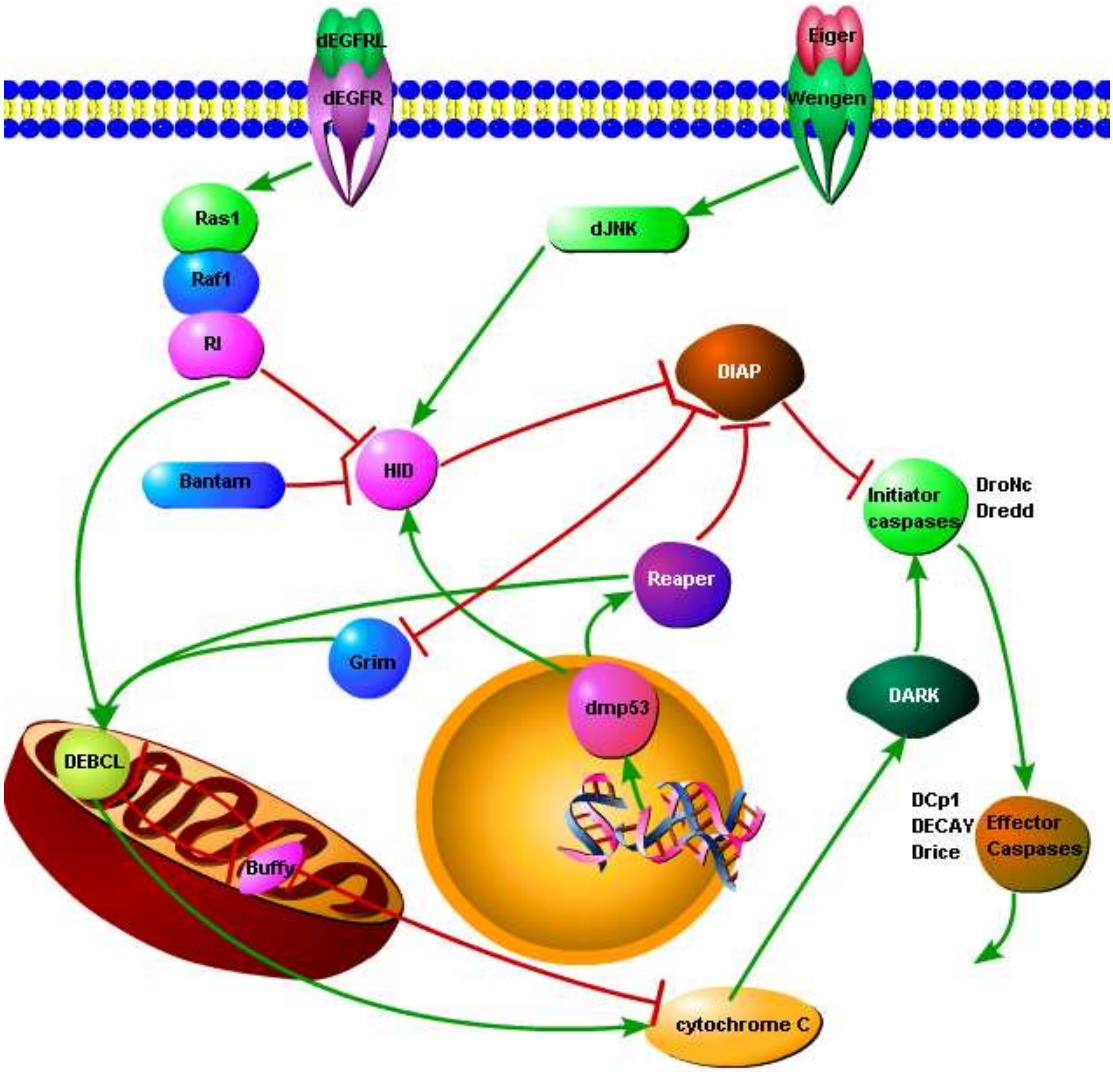


Figure 1.2: Apoptosis pathways in *Drosophila.* Programmed cell death involves the H99 death-inducing genes. Complex signals control the activity of these genes. The anti-apoptotic Bantam encodes miRNA that functions by targeting HID. The H99 genes induce Apoptosis in a Caspase-dependent manner. They act by inhibiting the negative regulators DIAP1 (*Drosophila* Inhibitor of Apoptosis-1) and DIAP2 (*Drosophila* Inhibitor of Apoptosis-2). Caspase activation can also be controlled via the formation of the Apoptosome-Like complex containing DARK (Drosophila Apaf-1-Related Killer). Caspase activation can be further regulated by the conformational change of the mitochondria leading to the release of Cytochrome-C. The exact role of cytochrome C in *Drosophila* apoptosis is not known (Richardson and Kumar, 2002).

Dronc and Dredd both require the apoptosome in order to be activated. DARK also possesses a WD40 domain and has been shown to bind with cytochrome C which is released from the mitochondria as a result of various death inducing signals. However, RNAi knockdown studies of cytochrome C shows that only a slight increase (2 fold) in DARK dependent apoptosis is observed in the presence of cytochrome C (Kornbluth and White, 2005).

1.4. Protein regulation and transcriptional response to stress: p53.

The tumour suppressor protein p53 was first identified in 1979 where it was found to interact with viral oncogenes T antigens (Lane and Crawford 1979 as reviewed by (Lane and Benchimol, 1990). Following DNA damage the p53 protein acts as a transcription factor that plays a role in Cell cycle regulation, apoptosis and the repair of DNA damage. Initially, only two mRNA splice variants were predicted arising from alternate splicing of introns 9 and 2. However, a further seven different human p53 protein isoforms have been identified, at least one of which arises from an internal promoter in intron 4 (Bourdon et al., 2005). These alternative forms of human p53 are expressed in a regulated manner, being tissue specific and having seemingly different cellular localizations and functions (Bourdon et al., 2005).

The *Drosophila melanogaster* ortholog of p53 was identified in 2000 due to its homology with human p53. This high degree of homology is restricted to the DNA binding domain, with the N and C termini showing a very low degree of homology (Ollmann et al., 2000) (Brodsky et al., 2000) (Jin et al., 2000). The Dmp53 sequence was also found to contain oligomomerisation sites and was able to substitute for

human p53 (Brodsky et al., 2000). Initially a single 385 amino acid Dmp53 protein was isolated from *D. melanogaster* (Ollmann et al., 2000) (Brodsky et al., 2000) (Jin et al., 2000). However, further studies revealed that three isoforms of Dmp53 were present, with the initial protein being an N terminally truncated version arising from an internal promoter. The largest isoform is 110 amino acids longer than the initial Dmp53 and contains two additional exons. The third Dmp53 is a splice variant of this larger form and is only 110 amino acids long (Bourdon, 2007). By aligning the sequences of *dmp53*, human p53 and the human p53 family members, p63 and p73, it was ascertained that the larger form of Dmp53 is the *Drosophila* equivalent of the full-length human p53. A five residue sequence was identified in the N terminal transactivation domain of Dmp53 that is conserved across species and in p63 and p73 (Bourdon et al., 2005).

The human p53 protein is made up of five functional domains; the N terminal transactivation domain, a proline rich Src homology 3 domain, the DNA binding domain, the tetramerization domain and the c terminal regulatory domain (see figure 1.3)(Römer et al., 2006). The *Drosophila* protein has an N terminal region that is rich in basic residues, which is similar to that of the human p53 (Brodsky et al., 2000). This may indicate that it serves a similar function. However, Dmp53 does not contain a Src homology domain and contains only one PXXP sequence (Ollmann et al., 2000) while human p53 contains 5 (Römer et al., 2006) (see figure 1.3).

1.4.1 DNA binding domain

The DNA binding domain consists of two structural motifs that interact with the major and minor grooves of the target DNA molecule. The overall structure of the DNA binding domain as determined by NMR is a β-sandwich, made up of two antiparrallell β sheets joined by a small hairpin. There is a long helix at the c terminus, which buts up against hairpin. Two smaller helices are present within the domain, one of which associates with a zinc ion (see figure 1.4) (Cañadillas et al., 2006). DNA sequences recognized by p53 consist of a double stranded palindromic sequence is separated from a second identical site by 13 bp. This sequence is 10 bp in length and can therefore be divided into 4 5 bp sequences. In order to interact with the DNA strand a tetramer of four p53 molecules is needed with each molecule interacting with one of these 5 bp sequences (Joerger and Fersht, 2008). Residues that are conserved between human p53 and Dmp53 include surface residues that function in DNA binding and those which may function to stabilize the tertiary fold of the domain (Ollmann et al., 2000).

1.4.2 Post-translational modifications of p53

A major way in which the stability of p53 is affected is through post-translational modifications. These modifications can occur on the c terminal regulatory domain and include ubiquitination, summolytion, acetylation, phosphorylation, methylation and neddylation. The binding of other proteins and non-specific DNA to the c terminus is also able to influence p53 activity (Römer et al., 2006). In addition to regulating the stability of p53, these posttranslational modifications affect the ability of p53 to functions as a transcription factor. The ability of p53 to bind DNA and components of

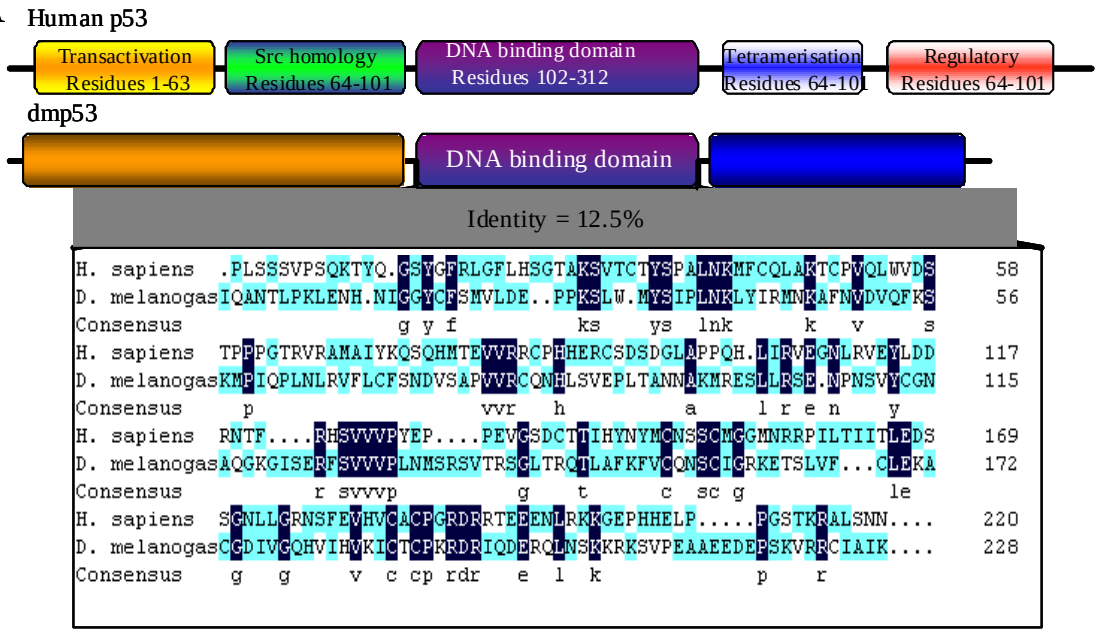
transcriptional machinery is aided by posttranslational modifications. There are generally two regions within p53 where modifications occur. Once again the c terminus, which can be phosphorylated, acetylated, glycosylated and sumolated (Sutcliffe and Brehm, 2004). The N terminal region, which can be phosphorylated (Sutcliffe and Brehm, 2004), neddylated (Harper, 2004), acetylated or methylated (Ivanov et al., 2007).

1.4.3 Regulation

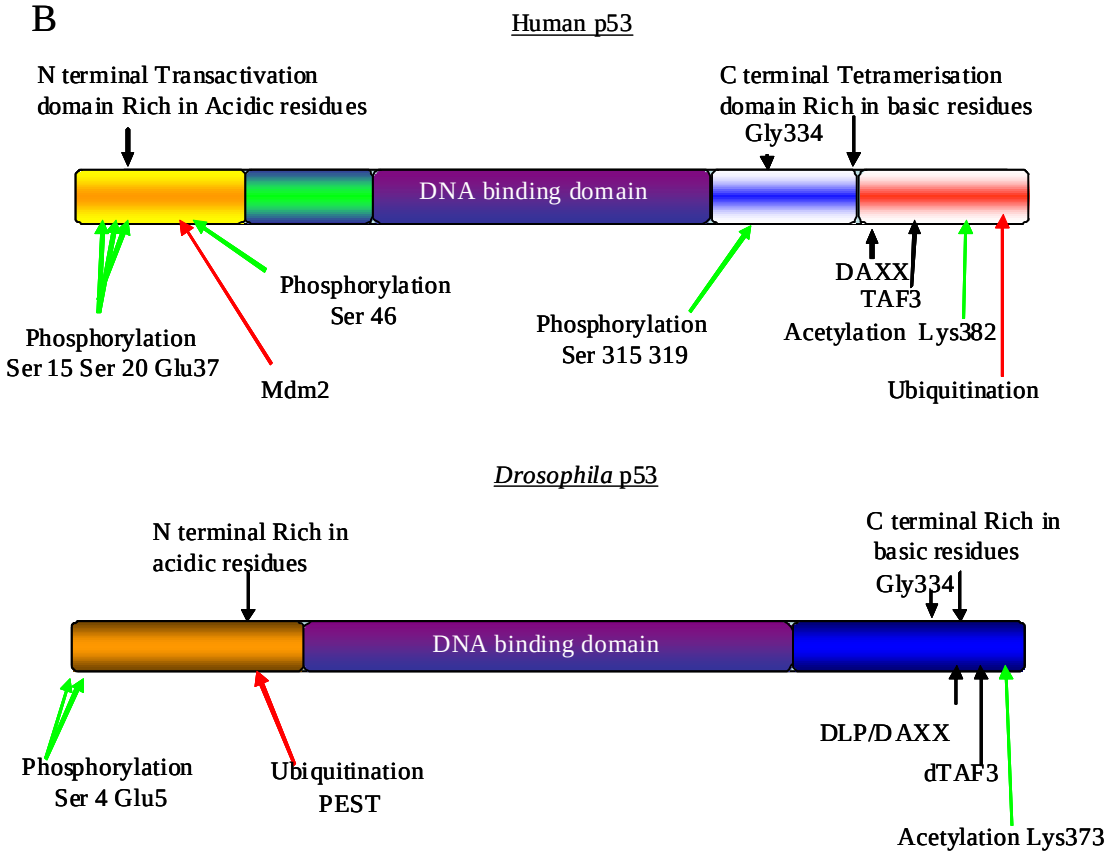
Like mammalian p53, Dmp53 requires co-factors to regulate its activity. One of these is the DAXX co-factor is a death domain carrying protein, which has a *Drosophila* homolog DLP. This co-factor has both pro and anti apoptotic functions. The fly and mammal proteins function in a similar way by binding p53/Dmp53 directly through its c terminus. DLP affects pro-apoptotic signalling via ARK with null mutants showing decreased longevity and fecundity (Bodai et al., 2007).

p53 is a labile heat sensitive protein that has a high turnover rate under normal cellular conditions (Römer et al., 2006). Generally changes in the amount of p53 present in the cell depend on the stabilization of the protein due to post-translational modifications. The most stable domain is the DNA binding domain, but the loss of the zinc ion stabilizing the two large loops of the minor groove binding structure, further decreases stability and increases aggregation (Joerger and Fersht, 2008).

A


Human p53
Transactivation Residues 1-63
Src homology Residues 64-101
DNA binding domain Residues 102-312
Tetramerisation Residues 64-101
Regulatory Residues 64-101
dmp53
DNA binding domain
Identity = 12.5%
H. sapiens .PLSSSVPSQKTYQ.GSYGFRLGFLHSGTAKSVTCTYSPALNKMFCQLAKTCPVQLWVDS 58
D. melanogasIQANTLPKLENH.NIGGYCFSMVLDE..PPKSLW.MYSIPLNKLYIRMNKAFNVDVQFKS 56
Consensus g y f ks ys lnk k v s
H. sapiens TPPPGTRVRAMAIYKQSQHMTEVVRRCPHHERCSDSDGLAPPQH.LIRVEGNLRVEYLDD 117
D. melanogasKMPIQPLNLRVFLCFSNDVSAPVVRCQNHLSVEPLTANNAKMRESLLRSE.NPNSVYCGN 115
Consensus p vvr h a l r e n y
H. sapiens RNTF....RHSVVVPYEP....PEVGSDCTTIHYNYMCNSSCMGGMNRRPILTIITLEDS 169
D. melanogasAQGKGISERPSVVVPLNMSRSVTRSGLTRQTLAFKFVCQNSCIGRKETSLVF...CLEKA 172
Consensus r svvvp g t c sc g le
H. sapiens SGNLLGRNSFEVHVCACPGRDRRTEEENLRKKGEPHHELP.....PGSTKRALSNN.... 220
D. melanogasCGDIVGQHVIHVKICTCPKRDRIQDERQLNSKKRKSVPEAAEEDEPSKVRRCIAIK.... 228
Consensus g g v c cp rdr e l k p r

B


Human p53
N terminal Transactivation domain Rich in Acidic residues
C terminal Tetramerisation domain Rich in basic residues
Gly334
DNA binding domain
Phosphorylation Ser 46
Phosphorylation Ser 15 Ser 20 Glu37
Mdm2
Phosphorylation Ser 315 319
DAXX
TAF3
Acetylation Lys382
Ubiquitination
Drosophila p53
N terminal Rich in acidic residues
C terminal Rich in basic residues
Gly334
DNA binding domain
Phosphorylation Ser 4 Glu5
Ubiquitination PEST
DLP/DAXX
dTAF3
Acetylation Lys373

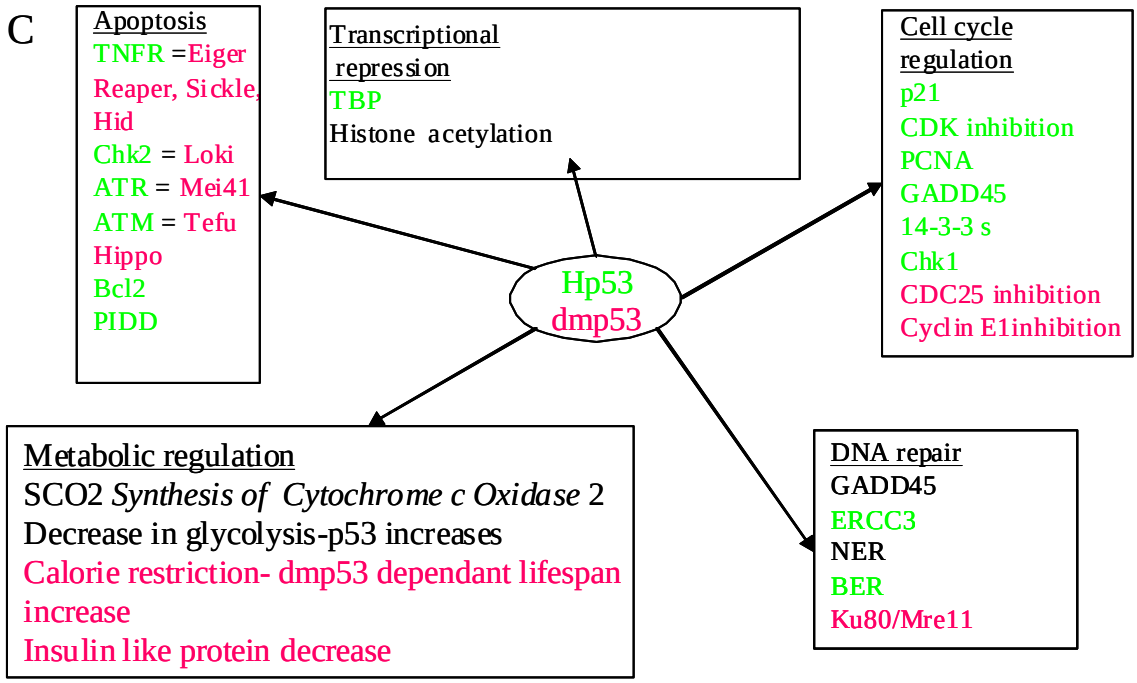


Figure 1.3: The different domain structures, regulation and functions of Dmp53 and human p53. (A) The human p53 protein is made up of five functional domains; the N terminal transactivation domain, a proline rich Src homology 3 domain, the DNA binding domain, the tetramerization domain and the c terminal regulatory domain (Römer et al., 2006). Both Dmp53 and human p53 have a high number of acidic residues present in the N terminal, implying that they serve similar functions (Brodsky et al., 2000.) The Src homology domain is proline rich and contains five copies of the amino acid sequence PXXP. These sequences are known to interact in signal transduction pathways by binding to Src homology 3 domains (Römer et al., 2006). There is only a single PXXP sequence in Dmp53 (Ollmann et al., 2000) and it may be the high amount of proline is important for p53 function (Toledo et al., 2007). The DNA binding domain is highly conserved in both *Drosophila* and humans sharing about 24% sequence identity and 40% similarity over 207 amino acids. Residues that are conserved between human p53 and Dmp53 include surface residues that function in DNA binding and those which may function to stabilize the tertiary fold of the domain (Ollmann et al., 2000). There is no significant sequence homology between the c termini of Dmp53 and human p53 with the sequence responsible for tetramerisation being poorly conserved. However, both are rich in basic residues and both are predicted to contain similar two dimensional structures (Ollmann et al., 2000) and the critical hinge residue Gly334 is conserved in *Drosophila* (Brodsky et al., 2000). (B)The stability and activity p53 is affected through post-translational modifications. These modifications can occur on the C terminal regulatory domain and the N terminal region and include ubiquitination, summolytion, acetylation,

phosphorylation, methylation and neddylation (Römer et al., 2006). Suspected acetylation sites in Dmp53 include Lysine 373 which is thought to be equivalent to acetylation site lysine 382 in human p53. Following DNA damage the serines at position 15 and 20 are phosphorylated as is the glutamate at position 37 in human p53. Equivalent sites in Dmp53 are the serine at position 4 and the glutamate at position 5 (Brodsky et al., 2000). (C) The different and common functions of Dmp53 and human p53 are illustrated in this diagram. Functions common to both molecules are written in black, those specific to human p53 are written in green and those specific to Dmp53 in red.

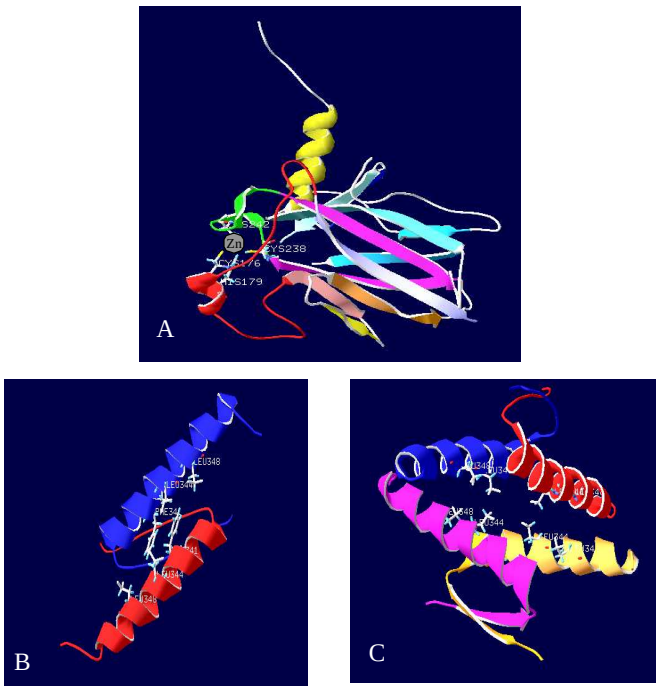


Figure 1.4: Human p53 structures. Structural information was obtained from the Protein Data Bank (Berman et al., 2000). The human p53 gene is composed of 192200 base pairs spanning 11 exons expressed as nine p53 protein isoforms. The protein is made up of five functional domains; the N terminal transactivation domain, a proline rich Src homology 3 domain, the DNA binding domain, the tetramerization domain and the c terminal regulatory domain. (A). The DNA binding domain consists of two structural motifs that interact with the major and minor grooves of the target DNA molecule. The overall structure of the DNA binding domain as determined by NMR is a β-sandwich, made up of two antiparrallell β sheets joined by a small hairpin. There is a long helix at the c terminus, which buts up against hairpin. Two smaller helices are present within the domain, one of which associates with a zinc ion. The major groove docking site is formed by the four and 5 antiparallel β sheet with a loop sheet helix motif. The zinc ion stabilizes the minor groove docking site. The tetramerisation domain consists of a β sheet followed by a α helix. (B) Initially two monomers form a dimer through associations in the antiparrallel β sheets and α helices. (C) The dimmers associate via the α helices, this interaction is stabilized by hydrophobic residues Leu 344 and Lue 348.

The number of proteins competing for a binding site upon the transactivation domain of p53 creates a means to control p53 and its downstream targets. The negative and positive regulators as well as the transcription activators will have to compete for the same sites based on their abundance and dissociation constants (Joerger and Fersht, 2008). The structure of the DNA binding domain is able to regulate the apoptotic and cell cycle arrest responses downstream of p53, by binding the recognition elements within the cell cycle control genes more tightly than those involved in apoptosis (Römer et al., 2006).

The localization of p53 within the cell is another means of regulation. The c terminus of p53 contains nuclear localisation signals that are recognizable by nuclear import factors. The N terminus of the protein may also associate with components of the cytoskeleton and microtubule network such as dyenin (Sutcliffe and Brehm, 2004). The nuclear export of p53 can be performed by the Mdm2 protein (see below); however, it is not an absolute requirement. The N terminus of p53 also contains a nuclear export sequence, which can be inhibited by phosphorylation (Sutcliffe and Brehm, 2004).

The topology of the DNA surrounding the binding sequence also influences p53s ability to bind DNA. By constraining the topology of DNA the affinity of p53 for the target sequence increases. The greatest affinity of p53 for its target sequences occurs due to negative supercoiling of target DNA. This may allow p53 to regulate or enhance its own activity in vivo, as p53 is able to remodel chromatin via the recruiting of histone acyltransferases (Jagelska et al., 2008).

1.4.4 Mdm2.

The protein coded by the *mouse double minute 2* gene is a well known oncogene that is over expressed in many tumours. It is an ubiquitin ligase and is known to interact with p53, pRb, and the growth suppressors p14/p19^{arf} (Deb, 2003). Mdm2 binds p53 at the N- terminus and catalyses the transfer of ubiquitin to several sites in the c terminus of p53 (Sutcliffe and Brehm, 2004). It also contains a nuclear localization sequence (NLS) and a nuclear export sequence (NES). This domain is also required for monoubiquitination. Once ubiquitinated p53 is exported from the nucleus and degraded by the 26s proteosome (Deb, 2003).

The binding of Mdm2 to p53 prevents the recruitment of transcription components and represses transcriptional activation by p53 (Oren, 1999). The two proteins also regulate each other through a negative feedback loop (Oren, 1999). The binding of p53 to Mdm2 is controlled by the state of phosphorylation of residues within or near the interaction domains on either p53 or Mdm2. Phosphorylation of these residues prevents p53 binding and degradation (Brooks and Gu, 2006). Mdm2 and p53 undergo structural rearrangements following their interaction. Eight amino acids within the amino terminal domain form a amphipathic a helix that projects into the hydrophobic binding pocket of Mdm2. Eight amino acids in the p53 interaction domain of Mdm2 forms a lid over the p53 binding pocket stabilizing their interaction (Brooks and Gu, 2006). The highly conserved MDM2 binding site was not identified in Dmp53. However, other regulatory elements are conserved (Brodsky et al., 2000). A PEST region at the N-terminus is responsible for Dmp53 regulation. PEST sequences correspond to proline (P), glutamic acid (E), serine (S), and threonine (T)-

rich regions in proteins. The function of PEST containing proteins is generally controlled by proteolysis, mostly via the ubiquitin pathway (Bereczki et al., 2008).

1.4.5 Functions of p53

1.4.5.1 Cell death

The main function of p53 is to act as a transcription factor to up-regulate the expression of genes involved in cell cycle arrest or in apoptosis. Mammalian TNFRs that are known targets of p53 are AP1 and Killer/DR5 (Sax and El-Deiry, 2003). Other membrane receptors that are up-regulated by p53 include the p53 apoptosis effectors related to PMP-22(PERP) protein, which initiates apoptosis by associating with the mitochondrial membrane influencing its permeability (Sax and El-Deiry, 2003). Yet another pro-apoptotic protein that is up-regulated by p53 is the P53-induced protein with a death domain (PIDD). The expression of this protein is induced by double strand DNA breaks (Sax and El-Deiry, 2003). The Bcl-2 family, are also transcriptional targets of p53. These include the pro-apoptotic Bax, PUMA and Noxa (Sax and El-Deiry, 2003).

Initial studies on Dmp53 indicated that it is indispensable for radiation induced apoptosis due to it being able to bind to an enhancer element in the promoter of the pro-apoptotic gene *Reaper* (Brodsky et al., 2000). This enhancer element increases Reaper expression following DNA damage, implicating *Reaper* as a direct transcriptional target of *Drosophila* p53 (see figure 1.5) (Brodsky et al., 2000). Mutation studies also showed that flies that lack *dmp53* are defective in damage and stress induced apoptosis. As predicted the radiation induced, expression of Reaper and

another pro-apoptotic gene sickle was deficient in these mutants. The identification of sickle as another target of Dmp53 explained why apoptosis could still occur when *reaper* was knocked out (Sogame et al., 2003).

The *Drosophila* homolog of Chk2 (loki) activated Dmp53 in response to UV irradiation induced damage (see figure 1.5). As there is no Mdm2 for loki (dmChk2) to free Dmp53 from, the kinase may accomplish p53 activation by directly phosphorylating Dmp53 (Peters et al., 2002). The activation of loki requires the *Drosophila* homolog of ATR (mei 41) (Brodsky et al., 2004) (see figure 1.5). Mutants of mei41 show decreased apoptosis, while mutants for ATM show increased p53 dependant apoptosis (Song et al., 2004). However, the presence of Loki and Dmp53 may not be an absolute requirement for UV irradiation induced apoptosis. A caspase dependant but Dmp53 and Loki independent apoptotic pathway was observed in *Drosophila* larvae exposed to ionizing radiation. This apoptotic response was slower than that induced by the Dmp53, chk2 pathway (Wichmann et al., 2006).

When the amount of p53 and Dmp53 targets was compared, Dmp53 was found to have substantially less targets than human p53. The downstream effectors of apoptosis in *Drosophila* are the pro-apoptotic genes *Reaper*, *Sickle* and *Hid.* These proteins appear to function in a dosage dependant mechanism as the transcription and expression of all three is stimulated following DNA damage (Brodsky et al., 2004). There is also a difference between the functional relationships between *Drosophila* E2F (dE2F) and Dmp53 due to the absence of Mdm2 and p14/ARF homologs in *Drosophila* (Moon et al., 2008).

1.4.5.2 Transcriptional repression

Like many transcription factors, p53 also has the ability to repress transcription. The use of transcription and translation inhibitors is unable to completely block p53 dependent apoptosis. Therefore, p53 is able to induce apoptosis without activating transcription, while at the same time p53 loses this ability once its proline rich domain has been removed (Ho and Benchimol, 2003).

Transcriptional repression may be performed in four separate manners.

1) Interference via p53 binding to the specific DNA recognition elements recognized by transcription factors. This occurs via direct competition for the DNA binding sequences or by binding to negative regulatory elements (Ho and Benchimol, 2003) .
2) Interference via p53s interaction with transcription factors without binding specific DNA recognition elements. This occurs by p53 binding to adjacent DNA sequences to those recognized by transcription factors and inhibiting or interfering with their ability to initiate transcription (Ho and Benchimol, 2003) (Koumenis et al., 2001).
3) Interference with transcription machinery i.e. preventing transcription factors such as the TATA binding protein. This interference seems to be due to a negative effect on the formation of the transcription initiation complex due to p53 blocking the interactions between TBP and Transcription Factor IIA that result in transcriptional initiation (Ragimov et al., 1993) (Ho and Benchimol, 2003) .
4) Altering chromatin structure and restricting the ability of transcription factors to gain access to specific DNA sequences (Ho and Benchimol, 2003).

1.4.5.3 DNA repair.

Cells that lack an active form of p53 are more compromised in DNA repair. Two downstream targets of p53, which are involved in the DNA repair pathway are the GADD45 and ERCC3 proteins (Garner and Raj, 2008). Despite mutant *dmp53* flies showing decreased levels of apoptosis, Jassim et al 2003 reported that following UV irradiation, the cells within the retina of these mutant flies displayed increased levels of apoptosis, suggesting that the DNA repair role is conserved in *Drosophila* (Jassim et al., 2003). The DNA repair pathways activated by p53 seem to be those of the nucleotide excision repair pathway (NER) (Smith et al., 2000) and the base excision repair pathway (BER) (Seo et al., 2002). B polymerase is involved in BER and seems to be the target of p53 in the DNA repair pathway. In this instance p53 does not stimulate B pol transcription. Rather, it seems likely that p53 forms a complex with B pol stabilizing it (Seo et al., 2002). Like human p53, Dmp53 aids in nucleotide excision repair by acetylating histones to allow chromatin relaxation and remodelling (Rebollar et al., 2006). Components of the Ku and Mre11 DNA repair complexes were also found to be downstream targets of Dmp53 (see figure 1.3). These complexes participate in the repair of double strand breaks via non-homologous end joining (Brodsky et al., 2004). Evidence also suggests that DNA repair may still proceed in the absence of p53 as p53 seems to play no role in the transcription coupled repair (TCR) pathway.

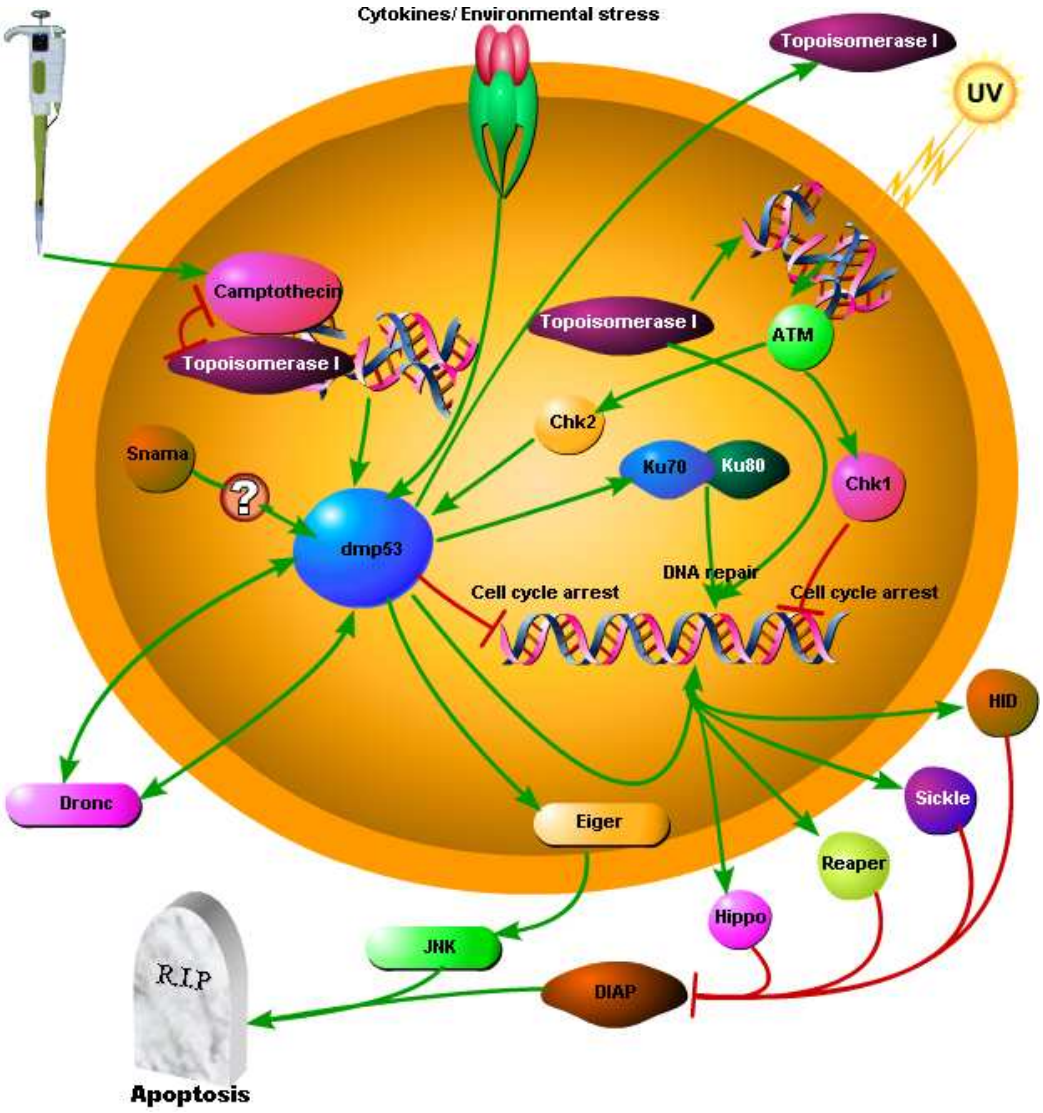


Figure 1.5: The signalling pathways of the *Drosophila melanogaster* orthologue of p53. Dmp53 initiates the transcription of the pro-apoptotic H99 genes *Reaper*, *Hid*, *Sickle, Grim* and *Jaffrac*. *Drosophila* orthologs of DNA damage signalling molecules such as Chk2 (loki), activates Dmp53 in response to UV irradiation through the *Drosophila* homolog of ATR (mei 41). Telomere fusion (dATM) decreases p53 dependent apoptosis. Dmp53 plays an important role in DNA repair and aids in nucleotide excision repair by acetylating histones to allow chromatin relaxation and remodelling (Brodsky et al., 2004; Rebollar et al., 2006). Components of the Ku and Mre11 DNA repair complexes were also found to be downstream targets of Dmp53. These complexes participate in the repair of double strand breaks via non-homologous end joining. The TNF pathway is also initiated via Dmp53 as is the Hippo pathway. Both these pathways lead to cell death following DNA damage. Although Dmp53 does not appear to regulate cell cycle progression in the traditional way, it does seem to play a role in compensatory proliferation, where it inhibits the Cell Division Cycle 25 protein homolog string (stg) (Wells et al., 2006).

1.4.5.4 Cell cycle arrest.

Active p53 is required for both G1 and G2 cell cycle arrest following stress. The major downstream target for cell cycle arrest is the CDK inhibitor $p21^{waf1/cip1}$, when this gene is mutated or lost normal development continues but cells are unable to undergo G1 arrest in response to DNA damage (Sax and El-Deiry, 2003). The first functional studies of Dmp53 indicated that it is dispensable for G1 cell cycle arrest (Brodsky et al., 2000). Over expression of wild type Dmp53 does not activate decapo which is the fly homolog of the CIP/KIP G2 cell cycle arrest factors stimulated by human p53 (Sutcliffe and Brehm, 2004).

Following cell death the organism must replace the lost tissue through compensatory proliferation. In order to study this process so called undead cells are generated. These are cells that have been damaged but are protected from cell death by the caspase inhibitor p35. These undead cells then stimulate proliferative growth (Wells et al., 2006) and require active Dmp53 in order to undergo compensatory proliferation. This activation of Dmp53 does not require DNA damage or other cell stress signals and seems to rely on the initiator caspase DRONC degrading a negative regulator of Dmp53. One of the results of this activation is the inhibition of the Cell Division Cycle 25 protein homolog string (stg). Cdc25 proteins control entry into and progression through various phases of the cell cycle. This explains the G2 cell cycle arrest that occurs due to the presence of undead cells (Wells et al., 2006).

A Dmp53 dependent G1 cell cycle arrest was observed during mitochondrial dysfunction. The AMP activated protein kinase (AMPK) responds to the change in the

ATP:AMP ratio (Hardie, 2004). Once activated by low levels of AMP –and therefore, low energy levels- AMPK phosphorylates and activates p53, which leads to cell cycle arrest to prevent cell proliferation (Bensaad and Vousden, 2007). AMPK then activates Dmp53, which in turn down regulates Cyclin E1 causing a G1 cell cycle arrest. Therefore under abnormal conditions reflecting a mitochondrial dysfunction, Dmp53 can induce G1 cell cycle arrest (Mandal et al., 2005) (Owusu-Ansah et al., 2008).

1.4.5.5 Metabolic role of p53.

In addition to its role as an anticancer molecule, p53 has also been identified as playing a role in regulating aerobic respiration. It performs this function by regulating mitochondrial oxygen consumption via the *Synthesis of Cytochrome c Oxidase* 2-assembly protein. SCO2 is a transcriptional target of p53 and plays a role in the assembly of the cytochrome c oxidase complex. Any defects in p53 expression consequently lead to a defect in SCO2 transcription and cells compensate for this by increasing the rate of glycolysis (Ma et al., 2007). This has been shown experimentally by the inverse relationship between p53 levels and the fraction of ATP derived from glycolysis (Matoba et al., 2006) and is suspected to be the explanation for the Warburg effect (Warburg 1930).

Glycolysis can be regulated through the activity of the three enzymes that catalyse those reactions with the large negative change in free energy. The three regulated enzymes are hexokinase, phosphofructokinase, and pyruvate kinase. The transcription of pyruvate kinase can be influenced indirectly by p53. This is due to p53 being a transcriptional regulator of the TP53-induced glycolysis regulator (TIGAR). TIGAR

regulates glycolysis by degrading fructose-2,6-bisphosphate, an allosteric activator of pyruvate kinase (Bensaad et al., 2006).

Reduction in Dmp53 expression normally results in flies that are sickly and have decreased lifespan. However, if the loss of Dmp53 is restricted to the nervous tissue, the lifespan of the flies can increase as much as 58% in females and 32% in males. Over expression of Dmp53 in the neurons during development is lethal and results in increased oxidative damage. These effects on lifespan are not observed when downstream targets of Dmp53 are over expressed (Bauer et al., 2005). In a similar fashion calorie restriction increases lifespan through a Dmp53 inactivating or down-regulating pathway (Bauer et al., 2007). Dmp53 may be involved in regulating the lifespan of *Drosophila* in two separate pathways in separate tissues. A decrease in Dmp53 in the fat body results in a decrease in the transcription and expression of insulin like protein (ILP), which in turn leads to a down regulation of signalling via the insulin like growth factor signalling pathway. This leads to a decrease in the nuclear translocation of forkhead transcription factor FOXO and an increase in lifespan (Bauer et al., 2007).

Histone acetylation is required for Dmp53 induced apoptosis during development (Miotto et al., 2006). Here the primordial germ cells undergo programmed cell death, which is Dmp53 dependant, but also requires the activity of Outsiders, a monocarboxylate transporter which is thought to act upstream of p53. Outsiders catalyses the proton linked transport of carboxylate and is localized to the mitochondrial or plasmamembrane. Some of its substrates include pyruvate and lactate (Yamada et al., 2008). Pyruvate transport has been identified as playing a role

in apoptosis, as pyruvate uptake by cells through the action of monocarboxylate transporters leads to a pyruvate dependant inhibition of histonedeacetyl-transferase. This in turn leads to up-regulation of Dmp53 and therefore an increase in apoptosis (Thangaraju et al., 2006).

1.4.6. p53 ancestry.

The most pleisiomorphic organism where a p53 homolog has been identified was the nematode *Caenorhabditis elegans.* This protein was named CEP-1 and it is the only p53 like molecule identified in *C. elegans* thus far. Cep -1 is required for radiation and other stress responses (Schumacher et al., 2001). There is no Mdm2 homolog in the nematode but other regulation pathways namely ATM and ATR are conserved (Lu and Abrams, 2006). The more closely related model organism *Danio rerio* contains a p53 protein that shares 48% similarity to human p53. It also contains an MDM2 homolog, the p53 family members p63 and p73 and the cell cycle regulator p21 is a downstream target of the zebra fish p53 (Lu and Abrams, 2006). These results seem to imply that p53 evolved at the same time as animals and while some of the function and signalling pathways are ancient, the appearance of Mdm2 and Mdm4 appears to have occurred after the evolution of chordates (Lu and Abrams, 2006).

Two p53 family members, p63 and p73, also serve a pro-apoptotic and cell cycle arrest role. They display a high degree of sequence similarity but are unable to substitute for each other. These two proteins are found together with p53 in all chordates (see figure 1.6). However, in invertebrates all three are only found in molluscs, where it is not possible to classify the p63 or p73 proteins as being the most

homologous to the mammalian p63 or p73 proteins. All other invertebrates possess only one family member, and these cannot be classified as p53, p63 or p73 as they show similarity in their DNA binding domains (see figure 1.6) (Ou et al., 2007). The arthropod and nematode p53 homologs show the inclusion of other domains which allow them to stabilize the formation of dimmers, allowing the more pleisiomorphic ancestral form to accomplish the same tasks as the mammalian p53s (Ou et al., 2007). All p53 gene members including Dmp53 contain internal promoters that give rise to multiple isoforms.

1.4.7 Proteins that interact with p53.

1.4.7.1 E2F-1/Retinoblastoma protein.

The retinoblastoma protein (Rb) was first identified as a 110 kDa protein found in the nucleus, since then a further two Rb like proteins have been identified, p107 and p130. This protein suppresses cell proliferation and induces cell cycle arrest through the formation of E2F-RB complexes (Sun et al., 2007). The E2F family of transcription factors, are involved in the control of cell-cycle progression, DNA replication, mitosis, the mitotic checkpoint, DNA-damage checkpoints, DNA repair, differentiation, development and apoptosis (Polager and Ginsberg, 2008). In mammals, the E2F family comprises eight genes (E2F1–8) (DeGregori and Johnson, 2006), while *Drosophila* has two (dE2F1-2) (Sutcliffe et al., 2003). In *Drosophila* dE2F1 serves the function of an activating E2F, while dE2F2 performs the function of a repressing E2F (Sutcliffe et al., 2003).

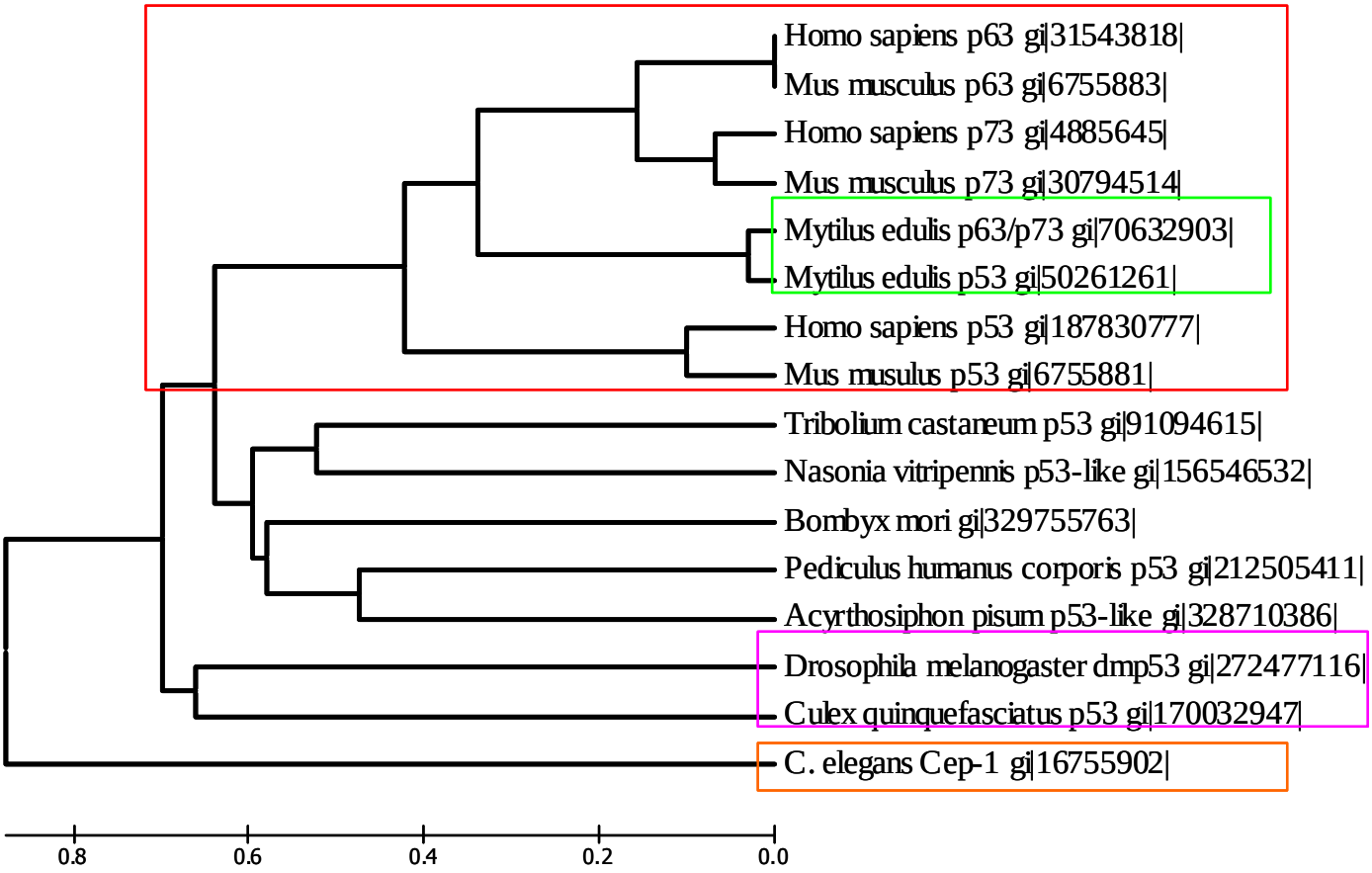


Figure 1.6 Homology amongst members of the p53 family. The sequences were aligned using the Multiple sequence comparison by log-expectation algorithm (MUSCLE) (Edgar, 2004)**.** The tree was constructed using the neighbour joining method. The most *C. elegans* p53 analogue Cep-1 is the most distantly related protein. The tree also shows the two dipteran sequences (marked with a purple box) sit together on separate branch to the other insect sequences. The red box marks the point where the p53 family diverged to three separate genes p53, p63 and p73. This must have happened before the divergence of chordates and invertebrates, as the molluscs (marked in green) have three forms. It is difficult to assign the names p63 or p73 to these proteins as they all share significant homology. The other insect sequences group more closely together.

In *Drosophila* two different mutants were initially described in cells which fail to arrest in G_1. The first *dacapo* (*dap*) (de Nooij *et al*., 1996) coded for a CIP/KIP-type cyclin-dependent protein kinase inhibitor homologue and the second *fizzy-related* (*fzr*) (Sigrist et al., 1995) coded for a CDC20. The *Drosophila* homologue of Rb (RBF) shares sequence similarity with the human Rb proteins (Du and Dyson, 1999). RBF1 was found to be negatively regulated by decapo and like mammalian E2F1, inactivation of dE2F1 prevents DNA synthesis, while over expression results in cells entering S-phase and undergoing apoptosis (Sutcliffe et al., 2003). RBF1 is also negatively controlled by cyclins or CDK complexes. Therefore, like mammalian signalling systems CDK complexes disrupt the EF-pocket protein complex (Polager and Ginsberg, 2008).

In *Drosophila* there is a direct link between the activator and repressor E2Fs. Deletion of the activator dE2F1 leads to cell-cycle-progression and proliferation defects. These flies can be rescued by the additional deletion of the repressor dE2F2 (Frolov et al., 2001). However, the relationship between dE2F1s and Dmp53 differs between flies and vertebrates due to the absence of Mdm2 and p14/ARF in *Drosophila*, both of which are required for E2F signalling (Moon et al., 2008). In flies p53 and E2F co-operate during DNA damage induced apoptosis. Removal of dE2F1 or Dmp53 decreases the apoptotic signal. This suggests that they function independently of each other, regulating pro-apoptotic genes but converge on common targets to strengthen a pro-apoptotic DNA damage induced signal (Moon et al., 2008). These targets are most likely the H99 genes *reaper* and *hid*, as dE2F1 can induce their expression but is not required for low level basal expression in a stress free background (Moon et al., 2008).

1.4.7.2 Snama (RBBP6).

Another protein that has been reported to interact with p53 is the retinoblastoma binding protein 6 (RBBP6). This protein has been characterized in the mouse (Witte and Scott, 1997) , human (Sakai et al., 1995) and *Drosophila* (Mather et al., 2005). Orthologs have been found in most eukaryotes (Mather et al., 2005) (Pugh et al., 2006). Generally, they consist of an ubiquitin-like domain (DWNN-Domain With No Name), a RING-finger like motif and p53- and Rb1- binding domains (Pugh et al., 2006) .

The DWNN domain was first identified as being present in a group of proteins identified in a promoter trap mutagenesis screen for cells resistant to cytotoxic T lymphocyte killing. This trap involved disrupting genes randomly with a promoter-less retrovirus, targeting genes involved in immune pathways. By identifying clones that were resistant to CTL killing, genes responsible for resistance, were identified (Mather, 2005). One of the novel genes identified was named Domain With No Name (DWNN) (Skepu, 2005). When these cell lines were also screened for resistance to apoptosis induced by staurosporine, three lines were isolated and DWNN was also identified in one of these lines, (Pretorius, 1999) as cited by (Mather, 2005). The DWNN domain may function by interacting with other proteins such as Rb and p53. GFP-DWNN fusion proteins localise to the nucleus and DWNN domains have been found to associate with higher molecular weight proteins (Seameco, 2004).

The DWNN domain is found throughout eukaryotes and is absent in the prokaryotes. In most organisms it is found as a structural motif of a larger protein however the

domain exists as a single protein in higher chordates (humans). Despite only sharing 22% identity with ubiquitin DWNN has an ubiquitin like fold with a similar 3 dimensional structure, but the *Drosophila* form lacks the di-glycine necessary for cleavage by hydrolases. This di-glycine is found in the mouse and human forms (Mather et al., 2005). The existence of DWNN as a standalone protein implies that it may therefore act like a ubiquitin like modifier (Pugh et al., 2006).

Another DWNN containing protein PACT is a 250-kDa nuclear protein and it interacts with both p53 and Rb in vitro and in vivo (Simons et al., 1997) (Witte and Scott, 1997). Null mutants of PACT are not viable, but can be rescued by simultaneously knocking out p53 (Li et al., 2007). An alternatively spliced version of PACT is involved in cell cycle arrest and is responsible for camptothecin-induced apoptosis (Scott et al., 2003). PACT itself is a splice variant of full length RBBP6 corresponding to residues 207-1792 and most likely regulates p53 activity by interfering with the ability of p53 to bind DNA (Simons et al., 1997). PACT also interacts with Mdm2, suggesting that it is a negative regulator of p53 and enhances Mdm2-mediated ubiquitination and degradation of p53 (Li et al., 2007).

The *Drosophila* homolog of *rbbp6*, *snama* was identified by searching for the DWNN domain in the *Drosophila* genome. The cDNA of *snama* consists of nine exons with eight short introns –the longest being only 0.84 kBp making up an open reading frame of 1231 amino acids with a predicted molecular weight of 139 kDa. Analysis of Snama showed that like other members of this family the protein contains a C2HC zinc finger motif, a conserved cysteine-rich region near the zinc finger and a basic lysine-rich region that is also found in PACT (Mather et al., 2005).

Snama is expressed throughout development with its levels decreasing in the later embryonic stages. Double negative mutants of *snama* are not viable and die early on in development, dying between 48-72 hours into development(Jones et al., 2006) (Mather et al., 2005). Snama appears to be a negative regulator of apoptosis and due to its homology with RBBP6 may regulate Dmp53 and retinoblastoma protein (RBF) (Mather et al., 2005).

1.5 The transcriptional response to stress: Infection and immune response.

Due to their extreme proliferation and the environments in which they live, insects must be able to resist a large number of diverse pathogenic microorganisms including bacteria, fungi and protozoa (Boman, 1991; Bulet et al., 1999; Trenczek, 1997). However despite these facts very few encounters result in infection (Trenczek, 1997). A study on the use of entomopathogenic fungus to control insect pests showed that the infection rate is very low, between 0 and 3.71% (Bruck and Lewis, 2001).

Like all invertebrates insects lack the adaptive immune system, found only in vertebrates. The adaptive immune system is based on receptors that are generated by somatic mechanisms (Medzhitov and Janeway, 1997). Since insects are r-selected, having a short lifespan and generally only reproducing once producing many thousands of offspring, an adaptive immune system would not provide any significant advantages (Medzhitov and Janeway, 1997). Insect immunity more closely resembles the innate immune system of vertebrates. This is an evolutionary ancient system that provides an immediately available means of defense against pathogens. It also does

not require previous exposure to the pathogen (Vasselon and Detmers, 2002). The innate immune system has evolved to constantly recognize and react to structures commonly present in large groups of microorganisms. These structures are called PAMPs or pathogen associated molecular patterns (Medzhitov and Janeway, 1997). Once the microorganism has been recognized as non-self certain effector mechanisms will be activated to destroy the invading pathogen, and in vertebrates the innate immune system will also activate and orientate the adaptive immune system (Vasselon and Detmers, 2002).

The fact that insects are particularly resistant to bacterial infections led to the first studies on their immune system over a century ago (Hoffmann and Hetru, 1992). Kowalvsky (1887) and Cuenot (1896) identified the role of phagocytosis and capsule formation in insect defense (as cited by (Hoffmann and Hetru, 1992)). After the First World War this work was continued by Glaser (1918), Paillot (1919) and Metalnikow (1920), all of whom noted the appearance of bacteriolytic substances within the hemolymph of the insect after the insect was challenged with bacteria (as cited by (Hoffmann and Hetru, 1992)). However, it was not until 1981 that the anti-microbial peptide cecropin was isolated from the pupae of the cecropia moth *Hyalophora cecropia* (Steiner et al., 1981). Despite this in some insects the response to fungal infections is thought mainly to be a cellular response involving phagocytosis and encapsulation (Silva et al., 2000).

1.5.1 A brief summary of the insects immune system.

The insect's primary defence against infection is the physical barrier represented by the cuticle (Vilmos and Kurucz, 1998). Once this is pierced the humeral and cellular components of the innate immune system must protect the insect from infection.

1.5.1.2 Cellular response.

The insect innate immune system allows for rapid but non-specific responses to infection, by responding via the coordinated action of many sub-populations of hemocytes (Trenczek, 1997). Three classes of hemocytes regulate the immune response.

1) Crystal cells, which are involved in melanisation.
2) Plasmocytes that phagocytose and digest microorganisms.
3) Lamellocytes, which encapsulate parasites (Bangham et al., 2006).

Hematopoesis in *Drosophila* occurs in the embryonic and larval stages in lymph glands which form along the anterior region of the dorsal vessel in third instar larvae. Jak-STAT signaling pathways are required for immune induced differentiation of hemocytes in this gland (Kim and Kim, 2005) (Bangham et al., 2006).

The primary reaction of hemocytes towards smaller microorganisms is phagocytosis, which is a multi-step form of receptor-mediated endocytosis (Trenczek, 1997). It has been shown that all arthropods can remove bacteria via phagocytosis (Barnes, 1991). Insect phagocytosis involves a unique type of pattern recognition receptor known as Eater, which contains multiple EGF repeats (Bangham et al., 2006).

If a microorganism is too large to be phagocytosed, it can be encapsulated by layers of hemocytes or lamellocytes (Trenczek, 1997). These lamellocytes have a flattened shape and encapsulate parasite eggs and other large pathogens (Kim and Kim, 2005). Hemocytes can also aggregate to form nodules to trap a large number of microorganisms (Trenczek, 1997). In the mosquito *Culex quinquefasciatus* nodule formation is a secondary immune response that lags behind the initial phagocytotic response (Silva et al., 2000).

The third cellular response involves phenoloxidase hydrolysing phenols and the oxidation of o-phenols to form quinones (Trenczek, 1997). These reactive quinones mediate protein cross-linking to form melanin. Melanin is formed from dopamine and functions as a defense reaction in insects by immobilizing the pathogen. It is also required for wound healing (Russo et al., 1996) (Barillas-Mury et al., 2000) (Kim and Kim, 2005). This process also generates reactive oxygen species, which contribute to the killing of microorganisms (Trenczek, 1997). Apart from the phenoloxidase pathway, melanin formation in *Drosophila* requires the genes *pale* (*ple*) encoding tyrosine hydroxylase and *Dopa decarboxylase* (*Ddc*) encoding the enzyme Dopa decarboxylase. The p38 MAPK pathway in *Drosophila* controls the expression of Dopa decarboxylase in response to bacterial infection (Davis et al., 2008).

Phenoloxidase is present in the hemolymph as an inactive zymogen. It is activated by proteins that recognize lipopolysaccharide, β-1-3 glucan or similar characteristic structural molecules from pathogens (Barillas-Mury et al., 2000). These proteins that activate phenoloxidase are members of a serine protease cascade (Kim and Kim, 2005). The crystal cells themselves must rupture in order to release the phenoloxidase

products and the subsequent formation of melanin can be blocked by inhibiting RhoA function (Bangham et al., 2006).

Two forms of hemolymph clotting have been identified. The first involves clotting proteins catalyzed by calcium ions, and the second involves a serine protease cascade (Vilmos and Kurucz, 1998). Like the intermediates in the melanisation pathway, these serine protease pathway intermediates are toxic to other micro-organisms (Vilmos and Kurucz, 1998).

1.5.1.3 Recognition.

The pattern recognition theory states that immune recognition relies on products encoded by the genomes of the host and pathogen (Barillas-Mury et al., 2000). This means that any pathogen recognition event that results in an immune response would result in an advantageous selection on the host's genome, and a selective disadvantage on the pathogens genome. Therefore, any mechanism to avoid recognition would be advantageous to the pathogen. This has resulted in the evolution of an innate immune system that recognizes invariant molecular constituents essential for the pathogens survival, called PAMPS (Medzhitov and Janeway, 1997).

1.5.1.3.1 PAMPS.

These PAMPS include β 1-3 glucans and mannans from fungal cell walls, lipopolysacharide from Gram-negative cell walls and peptidoglycan from Gram-positive bacteria cell walls (Trenczek, 1997). The receptors that have evolved to recognize these PAMPS are known as Pattern recognition receptors (PRRs). These

PRRs are ancient molecules, which are found in both vertebrates and invertebrates (Barillas-Mury et al., 2000).

Peptidoglycan recognition molecules had been identified in mammals as early as 1986 (Silverman et al as cited by (Yoshida et al., 1996). The first homologous protein identified in insects was found in the silkworm *Bombyx mori* (Yoshida et al., 1996). The absence of this protein completely abolished the ability of peptidoglycan to activate the phenoloxidase cascade (Yoshida et al., 1996). A peptidoglycan recognition protein (PGRP) was also isolated from the moth *Trichoplusia ni* (Kang et al., 1998). This protein was strongly induced after bacterial challenge and expression was found to originate in the fat body (Kang et al., 1998). Two proteins with 43% sequence identity to *T. ni* PGRP were isolated from humans and mice, implying that PGRP is a highly conserved ancient component of the innate immune system (Kang et al., 1996).

13 PGRP genes have been identified in *Drosophila melanogaster.* These 13 genes can be divided into two classes based on their structure. The first family has short transcripts and encodes extracellular proteins, while the second group has long transcripts and encodes membrane-spanning proteins (Kim and Kim, 2005; Werner et al., 2000). However, only the short extracellular protein has been shown to bind peptidoglycan (Werner et al., 2000). Two isoforms of PGRP-LE exist and perform different functions. One is a short form which is found outside the cell and binds to diaminopimelic acid type peptidoglycan (Gram negative) and is necessary to activate the IMD pathway (See figure 1.7) (Bangham et al., 2006; Kim and Kim, 2005). The long form is intracellular and recognizes small peptidoglycan molecules released by

bacteria (Bangham et al., 2006). Another PGRP required for the activation of the Imd pathway is the transmembrane bound PGRP-LC (See figure 1.7). The short PGRP–SA is secreted and recognizes the Lysine type peptidoglycan in Gram positive bacteria. It is then involved in the activation of the Toll signalling pathway (Kim and Kim, 2005). Another type of peptidoglycan serves a catalytic amidase function in the removal of peptides from glycan chains resulting in the eventual down regulation of the immune response (Bangham et al., 2006). The final function of PGRP is demonstrated by PGRP-SB1 that can act as an antibacterial peptide by destroying peptidoglycan (Bangham et al., 2006).

β-1, 3 glucan is a component of fungal cell walls, while lipopolysaccharide is a bacterial surface component making these two molecules good target PAMPs for the innate immune system to recognize. Three gram negative binding proteins (DGNBP) have been isolated from *Drosophila melanogaster*, that bind to lipopolysacharide and β-1, 3 glucan, but not to peptidoglycan (Kim and Kim, 2005; Kim et al., 2000). Over-expression of this DGNBP greatly enhanced the inducibility of anti-microbial peptides (Kim et al., 2000). GNBPs are generally 50kDa proteins that contain a N-terminal β-1,3 glucan binding domain and a C terminal β glucanase like domain (Kim and Kim, 2005).

Other β-1,3 glucan recognition protein (βPGRP) have been found in a wide variety of arthropods including the silkworm, cockroach and crayfish (Yoshida et al., 1996), The *Anopheles gambiae* GNBP homolog (AgGNBP) is induced in response to bacterial and plasmodium infection (Barillas-Mury et al., 2000). The amino acid sequence of this AgGNBP shows 32.6% identity to the homologous protein found in the silkworm

B. mori, as well as 34.6% identity to a β1-3 glucanase from bacterium that lyse fungi (Dimopoulos et al., 1997). Another probable β-1-3 glucan recognition peptide, named hemocytin was identified in the silkworm *B. mori*. This peptide contains a lectin domain similar to those found in mammalian mannose binding peptides (Vilmos and Kurucz, 1998). Yet another probable fungal recognition peptide, is a large multiple domain protein Sp22D. It was cloned from *An. gambiae.* This protein contains two chitin-binding domains, a mucin like region, two lipoprotein receptor domains and finally a serine protease domain (Barillas-Mury et al., 2000).

Despite lipopolysacharide being a major component of bacterial cell walls, it is unable to initiate an immune response in *D. melanogaster*. However, it does illicit immune response gene expression in the silkworm (Tanaka et al., 2009) and army worm *M. sexta* (Rao and Yu, 2010). Similarly even though lipoteichoic acid (LTA) is a major constituent of the cell wall of Gram-positive bacteria, there are only a few reports of it being able to activate the immune system of insects (Rao and Yu, 2010). A multifunctional PRR was identified in *G. mellonella*. This protein was able to bind to lipopolysaccharides, lipoteichoic acid, and B-1, 3-glucan. It also functions as an opsonin that promotes the uptake of invading microorganisms into hemocytes (Kim et al., 2010).

Thiol ester proteins (TEPs) bind to the surface of pathogens and initiate a cascade that results in lysis or phagocytosis of pathogens. Four proteins of this family have been identified in *Drosophila*. They are secreted, have multiple splice forms and are upregulated via the Jak/STAT pathway during immune challenge by bacteria (Pal and Wu, 2009). The Drosophila homolog of the human Down syndrome cell adhesion

molecule (Dscam) has been implicated in pathogen recognition. Alternate splicing can generate up to 18000 variations of these proteins extracellular domain. Certain of these forms were found to bind *E. coli* in vitro. In Drosophila, it interacts with the adaptor Dreadlocks (Dock) and serine/threonine signalling protein Pak. Different spliceforms of Dscam might act like mammalian immunoglobulins by detecting specific pathogenic epitopes and marking them for phagocytosis (Pal and Wu, 2009).

1.5.2 Signalling pathways.

1.5.2.1 Toll signalling pathway.

The promoter regions for a number of the genes encoding anti-microbial peptides, and many of the peptides involved in immune response, were found to contain binding motifs that resemble those for members of the rel family of NF-κβ transcription factors (Goergel et al., 1993). For example the PGRP from *B.mori* was found to contain a NF-κβ like element (Yoshida et al., 1996), while proteins with structures similar to NF-κβ have been shown to be important for anti-microbial peptide gene regulation (Trenczek, 1997). Toll is a trans-membrane receptor with luecine rich repeats in its extra cellular region, while the intracellular region is homologous to the vertebrate interlukin 1 receptor (Levashina et al., 1999). Originally Toll was thought only to function in development, where the toll pathway is responsible for the establishment of the Dorsal-Ventral polarity in *D. melanogaster* embryos (Anderson et al., 1985), via Dorsal (Vasselon and Detmers, 2002).

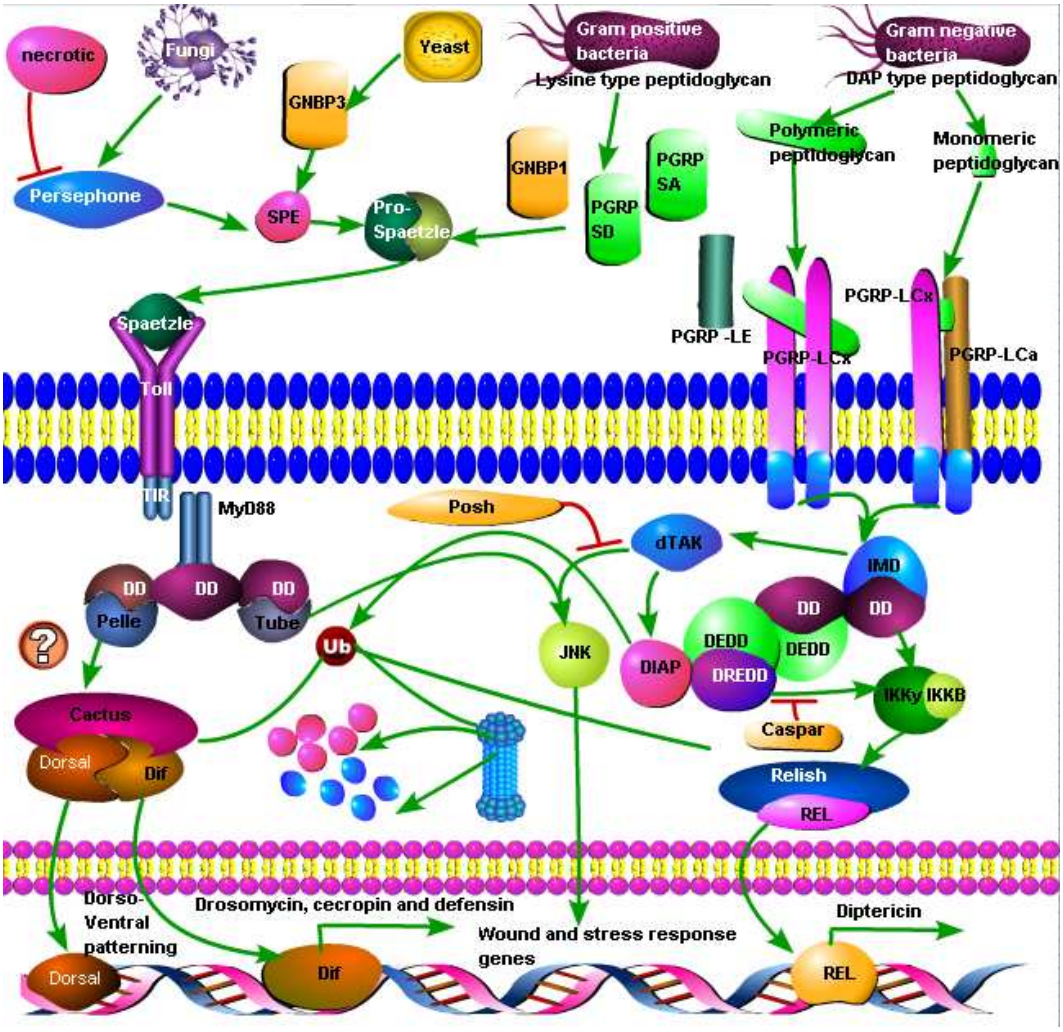


Figure 1.7: Insect cellular pathogen recognition pathways. Pathogens are recognised through PAMPS. Different pathogen recognition receptors recognize specific patterns. The spaetzle processing enzyme cleaves and activates spaetzle, which then activates Toll. Activated Toll dimerises allowing the TIR domain to recruit the dMyD88 adaptor protein. This then recruits the tube protein, which activates Pelle. Pelle phosphorylates cactus leading to its degradation, allowing the transcription factors dif and dorsal to translocate to the nucleus and activate transcription of Drosomycin, Cecropin and Defensin. Tube can also activate JNK resulting in the expression of wound and stress response genes. The activated IMD pathway recruits the Death Domain containing DEDD, which activates the MAP3K dTAK1. The IKK complex is activated via dTAk1 and phosphorylates Relish targeting it for degradation by the caspase DREDD. The resulting active Rel transcription factor is translocated to the nucleus where it activates transcription of antimicrobial peptides such as diptericin (Marmaras and Lampropoulou, 2009).

The Toll pathway involves the two-rel proteins Dorsal and Dif (Dorsal related immune factor) (Ip et al., 1993). Toll mutations were found to severely inhibit the ability of *D. melanogaster* to mount an anti-fungal response, while mutations in the Toll homologue 18-wheeler reduced the ability of *D. melanogaster* to mount an immune response against bacteria (Vasselon and Detmers, 2002).

Once an extra-cellular ligand binds to Toll, it causes a conformational change that results in the receptor becoming active and the recruitment of a second Toll molecule to form a dimmer (See figure 1.7). This activation results in a signalling cascade that interrupts a protein complex of the inhibitory protein cactus with dorsal/Dif (Means et al., 2000; Trenczek, 1997; Weber et al., 2005). This cascade is initiated by the Toll dimer recruiting and interacting with dMyD88 via a Toll/IL-1 receptor (TIR) domain. The N terminal Death Domain of dMyD88 then interacts with the Death Domains of the Tube protein. Tube is in close contact with Pelle due to Pelle's association with MyD88 via Death Domains. Tube then activates Pelle, which is the *Drosophila* homologue of the IL-1 receptor-associated kinase (IRAK) (See figure 1.7). This results in the phosphorylation and degradation of cactus, which results in the release of Dif and Dorsal (Wang and Ligoxygakis, 2006). Dorsal then translocates to the nucleus and activates the transcription of developmental and defence genes (Means et al., 2000; Trenczek, 1997). This is functionally equivalent to the vertebrate NF-κβ-Iκβ complex (Levashina et al., 1999). This pathway is initiated by the formation of the ligand for Toll. The ligand is created by a proteolytic cascade initiated by the recognition of PAMPS by recognition proteins (Vasselon and Detmers, 2002). The ligand is the cleaved form of the *spaetzle* gene product (Levashina et al., 1999). In *D. melanogaster* development Spaetzle cleavage is performed by the protease Easter,

which is itself cleaved and activated by the protease snake. However in the immune response neither of these proteases are required for the activation of the pathway (Vasselon and Detmers, 2002). The serine protease inhibitor Spn43AC (*necrotic*) down regulates the formation of spaetzle, and consequently the ability of the insect to mount an immune response (Levashina et al., 1999). In order to activate the serine protease inhibitor function of Necrotic the 80–100 amino acid long N-terminal must be cleaved. This cleavage occurs rapidly following Gram-positive bacterial or fungal infection (Pelte et al., 2006).

In order to cleave Spaetzle to activate the immune system, the serine protease Spaetzle processing Enzyme is required (SPE) (Jang et al., 2006). Flies that lack active SPE are more sensitive to infection, and during embryogenesis SPE is expressed in the lymph glands and fat body, both of which are organs involved in the immune response (Mulinari et al., 2006). SPE is not supplied maternally but is first expressed at early stages during embryogenesis at the tip of the germ band and dorsally in cells which will eventually form the fat body (Mulinari et al., 2006). SPE requires feedback from Toll in order to increase SPE transcription (Mulinari et al., 2006).

The NF-κB transcription factor Dif is involved in activating the transcription of cecropin by binding to NF-κβ motifs in the promoter region of the cecropin gene (Petersen et al., 1995), while Dif is a poor activator of diptericin transcription (Gross et al., 1996). The requirement for multiple motifs may be due to the fact that Rel proteins can form heterodimers, which increases the ability of these peptides to regulate transcription (Han and Ip, 1999). *Drosomycin* induction is mainly controlled

by Dif/Relish heterodimers, while *defensin* is activated by Dorsal/Relish heterodimers. *Attacin* can be activated by Relish homodimers, and Dif/Relish or Dorsal/ Relish homodimers (Han and Ip, 1999).

1.5.2.2. Imd pathway.

A recessive immuno-deficient mutant indicated the existence of a second regulation pathway. In *Drosophila melanogaster*, individuals containing this mutation show reduced expression of antibacterial peptides, while the expression of the anti-fungal drosomycin is not affected (Ferrandon et al., 1998; Schroder, 1999). Another Rel protein Relish was identified in *D. melanogaster*, which also has the ability to activate cecropin transcription (Dushay et al., 1996). Relish is under the control of the Imd pathway (Hoffmann and Reichhart, 2002). Unlike Dorsal and Dif, Relish contains ankyrin repeats. These ankyrin repeats may be the means for the regulation of relish activation, as no inhibitor relish complex has been identified. Relish may also serve a developmental function, as it is expressed in the early embryo (Dushay et al., 1996). Relish has been identified in the mosquito *Aedes aegypti* where it controls the expression of cecropin and defensin. The mosquito Relish was activated by the taking of blood meals (Shin et al., 2003). The protein coded for by the *imd* gene, is a 25kDa death domain containing peptide that shows some sequence similarity with the Tumor Necrosis Factor α receptor interacting protein, RIP (Hoffmann and Reichhart, 2002).

The Imd pathway is initiated by the PGRPs PGRP LCx and PGRP LCa (See figure 1.6). These form a homodimer of PGRP LCx to recognize polymeric peptidoglycan or a PGRP-LCx, LCa heterodimer to recognize monomeric peptidoglycan. The PGRP

receptors then signal via their N-terminal domains to activate IMD, which is a death domain protein similar to mammalian receptor interacting protein 1 (Aggarwal and Silverman, 2008). IMD recruits the fly homolog of FADD (DEDD) via its Death Domain. FADD activates the JNK pathway via the MAPK3 kinase dTAK1 (TGF-β activated kinase 1). The length of the activation of the JNK pathway is controlled by the Plenty of SH3s (POSH) RING-finger ubiquitin-ligase, which degrades TAK1(See figure 1.6) (Wang and Ligoxygakis, 2006). dTAk1 is then required for the activation if the IKK complex, which is made up of two subunits, the catalytic IKKβ sub unit and the regulatory subunit Kenny or IKKγ. Once activated the IKK complex phosphorylates Relish (Aggarwal and Silverman, 2008), targeting it for processing by the caspase DREDD (Wang and Ligoxygakis, 2006). However, ubiquitination is also required for the activation of the IKK complex, but this does not appear to be for targeting the IKK complex for proteosomal degradation. Once the IMD pathway has been activated IMD forms a complex with dFADD and DREDD. This complex then needs the ubiquitination enzymes bendless and dUEV1a, *Drosophila* homologs of Ubc13 and UEV1a, in order to activate the JNKKK member dTAK1. This then results in the phosphorylation of relish and its activation (See figure 1.7) (Zhou et al., 2005).

Ubiquitination is able to positively and negatively regulate this pathway. The IMD pathway is suppressed through the activity of an SCF E3 ubiquitin ligase. This E3 ligase seems to target relish or targets upstream of relish such as DIKK and the caspase Dredd (Khush et al., 2002). Members of the JNK pathway as well as proteins involved in apoptosis signaling are involved in IMD regulation. The inhibitor of apoptosis (IAP2) regulates a component of the JNK pathway. JNK signaling is initiated by dTAK1 signaling to hemipterous, the *Drosophila* MKK7/JNKK homolog.

Hemipterous then phosphorylates *basket* (dJNK), which activates *Drosophila* AP-1. JNK signaling via the Imd pathway has been linked to wound repair and stress response (Aggarwal and Silverman, 2008). The JNK pathway is also required for a sustained Antimicrobial response via the IMD pathway. The AP1 complex inhibits the activity of Relish. In order to terminate JNK signaling Relish also seems to require the IKK member TAK1 (Valanne et al., 2007). Pirk (poor immune response upon knock in) is a negative regulator of the Imd pathway and is induced following Imd activation. It down regulates the immune response by binding to Imd via a Pirk domain. Pirk also associates with the cytoplasmic domain of PGRP-LC and blocks the IMD signaling pathway (Kleino et al., 2008). Caspar is the *Drosophila* homolog of Fas associated factor 1 and it suppresses the Imd pathway by preventing the cleavage of relish to form active Rel (Kim et al., 2006).

Crosstalk between the two immune pathways occurs at the level of the NF-κB transcription factors Relish, Dif and Dorsal. These factors may even form heterodimers. This may be how the two pathways may sometimes initiate the expression of the same Antimicrobial peptides (Bangham et al., 2006). Another similarity between the Toll, IMD and Phenoloxidase pathways is their requirement for serine proteases and serine protease inhibitors. Loss of the *Anopheles gambiae* serpins SRPN2 and SRPN6 results in a loss of the melanin response to protozoan parasites (Bangham et al., 2006).

1.5.3 Anti-microbial peptides.

For the past 30 years researchers have been isolating and characterizing a multitude of antimicrobial peptides from a variety of plants and animals. In total there are about 1638 antimicrobial peptides identified to date, isolated from plants, vertebrates and invertebrates. Of these peptides 1281 are bactericidal; 483 are antifungal; 102 are anti-viral and 100 act against cancer cells (Wang and Wang, 2004). The initial interest in antimicrobial peptides began in 1980 when cecropin was isolated from the cecropia moth *Hyalophora cecropia.* This was still 50 years after the initial observations made in the 1920s that insects released a bacteriolytic substance into their blood when challenged with bacteria. Infection of insects with a microorganism elicits the synthesis of multiple anti-microbial peptides and proteins. These peptides and proteins are then secreted into the hemolymph (Trenczek, 1997).

Synthesis of these peptides normally occurs in the insect's fat body, which is a functional equivalent of the mammalian liver (Trenczek, 1997). However it has been found that some of these peptides are expressed elsewhere, such as the anterior midgut tissue (Lehane et al., 1997); barrier epithelia (Ferrandon et al., 1998); and from hemocytes (Trenczek, 1997) (Destoumieux et al., 1997) (Ehret-Sabatier et al., 1996). All anti-microbial peptides from insects seem to possess the common attribute, that they are highly basic, which facilitates their interaction with the microbial cell membrane (Lauth et al., 1998). All of these anti-microbial peptides are also synthesised as precursor peptides that can be up to five times larger than the mature peptide (Barra et al., 1998).

Anti-microbial peptides from insects can be divided into classes based on their amino acid sequence and structural characteristics. These classes are

1) The linear amphipathic α helix forming peptides (Bulet et al., 2004).
2) The cystine rich or cyclic anti-microbial peptides.
3) The lysozymes.
4) The proline rich peptides (Otvos, 2002)
5) The glycine rich peptides (Rees et al., 1997).

1.5.4 The cost of immunity.

Cytotoxic defence systems such as the pro-phenoloxidase system may be equally reactive to the insects own tissue, defences against this include the basal lamina and the tight multi enzyme control of the pro-phenoloxidase system. However the malphigian tubules cannot be covered by protective tissue and are damaged by the pro-phenoloxidase toxins. This results in apoptosis and the melanisation of tissues surrounding the malphigian tubes. The final cost of this immune response is a 15% reduction in the lifespan of the organism. The TNF factor Eiger may be involved in the production of the autoreactive effect on cells (Sadd and Siva-Jothy, 2006). Continuous activation of the immune system decreases the insect's ability to perform other demanding activities. This was demonstrated using bumblebee workers, where foraging workers were less able to mount an immune response than non-foraging individuals (Konig and Schmidt-Hempel, 1995). Another study also using bumblebees showed that infected workers must increase their food intake in order to support increased immune activity (Moret and Schmid-Hempel, 2000). These studies reinforce the necessity for a low cost immune system. Mating has also been found to

decrease the pro-phenoloxidase response in *Tenebrio molitor*. This immunosuppressive signal was the juvenile hormone, which is known to reduce insect lifespan, but is required for gametogenesis and spermatophore production. Therefore, there is a tradeoff between reproductive ability and the phenoloxidase pathway, that leads to a decreased lifespan of the organism (Rolff and Siva-Jothy, 2002).

1.6 Integrated Stress response.

No stress response pathway occurs in isolation from other stress responses (See figure 1.8). Any stress from immune challenge to osmotic or physical stress initiates a multitude of responses that tie into each other. This gives rise to an integrated stress response which may to help the organism to cope with the multiple symptoms that are caused by any single stressor. For instance bacterial infections seem to initiate the transcription of detoxification enzymes that may help to remove any endotoxins that result from the lysis of bacteria. At the same time microbial infection leads to the activation of the JNK and p38 pathways which lead to a multitude of effects including rapid induction of antimicrobial peptides and melanin production (See figure 1.8).

Physically stressing *Galleria mellonella* larvae prior to infection, results in increased antimicrobial peptide expression. The antimicrobial peptides that were expressed showed no bias to target certain pathogens. This seems to be a protective response where a basal expression of a general antimicrobial response may prevent infection. Physical stress may also result in the damage to the cuticle which would result in the mounting of an immune response (Mowlds et al., 2008).

Fragmented chromosomal DNA (the end-point of the apoptotic response) also stimulates the innate immune system resulting in the transcription of antimicrobial peptides diptericin and attacin, which are under the control of the Imd pathway. The activation of the immune pathway by this means requires that there is an accumulation of DNA that is normally processed by caspase activated DNase or engulfed by nucleosomes (Mukae et al., 2002).

The integration of an organisms stress responses is accomplished by signaling networks. The initial components of this signaling network are the sensing proteins such as those that detect nutrient availability, pathogens, osmotic stress, heat shock, DNA damage, protein misfolding, chemical toxins and oxidative stress. These signaling molecules then activate response elements, sometimes directly or via signaling intermediates (Vermeulen and Loeschcke, 2007). The integration of these pathways occurs at the level of these intermediates and effectors. This makes sense as it allows for multiple responses to a single stressor so they can respond to the multiple effects of the stressors.

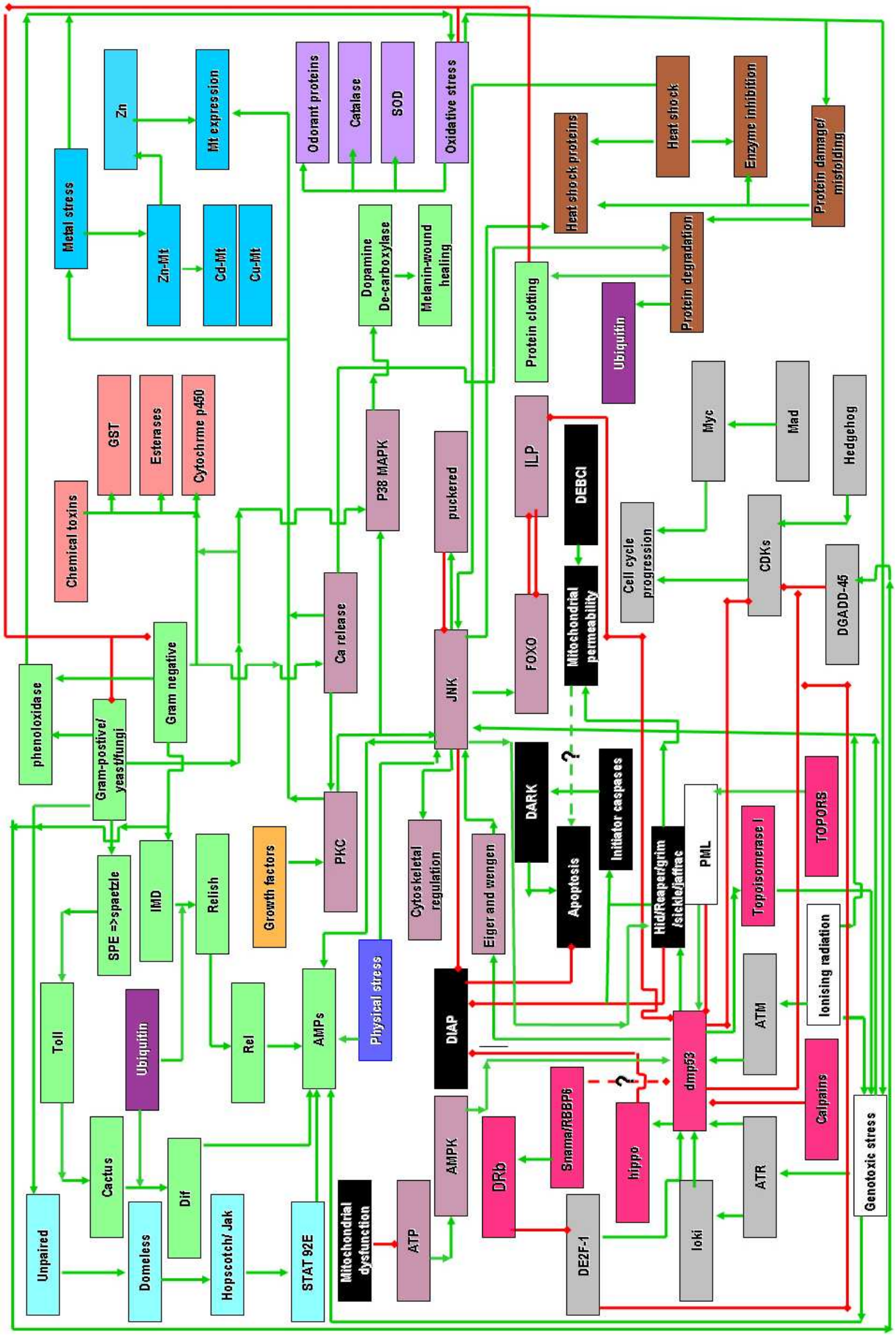
1.7 *Euoniticellus intermedius.*

The Coleoptera is the largest order of living organisms; between the adults and their larvae this order utilizes every terrestrial and freshwater habitat. They vary greatly in their diet, consuming plants and animals, as well as carrion and dung (Scholtz and Holm, 1996).*Euoniticellus intermedius* is a small brown beetle approximately 8-10mm long. It is found abundantly within the grassland regions of South Africa (Doube, 1991), as well as in tropical savanna throughout Africa (Hanski and

Cambefort, 1991). The species can be differentiated from other small dung beetles by the light colour of their hind femur and the symmetrical dark markings on their pronotum. Males possess a curved blunt horn (Marina, 1990).

Evolutionary dung beetles split into two groups, representing different techniques to protect the embryos from other organisms. *E. intermedius* is a tunneller. The beetle makes tunnels beneath the dung pat and brood balls are placed along the length of the tunnel separated by compact soil. In drier soil the broodballs are deposited in clumps at the end of the tunnels (Barkhouse and Ridsill-Smith, 1986).

The nesting behavior of *E. intermedius* changes depending on environmental conditions. This may be one of the factors that account for ability to thrive throughout Africa (Cambefort, 1991b). It builds its nests at varying depths in soil below a dung pat. The beetle buries a large amount of dung to use in the preparation of brood balls (Cambefort and Hanski, 1991). Typically it builds two types of nests. In the warm summer season it builds a nest that consists of two branched galleries that are parallel to the ground. The two branches come together to form a short vertical shaft that leads to the surface (Rougon and Rougon, 1991). Along these galleries mounds of dung are deposited to form sausage shaped brood “balls”. In the rainy season, the soil has higher water content, and the beetle adapts to this by building nests that contain more highly branched galleries (Rougon and Rougon, 1991), with a single brood ball in each branch (Rougon and Rougon, 1991).


Zn
Mt expression
Odorant proteins
Catalase
SOD
Oxidative stress
Heat shock
Enzyme inhibition
Heat shock proteins
Protein damage/ misfolding
Metal stress
Zn-Mt
Cd-Mt
Cu-Mt
Dopamine De-carboxylase
Melanin-wound healing
Protein clotting
Protein degradation
Ubiquitin
GST
Esterases
Cytochrme p450
P38 MAPK
puckered
ILP
DEBCl
Myc
Mad
Hedgehog
Chemical toxins
Ca release
FOXO
Mitochondrial permeability
Cell cycle progression
CDKs
DGADD-45
phenoloxidase
Gram negative
Gram-postive/ yeast/fungi
JNK
DARK
Initiator caspases
Apoptosis
Hid/Reaper/grim /sickle/Jafrac
PML
TOPORS
Topoisomerase I
Ionising radiation
ATM
SPE =>spaetzle
IMD
Relish
Growth factors
PKC
Cytoskeletal regulation
Eiger and wengen
Toll
Ubiquitin
Rel
AMPs
Physical stress
DIAP
dmp53
Calpains
Cactus
Dif
Unpaired
Domeless
Hopscotch/ Jak
STAT 92E
Mitochondrial dysfunction
ATP
AMPK
DRb
Snama/RBBP6
hippo
DE2F-1
loki
ATR
Genotoxic stress

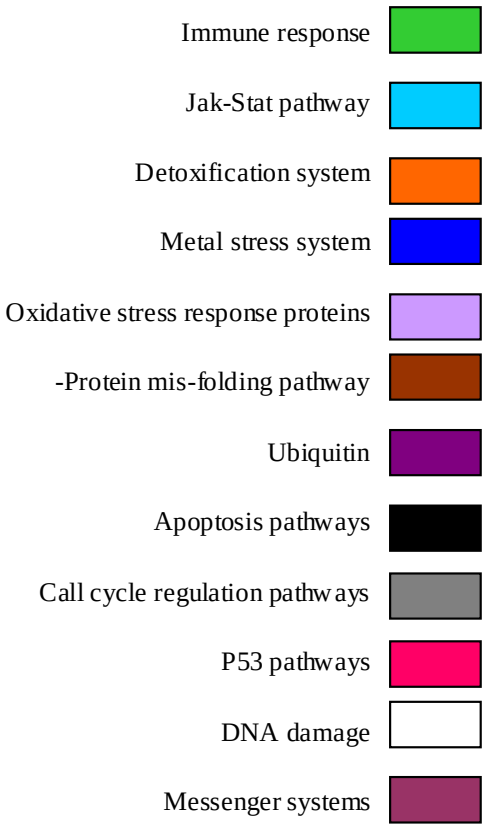


Figure 1.8: Integration of the Stress Response pathways:_This flow diagram illustrates the *Drosophila* stress response and shows the integration of the different stress responses. Components are grouped by the signaling pathway they are most often associated with.

For many dung beetle species there is little opportunity for rapid development in the larval stage due to limited resources within the brood ball. However *E. intermedius* has a rapid larval development and a high population growth rate. This makes *E. intermedius* is a r-selected species, displaying a high fecundity and opportunistic breeding (Cambefort, 1991a).. They achieve this by adapting the coprobiontic feeding strategy, where larvae use the microorganism present in the food (Cambefort, 1991b).

Adults live for about 1-2 months in the summer during which time they give rise to about 120 offspring (Marina, 1990). The life cycle of *Euoniticellus intermedius* is shown in figure 1.11. The adult female beetle has no differentiated oocytes in their ovariole when they emerge and are only ready to reproduce 4-8 days after emergence depending on temperature. Females can produce at least two eggs per day at 25°C and only occurs at temperatures above 19·6°C and below 32°C. Females can produce between 75 and 127 eggs in their lifetime (Tyndale-Biscoe, 1978). Brood balls contain one egg, weigh about 3g, and are buried up to 15cm below the ground surface (Marina, 1990). These embryos develop in the brood balls and hatch into larvae, which go through three larval instar stages and then develop into pupae. These stages of development have been described previously (Blume, 1984). The immature development stages last for about 28-40 days (Marina, 1990). The species has a broad climatic tolerance (Cambefort and Hanski, 1991). Adults continue to breed through the winter months in warmer climates (Cambefort, 1991b), but only the larval stages survive through winter in the cooler climates (Marina, 1990). Due to the diet available within the brood ball, the larval development is fast, which allows for the rapid population growth rate (Rougon and Rougon, 1991).

E. intermedius was introduced into Australia and the United States due to its efficiency in burying and dispersing cattle dung where they have been highly successful (Cambefort, 1991a). The beetle shows a high degree of specificity for food, as 99% of all *E. intermedius* are captured in traps baited with cattle dung (Cambefort, 1991a).

The true dung beetles can be classified within three families within the superfamily Scarabaeoidea. These are the Scarabaeidae, the Geotrupidae and the Aphodidae (Cambefort and Hanski, 1991). *E. intermedius* is a member of the Scarabaeidae family, subfamily Coprinae, tribe oniicellini (Cambefort and Hanski, 1991) (Cambefort, 1991b) (Scholtz and Holm, 1996). The most closely related model organism to *E. intermedius* is the red flour beetle *Tribolium castaneum*, whose genome has been sequenced (Gibbs, 2008). (See figure 1.9).

Most studies involving *E. intermedius* have focused on the beetles behaviour and ecology. These include secondary sexual characteristics and the use of this beetle as an indicator for the effect of insecticides spraying of cattle on dung beetles. In terms of development only a single study has been performed describing the larval and pupal stages (Blume, 1984). This study and one other also involved the characterisation of the adults nesting behaviour (Blume, 1984) (Barkhouse and Ridsill-Smith, 1986).

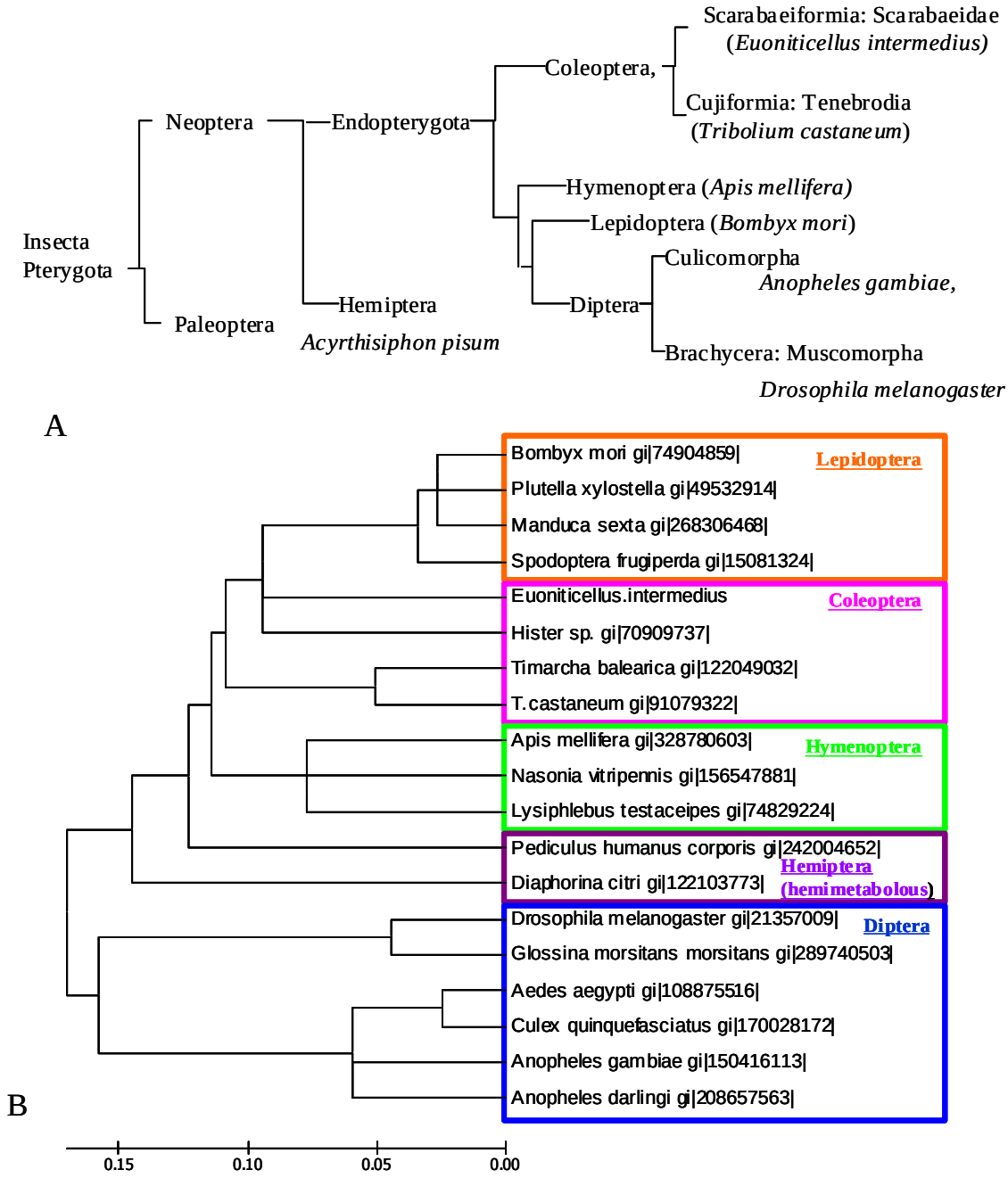


Figure 1.9: Phylogenetic trees showing relative location of *E. intermedius* to other arthropod model organisms. (A) Depicts the accepted taxonomic tree of the insect group. Orders containing model organisms are depicted showing their relative location to *Euoniticellus intermedius.* The aphid is the only model hemimetabolous insect that has been studied thus far. (A) Also shows that the closest model organism to the dung beetle is the tenebroid beetle *Tribolium castaneum*. Both are polyphagus beetles, lacking a prothoracic pleuron. They then divide into the families of Scarabaeformia and Cujiformia. (B) Is an alignment of ribosomal protein L18a sequences using the MUSCLE algorithm. The tree was constructed using the neighbour joining method. This tree confirms these relationships. The individual orders are marked in coloured boxes.

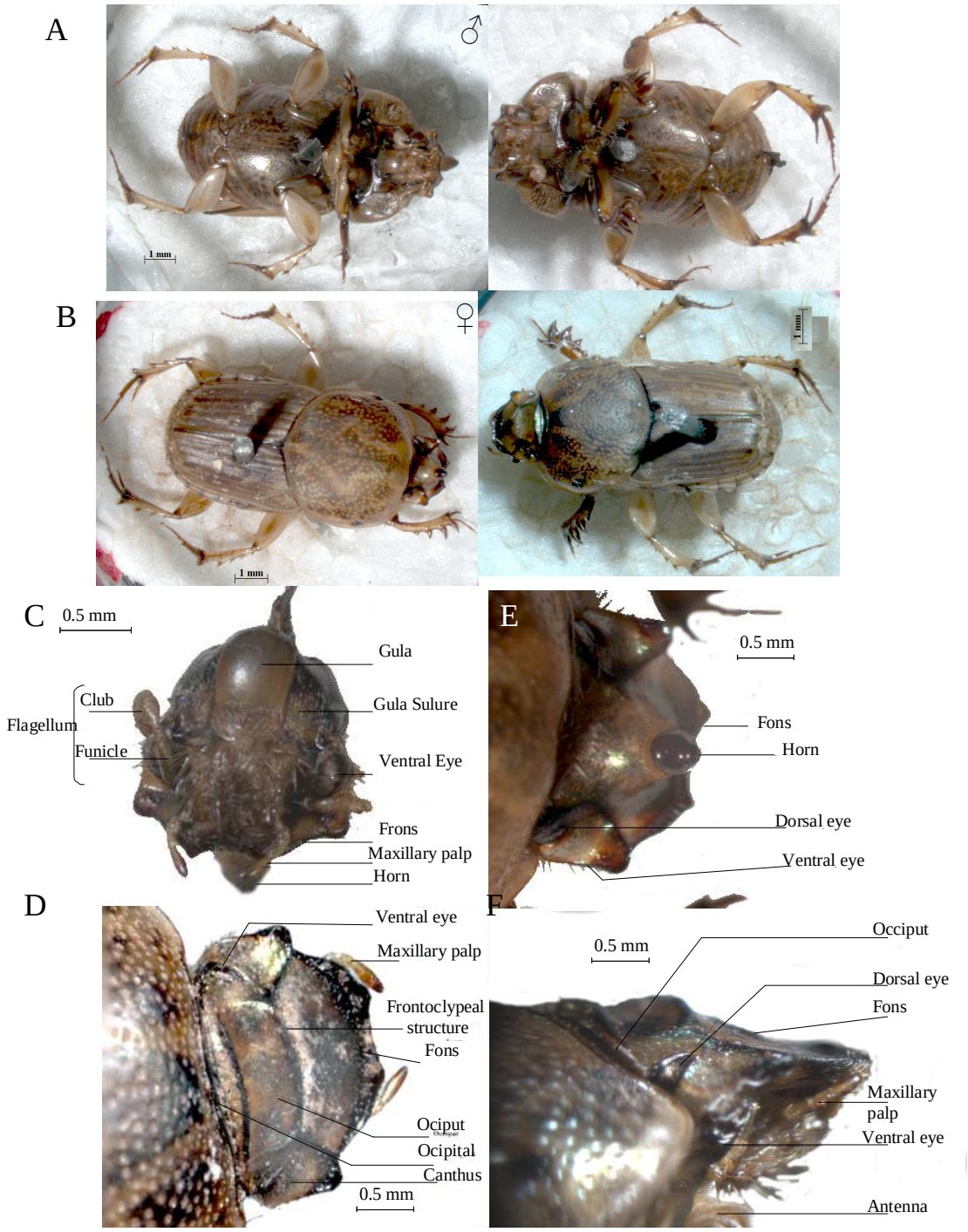


Figure 1.10: External Morphology of *E. intermedius.* **(**A) Adult male (B) adult female (C) (D) Head of adult female. (E)(F) Head of adult Male.

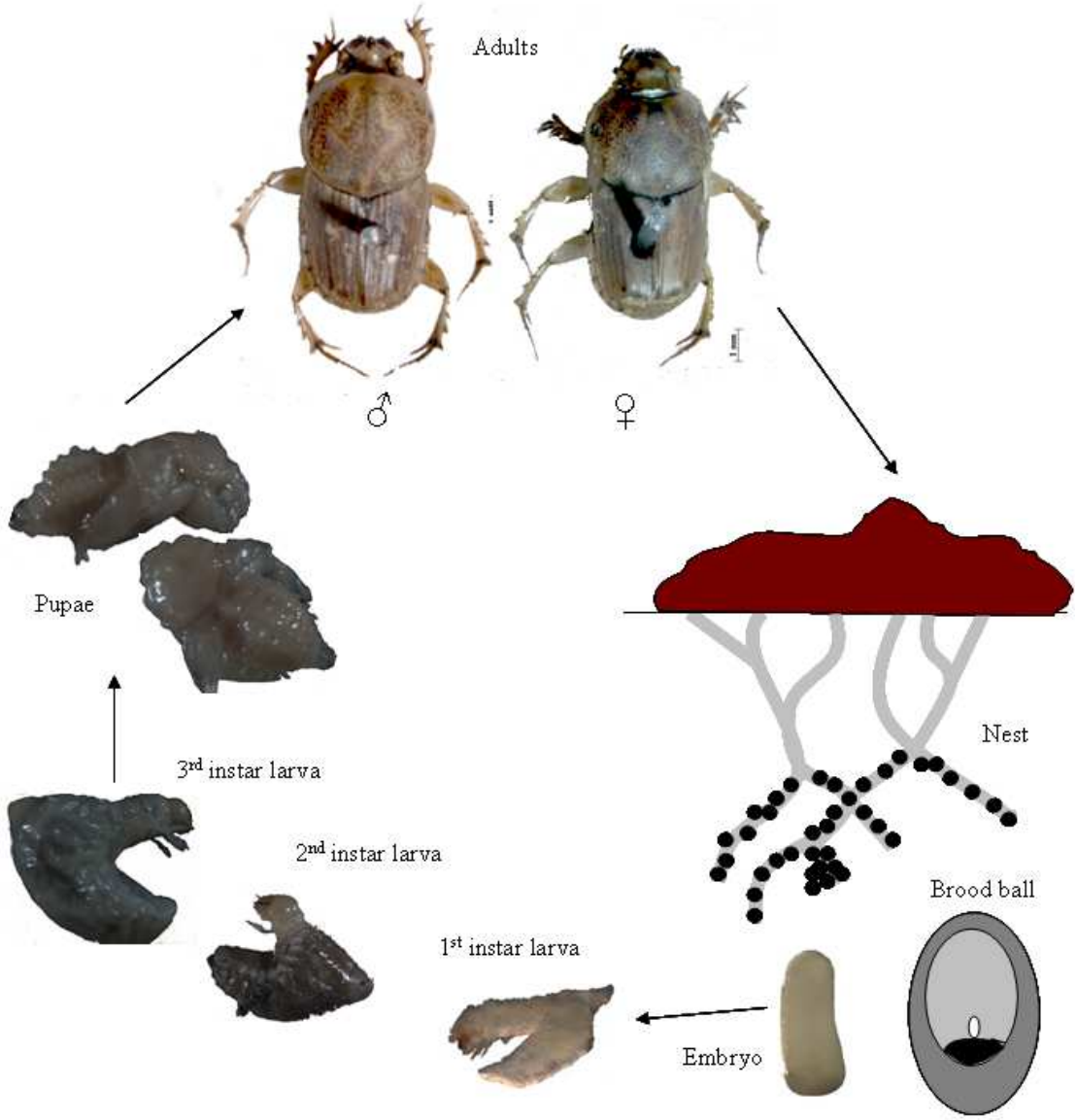


Figure 1.11: Life cycle of *E. intermedius.* Adult female beetles are able to produce at least two embryos per day depending on the temperature. These embryos are placed into broodballs, which are buried along tunnels dug by adult beetles of both sexes beneath the dung pat. These embryos take approximately 5 days to develop into larvae. The three larval stages last approximately 3 weeks, before forming a pupa.

1.8. *Drosophila melanogaster.*

Since 1910 when Thomas Hunt Morgan used *Drosophila melanogaster* in experimental studies of heredity at Columbia University, the fly has been an ideal model organism for genetic studies in eukaryotes. This is because they are easy to care for, have quick generation times and a high fecundity. Despite their complexity they have only four chromosomes that are easily visualized making genetic studies with them easy. Finally the complete sequencing of their genome and the availability of many techniques facilitate genetic manipulations. *Drosophila melanogaster* is also being used as a genetic model to study many human diseases such as Parkinson's, Alzheimer's, and Huntington's. This is due to the fact that about 75% of all genes in humans that cause disease have a homologous match in the fruit flies. The fly is also being used to study stress response mechanisms such as aging and oxidative stress, immune response, starvation, heat shock as well as toxicology.

1.9. Aims of this study.

The Aims of this study were to analyse the gene expression changes that constitute a stress response in two separate insect species, to two different stressors. Namely the response of the fruit fly, *Drosophila melanogaster* to DNA damage and the response of the dung beetle *Euoniticellus intermedius* to infection.

This study explored the impact of camptothecin on normal cells using *D. melanogaster* as a model organism. Camptothecin was selected as a DNA damaging agent as its mechanism of DNA damage is well known. The fruit fly is well characterised and could prove to be a useful tool to study the pathological effects of

cancer drug treatments as well as the differences in the response of normal and abnormal cells to camptothecin induced DNA damage. One of the major aims of this study was to assess the role of the fruitfly RBBP6 homolog SNAMA in the response of normal cells to DNA damage. We have investigated changes in the expression of genes involved in apoptosis such as *Reaper, Snama* and *Dmp53* in wild type flies upon camptothecin treatment. Specifically an attempt was made to establish a role if any for the Snama protein in the regulation of Dmp53 following DNA damage in *Drosophila melanogaster*. Changes in the expression levels of these genes were detected at the RNA level through the use of Northern blots and Semiquantitative Reverse Transcription PCR. Changes in expression levels of these proteins were detected using western blots. Changes in the overall protein expression in *D. melanogaster* were monitored using 2D PAGE and Mass spectrophotometry. The effect of the drug on the reproductive ability of flies and their offspring was also studied.

Another aim of this study was to assess the immune defence pathways of the dung beetle *Euoniticellus intermedius.* In order to achieve this we studied the changes in the protein expression pattern from beetles following infection with fungi. Specifically, attempts were made to isolate antimicrobial peptides from immune challenged *E. intermedius.* Proteins were extracted from whole beetles as well as from the hemolymph only. Changes in protein expression patterns and levels were monitored using HPLC, 2D PAGE and mass spectrophotometry. Additionally the presence of antimicrobial peptides was monitored by performing bacterial inhibition assays. Protein sequences obtained from mass spectrophotometry were used to design primers and isolate the cDNA coding for these proteins using RT-PCR.

Chapter 2

Material and Methods

2.1 Materials

- 2.1.1: Media
- 2.1.2:Buffers and solutions
- 2.1.3: Stains
- 2.1.4: Living organisms
- 2.1.5: Primers
- 2.1.6: Antibodies

2.2 Methods

- 2.2.1 Organism maintenance and challenging
- 2.2.2 Genomic DNA preparation
- 2.2.3 Topoisomerase I assay
- 2.2.4 Acridine orange staining
- 2.2.5 Protein extraction
- 2.2.6 Protein purification
- 2.2.7 SDS-PAGE analysis
- 2.2.8 Western blotting
- 2.2.9 TCA precipitation
- 2.2.10 Two-dimensional gel electrophoresis and Mass Spectrophotometry
- 2.2.11 GST assay
- 2.2.12 Inhibition assays
- 2.2.13 Chemical characterization of inhibitory sample
- 2.2.14 RNA isolation
- 2.2.15 Northern blots
- 2.2.16 Design of degenerate primers and RT-PCR
- 2.2.17 General Molecular Biology techniques
- 2.2.18 Quantitative PCR

2. Material and Methods

2.1 Materials.

2.1.1 Media.

Buffer	Components in buffer	Supplier
Apple Juice agar	1.25% sucrose 2% agar 0.025% streptomycin 0.25% methyl paraben 25% (v/v) Apple juice	 Sigma cat# S6501 Sigma cat# H6654
Cornmeal Molasses agar	1% sucrose 0.75% agar 5% molasses 7.5% cornmeal 2.5% yeast 0.05% methyl paraben	 Sigma cat# H6654
Larval glucose agar	7.5% glucose 2.5% agar 7.5% yeast 0.15% methyl paraben 0.025% streptomycin	 Sigma cat# H6654
Radial diffusion assay culture media	3% Tryptic soy broth	Merck cat# HG00C16.500
Underlay agar	0.03% Tryptic Soy Broth 1% agarose 0.02% Tween	
Overlay agar	6% Tryptic Soy Broth 1% agarose	

2.1.2 Buffers and solutions.

Buffer	Concentration in buffer	Supplier
Extraction buffer	0.1% Trifluoroacetic acid 10 μg/ml Aproptinin 20 μM Phenylthiourea 1 μM Leupeptin 100 μg/ml PMSF	Sigma cat#27242 Sigma cat#A1153 Sigma cat#P7629 Roche cat#1017101 Sigma cat#P7626
Western Stripping buffer	2 % SDS 62.5 mM Tris HCL pH 6.8 100 mM β-mercaptoethanol	
2D PAGE equilibration buffer I	6 M urea 2% SDS 0.375 M Tris HCL pH 8.8 20% glycerol 2% DTT	
2D PAGE equilibration buffer II	6 M urea 2% SDS 0.375 M Tris HCL pH 8.8 20% Glycerol 2.5% iodoacetamide	
2D PAGE rehydration buffer	8 M urea 2% CHAPS 50 mM DTT 0.2% Bio-lyte 0.5 % Bromophenol blue	 Sigma cat# C3023 Bio Rad cat# 1632094
RIPA buffer	1% Nonidet p-40 0.1 % SDS 0.5% Sodium deoxycholate made up to volume with PBS	
Tris tricine Anode buffer (10x) pH 8.9	1 M Tris 0.225 M HCl	
Tris Tricine Cathode buffer (10X) pH 8.25	1 M Tris 1 M Tricine	

	1% SDS	
Tris Tricine Gel Buffer (3X) pH 8.45	3 M Tris 1 M HCL 0.3% SDS	
Camptothecin	50 mM In DMSO	Sigma cat# C9911
Methyl Pyruvate	90% Meythyl pyruvate	Aldrich cat#371173
Staurosporine	2mg/ml in DMSO	Sigma cat# S4400
Drosophila Embryo Nuclear Protein Extraction Buffer I	1 mM DTT 0.1 mM EDTA 15 mM HEPES-KOH, pH 7.6 10 mM KCl 5 mM $MgCl_2$ 100 μg/ml Phenylmethylsulfonyl Fluoride (PMSF) 2 μg/ml Aprotinin 0.5 mM EGTA 50 μg/ml Leupeptin 0.35 M Sucrose	
Drosophila Embryo Nuclear Protein Extraction Buffer II	1 mM DTT 50 μg/ml Leupeptin 0.1 mM EDTA 15 mM HEPES-KOH, pH 7.6 10 mM KCl 5 mM $MgCl_2$ 0.8 M Sucrose 2 μg/ml Aprotinin 0.5 mM EGTA 100 μg/ml PMSF	

2.1.3 Stains.

Stain	Concentration	Supplier
Shevchenko, (1996) Silver stain		
Fixative	50% Methanol 5% Acetic acid	
Wash	50 % Methanol	
Sensitisation solution	0.02% Sodium thiosulphate pentahydrate	
Silver nitrate solution	0.1% $Ag(NO_3)_2$	
Developer	0.1% Formaldehyde 2% Sodium carbonate	
Destain	10% Nitric acid	
Conventional silver stain		
Fix	45% Methanol	
	12% Acetic acid	
Wash	5% Methanol	
	7% Acetic acid	
Sensitiser	10% Glutaraldehyde	
Silver Stain	0.2% Silver nitrate	
Wash	10% Sodium carbonate	
Developer	2% Sodium Carbonate	
	0,01% Formaldehyde	
Radial Diffusion assay destain	2% DMSO	
	20% Acetic acid	
Radial diffusion assay stain	27% Methanol	
	15% Formaldehyde	
	0.002% Coomasie Blue	
Acridine orange	Acridine orange 5ug/ml in 0.1 M	Sigma cat#A-6014
	sodium phosphate buffer	
	Equal volume of Heptane	

2.1.4 Living organisms.

Euoniticellus intermedius	Prof Marcus Byrne of the school of Animal Plants and Environmental Sciences at the University of the Witwatersrand
Drosophila melanogaster Wild type Canton S	
$y^1 w^{1118}; p53^{5A-1-4}$	3.3kb deletion in p53 gene. Obtained from Bloomington Drosophila Stock Center at Indiana University
Escherichia coli	Prof Jean-Marc Reichhart of the Institut de Biologie Moléculaire et Cellulaire, Strasbourg, France
Micrococcus luteus	
Beauveria bassiana	
Bacillus subtilus	Ms Monika Mathies of the School of Molecular and Cell Biology at the University of the Witwatersrand
Serratia marcesans	
Morganella morganii,	Department of Microbiology from the Medical school at the University of the Witwatersrand
Candida albicans	
Staphalycoccus aureus	

2.1.5 Primers.

Primer name	Sequence 5' → 3'
Snama primers	
DWNN forward	TTCATATGGATCCATGTCGGTACACTAT
Ringfingertail	CGAACAAAGCTTCTCCTTGCAATCG
DWNN1	GCATGCATGTCGGTACACTAT
DWNN2	AAGCTTGGCGATGGGGATGCG
DWNN3	AAGCTTCTACTTCTTTGATTT
Real timeSnama forward	ATGCGGCTGAGAACGAATC
Real timeSnama Reverse	TCACTATCCTCTGACTCCAATAAG
Snama probe NED	CCTTTTGTGACGACTGTGTGCGAACCTC
Dmp53 primers	
Drosp53 for	TAGCCGGAATTCATGTATATATCACAGCCAATG
Drosp53 rev	CCAGATGAGCTCTCATGGCAGCTCGTA
Real time Dmp53 forward	CATTCAGATCCAGGCGAACAC
Real Time dmp53 reverse	GGCGGCTCATCCAGAACC

Reaper for	AAACCAGAATTCATGGCAGTGGCATTCTAC
Reaper rev	TTACTCGAGCTCTCATTGCGATGGCTTGCG
RP49 primers	
Realtime rp49forward	CCAGTGGGATCGATATG
Realtimerp49reverse	CCAGGAACTTCTTGAATCC
RP49 probe VIC	ACAACAGAGTGCGTCGCCGCTTC
M13 promoter primers	
M13 Forward	GTTTTCCCAGTCACGAC
M13 Reverse	CAGGAAACAGCTATGAC
Primers based upon peptide sequences obtained from MALDI-TOF analysis of *E. intermedius* proteome	
2102a	ACTGGTGGTATGAATGCTGACGGT
2203a	ACTAGCTATCCAGGTAATTTGCCA
3004a	TTGGACGCTTTGGGTCAGGAATGT
3304a	GTTGTTTTGACTGCTGCTCATGCT
4001a	GAAGCTAGCACTTTGGCTGAATTT
4002a	GTTAGCAGCGCTACTTTTGTTAAA
5102Ua	TTGTATACTGCTGAACAGTTGGAA
5102Fa	GGTTTGACTCCAGAACAGTTGGAA
5310a	TATGCTCCAGGTGGTGTTGCTAGC
6005a	TTGAGCCAGTTTGACGACGACGTT
6203a	AGCCAGACTTTGGAAACTTTGGCT
7106a	AGCTTGTTGGACAGCTATCCAACT
7301a	GCTAGCGTTTTGAATGCTGACACT

2.1.6 Antibodies.

Anti-Snama raised in Rabbits	The Anti-Snama was produced in Rabbits by Professor Theresa Coetzer of the School of Biochemistry, Genetics and Microbiology at the University of KwaZulu Natal using heterologous protein produced by Mr Brent Oosthysen. The antibody was then purified by Mr Brent Oosthuysen
Anti *Drosophila* p53	Santa Cruz Biotechnologies cat#Dd21
Anti actin	Santa Cruz Biotechnologies cat#C-11

2.2 Methods.

2.2.1 Organism maintenance and challenging.

2.2.1.1 Beetle maintenance.

Beetles were bred in 160 mm x130 mm x 130 mm plastic containers that were halfway filled with soil. Cow dung was placed on the soil and single or multiple breeding pairs of beetles (1 male and 1 female) were placed within the containers. Every 3-4 days, fresh cow dung was placed within the container and once a week the containers were sieved and any broodballs were removed. If the breeding pair survived they were placed in a new container with fresh soil and dung.

Collected broodballs were placed in a large 400 x 300 x 200 mm plastic container and these were covered with compacted soil. A wet sponge was then placed on the soil to keep it moist. Once beetles began to emerge small plastic dishes filed with dung were used as traps to capture them. These were used as new breeding pairs or in experimental work.

2.2.1.2 Fly maintenance.

All flies were reared at 25°C on standard cornmeal agar food plus yeast. *Canton-S* was used as the wild-type strain in all experimental work. The mutant fly line y^1 w^{1118}; $p53^{5A-1-4}$ was used to determine the effect camptothecin had on p53 null mutant flies. These flies contain a 3.3kb deletion in p53 gene.

2.2.1.3 Pathogens and Toxins.

2.2.1.3.1 Bacterial pathogens.

All species of bacteria were maintained on LB agar plates containing no antibiotics. Bacterial cultures of *M. luteus* and *E. coli* were prepared by inoculating 2ml of LB broth and growing the culture until the cultures A_{550} were equal to 0.6. Beetles were then immobilised using CO_2, and as many mites as possible were removed using a heated inoculation loop. A steel needle was then heated over a flame and this was used to pierce the beetle's exoskeleton just behind the second leg. A 25 ul Hamilton syringe was used to inject some of the bacterial suspension into the hole created by the needle. The beetles were then moved to a substitute habitat after infection to decrease the chance of any other pathogen that was present in the soil or dung from infecting the beetle. The beetles were left overnight in order to mount an immune response.

2.2.1.3.2 Fungal pathogens

Beauveria bassiana cultures were initiated by cutting a fungal plug out of the original plate and placing them onto a fresh malt agar plate. The fungus was allowed to grow until it covered the plate. *B. bassiana* fungal spores were collected by adding sterile de-ionised water to the malt agar plate containing the fungal lawn. The spores were then suspended by rapid agitation with a sterile spatula. This suspension was filtered through sterile glass wool, and collected in a sterile tube. Spore number was determined using a haemocytometer, and diluted to $2x10^7$ spores/ml using 50%

glycerol. This spore suspension was used to inoculate fresh malt agar plates for the purpose of infection and antimicrobial assays.

2.2.1.3.3 Beetle infection.

Beetles were placed on a fungal plate in the formula of 1 beetle per 6.36 cm^2 of plate area. The plate containing the beetles was shaken for 5 minutes and then the beetles were removed and placed on a fresh malt agar plate and left overnight to mount an immune response. To enhance infection during this procedure a spore suspension of 1000000 spores/ml was sprayed onto the beetles and dung using a spray bottle with the nozzle set to produce a fine spray.

Control beetles were not infected with any pathogen; however for the bacterial infections a hole was still punched in the beetle's exoskeleton, but no bacteria were introduced. For the fungal infection, beetles were shaken on an empty plate with no fungus present. Beetles were transferred to an empty Lb plate and left in a dark container overnight at 25^0C.

2.2.1.3.4 Camptothecin and methyl pyruvate exposure of flies.

Camptothecin was used to treat Canton S as well as p53 mutant flies. They were fed camptothecin at a concentration of 1 mM by mixing it into yeast blobs. 0.5 ml of yeast paste was measured using a syringe and placed into a vial containing cornmeal agar. To this 10 μl of 50 mM camptothecin was added and stirred into the yeast. A negative control was created by adding nothing to the yeast in a third separate vial. This same

procedure was followed when the flies were exposed to methyl pyruvate at a concentration of 10%.

2.2.1.4 Mating of flies exposed to camptothecin.

To investigate the effect of camptothecin on the reproductive biology of flies, mating experiments were set up by mating five virgin female flies with a single male of a similar age. The flies were placed in duplicate vials according to the combinations of camptothecin treatment and sex. Flies were given food treated with 1 mM camptothecin or with DMSO for two days and then removed to new vials containing untreated food. They were then mated in the following groups. Untreated females with untreated males; treated females with untreated males; untreated females with treated males; treated females with treated males; DMSO fed females with DMSO fed males; DMSO fed females with untreated males; untreated females with DMSO fed males; DMSO fed females with camptothecin fed males; camptothecin fed females with DMSO fed males.

The number of embryos present on the feeding plates, were then counted every 24 hours starting from the time that the flies were first mated and transferred to untreated food. Embryos from day one were then transferred to cornmeal media and the number of pupae developing from the embryos was counted as well as the number of adults to finally emerge. Successful development was then calculated in terms of percentage of embryos that reached the pupal stage and the percentage of embryos to reach adulthood. Mating experiments were then repeated including samples treated with methyl pyruvate and camptothecin and methyl pyruvate. This was done to see if providing the flies with the end product of glycolysis would prevent the reproduction

anomalies that were observed. In total this was performed to give a sample size of six biological replicates for each sample. These replicates were then used to obtain the mean standard deviation and standard error values for the number of embryos produced and the number of embryos that reached the adult and pupal stages.

Finally these experiments were repeated with flies that lack *Dmp53* altogether. The mutant line y^1 w^{1118}; $p53^{[5A-1-4]}$ contains a 3.3 kb deletion in the *Dmp53* gene. These flies were exposed to camptothecin in order to establish whether the phenotypic responses were related to the presence of Dmp53. These exposures were repeated to give a sample size of three biological replicates.

In order to test for significant differences in the number of embryos produced or the percentage of embryos that survived to adulthood, a Tukey's test was performed in conjunction with a one way ANOVA and a student's-t test.

2.2.1.5 Validation of *dmp53* double mutants.

The presence or absence of *Dmp53* in the mutant line y^1 w^{1118}; $p53^{[5A-1-4]}$ was confirmed through the use of *Dmp53* specific primers.

PCR was then performed as follows.

DNA sample	400 ng
Primers	0.3 µM
10X PCR buffer with 15 mM $MgCl_2$	1X
dNTP mix	2.5 mM
Expand High Fidelity enzyme mix	0.1 U/µl

The cycle conditions were: denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 68°C for 1 minute over 35 cycles.

2.2.1.6 Mortality assays.

The mortality rates of untreated flies and flies exposed to camptothecin were observed. One hundred newly eclosed males and one hundred newly eclosed females were exposed to camptothecin. Another two hundred flies were left untreated. The mortality rate was observed, recorded and the data was used to perform a Kaplan Mier survivability plot.

2.2.2 Genomic DNA preparation.

Genomic DNA was prepared using a protocol based on the method described by Bender (1983). Flies were collected and homogenised in 100 μl grinding buffer. After a 30 minute incubation at 70°C, 35 μl of 8 M KOAc was added and incubated on ice for 30 minutes to pellet the cellular debris and protein. The DNA was the precipitated with isopropanol and washed with 70% ethanol before being resuspended.

2.2.3 Topoisomerase I assay.

2.2.3.1 Preparation of cytoplasmic and nuclear protein extracts from *Drosophila* embryos.

Wild type flies were exposed to 1 mM camptothecin and embryos were collected and dechorionated in 5% bleach. They were then homogenized in nuclear protein buffer 1

with inhibitors and centrifuged at 7,700 X g for 15 min at 4°C to pellet the yolk and nuclei. The supernatant containing the cytoplasmic extract was removed and stored. The top white layer of the pellet containing the nuclei was removed and homogenized in nuclear extract buffer I with protease inhibitors. This homogenate was then layered onto nuclear extraction buffer II and centrifuged at 1,310 X g for 30 min at 4°C. The pellet was then resuspended in Topoisomerase buffer.

2.2.3.2 Assay for topoisomerase I activity.

The presence or activity of topoisomerase I in the protein extracts was tested for by determining the ability of the extracts to alter the topology of supercoiled plasmid DNA. The assay was performed with 2.5 μg of supercoiled pUC18 DNA incubated with 5 μl of purified nuclear or cytoplasmic embryo protein extracts. This was replaced by water in the negative control. The reaction was performed using 1X Topo I Reaction Buffer with the final volume being 50ul. The reaction was incubated at 30°C for 30 min and stopped via the addition of Stop Solution. Five biological replicates were performed and the results of the assay were analysed using a chi squared distribution with the expected number of bands versus the observed number of bands for each of the treatments.

2.2.4 Acridine orange staining.

To demonstrate the occurrence of apoptosis, embryos were stained with acridine orange as described by (Abrams *et al.*, 1993). Briefly, embryos were collected from apple juice agar plates and dechorionated in bleach for 2 minutes. They were then placed in an equal volume of heptane and 5 μg/ml acridine orange (Sigma cat#A-

6014) in a 0.1 M sodium phosphate buffer, pH 7.2 (0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4). The staining reaction was left to proceed for 5 minutes with shaking. Embryos at the interphase between the stain and heptane were removed and placed on a cavity slide containing halocarbon oil. The slides were then viewed and photographed using the Axioskopmot 2 fluorescence microscope. Five biological replicates were performed for each sample with the number of embryos with abnormal levels of apoptosis being counted. The means of these numbers were compared between the treatments using a Tukey's test performed in conjunction with a one way ANOVA.

2.2.5 Protein extraction.

Proteins were extracted from *D. melanogaster* in one of two ways depending on the intended use for the sample. For western blots, flies were homogenised in 5 X SDS-PAGE sample buffer in a ratio of 10 flies per 50 µl of sample buffer. Samples were then centrifuged at 13000 x g for 30 minutes and the supernatant was moved to a fresh tube. Samples for 2D PAGE were prepared by homogenizing flies in RIPA buffer containing protease inhibitors.

Before the extraction of protein from the beetles, their sex was recorded and the males were checked for nematode parasites. The beetles were killed and placed into a mortar and ground under liquid nitrogen. The ground beetles were then transferred to a tube and peptide extraction buffer was added in the ratio of 1ml per 2 beetles. The extraction buffer was based on acidic extraction and contained 0.1% trifluoroacetic acid to obtain the necessary pH of 3. It also contained 10µg/ml aprotinin and 15 µM phenylmethylsulfonyl fluoride as protease inhibitors. Lastly it contained 20 µM

phenylthiourea to prevent melinisation by sequestering the copper ions needed for the catalytic activity of tyrosinase, which is the major enzyme involved in the formation of melanin (Nappi, 2005). The tube containing the ground beetles in extraction buffer was placed on a suspension mixer for 30 minutes at 4^0C. It was then spun at 13000 x g for 30 minutes at 4^0C. The supernatant was collected and placed in a new tube.

The peptide concentration was established using the Bradford method (Bradford, 1976). A standard curve was constructed using 10 μl of a range of standard bovine serum albumin concentrations, and 1ml of Bio-Rad dye reagent.

2.2.5.2 Collection and preparation of hemolymph samples.

Hemolymph was extracted from beetles before and after fungal infection. The exoskeleton of the beetle was pierced using a heated tungsten spike. A 10 μl microsyringe was then used to withdraw between 2 and 5 μl of hemolymph from each beetle. The extracted hemolymph was diluted 1:4 in extraction buffer. The protein concentration was measured by the Bradford method (Bio-Rad cat# 500-0001) (Bradford, 1976).

2.2.6. Protein purification.

2.2.6.1 High Salt Cation exchange column.

The cation exchange matrix consisting of a methacrylate polymer containing sulfonate functional groups was packed into the 0.5 mm diameter columns, to produce a 1ml void volume column. The column was connected to a peristaltic pump and the flow

rate was adjusted to 1ml/min. The crude protein extracts were added to the column and pumped through. One void volume of 0.05 % Trifluoroacetic acid was then added to the column. The flow through from both these elution steps was collected, and combined. In order to further separate the sample into different fractions, 0.1 mM Tris pH 7.0 buffer was pumped through the column. The low pH of the 0.05% Trifluoroacetic means that proteins with a pH greater than 3 would remain positively charged and bound to the column. The tris buffer would elute all those proteins with a pI greater than 7.0.

Finally 1 M NaCl was added to the column to elute all the proteins still bound to the column. This sample was expected to contain most of the antimicrobial peptides and the majority of this sample was then applied to the c18 columns.

2.2.6.2 C18 Sep pak vac column.

The C18 columns were 0.8 ml void volume columns from Waters. The purification procedure involved the use of three different concentrations of acetonitrile in 0.05% trifluoroacetic acid. These concentrations were 0%, 40 % and finally 80% acetonitrile. The columns were activated using methanol and washed with two void columns of 0.05 % trifluoroacetic acid. Following the addition of the sample to the column, 1 void of 0.05% trifluoroacetic acid was added to the column and the flow through was captured. This was followed by the serial addition of the various acetonitrile solutions in increasing order. Solutions were pumped through the column using a 20ml syringe inserted into the top of a tube leading through a rubber stopper, which was inserted into the top of the c18 column.

2.2.6.3 Cleaning and concentration of samples.

Acetonitrile was then removed from the samples by exposing them to a stream of nitrogen gas for approximately 30 minutes. Samples were then lyophilized in a freeze dryer to concentrate the samples.

2.2.6.4 Molecular weight cut off purification.

Samples were further purified and concentrated using a stirred cell concentrator with a 10kDa molecular weight cut off membrane. A nitrogen gas pressure of 200 kPa was used to drive the cell. The stirred cell was operated according to the manufacturers instructions. This would result in all the proteins smaller than 10 kDa being pushed through the membrane and being collected in the tube.

2.2.6.5 Reverse phase HPLC.

HPLC was performed on a Shimadzu VP series machine. The run consisted of an initial 5 minutes with no acetonitrile following sample loading. The acetonitrile gradient was increased at a constant rate of 2.4% per minute such that 30 minutes into the run the acetonitrile concentration was 60 %. This percentage concentration was kept constant for five minutes and then decreased to 0% over 5 minutes at a constant rate of 12% decrease per minute. The entire length of the run was 40 minutes. The eluant was collected in separate tubes at 1ml per tube per minute. At the end of the HPLC run there were 40 samples each named with a suffix describing the minute when the sample eluted.

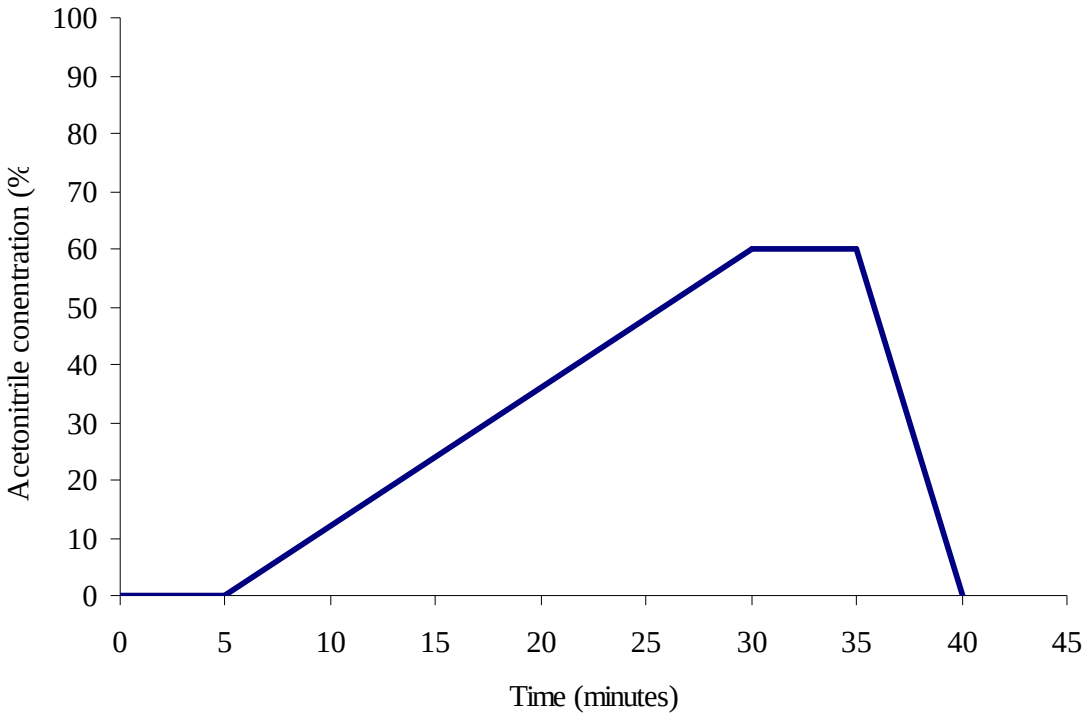


Figure 2.1: Acetonitrile gradient for Reverse Phase HPLC. The acetonitrile concentration profile of the 40-minute HPLC run shows the initial 5 minutes with 0% acetonitrile concentration. The acetonitrile concentration was then increased at a constant rate of 2.4% per minute. At 30 minutes the acetonitrile concentration was 60 %. This percentage concentration was kept constant for five minutes and then decreased to 0% over 5 minutes at a constant rate of 12% decrease per minute.

2.2.6.6 Peptide concentration.

The concentration of the peptide samples was determined using the Bradford method and a UV VIS spectrophotometer. The final standard curve covered the peptide range of 0.2 mg/ml to 2 mg/ml and involved an assay volume of 1ml. Seven separate assays were performed and these were used to calculate the mean absorbance readings as well as standard deviation and error readings. These values were then used to construct the final standard curve.

2.2.7 SDS--PAGE analysis.

2.2.7.1 Size estimation and banding pattern.

In order to identify changes in the peptide samples differing in the stage of purification as well as in inhibitory ability, the samples were electrophoresed on 12, 15 and 20% SDS polyacrylamide gels (Laemmli, 1970). Protein bands were visualised by staining the gels with coomasie or silver. The gels were scanned on the GS800 densitometer and the banding patterns were analysed using the quantity one software. This software was also used to determine the molecular weight of the different bands located on the gels.

2.2.7.2 Tris Tricine SDS-PAGE.

To improve the resolution of SDS-PAGE and 2D PAGE, Tris Tricine gels were run according to (Schagger et al 1987) with some alterations (Schagger 2006). The separating gels were 16% gels with a 4% stacking gel. The gels were run using at a constant power of 10 watts (100mAmp and 100V).

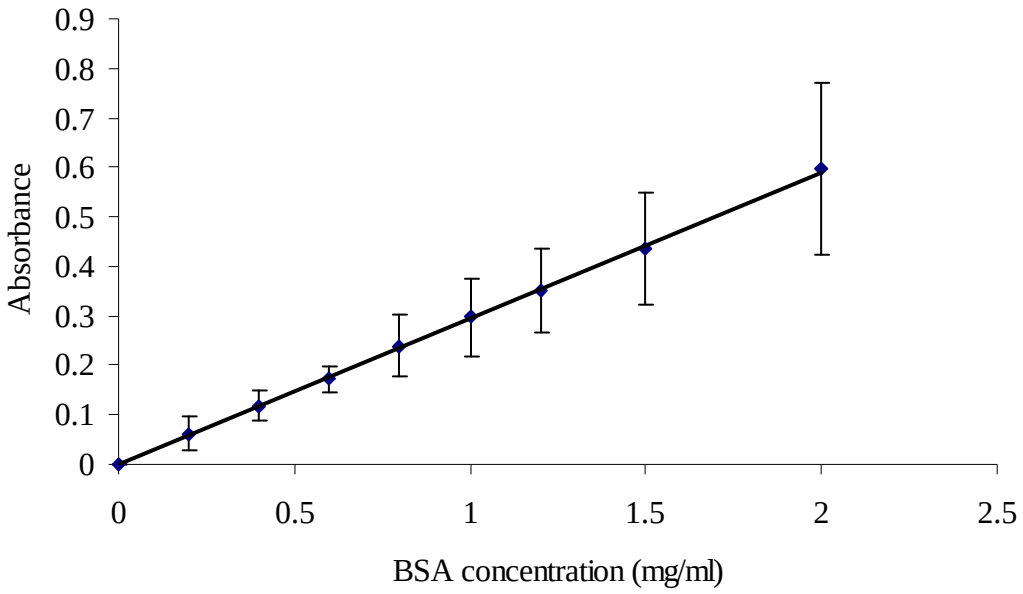


Figure 2.2: Standard curve for protein concentration determination using the Bradford assay. The standard curve for protein concentration determination consisted of 10 replicates. The mean values were entered into the spectrophotometer and this was used as the actual curve. The curves equation is given as y = 0.2956x - 0.00004 and an $R^2 = 0.9994$.

2.2.8 Western blotting.

Proteins samples were separated by SDS-PAGE and transferred using Towbin buffer to a Hybond– P: PVDF membrane (Amersham Bioscience Cat#1487) in a Hoefer mini VE blotting cassette (Amersham Bioscience cat# PH 80-6418-96). Non-specific binding was prevented by blocking in SuperBlock™ Dry Blend Blocking buffer (Pierce cat #37545). The blot was probed with either a 1:5000 dilution of anti-Dmp53 (Santa Cruz Biotechnology p53 d-200, 200 μg/ml) for 1 hour or a 1:5 000 dilution of anti-Snama antibodies. The p53 (d-200) antibody is a rabbit polyclonal antibody raised against amino acids 186-385 of p53 of *Drosophila melanogaster* origin. The anti- Snama antibody is a chicken polyclonal antibody raised against the DCM region of heterologously expressed denatured Snama. A goat polyclonal anti-actin (C-11) IgG (200 μg/ml) raised against the C-terminus of Actin of human origin was used as an internal control. Following two ten minute washes PBS-Tween, the membrane was incubated for an hour in a 1:10000 dilution of an anti-chicken IgG peroxidise conjugate produced in rabbit (Sigma Aldrich cat# A9046) to detect anti-p53. In order to detect anti-Snama, the membrane was incubated for an hour in a 1:10000 dilution of an anti-rabbit IgG peroxidise conjugate produced in goats (Sigma Aldrich cat# A 0545), while the anti actin was detected by incubating the membrane for an hour in a 1:5000 dilution of anti-goat IgG peroxidise conjugate, produced in rabbits (Pierce biotechnology cat# 31402). The secondary antibodies were removed and the membrane was washed 6 times 10 minutes each with PBS-Tween.

The blot was stripped according to the manufacturer's instructions. It was then blocked once again and probed with the alternate primary antibody. The specifically

bound probe was detected using the SuperSignal® West Pico chemiluminescent substrate (Pierce Cat# 37077) as per manufacturers' instructions.

2.2.9 TCA precipitation.

Before peptide or protein samples could be analysed using 2D PAGE, they had to be precipitated with trichloroacetic acid to remove contaminants. An equal volume of 20% TCA was added to the protein sample. This was then incubated for 30 min on ice and spun down at 1300g for 15 minutes at 4 degrees. The pellet was washed with ice-cold acetone. The pellet was then air dried and resuspended in Rehydration buffer.

2.2.10 Two-dimensional gel electrophoresis and Mass spectrophotometry.

2.2.10.1 Two-dimensional gel electrophoresis.

In order to further separate and isolate individual proteins of interest for mass spectrophotometry, differently treated samples were run on a 2D PAGE gel. The first dimension was performed on IPG strips for use in a Protean IEF system. Five flies were ground in RIPA buffer and large debris was excluded by centrifugation and protein concentration was determined using the Bradford assay (Bio-Rad cat# 500-0001). Equal amounts of each sample were purified and concentrated using a 2D PAGE sample preparation kit (Bio Rad cat# 163-2130) or through TCA precipitation. The resulting pellet was re-suspended in 125 ul of 2D re-hydration buffer (8M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-lyte, 0.5 % Bromophenol blue). The IEF strips (Bio-Rad cat# 1632099) were placed face down into the sample and covered with mineral oil and passive re-hydration was allowed to occur for 12 hours. The strips

were then moved to a focusing tray where isoelectric focusing was performed according to the following program: An initial low voltage (250V) 20 minute linear ramping step, followed by a high voltage (4000V) 2 hour linear ramping step. The final step was a rapid ramping step for 10000 volt-hours (vh). Hemolymph protein extracts and whole beetle extracts were prepared in the same fashion. Isoelectric focusing was performed with the program being altered for whole beetle extracts to include a second 500v 30-minute low voltage linear ramping step. Three biological replicates were performed for both the fly whole protein extracts and the beetle hemolymph extracts.

Once isolelectric focusing was complete the strip was removed from the focusing tray and placed in a new re-hydration tray where it was covered with equilibration solution 1 (6 M urea, 2% SDS, 0.375 M Tris HCl pH 8.8, 20% glycerol, 2% DTT). After equilibration for 10 minutes, solution 1 was replaced with equilibration solution 2 (6 M urea, 2% SDS, 0.375 M Tris HCl pH 8.8, 20% Glycerol, 2.5% iodoacetamide) for a further 10 minutes. The strips were then briefly placed in SDS running buffer and then placed on top of a 12% polyacrylamide separating gel. They were then subjected to electrophoresis for 2 hours at 150 volts and stained with Coomasie blue.

2.2.10.2 Image Analysis.

The gels were digitised using a PDQuest 2-D Analysis Software Version 6.2 (Bio Rad cat#170-9630). Spot detection was carried out with a sensitivity setting of 112.21 using the camptothecin treated and fungal infection gels as the master gels. Spots were initially matched using the programs automated matching function. Extended matching was then performed using the classical matching function. Both these

methods involve matching spots based on the position of landmark spots. Manual spot matching and analysis was then performed across the gels images.

Normalization was performed automatically by the program, based on the total quantity in all valid spots option. This method assumes that changes in density average out across the gels being analysed.

Briefly the normalisation formula used is as follows

$$\text{Normalised spot quantity} = \frac{\text{Raw spot quantity} \times \text{Scaling factor}}{\text{Normalisation factor (total quantity in all valid spots)}}$$

The scaling factor used was 10^6 parts per million.

The density values obtained for each of the three biological replicates were used to calculate the mean OD as well as the standard deviation and error for each spot.

2.2.10.3 MALDI-TOF Mass Spectrometry.

All mass spectroscopy analysis was performed at the Plateforme Protéomique of the Institut de Biologie Moléculaire et Cellulaire in Strasbourg. Selected spots were manually excised from the gels and placed in a 5% acetic acid solution. After tryptic in-gel digestion following the method of Rabilloud et al, the proteins were analysed by matrix laser desorption-time-of-flight (MALDI-TOF) and mass spectroscopy. NanoLC-MS/MS analysis of the digested proteins was performed using a CapLC capillary LC system (Waters, Altrincham, UK) coupled to a hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Q-TOF Micro, Waters). The sample (5 µL) was first concentrated and cleaned in a C18 PepMap precolumn cartridge (LC Packings) and then separated on-line by the analytical reversed-phase capillary column (Pepmap C18, 75 µm i.d., 15 cm length; LC

Packings) under a 200 µL min^{-1} flow rate. The gradient profile used consisted of a linear gradient from 97% A (97.9% H_2O; 2% ACN, 0.1% [v/v] HCOOH) to 95% B (98% ACN, 1.9% H_2O, 0.1% [v/v] HCOOH) in 45 min followed by a linear gradient to 95% B in 3 min.

The spray system (liquid junction) was used at 3.6 kV. Mass data acquisitions were piloted by MassLynx 4.0 software (Waters). NanoLC-MS/MS data were collected by data-dependent scanning, that is, automated MS to MS/MS switching. Fragmentation was performed using argon as the collision gas and with a collision energy profile optimised for various mass ranges of ion precursors. Four ion precursors were allowed to be fragmented at a time. Mass data collected during a NanoLC-MS/MS analysis were processed and then submitted to *de novo* sequencing. Fragmentation spectra were loaded onto the Peptide Sequencing software (BioLynx, Waters) and the sequences were processed manually before being submitted to database searches.

2.2.10.4 Statistical and Bioinformatic analysis of protein sequencing data.

For the 2D PAGE analysis of flies exposed to camptothecin the peptides were identified using the MASCOT program based on the mass peptide fingerprint obtained for the tryptic digest of the peptides (Perkins et al., 1999).

The peptide fragments obtained from the MS/MS analysis of the fungal infected and uninfected *Euoniticellus intermedius* proteins were assigned an identity in using three different database searches. Firstly a MASCOT search was performed to obtain a probability based identification of the protein based on the sequences obtained.

Secondly a BLAST search was performed using the NCBI databases in order to identify proteins that contained similar sequences to those identified by MS/MS. Finally BLAST searches were carried out using the Flylab genome base database consisting of EST sequences of the adult transcriptome.

Proteins were organised into functional classes using gene ontology based on biological processes. Functional enrichment was performed using a Fisher's exact test, with the sum of the mean OD values for each functional class being compared to the total OD for all proteins detected in both the camptothecin treated and untreated group. Increases in the expression of individual peptides was analysed using a student's two sided T- test as well as an F- test of the equality of two variances.

2.2.11 GST assay.

An enzyme assay of one of the identified proteins was performed in order to confirm that the changes in the optical density values observed in the 2D PAGE gels, did reflect real changes in protein amount and therefore activity. A Glutathione S Transferase assay was chosen as it is a low cost, fast and well established assay. The assay was performed as per the method of (Habig et al., 1974). The basis of the assay is the conjugation of reduced glutathione to 1-Chloro 2,4-dinitrobenzene (CDNB) which produces a dinitrophenyl thioether which can be detected by spectrophotometer at 340 nm. Therefore increased GST activity leads to an increase in the absorbance at 340nm.

Glutathione –SH + CDNB -> Glutathione –S-CDNB

In order to determine the activity of the GST the slope of the linear region of the curves were determined using the following equation.

$$DA340 = \frac{A340\ (Time\ 2) - A340\ (Time\ 1)}{Time\ 2\ (min) - Time\ 1\ (min)}$$

One unit of enzyme will conjugate 10.0 nmol of CDNB with reduced glutathione per minute. GS-DNB extinction coefficient at 340 nm 0.0096 μM-1 cm-1.

$$GST\ activity\ \mu mol/min/mL = \frac{A340\ min\text{-}1 \times 0.3\ mL\ Reaction\ Volume}{0.0096\ \mu mol\text{-}1\ cm\text{-}1 \times 1000\ mL \times 1\ cm \times 0.01 ml.}$$

Six biological replicates were performed and these were used to obtain a mean value for the GST activity as well as standard deviation and standard error values.

2.2.12 Inhibition assays.

2.2.12.1 Solid and liquid media inhibition assays.

Inhibition assays were performed using *E. coli,* to represent Gram negative bacteria and *M luteus* to represent Gram positive bacteria. Fungal pathogens used in the inhibition assays were *B. bassiana* and *S. cerevisae.*

Both solid inhibitions on agar plates as well as liquid inhibitions in multiwell plates were performed. The solid media inhibitions were based on the addition of 100μg of peptide to filter paper discs that were placed onto plates that had been streaked with bacteria. The diameter of any clearings and the thickness of the agar were recorded for the calculation of the MIC at a later stage. Three biological replicates were performed for each set of samples against each micro-organism. This was used to obtain the means, standard deviation and standard error values for the diameter of the clearings.

Liquid assays were based on A_{600} readings of inoculated LB media with and without the addition of 100µg of peptide samples. The effect of the peptide on the growth of the bacteria was determined using the following formula (Barbault et al., 2003).

$$\frac{A_{595}\text{ (test well)} - A_{595}\text{ (medium)}}{A_{595}\text{ (well without peptide)} - A_{595}\text{ (medium)}} \times 100$$

Cultures were also spread plated on LB plates and the colonies on these plates were counted, and compared with each other using the following formula.

$$\frac{\text{Colony count test plate}}{\text{Colony count control plate}} \times 100$$

Three biological and three technical replicates were performed for each sample against each of the pathogens. The technical replicates were used to generate a mean for each sample. These means were then used to generate a mean for all the biological replicate values. These values were also used to generate standard deviation as well as standard error values.

2.2.12.2 Radial diffusion assays.

Radial diffusion assays were based on the technique of Leher et al 1991. Briefly 50ml Tryptic Soy Broth cultures of *E.coli* or *M luteus* were grown for approximately 18 hours at 37^0 C. 50 µl of this culture was used to inoculate a fresh 50ml Soy broth culture, which was grown for a further 3 hours at 37^0 C. The culture was then spun down and washed and then resuspended in cold 10 mM sodium phosphate buffer. The optical density was then measured at 620 nm, and this was used to calculate the volume of culture that would contain $4x10^6$ colony forming units per ml using the following formula.

0.2 $O.D_{620}$ = $5x10^7$ CFU/ml.

This Volume of bacteria culture was then added to 15ml of underlay agar. Once the plate had set, holes were punched in the agar and peptide samples as well as a negative control consisting of 0.1% TFA as well as a positive control consisting of tetracycline was added to the holes. The plates were allowed to incubate for 3 hours at 37^0 C. 15 ml of overlay agar was then added to the plate. The plates were incubated for 18 hours at 37^0 C. The plates were then stained overnight, destained and the diameter of the clearing was then measured (0.1mm =1U). Three technical replicates and six biological replicates were performed for each sample. The technical replicates were used to generate a mean for each sample. These means were then used to generate a mean for all the biological replicate values. These values were also used to generate standard deviation as well as standard error values.

2.2.13. Chemical characterization of inhibitory sample.

2.2.13.1 Proteinase K assay.

Inhibitory samples were treated with protienase K in order to establish if a protein is responsible for the inhibition of bacterial growth. The sample was treated with proteinase K as per the manufacturer's instructions. Basically proteinase K was added to the sample to a final concentration of 100µg/ml. The same volume of water was added to a negative control sample. Two final controls consisted of 100µg/ml of proteinase K diluted to the same volume as the other samples with water and the same volume of water with nothing further added. The four reactions were incubated at 37 ^{0}C for 15 minutes and were then heated to 70 ^{0}C for 15 minutes in order to inactivate

the proteinase K. Solid and liquid inhibition assays were performed on all four samples. The liquid inhibitions had a further control that consisted of ampicillin at the concentration of 100 μg/ml.

This assay was repeated to give three biological replicates. These values were then used to calculate the mean zone of clearing for each sample as well as the standard deviation and error values.

2.2.13.2. Heat stability of inhibitory peptide.

Due to the structure of certain families of antimicrobial peptides they tend to be very heat stable. Therefore the heat stability of the antimicrobial samples was determined using liquid media inhibitions assays. Six aliquots of an inhibitory sample were heated to six different temperatures, five of them ranging from 50-90 ^{0}C and one at 20 ^{0}C. The samples were cooled on ice and then used in a liquid inhibition assay using ampicillin and the room temperature (20 ^{0}C) sample as controls. The successfulness of these inhibition assays was established using spectrophotometry and colony counts.

This assay was repeated to give three biological replicates. These values were then used to calculate the average inhibitory activity for each sample as well as the standard deviation and error values.

2.2.14 RNA isolation from flies and beetles.

The RNA extraction procedure was based on that of Chomcynski 1993, using the TRIzol LS reagent (Life technologies Gibco-BRL). Larvae and adults were both homogenized in 500µl of TRIzol. Adult beetles were homogenized in a ratio of two beetles per 500µl of TRIzol. RNA was then recovered as per the manufacturer's instructions. The RNA pellet was then dissolved at 65^0 C in 50 µl nuclease free water. Samples were snap cooled on ice and the concentration was determined on a spectrophotometer at 260 nm.

2.2.15 Northern blots.

Isolated and quantified RNA was then divided into 60 µg aliquots. These were then precipitated with 2.5 volumes of 100% ethanol. The ethanol was removed and the pellets were re-suspended in 60 µl RNA loading buffer. The samples were re-suspended by heating at 65^0 C for 10 minutes followed by snap cooling on ice. The RNA samples were then loaded in 20 µl triplicates onto a 1% agarose gel containing 6% formaldehyde and cast-using 1x MOPS buffer. The RNA was then transferred onto a Hybond™ nylon membrane by capillary transfer using 10 x SSC as the transfer buffer. Blotting was allowed to continue for 16 hours. The RNA was then fixed to the membrane by placing the membrane into a UV stratalinker, where the membrane was exposed to 120 000µjoules/cm^2 for 30 seconds.

2.2.15.1 Preparation of DNA probes.

Probes were prepared for all the following genes; *Rp49, Dmp53* and *Snama.* The *rp49* probe was obtained from the pJM954 vector containing the cDNA for the *Drosophila*

ribosomal protein *Rp49*. The *Rp49* fragment was excised from the plasmid using the restriction enzymes Apa1 and Hind III. The restriction digest was then run on a 1% agarose gel and the fragment was cut from the gel and purified using the Genelute gel cleanup kit. The *Dmp53* was excised from the pGEM-T-Easy-*Dmp53* construct (Zakwe unpublished) using EcoR1 and Xho1.Probes for *Snama* were created using the Polymerase Chain Reaction. *Snama* was amplified from the pot-2 vector containing the cDNA for the DWNN domain of Snama, using the DWNN forward and ringfingertail primers.

2.2.15.2 Labelling of DNA probes with [α ^{32}P] dCTP.

The rediprime ™II random prime labelling system was used to label the probes based on the manufacturer's instructions. Approximately 25ng of DNA was diluted to a volume of 45µl using TE buffer. The DNA was then denatured at 95^{o}C and snap cooled on ice. The denatured DNA was then added to the rediprime ™II reaction tube mixture consisting of dATP, dGTP, dTTP, random primers and Klenow enzyme. 50µCi of [α-P^{32}] CTP was then added to the tube and the reaction was incubated at 37^{0}C for 20 minutes. The reaction was stopped by the addition of EDTA to a final concentration of 0.02 mM.

2.2.15.3 Hybridisation, detection and stripping blots.

Blots were hybridised using the Ultra-hyb buffer (Ambion cat#8669) following the manufacturers protocol. The membrane was then placed in the buffer for 30 minutes at 42^{0}C to pre-hybridise. The DNA probes were denatured by heating to 95^{0}C and snap cooling in ice. 12.5 µCi of the probe was then added to the hybridization buffer.

Hybridisation was allowed to proceed overnight. The non-specifically bound radiolabels were removed by 2 washes with 0.1X SSC buffer at 65°C. Blots were wrapped in UV-transparent wrap and autoradiography was performed at –70°C.

The probe was removed from the blot using a boiling solution of 0.5% (w/v) SDS. The membrane was left to cool to room temperature and was then placed in hybridization buffer to pre-hybridised. The probing procedure was then repeated with a new probe.

2.2.16 Design of degenerate primers and RT-PCR.

The peptide sequences obtained from the MS/MS analysis of hemolymph protein extracts purified on 2D PAGE, were used to design primers using the *Drosophila melanogaster* codon usage. These primers were then used as the forward in RT-PCR reactions using adult beetle RNA. The reverse primer was an oligodt (17t) primer. RT-PCR was performed using the two-step Improm II kit from promega. First strand cDNA was produced using the oligo dt primer. The $MgCl_2$ concentration used in the cDNA synthesis was 3 mM.

Table 2.1: Amplification parameters of the second step RT-PCR reaction for different primers

Primer	Annealing temperature (C^0)	$MgCl_2$ concentration (mM)	Extension time (minutes)
2102a	63	2 mM	1
2203a	60	2 mM	1
3004a	62	2 mM	1
3304a	No band obtainable	No band obtainable	
4001a	60	2 mM	1
4002a	63	2 mM	1
5102Ua	61	2 mM	1
5102Fa	64	2 mM	1
5310a	63	1.5 mM	1
6005a	61	1.5 mM	1
6203a	60	2 mM	1
7106a	65	1.5 mM	1
7301a	60	2 mM	1.5

Table 2.2 details the modifications made to the PCR reaction components and conditions for each of the primers used in conjunction with oligo dT17, in order to obtain product.

2.2.17. General Molecular Biology techniques.

2.2.17.1 Competent cells.

Chemically competent *E. coli* XL-1 Blue cells were prepared using a calcium chloride based procedure (Mandel and Higa, 1970). A single bacterial colony was used to inoculate a LB culture, which was grown overnight and then diluted 100 times with LB. The culture was left to grow at 37ºC until an OD_{600} of between 0.5 and 0.6 was reached. The cells were then chilled and centrifuged at 5000 x g for 10 minutes at 4ºC. The pellet resuspended in half the culture volume of ice-cold 100 mM $MgCl_2$. This was followed by a 30 minute incubation on ice followed by centrifugation and resuspension in a tenth of the original culture volume of ice-cold 100 mM $CaCl_2$ containing 15% glycerol.

2.2.17.2 Ligations.

The DNA band of interest was extracted from an agarose gel stained with Sybr Gold and viewed on a dark reader to prevent DNA damage by UV light. Extraction was done using the Genelute agarose purification kit from sigma. Purified PCR products were ligated into the pGEM®-T Easy using the pGEM®-T Easy vector TA-cloning system.

2.2.17.3 Transformation.

Half of the ligation reaction mixture of 10µl, containing approximately 25 ng of vector with 75ng of insert, was added to 45µl of competent *E. coli* cells. The mixture

was then incubated on ice for 20 minutes and heat shocked for 90 seconds at 42ºC. The transformed cells were made up to 1 ml with LB and incubated for 1 hour at 37ºC. 100 µl of transformed cells were then spread on LB supplemented with ampicillin plates. These plates were then incubated overnight at 37ºC.

2.2.17.4 Colony Screening.

Plasmid DNA was prepared from small (2ml) overnight cultures of *E. coli* using the alkali lysis method (Birnboim 1979). This extraction was further purified using phenol: chloroform: isoamyl alcohol (25:24:1). The plasmid DNA was re-suspended in water. Restriction analysis reactions were performed to confirm the presence of an insert in the isolated plasmids. The corresponding 10x buffer constituted 10% of the total reaction with unit of enzyme for each µg of DNA. The reaction was carried out at 37^{o}C for 6 and the restricted DNA was analysed on a 1% agarose gel.

2.2.17.5 Colony PCR, sequencing of DNA and construction of phylogenetic trees.

The presence and size of inserts was also confirmed using colony PCR with M13 forward and reverse primers, which bind to either side of the multiple cloning site in the pGEM-T-Easy vector. Plasmids with confirmed inserts were then sent to Inqaba Biotechnical Industries (Pty) Ltd, and sequenced using the standard M13 primers.

Phylogenetic trees were constructed using the MEGA (Molecular Evolutionary Genetics Analysis) software (Tamura et al., 2011) and the MUSCLE algorithm (M**U**ltiple **S**equence **C**omparison by **L**og- **E**xpectation) (Edgar, 2004). Trees were

constructed using the neighbour joining method and a bootstrap test of phylogeny was carried out with 500 bootstrap replications (Efron, 1982) (Felsenstein, 1985).

2.2.18 Quantitative PCR.

2.2.18.1 RT –PCR to confirm RNA levels following DNA damage.

In order to confirm the Northern blot results RT-PCR was performed using the Access RT-PCR kit (promega cat#A1250) using volumes suggested by the manufacturer. RNA samples were collected from flies that had been exposed to camptothecin, methyl pyruvate and a combination of methyl pyruvate and camptothecin, as well as male and female flies that had remained untreated. RNA was also collected two days after treatment and these samples were referred to as recovery samples. RNA was also collected from treated and untreated larvae. RNA was quantified using the samples A260 readings on a nanodrop.

For the *Dmp53*, *Snama* and *Reaper* transcripts the cycling conditions were as follows: 50°C for 30 minutes; 94°C for 5 minutes; followed by 32 cycles (determined to be the midpoint of exponential amplification) of: 94°C for 30 seconds, 60°C for 40 seconds, 68°C for 70 seconds; and a final extension at 68°C for 5 minutes. For *Rp49* the annealing temperatures were lowered to 56°C.

2.2.18.2 Internal standard.

In order to perform semi-quantitative PCR the cycle number at which exponential amplification was occurring had to be established. Amplification reactions of *Rp49*

were used to establish this cycle number by performing 25, 30, 35 and 40 cycle reactions. These were then run on a 1% agarose gel and digitized on a GS800 calibrated densitometer (Bio Rad cat# 170-7980) and the resulting images were analysed using the Quantity one software. This was repeated to give three technical replicates for each sample. The process was also repeated using different samples to give three biological replicates. The average densities of the individual bands were used to plot a curve of product produced versus cycle number. The resulting graph was then used to determine the optimum cycle number where product formation was still occurring at an exponential rate.

2.2.18.3 qPCR.

RNA was extracted from flies exposed to camptothecin and used as a template for reverse transcription using the Improm RT-PCR kit (Promega cat# A380). The concentration of the resulting cDNA was established and equal amounts of template were used in the qPCR. qPCR was performed using Taqman probes (Applied Biosystems) with VIC labels for the *Snama* probe and NED label for the *Rp49* probe. The primers Realtime Snama forward, and reverse, Realtime rp49 forward and reverse and a predesigned assay for *Dmp53* (Applied biosystems cat#Dm02154336_g1) were used in the multiplex qPCR. The reaction was performed in a 25 µl volume, which included 50 ng/ µl of template, 100 nM of each forward primer and 80 nM of each reverse primer and 250 nM of each probe. The 2x Master Mix contained ROX as a passive reference. Amplification and detection were performed using an ABI Prism 7500 sequence detection system (Applied Biosystems cat# 4324018) using the following program 95°C denaturation to 54°C annealing with an extra 62°C extension

step. A melt curve was performed to establish the number of products formed. Accurate biological replicates could only be performed on the *Dmp53* and *Rp49* assay. Three biological replicates were performed and these values were used to calculate the mean value as well as the standard deviation and error.

Chapter 3

The response of *Drosophila melanogaster* to DNA damage

3.1 Introduction

3.2 The physiological effects of camptothecin exposure

3.3 Topoisomerase assays were inconclusive

3.4 Proteomic analysis of *Drosophila melanogaster* following camptothecin exposure

3.5 Methyl pyruvate supplementation

3.6 Effects of camptothecin exposure on *snama*, *dmp53* and *reaper* mRNA levels.

3.7 Changes in the expression pattern of Dmp53, Snama following camptothecin exposure

3.8 Functional roles of Dmp53 following camptothecin treatment

3.9 Discussion

3.1 Introduction.

One of the aims of this study was to monitor the DNA damage response in the model organism *Drosophila melanogaster*. DNA damage was initiated through the use of chemotherapy agent camptothecin. Camptothecin is a plant alkaloid isolated from the asterid *Camptotheca acuminata* (Lorence and Nessler, 2004). Poor solubility and high toxicity has limited the usefulness of camptothecin as an effective anticancer agent. Water soluble camptothecin derivatives were first approved for use as anticancer drugs during the mid-nineties (Lorence and Nessler, 2004),(Chung et al., 2006). Camptothecin is a topoisomerase poison as it inhibits the nuclear enzyme DNA topoisomerase I. This enzyme introduces transient single strand breaks supercoiled DNA, relaxing it. The enzyme re-ligates the nicked strand forming an intact DNA helix again. (Lorence and Nessler, 2004).

A key step in the process is the formation of a cleavable complex between the enzyme and DNA through covalent linkage between a tyrosine in the enzyme active site with the 3' end of the DNA strand. Camptothecin and its derivatives turn topoisomerase I into a cytotoxic poison by stabilising the cleavable complex. This prevents re-ligation, causing multiple breaks in genomic DNA. The camptothecin-DNA-topoisomerase complex is transient because camptothecin does not form covalent bonds with the enzyme (Lorence and Nessler, 2004) (Marchand et al., 2006). Concentrations of camptothecin of 1 µM have been shown to decrease the ability of human topoisomerase to relax supercoiled DNA by as much as 50 % (Holden et al., 1999). In *Drosophila* Topoisomerase I is an essential protein required during embryonic development. It is concentrated in the ovaries and is maternally inherited by the early

embryo (0-2 hours) with maximal zygotic expression in the 6-12 hour period (Lee et al., 1993).

Camptothecin has also been observed to initiate apoptosis in a caspase independent pathway via the formation of acidic organelles (lysosomes). This cell death pathway is also independent of changes in mitochondrial membrane permeability (Ondrousková et al., 2008).

Camptothecin, is known to have negative effects on fertility and reproductive ability in premenopausal women (Tanaka et al., 2008) and mice (Utsunomayi et al., 2008). It is not known whether *Drosophila,* and therefore, other insects will respond in the same way to camptothecin treatment as vertebrates do. However, observations made during the course of exposing the flies to camptothecin led to the monitoring of changes in the ability of the fly to produce embryos following camptothecin exposure. The effect on embryos produced by adult flies exposed to camptothecin was also monitored through fluorescent microscopy. Overall changes in the proteomic response of *Drosophila melanogaster* following camptothecin exposure were monitored through the use of 2D-PAGE combined with MALDI-TOF analysis of protein extracts. The effects of the camptothecin induced DNA damage on the transcription of *snama* and *dmp53* and *reaper* were assessed at the RNA level using Northern blots, semi-quantitative Reverse Transcriptase-PCR as well as Real Time PCR. Meanwhile the effects of camptothecin exposure on the expression levels of Dmp53 and Snama were assessed using Western blot analysis.

The exact role of Snama, the *Drosophila* DWNN containing protein is unknown. Its orthologues such P2P-R, PACT and RBQ1 are involved in cell cycle regulation, whereas the yeast ortholog Mpe1 is involved in mRNA processing. There is evidence that Snama may interact and regulate p53 (Rakgotho, 2007), possibly by ubiquitinating p53 and degrading it in a similar means to that of MDM2 (Brooks and Gu, 2006). Alternatively, Snama may behave as a RBBP6 homolog and negatively regulate p53 through an indirect manner utilizing different signalling networks. Disruption of the mice homolog PACT leads to an accumulation of p53 and increased apoptosis. PACT also enhances the MDM2-p53 association, thereby increasing p53 turnover (Li et al., 2007).

RBBP6 is able to act as an E3 ubiquitin ligase, where it ubiquitinates YB-1 (Y-box binding protein 1) leading to decrease in the levels of YB-1 (Chibi et al., 2008). This may also provide a means whereby RBBP6 is able to regulate p53 as YB-1 is known to repress p53 levels, its negative regulation by RBBP6 means that up-regulation of RBBP6 would lead to an increase in apoptosis. Since this contradicts the positive up-regulation of p53 due to ubiquitination of MDM2. This means that RBBP6 may act on p53 in two separate ways producing two different results (Chibi et al., 2008).

Snama also seems to play a role in development. Here expression is regulated by hedgehog signalling, though not exclusively. Hedgehog signalling is normally associated with cell proliferation and controls cyclin E and D transcription and therefore, cell cycle control (Jones et al., 2006). Loss of Snama seems to be implicated in aberrant DNA synthesis being associated with DNA over-replication (Jones et al., 2006).

In an attempt to understand the role of Snama in DNA damage as well as any role it plays in the regulation of p53, camptothecin was used to damage DNA. Total RNA was extracted and the transcription levels of *Dmp53* and *Snama* were monitored through the use of Northern blot analysis, semi-quantitative reverse transcriptase PCR and quantitative Real Time PCR. The changes in the expression of Dmp53 and Snama, following camptothecin treatment, were monitored through the use of western blot analysis.

3.2 The physiological effects of camptothecin exposure.

As camptothecin causes DNA damage it is potentially mutagenic. Rats exposed to high doses of a camptothecin analogue showed a wide array of symptoms including a high incidence of hair loss; decreased food consumption; high thymic atrophy; visible defects of thoracic and abdominal organs; increases in testosterone and finally a decrease in litter size as a result of an increase in the number of foetal deaths (Chung et al., 2006).

Following camptothecin exposure it was noted that the fecundity of the flies decreased. These observations were related to the number of embryos the affected flies were able to produce, the number of embryos that successfully developed to the larval stage, as well as the time needed for development. In order to obtain quantitative data for these effects, male and female flies were exposed to camptothecin separately. These populations of exposed flies were then mated with populations of untreated male and female flies and with themselves to generate

populations where one sex was treated while the other was not, or populations where both sexes were treated, or untreated.

The numbers of embryos produced by these populations was counted and the adults were removed to new vials. The number of pupae that finally developed from these embryos was then counted. These numbers were used as an indication of development time and success. Finally the numbers of adult flies of both sexes that died were recorded as an indication of the effect of camptothecin on mortality levels.

3.2.1 Camptothecin exposure results in a decrease in the reproductive ability of female *Drosophila melanogaster.*

Figure 3.1A is a representation of the effect of camptothecin on the number of embryos produced. Camptothecin treatment of female flies resulted in a significant decrease in the number of embryos being produced when compared to the untreated control flies ($p = 0.0039$). The low numbers of embryos produced by populations where females had been exposed to camptothecin, shows that the reduced ability to produce embryos is related to camptothecin affecting the female reproductive organs. The populations containing males that had been exposed to camptothecin initially showed little difference in the ability to produce embryos compared to untreated flies ($p = 0.7473$), with significantly higher numbers of embryos than populations with treated females (p value = 0.0098). Towards the later stages of the experiment the embryo production decreased in comparison to the untreated flies. However as can be seen by figure 3.3 this is probably due to increased mortality in the males exposed to camptothecin (see below). The DMSO fed flies show a slightly decreased ability to

produce embryos but this is not significant ($p = 0.6147$). Once again, this may be due to increased mortality.

Camptothecin also affected the ability of embryos to reach the pupal stage of development (figure 3.1B) with the number of embryos from the flies that had been treated with camptothecin that developed into pupae, being significantly lower ($p = 0.0057$) than those from untreated flies. However, these effects do not continue past the pupal stage, and the number of adults that emerge from pupae is not affected by camptothecin treatment ($p = 0.6238$). These effects are common to all populations where one of the sexes had been exposed to camptothecin. Despite camptothecin not affecting the ability of male flies to fertilise the females, it does seem to be lead to heritable damage of their offspring. Therefore, the reproductive cells of the male flies seem to also be affected by the camptothecin treatment. It was also noticed that embryos from fly populations where one or both of the sexes had been exposed to camptothecin, showed longer development times (data not shown) and took one to two days longer to reach the pupal stage than embryos from untreated flies.

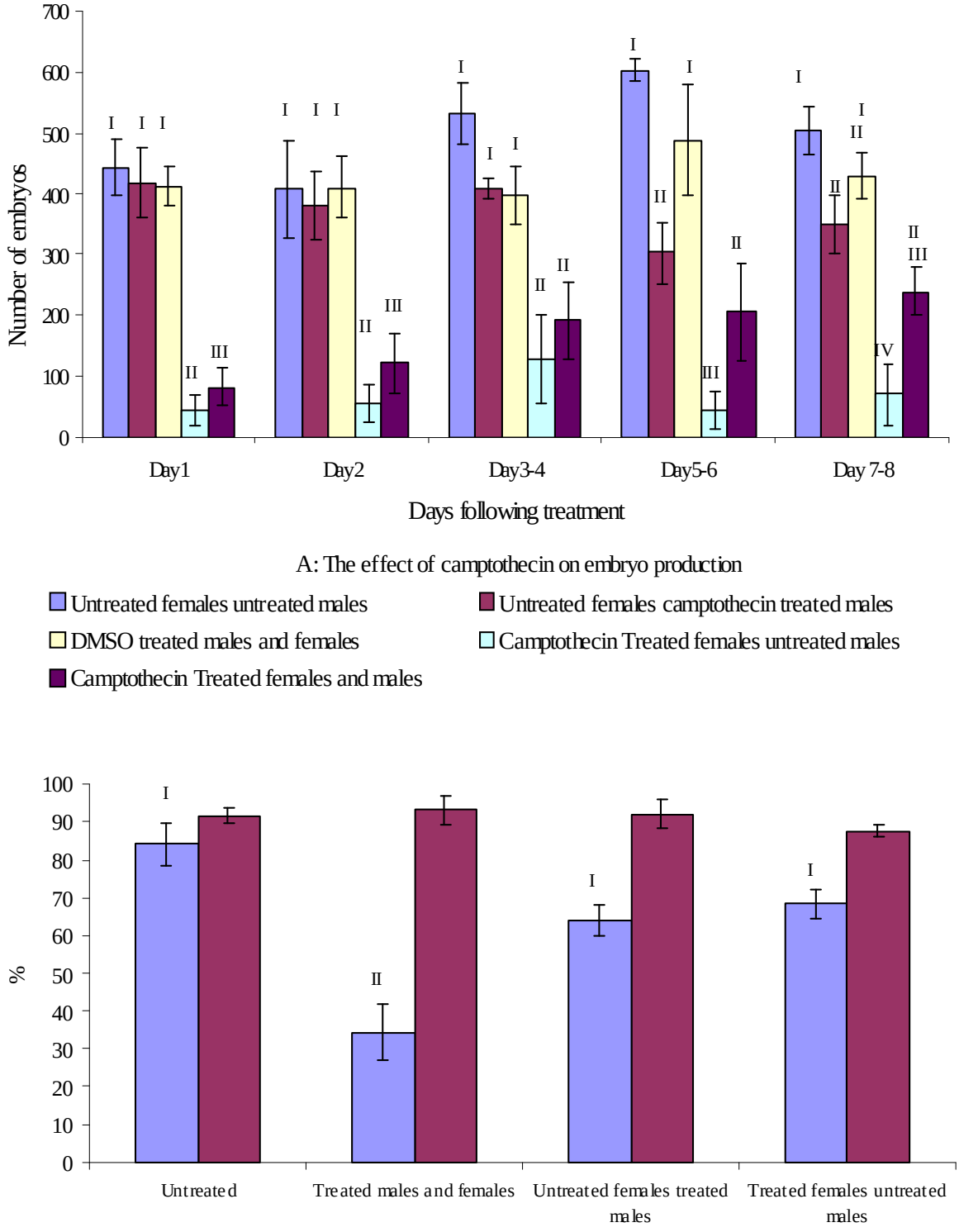


Figure 3.1: The influence of camptothecin on *Drosophila* fecundity and development times**.** (A). Populations containing females that had been exposed to camptothecin showed a significantly ($p = 0.003$) decreased ability to produce embryos. The error

bars reflect the standard error of the mean of biological replicates. The roman numerals represent homogenous groups where there is no significant difference between the means. The effects of camptothecin exposure on larval and pupal development are shown in (B) A significantly higher percentage of embryos produced when both sexes had been exposed to camptothecin, also failed to develop into pupa ($p = 0.057$). These effects were decreased but still significantly higher when only one of the sexes was exposed to camptothecin ($p = 0.046$). However, in all populations those embryos that managed to develop into pupae were equally successful at developing into adults ($p = 0.628$). The error bars reflect the standard error of the mean of biological replicates.

Increased development time is a classical indication of increased apoptosis in fly embryos (Li et al., 1999). Additionally these negative effects are transmitted from the fly male or female to the embryo, where they decrease the survival rate of the embryos. A situation similar to that seen in rats (Chung et al., 2007).

3.2.2 Staurosporine exposure has no effect on the female's fecundity.

In order to establish whether the observed decrease in embryo number is due to the specific action of camptothecin and not due to reduced energy and resource use for reproduction by flies responding to stress, flies were exposed to staurosporine. This toxin inhibits protein kinase C (Tamaoki et al., 1986) and therefore initiate apoptosis in a different manner to camptothecin. Following exposure the number of embryos produced was counted, and as figure 3.2 shows the number of embryos produced on days 1 and 2 remained high in the flies that were exposed to staurosporine, compared to untreated flies ($p = 0.4108$). This implies that initially there is no significant difference between the embryo production of staurosporine treated and untreated flies. Egg production decreased significantly at around day 3 ($p = 0.0127$), but this was due to increased mortality in the samples where the flies had been exposed to staurosporine, which consequently lowered the number of embryos produced.

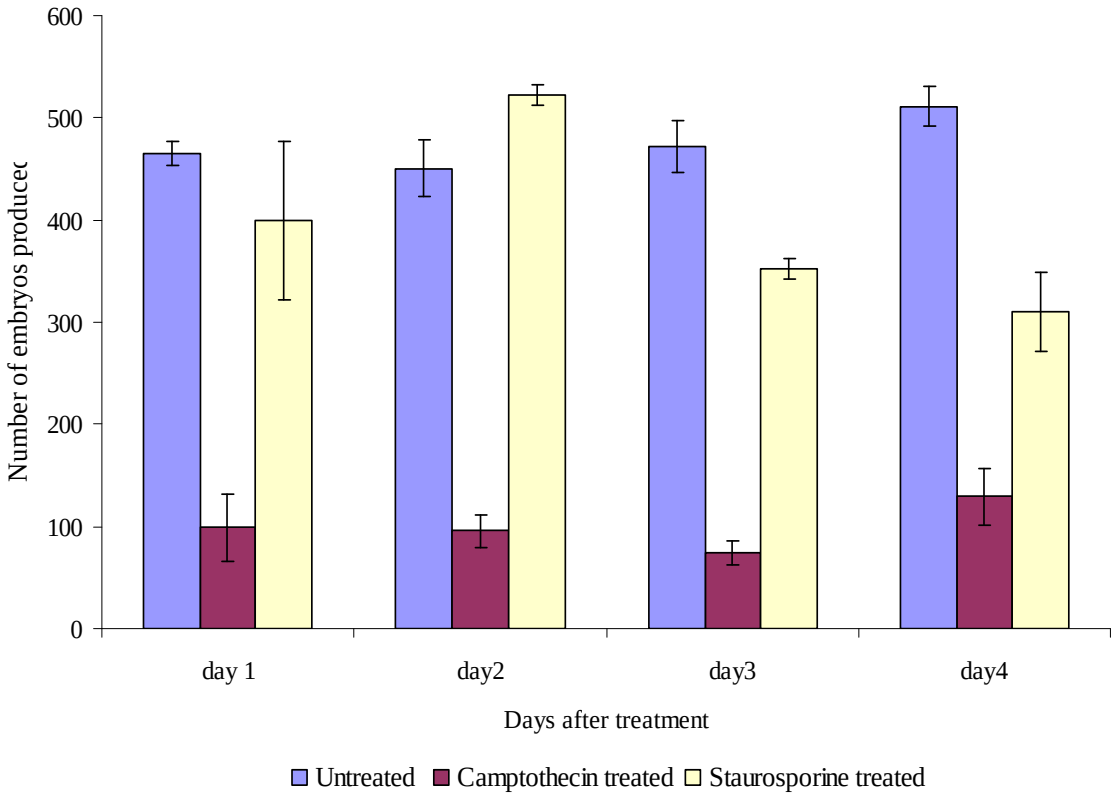


Figure 3.2: The effect of the protein kinase C inhibitor, Staurosporine on the ability of female flies to produce embryos. Exposure of flies to staurosporine did not result in a significant decrease in the number of embryos that were produced ($p = 0.4108$). This implies that the decrease in fecundity is a particular result of camptothecin exposure and is not due to any general stress response resulting in a decrease in reproduction. The error bars represent the standard error of the means of biological replicates.

3.2.3 Camptothecin decreases the survival probability of male and female flies.

Figure 3.3 is a Kaplan Meir survivorship plot of two hundred flies of different sexes that have been treated with camptothecin or left untreated. The plot clearly shows an increase in the mortality rate of flies of both sexes once they had been fed camptothecin. All populations show a similar survival probability until days 3-4 where the mortality rate of the treated males and females increases. This increases sharply again on day 5. After this the probability of survival stays constant until day nine. The increase in the mortality observed on day 9 in the untreated females and is probably due to a factor separate from camptothecin exposure. Therefore, it can be concluded that the mortality due to camptothecin peaks rapidly after exposure at around day 5. After this the flies seem to recover. However, on day 11-12 there is another sharp increase in mortality in those flies exposed to camptothecin. This seems unlikely to be due to direct effects caused by camptothecin and may be due to some secondary effects such as an increase in ROS or may be due to the high energy costs of the initial stress response. The difference in the survival probability between untreated males and females is unsurprising as *Drosophila* females tend to live longer than males (Tower, 2006).

Therefore, a consequence of camptothecin treatment is the increase in mortality resulting from fatal physiological stress in the form of DNA damage and the generation of ROS.

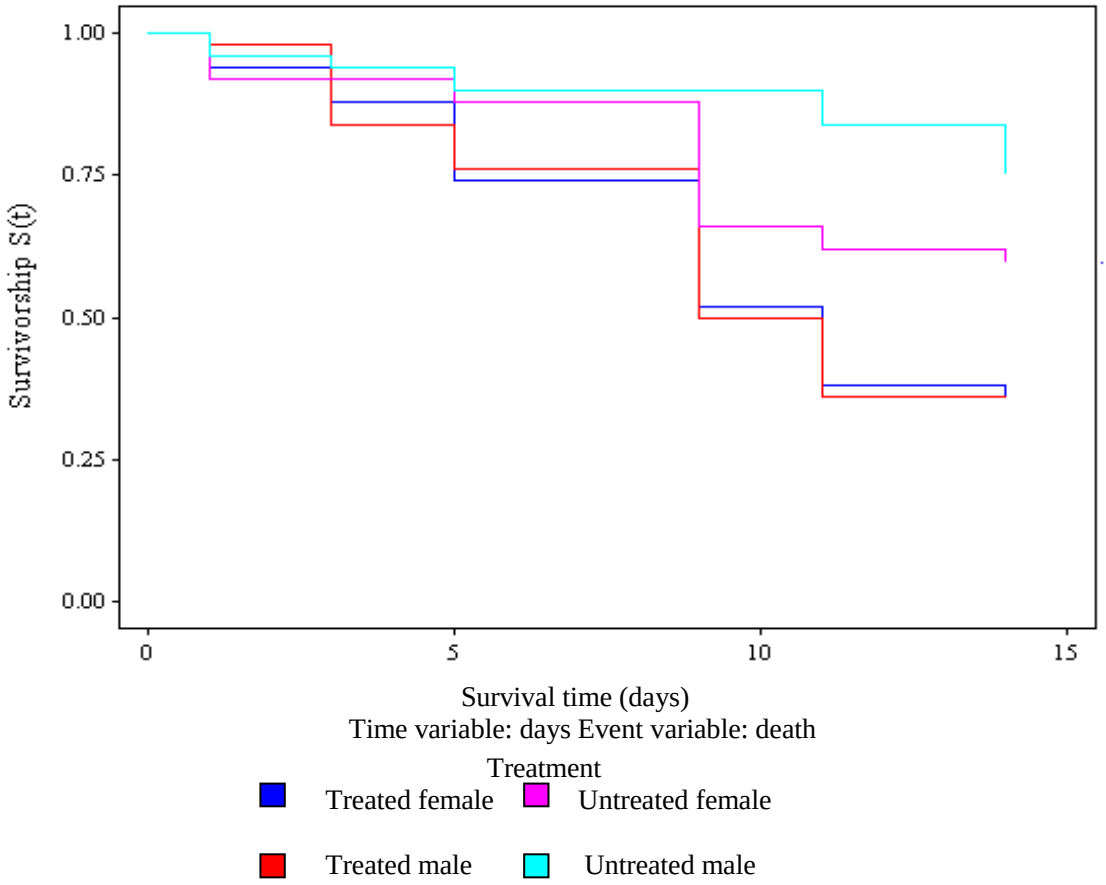


Figure 3.3: The Mortality rate of untreated flies compared to those exposed to camptothecin. A Kaplan-Meier product limit estimate of the survival functions of each individual fly grouped according to sex and treatment was performed at a 95% confidence interval. The resulting survivorship plot showed that both sexes fared poorly when treated with camptothecin. Untreated males and females both showed decreased survival probability on day 10. By the end of the experiment flies from both sexes only had a 37% chance of survival compared to 55% for untreated females and 75% for untreated males.

3.2.4 Higher levels of apoptosis occur in embryos produced by flies that have been fed camptothecin.

Acridine orange staining was used to monitor levels of apoptosis in embryos from camptothecin treated and untreated flies. Increased apoptosis may explain the higher mortality rate and longer development time in embryos from adults that were exposed to camptothecin. Acridine orange is a cationic fluorescent dye, which is cell-permeable. It interacts with nucleic acids via intercalation and electrostatic interactions. When bound to DNA it emits green light with a wavelength of 525 nm when excited at 502 nm. The intense green fluorescence of acridine orange (AO) is specific for cells dying of apoptosis where it binds to condensed chromatin or fractured fragments of DNA that form due to apoptosis (Abrams et al., 1993). This is due to the different excitation and emission wavelengths when (AO) associates with RNA and DNA. As the fragments of DNA in dying cells are more accessible more AO will associate with DNA than is possible in healthy cells.

The results depicted in figures 3.4, 3.5 and 3.6, show increased apoptosis in those embryos from flies that were fed camptothecin, compared to those from untreated flies. Apoptosis is first observed in the developing *Drosophila* embryo at what is regarded as stage 11 of development, which is approximately 7 hours after the egg is laid. In figure 3.4 the embryo from untreated flies (3.4A and B) is very young and is probably at stages 3- 4 in development, as there is a clear peripheral ring. In contrast to this the embryo from the treated flies (3.4 C and D) is featureless with a very high rate of apoptosis occurring throughout the embryo.

The embryos depicted in figure 3.5 are 3-6 hours old and should be at a stage in development (approximately stages 7-11) where many structures are visible. The embryo from untreated flies, shown in figure 3.5 (A and B) is approximately 6 hours old and is at stage 11 of development. It shows structures such as the foregut, hindgut and tracheal pits. There are no detectable signs of a high rate of apoptosis. This is not unexpected as apoptosis is only expected to occur at higher rates in the 7^{th} hour of development at about stage 11. In accordance with the literature the regions of the embryo where apoptosis is observed are the dorsal region of the head just anterior to the tip of the germ band (Abrams et al., 1993).

In contrast the embryos from flies treated with camptothecin show either a disrupted development with high levels of apoptosis (figure 3.5 C and D), or slower rates of development, with 3-6 hour embryos still resembling those at very early stages of development (fig 3.5 D and E). Figure 3.5 C and D shows an embryo at a slightly earlier stage of development than the embryo in figure 3.5 A and B. The proctodeal invagination has begun to form in this embryo. Apoptosis in the germ band at this stage is normally confined to the anterior region. However, widespread apoptosis is occurring throughout the germ band. Such widespread apoptosis should only occur much later and is tightly controlled and tissue specific. As mentioned previously the only other areas where apoptosis should be occurring at this developmental stage is the gnathal segments and clypeolabrum (Abrams et al., 1993).

Figure 3.6 depicts embryos that are between 6-9 hours old, with images (A)(B)(C) and (D) being embryos from flies that were not exposed to camptothecin while images (E)(F)(G) and (H) are embryos from flies that were exposed to camptothecin.

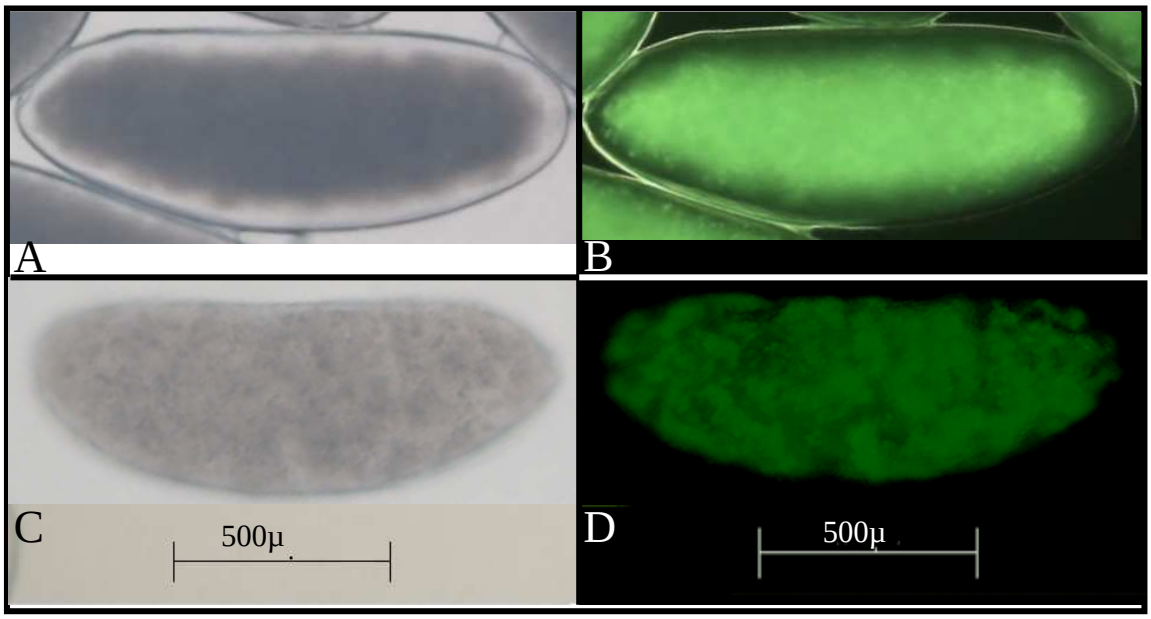


Figure 3.4: Acridine orange staining of 0-3 hour embryos to detect apoptosis**.** In order to detect an increase in apoptosis, 0-3 hour embryos were exposed to camptothecin (C) and (D) or left untreated (A) and (B). Figures (A) and (C) are images of the same embryos in (B) and (D) respectively. Images (B) and (D) were obtained using reflected fluorescent light at a wavelength of 480nm. Images (A) and (C) were obtained using light microscopy. The levels of apoptosis were based upon increased fluorescent intensity compared to the control embryos from untreated flies. There is a high level of apoptosis occurring throughout the embryo in (D). The peripheral ring clearly visible in (A) shows that this embryo from untreated flies is at stage 4 in development and there is no sign of apoptosis in the blastoderm nuclei at the periphery (B).

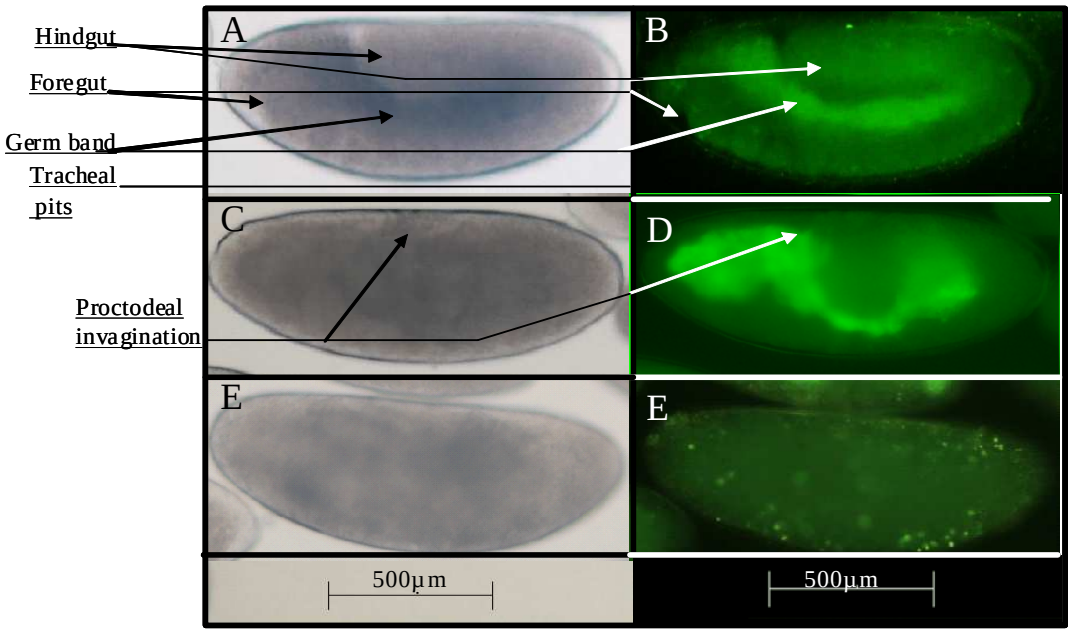


Figure 3.5: Acridine orange staining of 3-6 hour embryos to detect apoptosis. Images represent 3-6 hour embryos from flies that had been exposed to camptothecin (C), (D), (E) and (F) or from flies that remained untreated (A) and (B). Images (A), (C) and (E) are images of the same embryos in (B), (D) and (F) respectively. Images (A), (C) and (E) were obtained using reflected fluorescent light at a wavelength of 480nm. Images (B), (D) and (F) were obtained using light microscopy. The embryo in images A and B is at the 11th stage of development, and features that are characteristic of this stage can clearly be seen. The embryo in image C and D is at a slightly earlier stage of development but structures are not clear. The gut seems to be developing but there is a very high level of apoptosis occurring and the developing organ seems misshapen. The embryo depicted in images E and F appears to still be at a very early stage of development despite being 3-6 hours old. This implies that the embryo has an increased development time compared to wild type embryos. Higher levels of apoptosis are still visible in the embryos from the treated flies.

The embryos from unexposed flies show normal development and apoptosis for embryos of this age. By 7 hours after being laid the embryos have reached stage 12 of development. In stage 12 apoptosis intensifies beneath the developing epithelium of the gnathal segments through the procephalic lobe and within the clypeolabrum. Cell death is also observed in the posterior abdominal segments; along the ventral midline and beneath the dorsal ridge. Apoptotic cells also mark out a segmented pattern within the ventral region (Abrams et al., 1993). In 3.6 B the segmented pattern is clearly visible with a high degree of staining present. Apoptotic cells are also visible in the procephalic lobe and the clypeolabrum in 3.6 D. The area of the abdominal segments shows an intense green fluorescence in both 3.6 B and D.

Many of the 6-9 hour embryos from flies treated with camptothecin still show abnormal development and increased levels of apoptosis (figure 3.6 F). Other embryos do not seem to be in the correct stage of development, showing characteristics of embryos that are much younger than 6-9 hours. This is most likely due to increased levels of apoptosis when the embryo was younger. There are also much fewer viable embryos present 6-9 hours after laying than there were in younger samples of embryos from flies treated with camptothecin. This may be due to many of the embryos dying (figure 3.1B).

The effects of camptothecin on the levels of apoptosis were quantified by counting the number of embryos displaying higher levels or unusual patterns of fluorescence. This indicated higher or abnormal occurrences of apoptosis. The results of this analysis are depicted in figure 3.7.

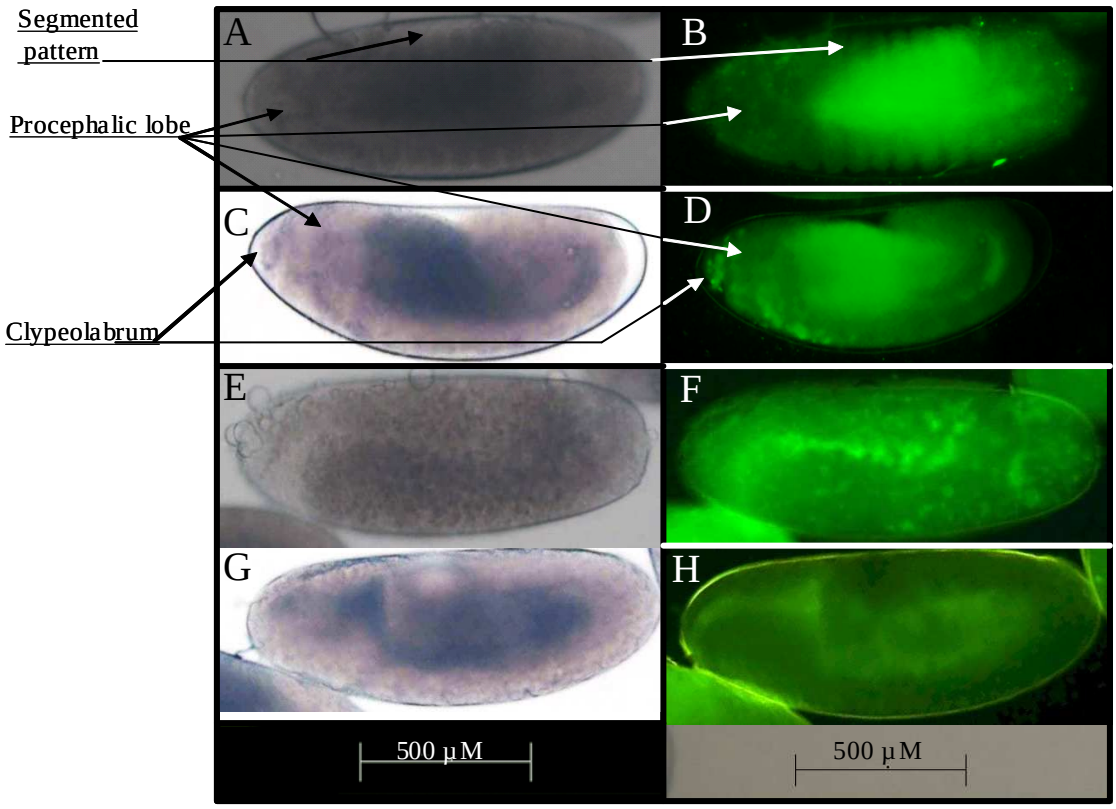


Figure 3.6: Acridine orange staining of 6-9 hour embryos to detect apoptosis. 6-9 Hour embryos were collected from flies that had been exposed to camptothecin (E), (F), (G) and (H) and from flies that had remained untreated (A), (B), (C) and (D). The ventral view of an embryo from untreated flies (A) and (B) demonstrates the normal pattern of apoptosis in stage 12 (7-9 hours), where the dying cells demarcate the segmented pattern within the ventral region. (A) and (C) also show that at this stage in development, apoptosis occurs in the procephalic lobe and within the clypeolabrum, as well as the posterior abdominal segments. In contrast the embryos from flies that have been exposed to camptothecin are still showing abnormal high rates of apoptosis (E). However, there is a higher percentage of embryos that are not displaying high rates of apoptosis. Rather these embryos display a much slower rate of development and appear to be at an earlier stage of development (G).

Embryos from untreated flies display significantly lower rates of abnormal apoptosis in both 0-3 hour and 3-6 hour embryos, while over 50% of the embryos from treated flies showed abnormal levels of apoptosis figure 3.7. An ANOVA analysis of these numbers confirmed that at the 95% confidence level there is a significant difference between the means of the numbers of apoptotic and non apoptotic embryos from untreated adults at 0-3 ($p = 0.02$) or 3-6 hours ($p = 0.001$) . However, for the 0-3 hour embryos there was no significant difference between the means of the embryos from treated embryos ($p = 0.403$) implying that there were equal numbers of embryos displaying normal and abnormally high levels of apoptosis. For 3-6 hour embryos none of the means were similar but the levels of abnormal apoptosis were the highest in embryos from treated flies. The situation at the later stages of development is slightly different (figure 3.7). For the first time there are two groups of means showing significant similarity. These are the embryos displaying normal apoptosis, from both treated and untreated flies ($p = 0.0607$), and the embryos displaying abnormal apoptosis, once again from both treated and untreated flies ($p = 0.0607$). Therefore, regardless of the treatment of the adult flies there are a higher number of embryos showing normal levels of apoptosis than there are showing abnormal levels of apoptosis. This shows that many of those embryos from treated flies that have survived past this point are recovering from the effects of camptothecin treatment of their parents and may continue to develop normally, albeit at a slower rate than those from untreated flies. Alternately these may represent a sub-set of embryos that were not severely affected by camptothecin treatment and are now the only embryos that have survived; however, the slower development times show that these embryos have still been negatively affected.

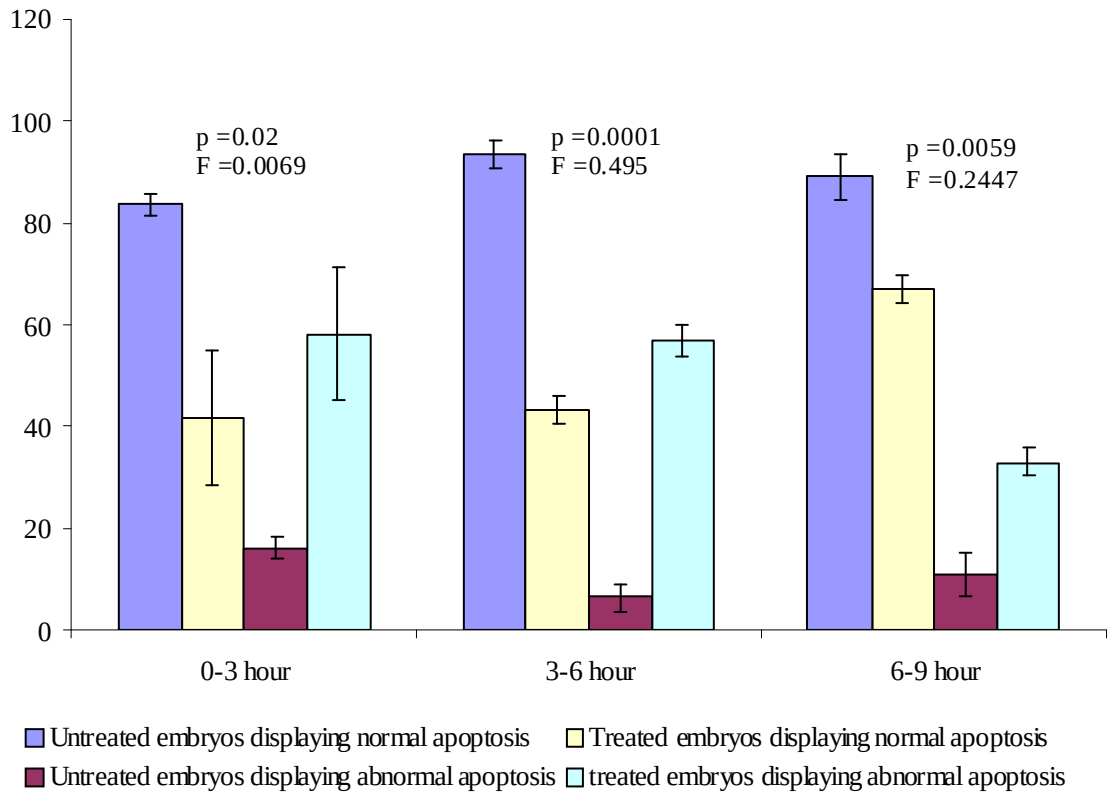


Figure 3.7: Proportions of embryos displaying normal or abnormal patterns of apoptosis. Embryos from untreated flies show significantly lower levels of abnormal apoptosis in the younger 0-3 and 3-6 hour embryos, with over 50% of the embryos from treated flies showing abnormal levels of apoptosis. This level drops dramatically in the 6-9 hour embryos, where the treated embryos show no significant increase in the number of embryos undergoing abnormal apoptosis. Here it could be assumed that most of the badly affected embryos have died and those that remain are viable and are recovering from the camptothecin exposure. The error bars represent the standard error of the means of biological replicates. The p values displayed were obtained through a student's two tailed t test comparison of the normal apoptosis numbers for the embryos from treated and untreated adults.

3.3 Topoisomerase I assays were inconclusive.

In *Drosophila* topoisomerase I is supplied maternally and is found at high levels throughout embryogenesis (Gemkow et al., 2001). The detrimental effects of camptothecin seem to be passed from the adult flies to their offspring. In order to monitor topoisomerase 1 activity, the nuclear protein component of early stage embryos whose parents had been exposed to camptothecin was tested for topoisomerase I activity via a relaxation assay (figure 3.8).

When puc18 was treated with *Drosophila* embryo cytoplasmic extract it resulted in the DNA being totally digested (data not shown). As can be seen in figure 3.8, the nuclear extract from embryos whose parents had not yet been exposed to camptothecin were able to partially relax the supercoiled puc18 DNA while those whose parents had been exposed to camptothecin did not show any ability to relax supercoiled DNA. This disappearance of the ability to relax supercoiled DNA may be due to the continued inhibition of DNA topoisomerase activity.

A chi squared analysis of replicate experiments was performed. The hypothesis was that one band would be present in samples treated with nuclear extract from embryos whose parents were untreated and three bands would be present in the other samples. The chi squared analysis showed that this could only be accepted at the 80% confidence level.

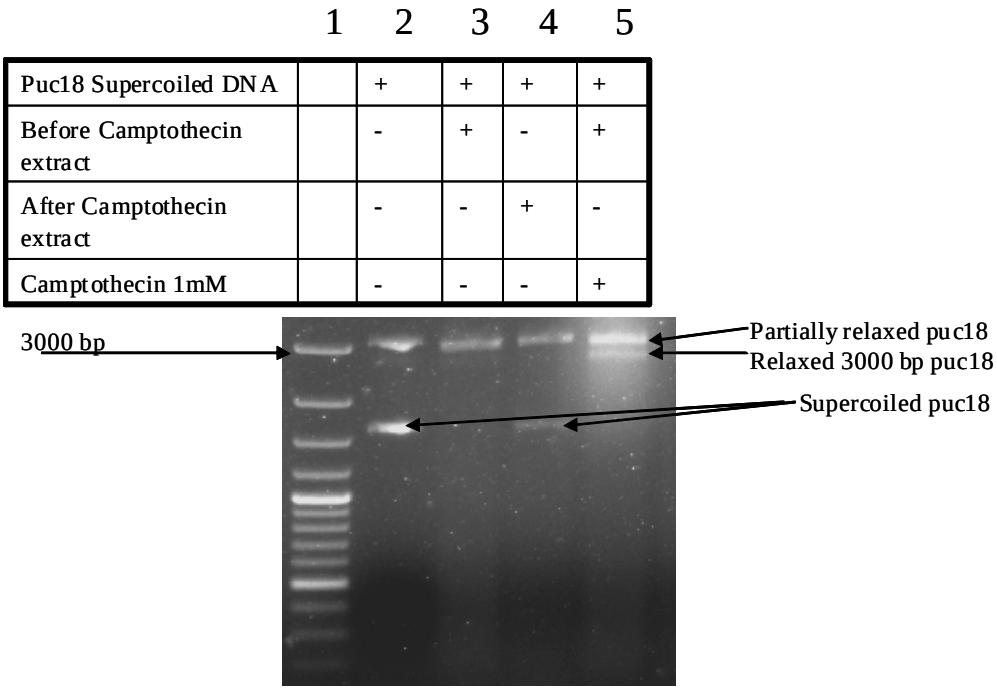


Figure 3.8: Topoisomerase relaxation assay. Topoisomerase I will catalyse the topological change in duplex DNA by nicking one strand and altering the linking number by allowing the unbroken strand to move through the broken strand. This can relax both negative and positive supercoiling (Stewart and Champoux 2001). Topoisomerase activity should convert supercoiled DNA to relaxed DNA. The box above the gel image describes what components were present in each reaction tube. Only the nuclear extract from embryos whose parents had not been treated with camptothecin was able to relax supercoiled DNA. Camptothecin treatment of this extract resulted in any topoisomerase present only being able to produce partial relaxation.

3.4 Proteomic analysis of *Drosophila melanogaster* following camptothecin exposure

A 2D-PAGE analysis of crude protein extracts from treated and untreated flies was performed (figure 3.9); in order to identify protein expression changes following camptothecin treatment. Proteins whose expression patterns had changed were excised and sent for mass spectroscopic analysis. This proteomic analysis following camptothecin treatment (figure 3.10), identified changes in the levels of proteins related to oxidative metabolism, protein folding, cytoskeletal proteins and anti-oxidant activity (table 3.1).

3.4.1 Camptothecin exposure results in a glycolytic flux

An alteration of the expression level of proteins involved in oxidative metabolism followed camptothecin exposure (figure 3.10 A). These included four glycolytic pathway enzymes namely Fructose-1,6 Bisphosphate; Triosephosphate isomerase; Enolase and pyruvate kinase, three citric acid cycle enzymes namely Aconitase; Knockdown (citrate synthase) and Dihydrolipoyl dehydrogenase and three other enzymes involved in metabolism namely UGP; ATP synthase; and bellwether. Three of the glycolytic enzymes Fructose-1, 6 Bisphosphate ($p = 0.00346$); Enolase ($p = 0.0072$) and pyruvate kinase ($p = 0.0111$) show a significant increase in expression following camptothecin treatment, while Triosephosphate isomerase shows a significant decrease in expression ($p = 0.0095$,) (figure 3.10 A).

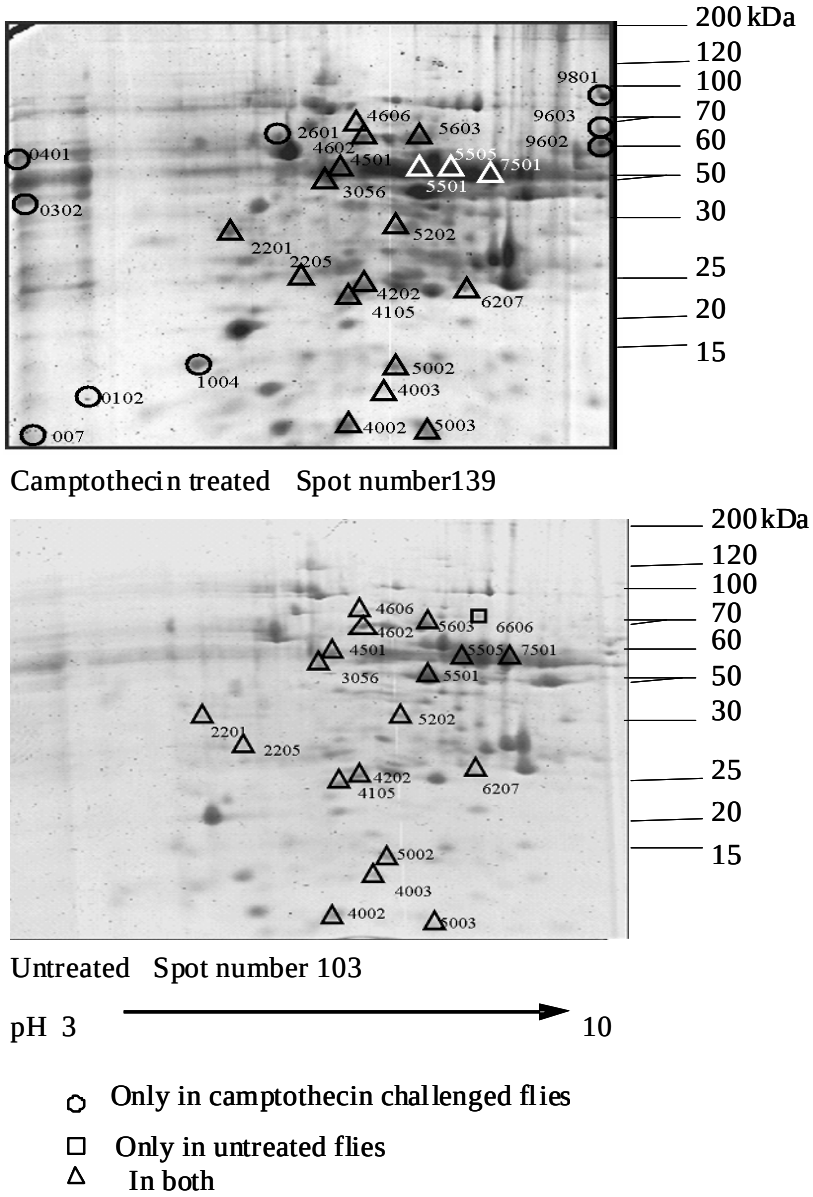


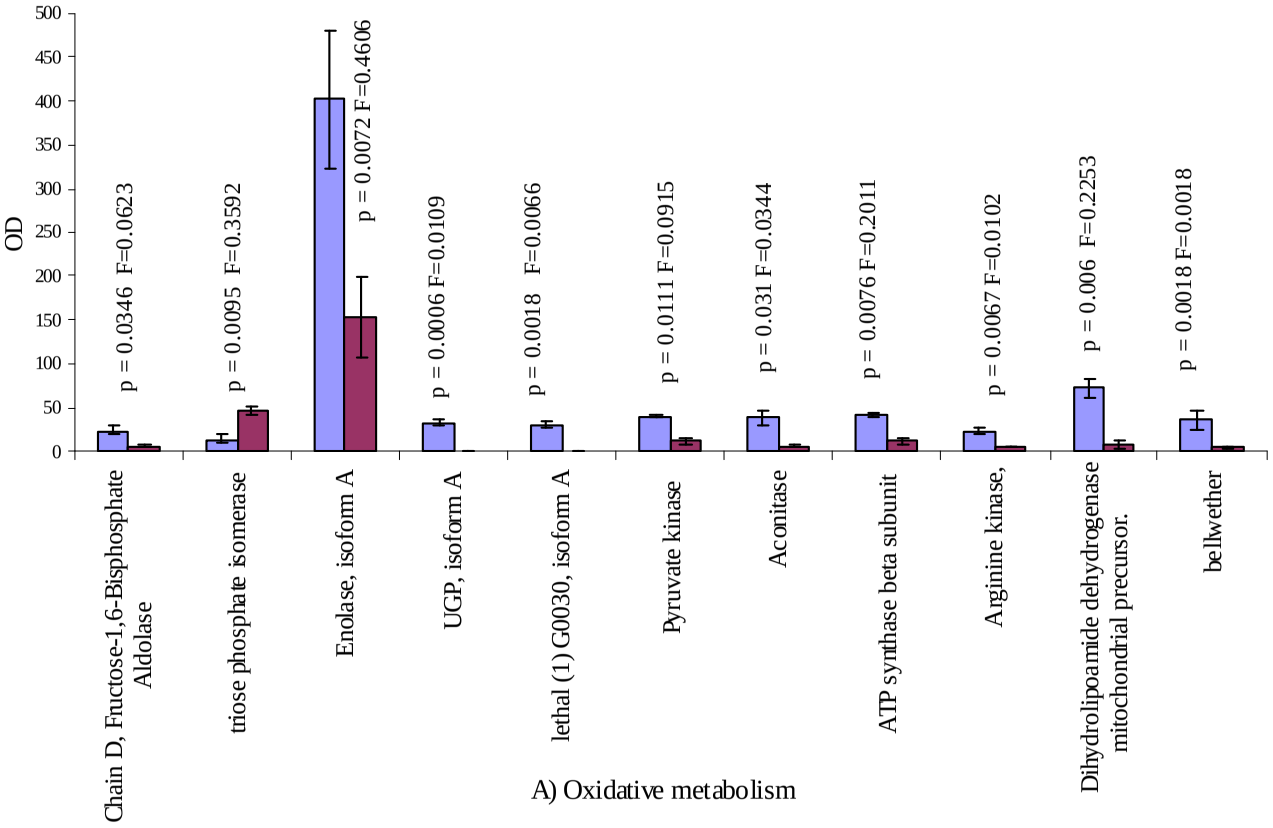
Figure 3.9: 2D gel electrophoresis of *D. melanogaster* crude protein extract before and after exposure to camptothecin. Proteins that were excised for further analysis are marked by one of three symbols based upon their occurrence and changes in optical density in differently treated samples. Proteins that occur in both samples are marked with a triangle. Those that only appear following camptothecin exposure are marked with a circle. Finally those that disappear following camptothecin exposure are marked with a square. The number of spots was much higher in the camptothecin treated sample, which reflects the large number of proteins that only appeared following camptothecin treatment.

As can be seen from the error bars in figure 3.10 A, this pattern emerged in all replicate gels. The ATP synthase components bellwether ($p = 0.0018$) -the alpha subunit of the mitochondrial ATP synthase- and the beta subunit of ATP synthase ($p = 0.0076$) are both significantly up-regulated following camptothecin exposure. This suggests an increase in ATP usage. The expression of the citric acid cycle enzymes aconitase ($p = 0.0381$), dihydrolipoyl dehydrogenase ($p = 0.006$) and citrate synthase ($p = 0.0001$) are also significantly increased ($p = 0.0001$) by camptothecin exposure. Other proteins involved with glycolysis whose expression is increased following camptothecin exposure are the uridylyltransferase UGP ($p = 0.0001$) and arginine kinase ($p = 0.067$).

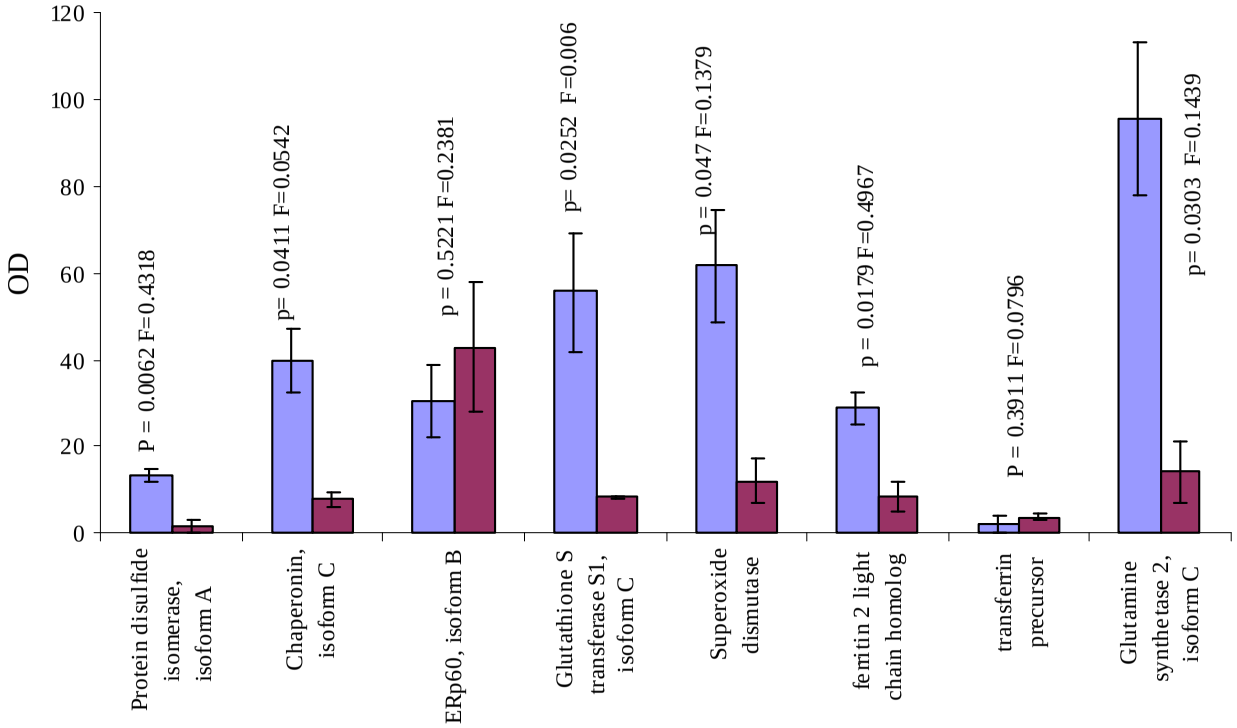
The functional enrichment analysis indicated that there is an overall significant increase in the expression of proteins involved in oxidative metabolism following camptothecin exposure ($p = 0.0116$).

3.4.2 Camptothecin exposure results in an increase in antioxidant and detoxification activity as well as cytoskeletal changes.

Changes in the levels of detoxification, cytoskeletal and proteins involved in the processing of incorrectly folded proteins, following camptothecin treatment were observed (fig 3.10B).



A) Oxidative metabolism



B) Protein folding and detoxification

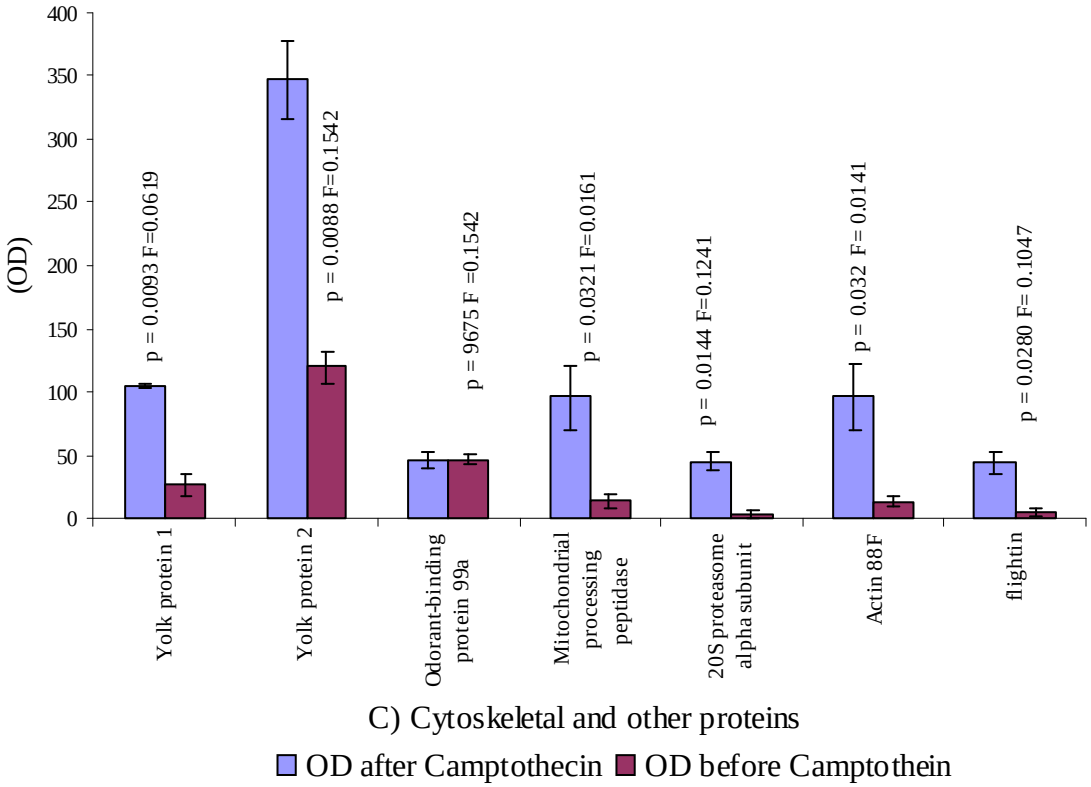


Figure 3.10: The optical densities of the proteins isolated from the hemolymph of fungal infected and uninfected beetles, identified using 2D PAGE and mass spectroscopy. (A) Represents those proteins involved in oxidative metabolism. These proteins are predominantly enzymes involved in glycolysis and the citric acid cycle and the expression of all but one of these proteins increases. (B) Represents those proteins involved in protein folding, antioxidant or detoxification activity. Not surprisingly the expression of chaperones increases following stressful conditions such as camptothecin exposure. (C) The expression levels of cytoskeletal and other proteins before and after treatment with camptothecin. The expression level of both cytoskeletal proteins increases following camptothecin treatment, and may reflect cytoskeletal changes as a result of apoptosis and changes in cell shape following camptothecin treatment. Both mitochondrial processing peptidase and the 20S proteosome play a role in ubiquitin linked protein proteolysis. The error bars indicate the standard error of the mean among three biological replicates. Students two tailed t test was performed to establish if changes in the mean of each proteins OD following camptothecin treatment were significant. The resulting p-values appear above each protein in figures A-C. Functional classes were assigned based on sequence similarity with proteins with known functions.

The increase in the expression of chaperones that aid in the correct folding of proteins would help prevent damage and misfolding of proteins caused by the exposure of cells to a toxic agent. There is a significant increase in the expression of protein disulfide isomerise ($p = 0.0062$) and chaperonin C ($p = 0.0411$) following camptothecin exposure. However, the highly similar Erp60, which has similar functions to that of protein disulfide isomerase appears to be down-regulated following camptothecin exposure, but this decrease is not significant ($p = 0.5221$) (figure 3.10B). Functional enrichment analysis indicates that there was no significant overall increase in the expression of proteins responsible for protein folding ($p = 0.99$).

Like many other chemotherapeutic agents, camptothecin treatment results in the generation of free radicals. Camptothecin has also been found to induce increased expression of co-enzyme Q10 and consequently higher levels of reduced coenzyme Q10 are found in cancer cell lines that have been treated with camptothecin (Fernández-Ayala et al., 2005). DNA damage also results in the generation of ROS. Toxic compounds such as insecticides, xenobiotics and environmental pollutants are metabolised by enzymes to aid in their deactivation and excretion. Two of the most common enzymes used to detoxify chemicals in insects are the Cytochrome p450s and Glutathione S transferases. Consequently, the expression of proteins involved in de-toxification and anti-oxidant activities increases following camptothecin treatment in order to remove this toxic chemical and deal with the excess ROS that has been generated (figure 3.10 B). Glutathione S transferases ($p = 0.0252$) as well as the enzyme superoxide dismutase ($p = 0.047$), which converts O_2 to hydrogen peroxide were found to be significantly up-regulated following camptothecin exposure.

The expression of the iron binding protein ferritin also increases significantly ($p = 0.0179$) following camptothecin exposure. Ferritin is known to play a role in the response of organisms to anoxia (Larade and Storey, 2004) and may protect the cell against ROS. Functional enrichment analysis indicates that there is a significant increase in the expression of detoxification proteins ($p = 0.007$).

The expression of the nitrogen metabolising enzyme Glutamine synthetase increases significantly ($p = 0.0303$) following camptothecin treatment. It has been observed that this enzyme is prone to damage by oxidative stress (Castegna et al., 2011). The Yolk proteins YP1 ($p = 0.0093$) and YP2 ($p = 0.0088$) also show a significant increase in expression following camptothecin treatment as do the cytoskeletal proteins actin ($p = 0.032$) and flightin ($p = 0.028$).

The levels of Mitochondrial processing peptidase (MPP) also increase following camptothecin exposure (fig 3.10C). MPP is responsible for the proteolytic cleavage of the N terminal pro-sequence that targets mitochondrial proteins encoded for in the nucleus, to the mitochondria. These pro-sequences consist of 20-60 residues and are normally positively charged. Following translocation into the mitochondria the prosequence must be removed to allow for correct folding (Gakh et al., 2002). The expression levels of the 20S proteosome, which is involved in the degradation of damaged, unneeded or misfolded proteins, is also significantly increased ($p = 0.0144$) following camptothecin exposure.

3.4.3. Some proteins have multiple identities using Mascot.

Many of the protein spots were assigned multiple identities using the mass fingerprint data (table 3.1). This is because each spot generated multiple peptide fingerprints, or had mass fingerprints that were similar to those of multiple proteins. Only *Drosophila* proteins were considered as valid probable proteins. The protein score obtained using Mascot was also used to evaluate the protein identifications. This protein score is equal to.

$$-10*\text{Log}(P)$$

P is the probability that the observed match is a random event, with protein scores greater than 67 being significant ($p<0.05$). Only protein hits that scored above or near this value were retained as possible identities for the unknown proteins.

3.4.4. Changes in optical densities mirror changes in the enzyme activity of Glutathione S transferase.

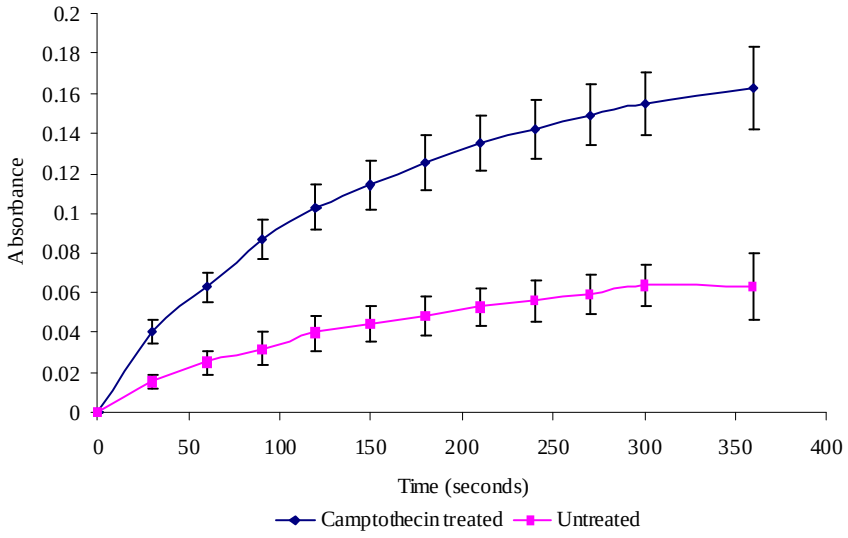
Due to the large degree of error seen in the optical density readings represented in figure 3.10, it was decided to perform an enzyme assay on one of the up-regulated proteins in order to establish if the observed increase in optical density did translate to an increase in the amount of protein present. Glutathione S Transferase was selected due to the ease and low cost of the assay. The assay shows a threefold increase in GST activity in the protein extracts from camptothecin treated flies. However, the 2D PAGE gels indicated that at least 6 times as much GST is present in the extracts from camptothecin treated flies compared to those that have not (figure 3.11 A and B).

Table 3.1 The identification of up or downregulated proteins using MALDI-TOF MS

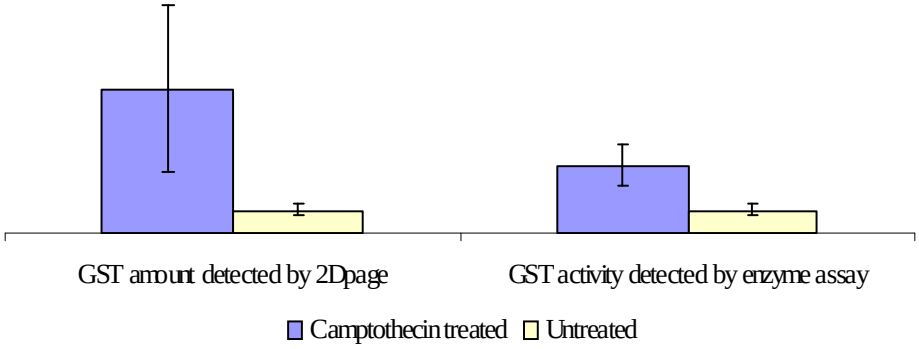
Sample number	Identity	Accession number	Mw	PI	Score	Error (ppm)
Oxidative metabolism						
0302	Chain D, Fructose -1-6-bisphosphate Aldolase	CG-31692-RA	39231	6.67	175	36
4105	Triose phosphate isomerase	CG-2171-RA	26942	6.00	220	36
5501	Enolase, isoform A	CG17654-PA	46861	6.10	152	401
9602	UGP, isoform A	CG4347-PA	58944	6.92	198	27
9602	Lethal (1) G0030, isoform A	CG3861-PA	51713	8.89	59	46
9602	Pyruvate kinase	RH07636p	57951	7.13	51	35
9801	Aconitase	CG9244-PB	86200	8.49	517	31
0401	ATP synthase beta subunit		53544	5.19	229	32
0302	Arginine kinase	CG32031-PD	40126	6.04	221	42
5603	Dihydrolipoamide dehydrogenase mitochondrial precursor	CG7430-PA	53565	6.41	114	-
9602	Bellwether	CG3612-PA	59612	9.09	167	38
9801	bellwether	CG3612-PA	59612	9.09	269	19
Protein folding						
2601	Protein disulfide isomerase, isoform A	CG6988-PA	56031	4.72	243	30
4606	Chaperonin, isoform C	CG7033-PC	58223	5.68	256	21
4602	ERp60, isoform C	CG8983-PB	55678	5.62	162	32
Antioxidant activity and detoxification						
2201	Glutathione S transferase S1, isoform C	CG8938-PC	27653	4.57	224	30
5002	Superoxide dismutase	CG11793-PA	15974	5.67	187	39
4202	Ferritin 2 light chain homolog	CG1469-PB	25483	5.90	63	-
6606	Transferrin precursor		72964	6.69	387	49
3506	Glutamine synthetase 2, isoform C	CG1743-PC	41962	5.42	177	32
Cytoskeleton						
3506	Actin 88F	CG5178-PA	42072	5.29	145	31

2205	Flightin	CG7445-PA	20643	5.30	124	30
Other						
4501	Yolk Protein 1	CG2985-PA	48739	7.19	275	19
5505	Yolk Protein 2	CG2979-PA	49744	7.74	248	34
7501	Yolk Protein 2	CG2979-PA	49744	7.74	373	35
5003	Odorant- binding protein 99a	CG18111-PA	16384	6.19	63	18
3506	Mitochondrial processing peptidase	CG3731-PA	52525	5.67	81	-
2205	20S proteasome alpha subunit		26995	4.83	83	23

The table above contains the data obtained for the proteins isolated using the 2D-PAGE analysis of samples isolated from flies that had been treated with and not treated with camptothecin. The spots were selected from a 2D-PAGE gel based on differences in optical density and therefore expression level. These spots were then analysed with MALDI-TOF mass spectrophotometry. This allowed for the identification of some of these proteins and also supplied the Mr and PI data. Some spots gave more than a single identification as the peptide fragments mass fingerprints matched more than one protein in the *Drosophila* database. Since protein scores greater than 67 are significant ($p<0.05$) only those identifications were the score exceeded or was close to this were kept as possible protein identifications.



A: GST activity assay



B: The relative activities and amounts of GST as determined by enzyme assays and 2D PAGE

Figure 3.11: GST enzyme assay for camptothecin and untreated fly extracts to confirm optical density changes do reflect changes in protein amount. (A) The camptothecin treated sample showed a significantly higher ($p = 0.0009$) GST activity than that of the untreated sample. The camptothecin treated samples had an activity of 0.15 µmol/min/ml while the untreated sample had an activity 0.051 µmol/min/ml. (B) The camptothecin treated flies therefore had threefold higher activities of GST. In contrast 2D page showed a 6 fold higher amount of GST present in camptothecin treated flies. The error bars indicate the standard error of the mean amongst biological replicates.

3.5 Methyl pyruvate supplementation.

As a result of the fluctuations in enzymes involved in carbohydrate metabolism, methyl pyruvate was used to supplement yeast. This would supply the flies with the end point of glycolysis and might prevent the glycolytic flux that was observed following camptothecin exposure. Methyl pyruvate supplementation may also restore the fecundity of female flies that had been exposed to camptothecin and improve the mortality rates of the treated flies. In order to establish the effectiveness of methyl pyruvate supplementation, *D. melanogaster* were fed yeast containing a mixture of methyl-pyruvate and camptothecin. Yeast containing just camptothecin or just methyl pyruvate served as a control.

3.5.1 Methyl pyruvate supplementation failed to significantly improve the reproductive ability of the female flies when both sexes were exposed to camptothecin.

In order to test if the presence of methyl pyruvate could reverse the effects camptothecin had on embryo development, newly eclosed males and females were separated into four populations. These populations were exposed to one of the following treatments: camptothecin; methyl pyruvate; a combination of both or untreated yeast. As shown in figure 3.12, the untreated populations as well as the camptothecin treated males and females behaved exactly as had been previously observed (figure 3.2) with no significant decrease in the number of embryos produced by the untreated female populations ($p = 0.6089$). Any camptothecin treated female populations produced significantly lower numbers of embryos ($p = 0.0033$).

The methyl pyruvate treatment had a significant detrimental effect on embryo number, regardless of which sex was treated ($p = 0.0104$ for both, $p = 0.0098$ for males and $p = 0.0185$ for females). This effect was not as severe as the effect camptothecin had and was short lived, with the flies recovering on the 3rd day after mating (figure 3.13). Methyl pyruvate treatment was able to partially rescue populations where only one sex was exposed to camptothecin. This was compared to those populations where the females had been exposed to camptothecin ($p = 0.009$). These populations also showed a faster rate of recovery. However, the population where both males and females were exposed to camptothecin and methyl pyruvate showed continued poor egg laying ability (p value = 0.4652), and unlike the camptothecin treated samples they had failed to recover as effectively by day eight.

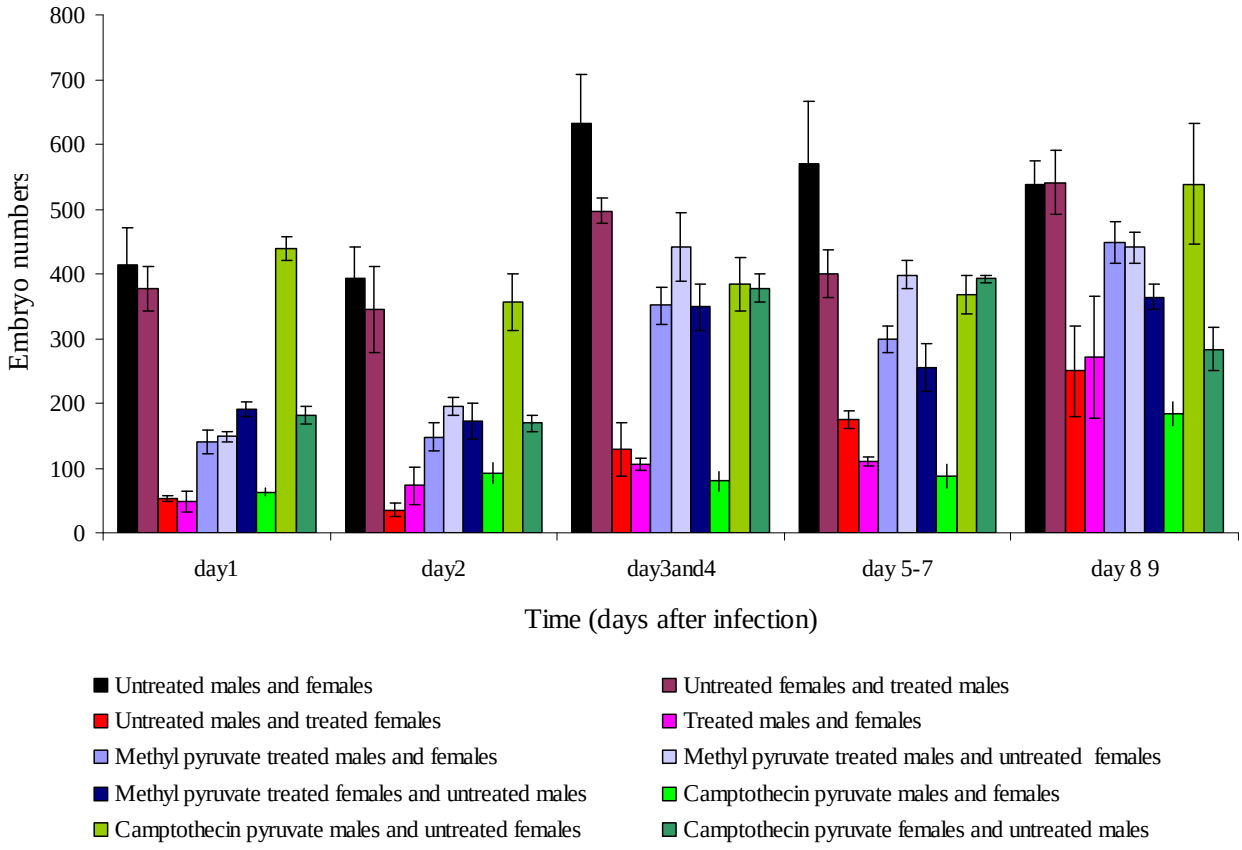


Figure 3.12: The effect of methyl pyruvate on embryo numbers. Once again the untreated population as well as those where the females were not treated with camptothecin produced the highest embryo numbers. The methyl pyruvate itself seemed to have a detrimental effect, regardless of which sex was treated. This effect was short lived, disappearing on the 3rd day after mating. Methyl pyruvate supplementation failed to significantly improve the reproductive ability of the female flies when both sexes were exposed to camptothecin. However the population where only the females were treated to camptothecin and methyl pyruvate showed a recovery on day 3 similar to the methyl pyruvate treated populations. The error bars represent the standard error of the means of biological replicates.

3.5.2 Methyl pyruvate is able to partially increase the viability of embryos produced by flies exposed to camptothecin.

As camptothecin treatment affected the viability of embryos produced by flies that had been exposed to camptothecin, the viability of embryos from adults exposed to methyl pyruvate and camptothecin and methyl pyruvate was also examined. Figure 3.13 shows the number of embryos that developed into pupae following treatment of the parent flies with camptothecin, methyl pyruvate and camptothecin and methyl pyruvate together. Methyl pyruvate alone had a small, insignificant detrimental effect on the number of embryos that survived to the pupal stage ($p = 0.2125$). In contrast camptothecin treatment once again resulted in the large detrimental effect that had previously been observed (Figure 3.1 B) ($p = 0.0001$). Supplementation with methyl pyruvate results in a significantly greater number of embryos surviving to the pupal stage ($p = 0.0249$) (figure 3.13). However, this was still significantly lower ($p=0.0013$) than the number of embryos that developed survived to the pupal stage in the untreated sample. This shows that methyl pyruvate is able to rescue some embryos from camptothecin induced death. These effects may be occurring in the adult flies and result in a higher number of viable embryos being produced or they may be occurring in the embryos themselves.

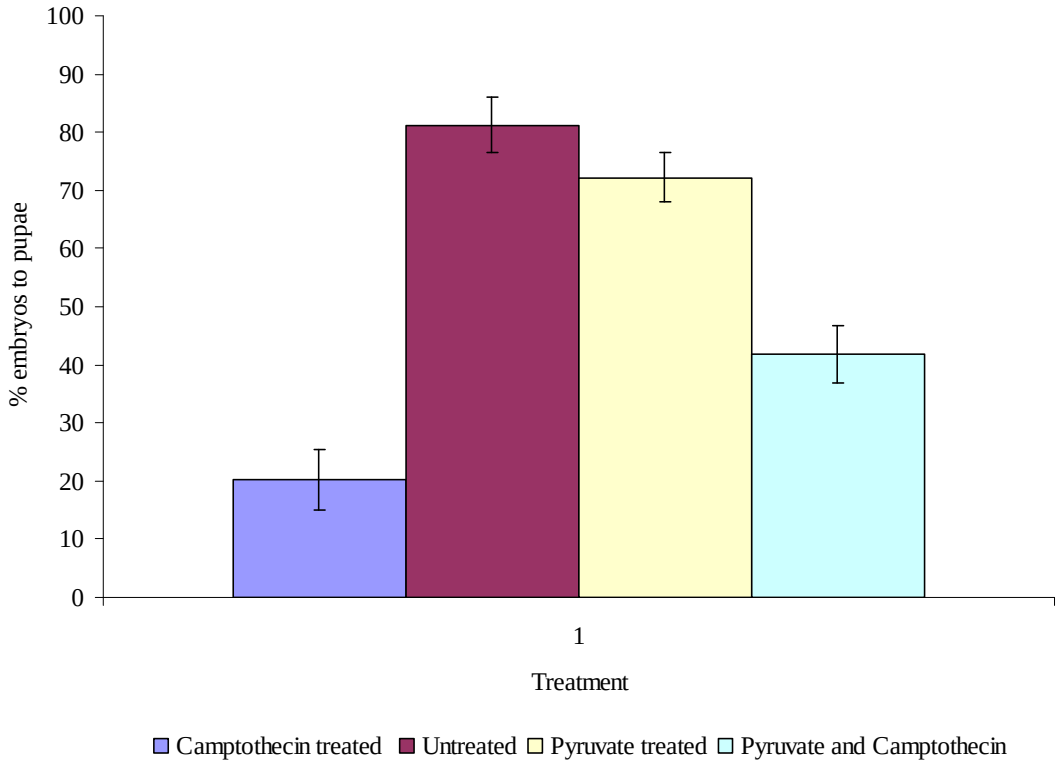


Figure 3.13: The ability of methyl pyruvate to rescue the camptothecin phenotype. The supplementation of camptothecin containing media with methyl resulted in an improved survival rate of the embryos, as a higher percentage developed to pupa. However, the methyl pyruvate on its own seemed to have a small detrimental effect on the ability of embryos to develop into pupae. The error bars represent the standard error of the means of biological replicates.

3.6. Effects of camptothecin exposure on *Snama* *Dmp53* and *Reaper* mRNA levels.

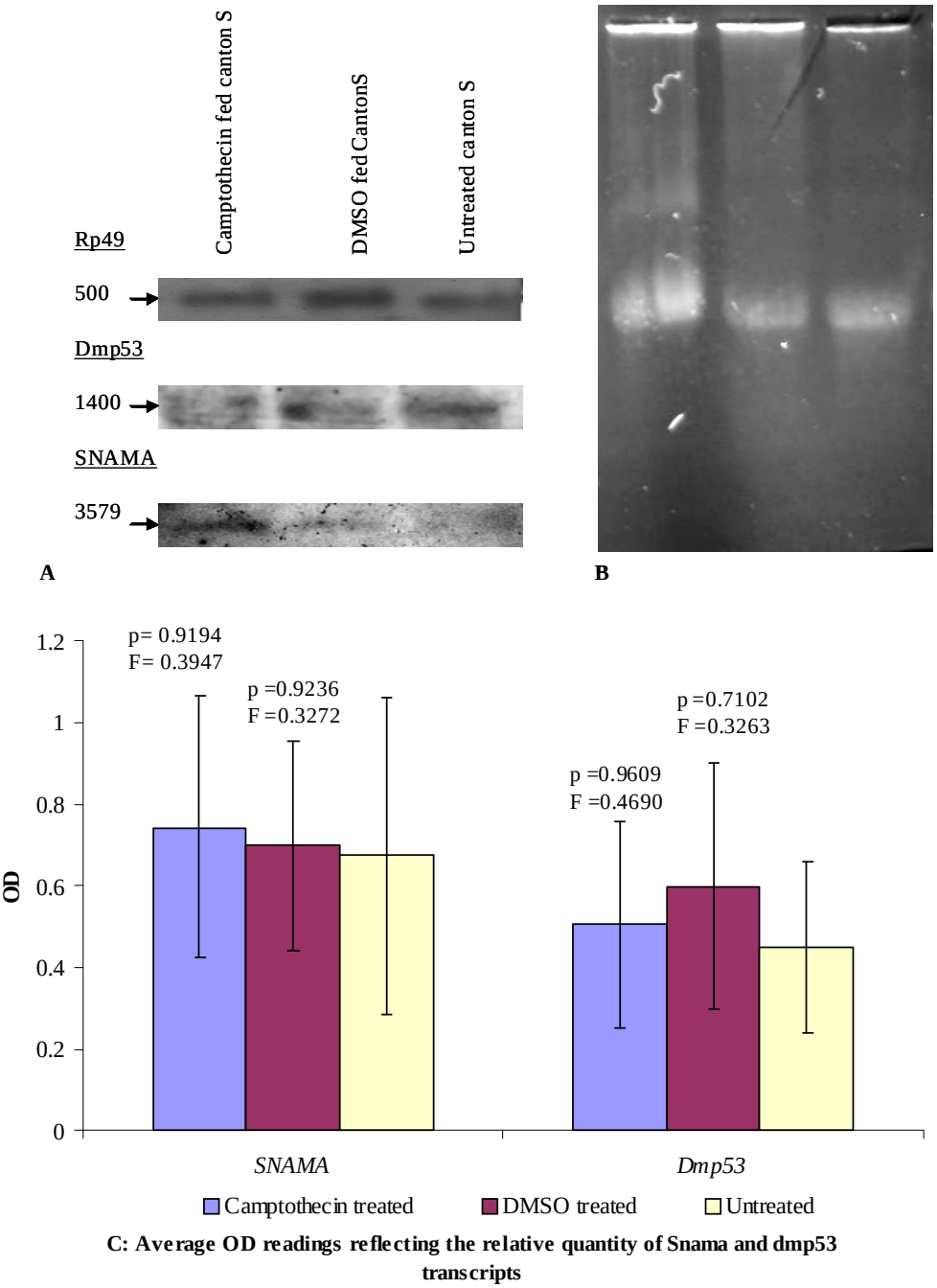
There are no reports on the result of camptothecin exposure on *Drosophila* and what role Dmp53 may play in this response. To determine if there was any interaction between the transcription levels of *Snama* and *Dmp53*, camptothecin was used to damage DNA and alter *Dmp53* transcription. Total RNA was extracted and the transcription levels of *Dmp53, Snama* and the pro-apoptotic gene *Reaper*, which is under the control of Dmp53, were monitored through the use of Northern blot analysis, semi-quantitative reverse transcriptase PCR and quantitative Real Time PCR.

Camptothecin treatment would be expected to increase p53 transcription as the presence of p53 is required for camptothecin induced apoptosis (Takeba et al., 2007). The evidence that Snama interacts with Dmp53 is based on pull down assays, where Dmp53 and Snama were shown to co-immunoprecipitate (Rakgotho, 2007). Heterologously expressed portions of Snama were able to function as an E3 ligase in an ubiquitin ligase assay. This region, known as the DCM (from the DWNN domain up to the RING finger), may therefore play a role in protein regulation (Antunes, 2008). It is not known whether this interaction happens in vivo. If Snama is able to catalyse the addition of ubiquitin or DWNN domains to Dmp53 then it may be involved in the negative regulation of Dmp53 in a similar manner to Mdm2. Especially as there is no known Mdm2 homolog in *Drosophila.* The identification of Snama as a homolog of RBBP6 implies that it may negatively regulate Dmp53 through an indirect manner utilizing different signalling networks.

3.6.1 Significant changes in the transcription of *Snama* and *Dmp53* following camptothecin exposure could not be determined using northern blots.

Northern blots were performed to establish the transcription patterns of *Dmp53* and *Snama* following camptothecin exposure. These blots were performed with total RNA, extracted from flies that had been fed 1 mM camptothecin mixed into yeast paste. *Rp49* Probes were used as an internal control.

Figure 3.14 A depicts the results of the Northern blots, as well as a duplicate of the formaldehyde gel used to perform the northern blot (figure 3.14 B). The gel shows that the total RNA samples used were intact. All three transcripts closely match the expected size with the smaller *Dmp53-PB* (FBpp0083753) transcript of around 1500 bp being detected. Figure 3.14 C is a graphic representation of the amount of transcript detected in each sample based on the optical density of the bands shown in figure 3.14 A. The *Rp49* control was used to correct for differences in the levels of RNA in each sample. Figure 3.14 shows that *Snama* transcript levels are lowest in the untreated adult flies and increases following DMSO or camptothecin treatment. However, the p values show that these changes are not significant for either the DMSO treatment (p=0.9609) or the Camptothecin treatment (p= 0.9194). Figure 3.14 also indicates that the levels of *Dmp53* transcripts decreased following camptothecin treatment as the same effect is observed in the DMSO treated sample, this increase in the transcription level of the shorter transcript of *Dmp53* (FBpp0083753) could be due to the presence of any foreign agent and not just the presence of camptothecin. One again these changes in the expression level of *Dmp53* are not significant with either the camptothecin (p= 0.9609) or DMSO treatment (p= 0.7102).



C: Average OD readings reflecting the relative quantity of Snama and dmp53 transcripts

Figure 3.14: The effects of camptothecin and DMSO treatment on the transcription of *Dmp53* and *Snama* in *Drosophila.* (A). The size of the bands was calculated using the stained duplicate of the formaldehyde gel (B). These sizes were approximations but closely matched the expected sizes of 534-800bp, 1500-1800bp, and 3939bp respectively (C) OD values were obtained using the Quantity one software package. None of the changes detected in *Snama* and *Dmp53* mRNA levels were statistically significant regardless of treatments.

The northern blot shows that both *Dmp53* and *Snama* are transcribed at very low levels compared to *Rp49,* irrespective of the samples treatment or origin. This agrees with the established expression pattern for Snama and Dmp53, both of which show reduced expression in adults (Bensaad and Vousden, 2007; Mather, 2005). The pattern observed for *Dmp53* and *Snama* mRNA levels after camptothecin treatment is the exact opposite of what was expected. According to the hypothesis that Snama negatively regulates Dmp53 to decrease apoptosis, it was presumed that camptothecin exposure would lead to DNA damage, followed by an increase in *Dmp53* transcription and an increase in apoptosis. The levels of *Snama* transcription would then decrease preventing it from negatively regulating Dmp53. However, as none of these changes can be considered statistically significant from the mRNA levels in the untreated flies, it is impossible to accurately interpret these results as changes in the transcription of these genes following camptothecin treatment.

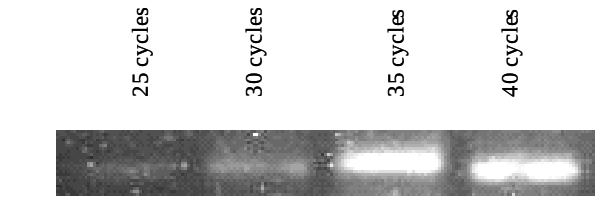
3.6.2 The exponential production of *Rp49* from a cDNA template occurs at approximately 32 cycles.

Since northern blots failed to establish the effects of camptothecin treatment on the presence and quantity of *Reaper*, *Dmp53* and *Snama* transcripts, a further attempt was made using semiquantitative Reverse Transcriptase PCR performed on total RNA extracted from flies that had been exposed to camptothecin and from untreated flies. Additionally, total RNA was also extracted from flies that had been exposed to methyl pyruvate and a combination of camptothecin and methyl pyruvate. This was done due to the glycolytic flux that was observed following camptothecin exposure. The end product of glycolysis may therefore have an effect in modulating the stress response

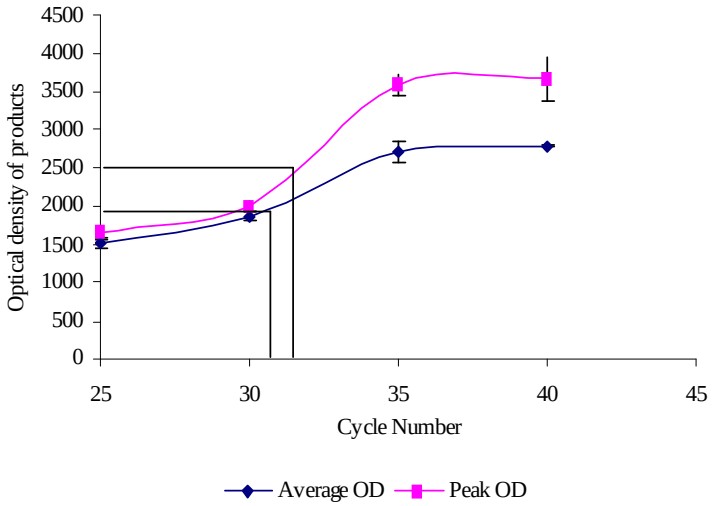
following camptothecin exposure and influence the transcription of *Snama*, *Dmp53* and *Reaper*.

In order to perform semi-quantitative RT-PCR, the cycle number at which the exponential amplification of the internal control (*RP49*) occurred, had to be determined. This is necessary as the amount of product formed should be due to the amount of template present and not due to any rate limiting component or condition. The cycle number at which the reaction was occurring at an exponential rate was determined by running the reaction for different cycle numbers. Starting at 25 cycles the reaction was run in increments of 5 cycles up to 40 cycles.

Equal volumes of the resulting products were electrophoresed on a 1% agarose gel and stained with ethidium bromide. The optical density of the resulting bands (figure 3. 15 A) were determined using the Quantity one analysis software. An ANOVA test was carried out on the optical density data and a Tukey's HSD (Honestly Significant Difference) test was used to compare the means. The 35 and 40 cycle OD readings showed no significant difference ($p = 0.6245$). The means of the 25 and 30 cycle OD readings are significantly lower than the 35 and 40 cycle OD means. These OD values were then plotted on a graph (figure 3.15 B) and the cycle number where the reaction was proceeding exponentially was determined to be 32 cycles. This cycle number was then used in all subsequent reactions.



A

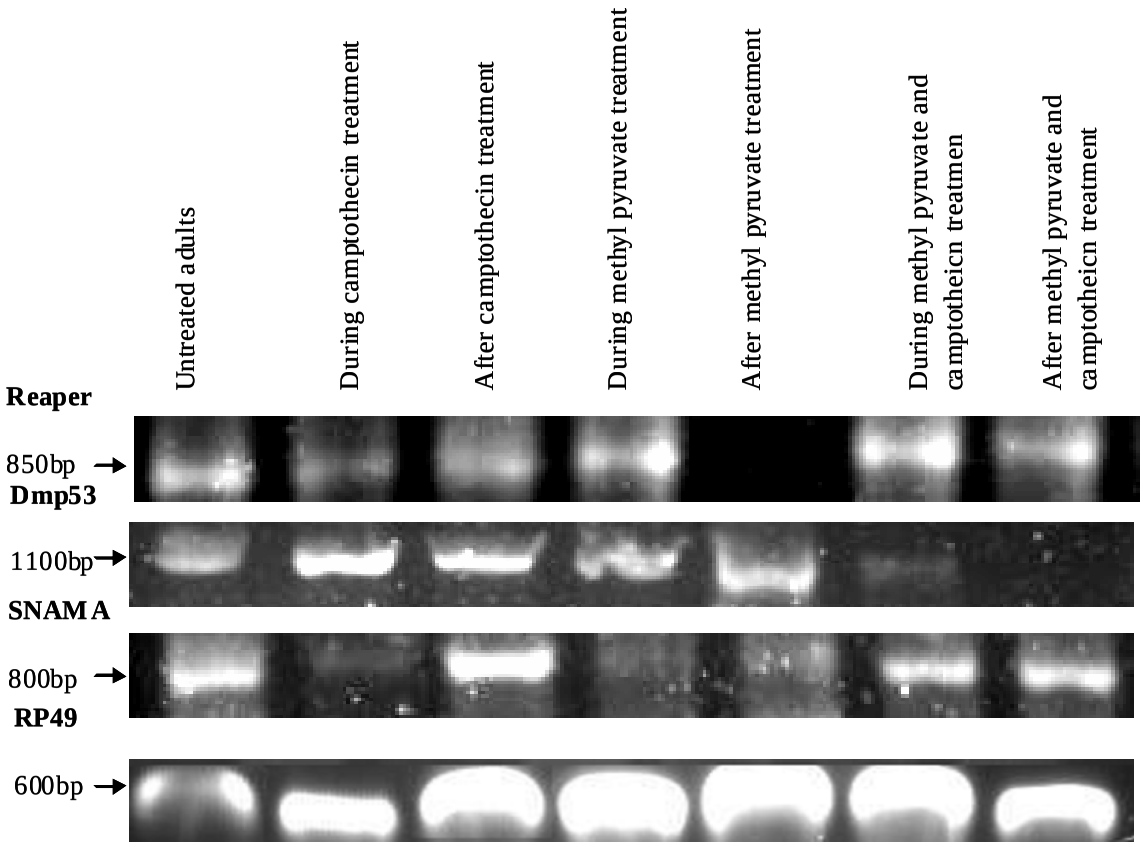


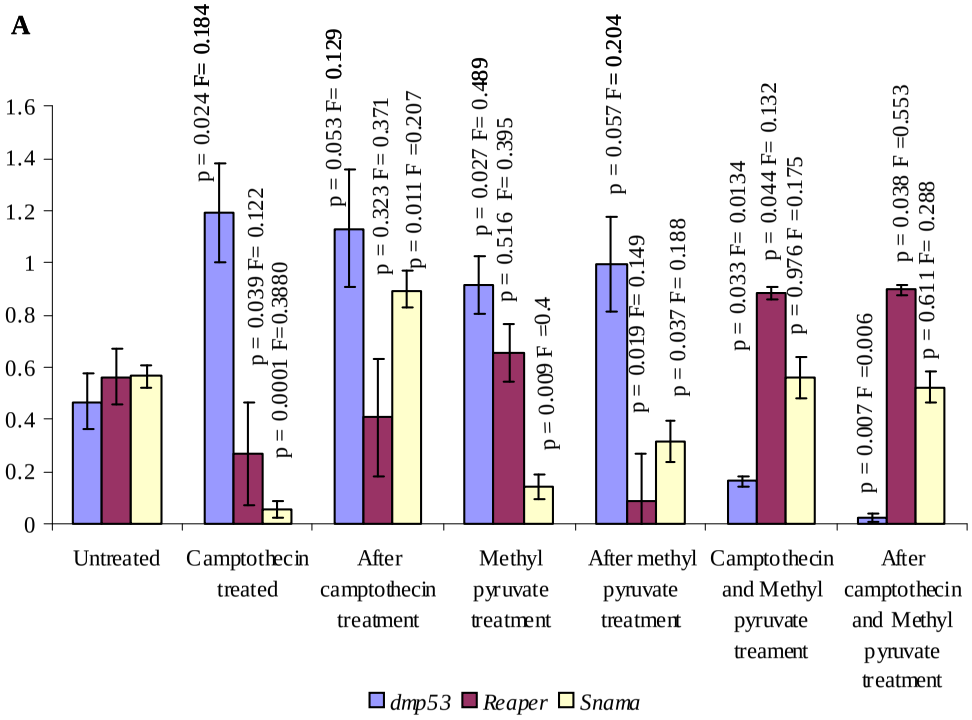
B: Determination of the optimum cycle number for the qPCR

Figure 3.15: Determination of the exponential phase of RT-PCR amplification of the internal control *Rp49*. (A) Shows a typical result of the RT-PCR using the internal control *Rp49*. Equal amounts of total RNA were used and the reaction was stopped at different cycle numbers. An equal volume of product was run on a 1% agarose gel. (B) The Optical density of the resulting bands determined the amount of product formed. These values were plotted to determine the cycle number at which product formation is occurring exponentially.

3.6.3 *Reaper* transcription patterns do not match that of *Dmp53*.

Reaper is a direct downstream target of Dmp53 and the transcription of *Reaper* is activated by Dmp53 following irradiation (Brodsky et al., 2000). It was expected that the levels of *Reaper* mRNA would reflect the levels of *Dmp53* mRNA. That is an increase in *Dmp53* transcription would result in an increase in *Reaper* transcription. However, the expected relationship between *Reaper* and *Dmp53* was not observed (Figure 3.16). *Reaper* mRNA is detected when *Dmp53* transcripts are not and intense *Dmp53* mRNA levels are accompanied by faint *Reaper* mRNA levels (figure 3.16). The lack of a direct relationship between the levels of transcription between these two genes is re-enforced by the graphic representation of the optical density values obtained (figure 3.16 B). This figure demonstrates that following camptothecin treatment there is a significant increase in the mRNA levels of *Dmp53* ($p = 0.0236$) while *Reaper* transcript levels decrease significantly ($p = 0.0391$). This absence of a relationship between *Dmp53* and *Reaper* is not due to a delayed response in the initiation of *reaper* transcription by p53. *Reaper* levels are not significantly different from the untreated samples after camptothecin treatment ($p = 0.323$) while the *Dmp53* mRNA levels show a slight but not significant increase compared to the levels in the untreated samples ($p = 0.0532$). The regulation of Reaper by Dmp53 may not be apparent at the mRNA level. These results also suggest that apoptosis induced by an increase in *Reaper* transcription does not occur following camptothecin treatment.





B: Relative quantity of *Reaper*, *Snama* and *Dmp53* transcripts based on optical density readings

Figure 3.16: The semi-quantitative Reverse Transcriptase-PCR analysis of the level of RNA transcripts present for *Reaper, Dmp53, Snama* and the control *Rp49*. The results of the semi-quantitative RT-PCR analysis of the four different transcripts are shown above. *Rp49* is present in each lane at nearly the same intensity. (B). Following camptothecin treatment the level of *Snama* transcripts decrease while that of *Dmp53* increases. Despite this the transcription level of *Reaper*, the downstream target of *Dmp53* also decreases. Both *Reaper* and *Snama* transcript levels increase in the recovery period while *Dmp53* levels return to levels not significantly greater than in the untreated flies. This pattern is repeated during methyl pyruvate treatment with *Snama* mRNA levels decreasing while those of *Dmp53* increase. *Reaper* mRNA levels are unaffected by methyl pyruvate exposure but is virtually absent in the recovery period, while *Dmp53* mRNA levels returning to the levels in untreated flies. A different pattern emerges when flies are exposed to methyl pyruvate and camptothecin. The level of *Dmp53* transcripts decreases and is virtually absent in the recovery period, while *Reaper* transcription increases and remains high in the recovery stage. *Snama* transcript levels remain unchanged with respect to the untreated flies during and after treatment with camptothecin and methyl pyruvate. Therefore, *Reaper* transcription levels do not mirror those of *Dmp53*, while the levels of *Snama* transcripts seem to show an inverse relationship to those of *Dmp53*. The error bars indicate the standard error of the means of biological replicates. The change in transcription levels following treatments were analysed using a student's t test at the 95% CI.

This lack of a relationship between *Dmp53* and *Reaper* is also observed in the methyl pyruvate treated samples. Here the levels of *Dmp53* mRNA increase significantly ($p = 0.0272$) and then once again decrease to a level not significantly different from that in the untreated flies ($p = 0.0573$). However, *Reaper* transcripts remain unchanged during methyl pyruvate exposure ($p = 0.516$) and decrease to barely detectable levels during recovery from methyl pyruvate treatment ($p = 0.0109$). This suggests that even though the methyl pyruvate is acting as a stressor and increasing the levels of *Dmp53* transcription, there is no increase in Reaper dependent apoptosis.

When the flies were treated with a combination of camptothecin and methyl pyruvate the levels of *Dmp53* mRNA dropped significantly ($p = 0.033$), virtually disappearing from flies that were recovering from the combined treatment ($p = 0.0061$). Conversely *Reaper* transcript levels increased significantly following the combined treatment ($p =0.044$) and remain high throughout the recovery phase ($p = 0.0379$).

3.6.4 Methyl pyruvate alters the transcription of *Dmp53* and *Snama*.

As seem in section 3.5.1 methyl pyruvate supplementation failed to rescue flies from the camptothecin phenotype with respect to the decreased fecundity that was observed following camptothecin exposure. Since camptothecin induces a p53 dependent apoptotic response and if we assume that increased apoptosis is responsible for the decrease in fecundity, we would expect that methyl pyruvate supplementation has no effect on the transcription levels of *Dmp53*. However, if methyl pyruvate can alter the DNA damage stress response resulting from camptothecin treatment, it may also be

able to restore the levels of *Snama*, *Reaper* and *Dmp53* transcription to pre-camptothecin treatment levels.

Methyl pyruvate treatment or camptothecin treatment alone equally increased *Dmp53* mRNA levels ($p = 0.267$) (figure 3.16). During the recovery phase post methyl pyruvate treatment, the levels of *Dmp53* mRNA remain high in both samples ($p=0.664$). Under the same conditions *Snama* mRNA levels are decreased to statistically equal levels following camptothecin or methyl pyruvate treatment alone ($p = 0.207$). The levels of *Snama* mRNA remain low during recovery from methyl pyruvate exposure while it increases to a high level during the recovery from camptothecin treatment ($p = 0.0041$). This suggests that methyl pyruvate alone is inducing a stress response and could be leading to increased levels of apoptosis. However, if *Reaper* mRNA levels are used as an indication of apoptosis then there is a significantly higher level of *Reaper* transcripts in the methyl pyruvate treated sample compared to the camptothecin sample ($p=0.05$). This situation is then reversed as the levels of *Reaper* increase following camptothecin exposure and decrease following methyl pyruvate exposure ($p= 0.0179$).

The combined effect of camptothecin and methyl pyruvate exposure seems to indicate that the methyl pyruvate is protecting the cells from camptothecin induced apoptosis, as the level of *Dmp53* mRNA decreases significantly compared to the untreated sample ($p = 0.033$) and camptothecin treated sample ($p = 0.0127$) during the combined camptothecin and methyl pyruvate treatment. This decrease continues to proceed to such an extent that in the recovery stage, *Dmp53* transcripts are virtually absent ($p=0.0067$). However, the levels of *Snama* mRNA remain unchanged during

(p= 0.978) and following (p =0.0608) exposure to a combination of methyl pyruvate and camptothecin. *Reaper* levels also increase significantly (p=0.030) and remain high (p = 0.0271) during and after treatment of flies with a combination of camptothecin and methyl pyruvate. This seems to indicate that methyl pyruvate is protecting the flies from a p53 dependent increase in apoptosis and in circumstances of decreased p53 dependent apoptosis, the levels of *Snama* transcription remain at a steady level. However, the elevated levels of *Reaper* mRNA and the lower embryo numbers indicate that apoptosis is still occurring.

3.6.5 *Snama* and *Dmp53* demonstrate an inverse transcriptional relationship following stress exposure.

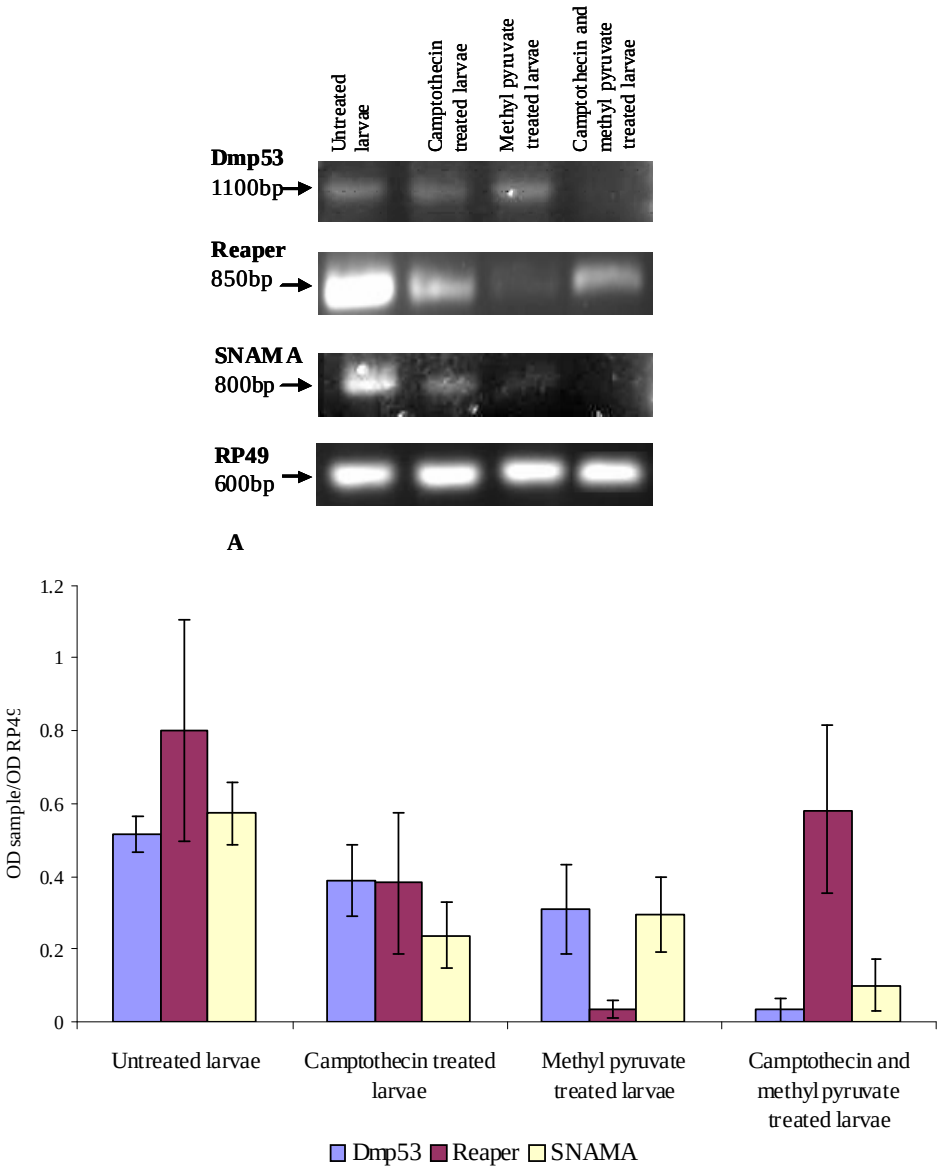
A common feature across all these results is the inverse relationship between *Snama* and *Dmp53* where mRNA levels of one will increase while the mRNA levels of the other decrease, following exposure to a potentially DNA damaging stressor. If Snama is a negative regulator of p53 and camptothecin induces a p53 dependent apoptotic response, a significant decrease in *Snama* transcripts would be expected to occur following camptothecin exposure and this was indeed the case (p = 0.001) Likewise the transcription of *Dmp53* was observed to increase significantly following camptothecin treatment (p =0.0236). During recovery from camptothecin *Dmp53* transcript levels decrease while those of *Snama* increase.

3.6.6. Levels of *Dmp53* transcription do not change in larvae treated with camptothecin, while *Snama* and *Reaper* transcription is highest in untreated larvae.

As both Snama and Dmp53 are expressed at higher levels in the larval stages of development (Brodsky et al., 2000; Mather et al., 2005; Ollmann et al., 2000), an attempt was made to monitor changes in transcription levels of *Dmp53, Snama* and *Reaper* in larvae produced by flies that had been exposed to camptothecin, methyl pyruvate and a combination of both.

As can be seen in figure 3.17, neither camptothecin ($p = 0.3218$), nor methyl pyruvate ($p = 0.229$) treatment of the adult flies, seemed to have any effect on the levels of transcription of *Dmp53* in their larval offspring. The levels of *Snama* mRNA were significantly lower in larvae from adults that had been treated with camptothecin ($p = 0.026$), while methyl pyruvate treatment led to an in-significant ($p = 0.089$) decrease in transcripts. Both *Snama* ($p = 0.0059$) and *dmp53* ($p = 0.0022$) transcripts were reduced to very low levels in the RNA extracted from larvae whose parents had been treated with both methyl pyruvate and camptothecin. More importantly, the larval samples do not show the inverse relationship between *Snama* and *Dmp53* that is observed in adults. The larvae do exhibit the previously observed pattern of decreasing levels of *Snama* mRNA following stress.

Figure 3.17 shows the mRNA levels of the pro apoptotic *Reaper* larvae remaining statistically unchanged following exposure of the parental flies to camptothecin ($p = 0.3211$) or a combination of both camptothecin and methyl pyruvate ($p = 0.604$).



B: Average quantity of *Dmp53*, *Reaper* and *Snama* transcripts based on OD readings

Figure 3.17: The semi-quantitative Reverse Transcriptase-PCR analysis of the level of RNA transcript levels of *Snama*, *Reaper* and *Dmp53* in larvae following camptothecin and methyl pyruvate exposure of parental flies. (A) Shows the results of the semi-quantitative RT-PCR on a 1% agarose gel. (B) Shows the average optical densities obtained via the analysis of the bands in (A). No clear relationship between *Dmp53* and *Reaper* is present. *Snama* transcription in the larvae seems to be similar to the adult with a high transcription level in the untreated larvae and decreases following all the treatments. Both *Dmp53* and *Snama* transcripts could not be detected in larvae treated with both camptothecin and methyl pyruvate. The error bars represent the standard error of the means of biological replicates.

Levels of *Reaper* mRNA are lower in larvae produced by adults that were fed methyl pyruvate ($p = 0.0066$). As in adult flies, no clear relationship between *Dmp53* and *Reaper* transcription was present. Taken in combination with the lower survival rates during the development of embryos from flies that have been exposed to camptothecin, these results suggest that camptothecin treatment of the adults has altered larval development through Reaper dependent apoptotic pathways.

3.6.7. Real Time PCR results confirm those of the semi-quantitative RT-PCR.

Real Time PCR was used to confirm the relative quantity of *Dmp53* and *Snama* transcripts following camptothecin treatment. The results as seen in figure 3.18 confirm the inverse relationship between *Snama* and *Dmp53* transcription levels. They also reinforce the results obtained for the semi-quantitative PCR as the mRNA levels of *Snama* drop ($p= 0.003, 0.0009$) while those of *Dmp53* increase ($p= 0.601, 0.0117$) following camptothecin treatment. The increase in *Dmp53* mRNA levels during camptothecin treatment is slight and insignificant; this is followed by a large significant increase in the recovery treatment.

The Real Time PCR results suggest that the flies do not recover from camptothecin exposure in the 24 hour period post camptothecin treatment, with the levels of *Dmp53* and *Snama* transcripts not returning to levels near pre-camptothecin levels. Neither do they stay steady. Instead the levels of *Snama* mRNA, continue to fall, while the levels of *Dmp53* transcripts continue to rise. This is in contrast to the semi-quantitative Reverse Transcriptase PCR results which suggest that the flies do begin to recover.

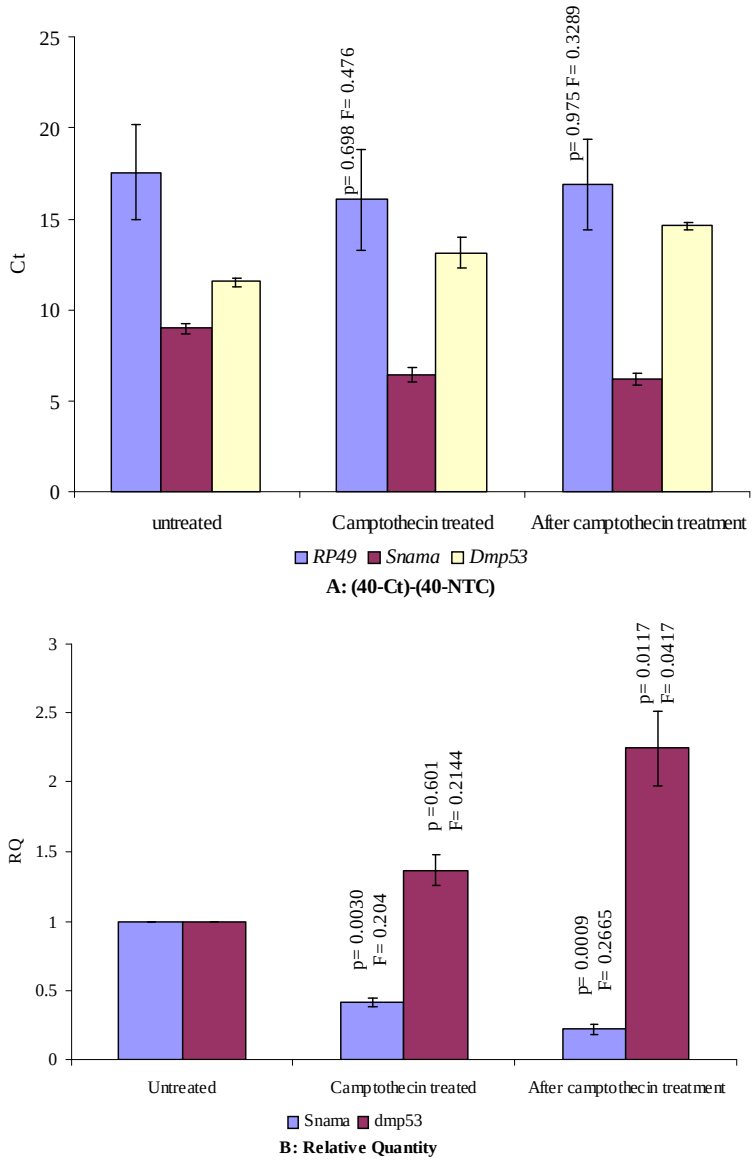


Figure 3.18: Real time quantification of *Snama* and *Dmp53* transcripts in flies that have been left untreated, treated with camptothecin and after Camptothecin treatment. (A) Shows the quantity of the mRNA transcripts for each of the genes as a function of the rate they reached the cycle number threshold less this number for the No Template Control. (B) The pattern of transcription following camptothecin treatment is similar to that seen with the semi-quantitative PCR. Levels of *Snama* mRNA decreases following camptothecin exposure while the levels of *Dmp53* transcripts climb. However, there are no signs of recovery as *Snama* transcript levels continue to drop while those of *Dmp53* continue to climb. The error bars represent the standard error of the means of biological replicates. The p values are the result of a student's -t test comparing the two treatments to the untreated flies.

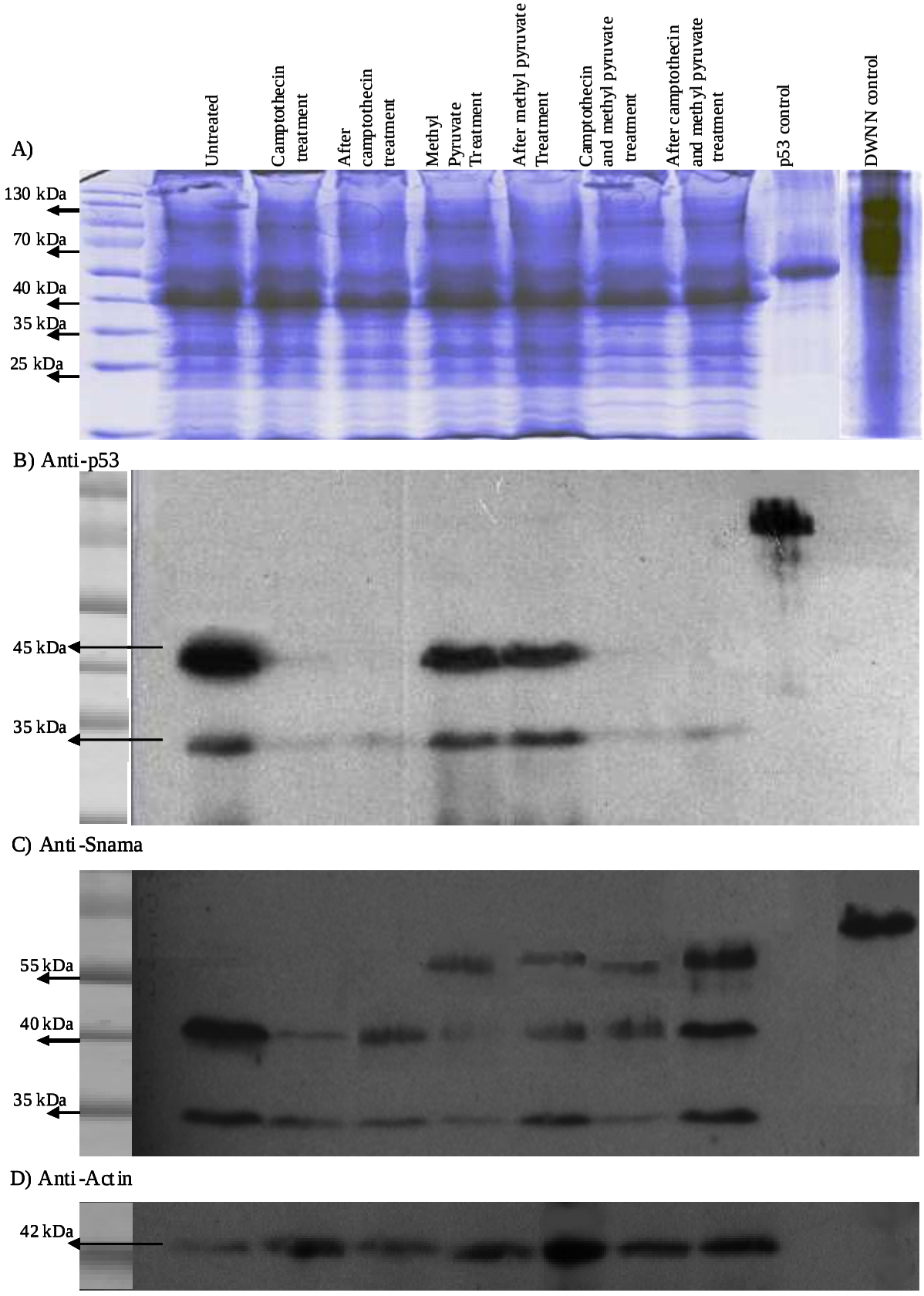
3.7. Changes in the expression pattern of Dmp53, Snama following camptothecin exposure.

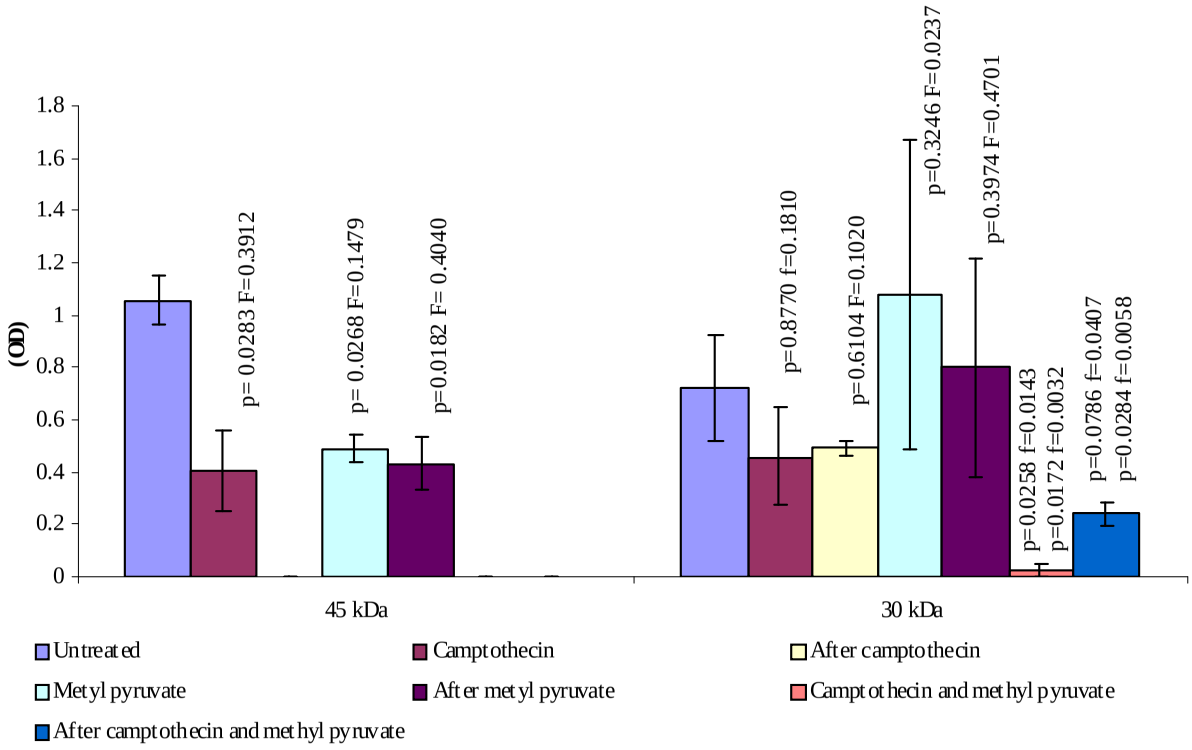
The Northern blots and RT-PCR supplied experimental data concerning the levels of *dmp53*, *snama* and *reaper* gene transcription during and after exposure to camptothecin, methyl pyruvate and a combination of both. However, mRNA levels may not reflect the levels of Dmp53, Snama or Reaper proteins (Greenbaum et al., 2003b; Remedios et al., 1996) in the fly following exposure. Proteins are the functional product of genes and therefore, the proteome is a more useful tool in determining cell function. The relationship between mRNA and protein levels is complex with different studies reporting various levels of correlation between the two. Positive highly significant correlations between the changes in protein and mRNA levels have been reported (Anderson and Seilhamer, 1997) (Orntoft et al., 2002). Other studies report only limited correlations between mRNA and protein expression levels (Greenbaum et al., 2003a) (Chen et al., 2002), while other studies found no correlation at all (Lichtinghagen et al., 2002). These low levels of correlation are most likely due to post transcriptional modification of proteins and protein turnover rates (Greenbaum et al., 2003a). These proteins could be regulated at the protein level through degradation or by regulating protein activity. In order to establish the levels of these proteins following exposure, western blots were performed on protein samples extracted from flies before, during and after exposure to camptothecin, methyl pyruvate and a combination of both these chemicals.

3.7.1 Anti Dmp53 detects two band of the correct size.

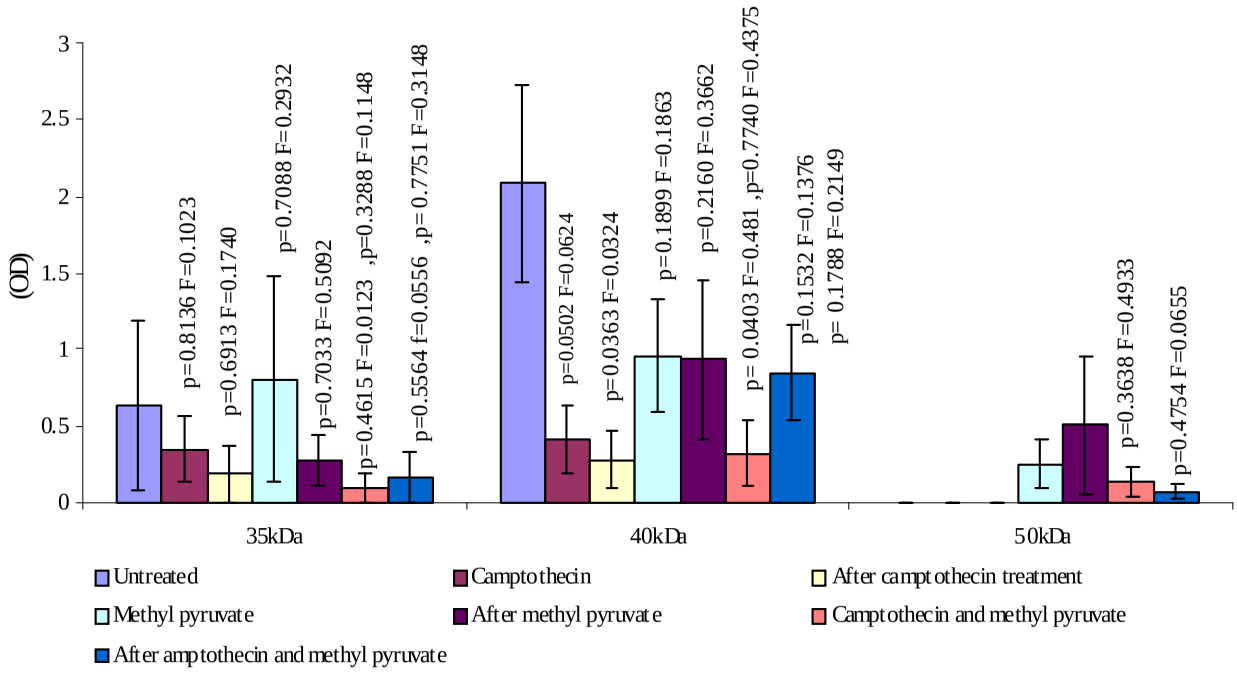
Anti-Dmp53 antibody is detects two bands at 30 and 45 kDa. These bands are close to the expected size for Dmp-53-PD (FBpp0300896) and Dmp53-PA (43.7 kDa) (AAF5607) respectively (figure 3.19 A). The untreated flies have significantly higher expression of the 45 kDa band than flies that have been treated with camptothecin ($p = 0.0283$ $F = 0.3912$) or methyl pyruvate ($p = 0.0268$ $F = 0.1479$). It is completely absent in flies that have been allowed to recover from camptothecin treatment, as well as from flies that had been exposed to a combination of camptothecin and methyl pyruvate. This differs from the transcription patterns observed in the semiquantitative RT-PCR (figure 3.16) and Real Time PCR (figure 3.18). This expression pattern is also unexpected, as exposure to stress would be expected to increase the expression of Dmp53.

A smaller 30 kDa band was detected in all flies regardless of treatment (figure 3.19B and E). There is no significant difference between the expression levels of this protein in most of the treated flies compared to the untreated flies. However, when flies were exposed to a combination of both camptothecin and methyl pyruvate, the expression of this protein is lower than in the untreated flies (During treatment $p = 0.0258$ $f = 0.0143$ and after treatment $p = 0.0786$ $f = 0.0407$) or in those flies that were exposed to camptothecin alone (During treatment $p = 0.0172$ $f = 0.0032$ and after treatment $p = 0.0284$ $f = 0.0058$).


A)
Untreated
Camptothecin treatment
After camptothecin treatment
Methyl Pyruvate Treatment
After methyl pyruvate Treatment
Camptothecin and methyl pyruvate treatment
After camptothecin and methyl pyruvate treatment
p53 control
DWNN control
130 kDa
70 kDa
40 kDa
35 kDa
25 kDa
B) Anti-p53
45 kDa
35 kDa
C) Anti-Snama
55 kDa
40 kDa
35 kDa
D) Anti-Actin
42 kDa



E: Determination of the quantity of Dmp53 based on optical density



G) Determination of the quantity of Snama based on optical density

Figure 3.19: Western blot analysis of *Drosophila melanogaster* treated with camptothecin, methyl pyruvate and both camptothecin and methyl pyruvate. Positive controls exist of a recombinant GST-DWNN Catalytic Domain fusion protein and a recombinant GST-Dmp53. (A) Is a duplicate gel. (B)(E) Anti Dmp53 detects a 45 and 30 kDa band. The 45 kDa band is similar in size to the 43.7 kDa Dmp53-PA while the smaller 30 kDa band is near to the 36.1 kDa size for the smaller transcript Dmp-53-PD. This 45 kDa band vanishes during the recovery from camptothecin treatment. It is also undetectable in flies during and after treatment with a combination of camptothecin and methyl pyruvate. The expression level of the smaller 30 kDa band in most of the treated flies is not significantly different from the level in the untreated flies. The exception is those flies exposed to a combination of both camptothecin and methyl pyruvate. (C) Anti-Snama detects the Snama positive control, a 35 and a 40 kDa band in all flies regardless of treatment while a larger 50 kDa is detected only when flies are exposed to methyl pyruvate. (F) The 35 kDa band is detected at statistically the same level in all flies regardless of treatment. The 40 kDa protein is detected at significantly lower levels in flies treated with camptothecin and during treatment with both camptothecin and methyl pyruvate (D) Anti actin detects a band of the correct size at approximately 41.8 kDa for *D. melanogaster* actin. In (E) and (F) the error bars indicate the standard error of the means of biological replicates. The p values are the result of two tailed t-test analysis between the untreated and all treated flies, as well as between the camptothecin treated and combination of camptothecin and methyl pyruvate treated flies.

3.7.2 Anti-Snama detects 3 different bands, none of which are the correct size.

The anti-Snama antibody detects a 35 and a 40 kDa band in all flies regardless of treatment (figure 3.19 C). While there is no significant difference in the expression level of the 35 kDa band regardless of treatment, there is a significant decrease in the level of the 40 kDa protein in flies treated with camptothecin ($p = 0.0502$ $F = 0.0624$) as well as in flies treated with both camptothecin and methyl pyruvate ($p = 0.0403$ $F = 0.481$) (figure 3.19 F). Anti-snama also detects a larger 50 kDa protein in flies that have been exposed to methyl pyruvate or a combination of methyl pyruvate and camptothecin. This band is detected in flies that are still undergoing treatment as well as in flies that are recovering from treatment. There is no significant difference between the expression level of this 50 kDa protein regardless of treatment or stage of recovery. The common factor amongst all the flies that express this protein seems to be exposure to methyl pyruvate. The expression of these proteins does not match the transcription patterns observed in the semiquantitative RT-PCR (figure 3.16) and Real Time PCR (figure 3.18). However, neither do they match the expected protein size of 139 kDa. The larger 50 kDa band is near to the 55.6 kDa predicted size of a theoretical splice variant of Snama (Snama-PB).

3.8. Functional role of Dmp53 following camptothecin treatment.

In order to establish what role Dmp53 plays in vivo following camptothecin treatment, the drug was used to treat flies that lack an active Dmp53. The mutant fly line y^{1} w^{1118}; $p53^{5A\text{-}1\text{-}4}$ contains a 3.3kb deletion in *dmp53* gene. These flies show an increased ability to survive UV irradiation (Qi et al., 2004) and a higher mortality rate

during development (Jaklevica and Su, 2004). In order to establish what role dmp53 plays in the effect that camptothecin has on the ability of the flies to reproduce, or to develop to adulthood, y^1 w^{1118}; $p53^{5A-1-4}$ mutant flies were separated into males and females and exposed separately to camptothecin or left untreated. The response of these flies to treatment was then monitored.

3.8.1 Camptothecin exposure has no adverse effects on the fecundity or development of p53 null mutant flies.

As can be seen from figure 3.20 A, camptothecin had no significant negative effect on the ability of the mutant flies to produce embryos ($p = 0.3673$) compared to the wild type flies, which displayed a significant decrease in embryo production when female flies were fed camptothecin $p = 0.0001$).

The embryos produced by the mutant flies that were exposed to camptothecin did not display any significantly reduced ability to reach adulthood (Figure 3.20 B) ($p=0.000$). They did show a slightly higher mortality rate than the wild type flies. This was expected due to the complete loss of *dmp53*. This higher mortality occurred in the pupae as well as the embryos and larvae.

To confirm that the $p53^{5A-1-4}$ mutant strain did indeed have a 3300 bp deletion of *dmp53*, PCR was performed with *Dmp53* specific primers using genomic DNA - extracted from wild type and $p53^{5A-1-4}$ - as a template. The *Dmp53* primers amplify a 1200bp fragment from wild type flies but failed to amplify a product from $p53^{5A-1-4}$ flies (figure 3.21). The presence of good quality intact genomic DNA for both

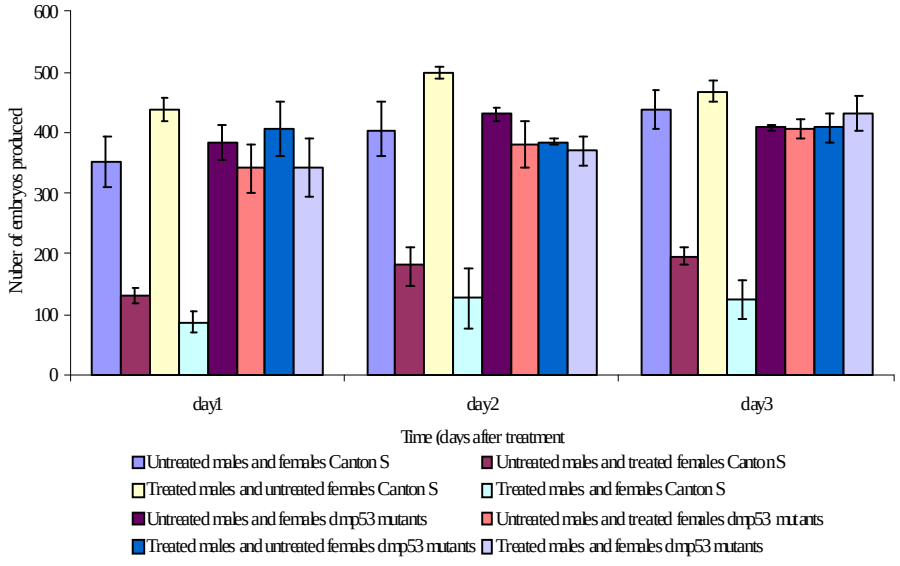
samples was demonstrated via the successful amplification of a product of the correct size for *Rp49,* using primers specific for *Rp49*. This implies that these flies were indeed *dmp53* null mutants and the effects observed in figure 3.21 is indeed due to a lack of active Dmp53.

3.9 Discussion.

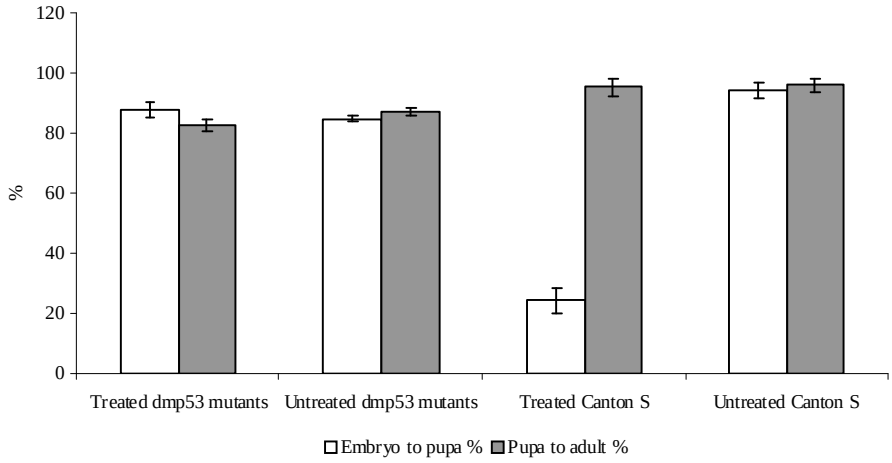
As camptothecin is a DNA damaging agent, it is a potential mutagen, with toxicity in mammals manifesting itself in a wide variety of pathologies. These include hair loss; decreased food consumption; high thymic atrophy; visible defects of thoracic and abdominal organs; increases in testosterone and finally a decrease in litter size as a result of an increase in the number of foetal deaths (Chung et al., 2006).

3.9.1. DNA damage has negative effects on fecundity, lifespan and the viability of *D. melanogaster* offspring.

Camptothecin treatment had severe and highly noticeable effects on the fecundity, development and mortality of *D. melanogaster*. Embryo production decreased sharply after treatment. This effect on fecundity was confined to female flies and was due to some direct effect produced by the action of camptothecin. It is already known that camptothecin negatively impacts gonads, as cells which proliferate rapidly are the main targets of anticancer drugs (Tanaka et al., 2008). Therefore, chemotherapy often results in gonadal impairment with alkylating agents causing the most severe damage ((Meirow, 2000) ,(Howell and Shalet, 1998), (Gradishar and Schilsky, 1988)).



A: The effect of camptothecin on dmp53 null mutant flies ability to reproduce



B : The effect of camptothecin on the development of dmp53 null mutant flies

Figure 3.20: The effect of camptothecin on *dmp53* null mutant flies. Figure (A) shows that camptothecin has no effect on the ability of the *Dmp53* null mutant flies to produce embryos. However, embryo production is slightly lower in the *Dmp53* null flies than in untreated wild type flies. Figure (B) shows that camptothecin has no effects on the development of the embryos produced by p53 null mutant flies that were exposed to camptothecin. This is in stark contrast to treated wild type flies. The error bars represent the standard error of the means of technical replicates of biological replicates.

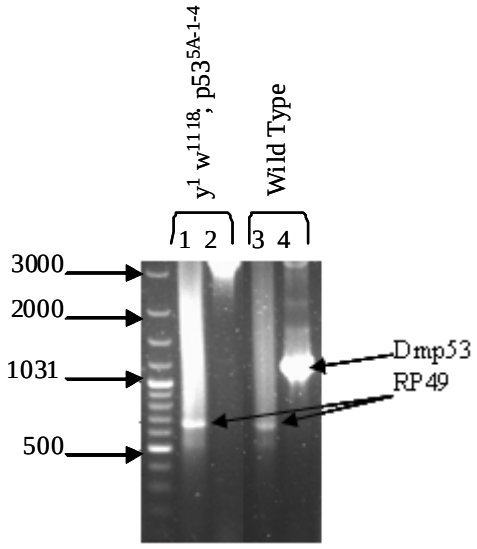


Figure 3.21: Confirmation of the genotype of y1 w118 p53 5A-1-4 mutants through PCR. The PCR confirms that the y1 w118 p53 5A-1-4 mutants lack *Dmp53*. The *Dmp53* specific primers fail to amplify the gene from the y1 w118 p53 5A-1-4 DNA extracts, while *Dmp53* is amplified from the wild type flies. This is not due to the quality of the extracted DNA as the RP-49 control primers do successfully amplify *Rp49* in both the wild type and the mutant DNA extracts.

Through the use of null Dmp53 mutant flies, homozygous for a 3.3 kb deletion of the *Dmp53* gene, it was shown that the decrease in fecundity following camptothecin treatment is Dmp53 dependent. Examination of camptothecin treated female ovaries, showed them to be abnormally small and with lowered structural integrity (Ntwasa private communication), which is indicative of increased levels of apoptosis. This implies that the decreased fecundity is a result of an increase in Dmp53 dependent apoptosis in the ovaries. Another toxin, the protein kinase C inhibitor staurosporine had no effects on fecundity. Therefore, the observed decrease in fecundity is due specifically to the DNA damaging effects of camptothecin.

Camptothecin treatment leads to an equal increase in the mortality of both sexes, despite the concurrent increase in detoxification genes. This may be due to the increased energy used to express the detoxification enzymes, or the failure of the detoxification systems to completely rescue the flies. Additionally camptothecin treatment of adult flies led to offspring that developed slower and displayed higher than normal levels of apoptosis that occurred at inappropriate stages of development. This situation has also been observed in rats treated with camptothecin (Chung et al., 2007).

Camptothecin itself could be transferred to the embryos in small amounts, as other toxins are known to enter the *D. melanogaster* embryo following exposure (Stallings et al., 2006). Topoisomerase assays failed to consistently show a lack of topoisomerase activity in these samples (figure 3.8). However, there was still some evidence that active topoisomerase is absent from these embryos. Since camptothecin is used as a chemotherapeutic agent it should be able to cross membranes and into the

embryos from the adult female where it may continue to inhibit topoisomerase activity. The ring canals that connect the developing oocytes would also aid in the transport of camptothecin between cells. The defects in development may be due to topoisomerase I no longer being supplied in high enough quantities to the embryo in the ovary, as it has been depleted through the action of camptothecin. Previous studies using Western blot analysis show about 90% of Topoisomerase I was found linked to DNA following camptothecin treatment. This also resulted in increased ubiquitination of topoisomerase with a 75% decrease in topoisomerase I levels (Desai et al., 1999).

Ovaries that were surgically removed from patients treated with camptothecin displayed an absence of growing follicles. The high levels of apoptosis in these cells following treatment with camptothecin, was found to be due to Fas ligand expression (Utsunomayi et al., 2008). If camptothecin signalling stimulates specific Eiger expression in the *Drosophila* ovaries, it would explain why the defects observed were confined to the ovaries as well as the poor condition of the ovaries themselves. Camptothecin treatment also increased the transcription of *Dmp53* as seen in the real time and semi-quantitative PCR. This could lead to an increase in Eiger expression, activating the Wengen pathway and resulting in an increase in Hid dependent apoptosis.

Camptothecin rapidly induces ovarian failure in premenopausal woman, with symptoms including decreased sex hormone production (Tanaka et al., 2008), as well as an increase in the levels of follicle stimulating hormone and lutenizing hormone levels (Utsunomayi et al., 2008). The major hormones in insects that are responsible for development and reproduction are ecdysteroids (Simon et al., 2003). Removal of insect ovaries leads to a decrease in the synthesis of juvenile hormone (JH) and an

increase in JH esterase activity (Renuci et al., 1990). Following stress both JH and 20 ecdysterone are up-regulated, resulting in decreased fecundity due to delayed oocyte maturation, increased levels of egg re-absorption and decreased oviposition (Gruntenko and Rauschenbach, 2008). The two hormones regulate each other's levels by negatively impacting hormone degradation. However, if juvenile hormone is absent the remaining 20E induces apoptosis in the nurse cells as well as leading to an accumulation of yolk proteins in the hemolymph due to oocyte re-absorption (Soller et al., 1999). A disturbance in the hormone levels is implied in this study due to the increased levels of yolk protein detected using 2D-PAGE. This may then result in the decrease in embryo numbers due to oocyte re-absorption or increased apoptosis in the nurse cells.

The negative effects of camptothecin on the offspring of exposed flies seem to have ceased by the larval stage. *Dmp53* RNA levels have returned to normal and the development studies indicate that at some stage there must be near to complete recovery in one of the larval stages. However, *Reaper* and *Snama* transcript levels are both lower in the larvae from the camptothecin treated flies. This implies that camptothecin treatment of the adults is still exerting an influence on the larvae. This difference in the levels of *Reaper* transcripts may be due to a time based induction of Reaper expression and apoptosis. Reaper is expressed in the developing neurons of larvae, with loss of function *Reaper* mutants displaying enlarged central nervous systems (Peterson et al., 2002). At this stage of development Reaper expression is most likely under the control of the Ecdysone receptor signalling cascade (EcR) (Brodsky et al., 2000). This hormone regulates the metamorphosis from larvae to adult and puparium formation is preceded by a sharp rise in the levels of this hormone (Bangs and White, 2000). During the initial larval stages the levels of ecdysone

increase leading to increased Reaper expression and apoptosis. The hormone levels drop during the mid-prepupal stages and this is followed by a late stage spike of hormone secretion which triggers puparium formation (Buszczak and Segraves, 2000). The lag in development observed in embryos from flies exposed to camptothecin would therefore imply that even though the embryos were the same age they may be at different stages of development and would be expressing Reaper at different levels.

3.9.2 The Stress response of normal cells to DNA damage by camptothecin results in a glycolytic flux.

Proteomic analysis of wild type *D. melanogaster* during recovery from exposure to the DNA damaging agent camptothecin, revealed changes in the expression of new genes. When the proteomic responses of these normal *Drosophila* cells recovering from camptothecin treatment (figure 3.9 and 3.10), are compared to those of human cancer cells, undergoing camptothecin treatment (Parenta et al., 2009) (Yu et al., 2007) some similarities are observed. This similarity is interesting because it demonstrates a similar but not identical pattern of protein expression occurs in human cancer cells and normal *D. melanogaster* cells. In both cell types, camptothecin treatment led to the increase in the expression of cytoskeletal proteins, antioxidant and detoxification enzymes, metabolic proteins and proteins involved in protein degradation (Hull and Ntwasa, 2010) (Parenta et al., 2009) (Yu et al., 2007). Notably those proteins involved in metabolism were specifically increased in *D. melanogaster*. It is also important to note that not all the spots present on the 2D-PAGE were analysed by mass spectrometry.

The increase in the expression of enzymes involved in glycolysis and the Krebbs cycle is significant. This implies that following camptothecin exposure the flies are undergoing a glycolytic flux that involves an increase in enzymes from multiple metabolic pathways such as the TCA cycle and glycogenesis. This metabolic shift is also different to that observed in cancer cells, where there is a shift to the rapid but less productive glycolytic cycle (Warburg, 1956).

In cancer cells an increase in the ability to resist apoptosis caused by DNA damage is linked to an increase in the expression of glycolytic enzymes or an increase in the energy use of the cell and consequently increased energy production. This increase is greater than that normally observed in untreated cancer cells (Shin et al., 2009). Additionally, apoptotic stimuli are known to induce an increase in cellular ATP levels. Energy is required for the orderly progression of apoptosis and for DNA damage repair (Zamaraeva et al., 2005). An increase in the amount of ATP present in damaged cells also favours apoptosis over necrosis (Grusch et al., 2002).

As in cancer cells, the normal cells recovering from DNA damage in this study, may require an increase in the amount of available energy, necessitating the increased expression of metabolic enzymes. This energy may be required by *D. melanogaster* to repair DNA damage or to aid in increased protein turnover. It may also be required for increased gene expression or even to supply energy required for an increase in apoptosis.

The increase in the expression of arginine kinase (figure 3.10) during the recovery period may function to generate an energy reserve in invertebrates by catalysing the

transfer of a phosphate group to arginine to generate phosphoarginine (Wu et al., 2007). The resulting reserve may be required for high energy buffer systems, rapid motility, and most interestingly the regulation of glycolytic fluxes (Brown et al., 2004). Therefore, increased arginine kinase may act to create a reservoir of stored energy that will be used to generate an increase in the available energy in a short time, preventing any interruption in cell recovery.

Triphosphate isomerase (TPI) is the only protein involved in carbohydrate metabolism, whose expression levels decreases during the recovery from camptothecin treatment. It is worth noting that the temperature sensitive *wasted away* mutation in *D. melanogaster* results in flies that exhibit motor impairment, vacuolar neuropathology, and severely reduced lifespan (Gnerer et al., 2006). These pathologies are similar to those found in human TPI deficiency. Incidentally they also closely resemble the side effects of camptothecin treatment. Consequently, the inhibition of TPI by camptothecin treatment may provide an explanation for some of these side effects.

The range of metabolic enzymes affected during recovery from camptothecin exposure, shows that pathways have been affected in two separate cell compartments, namely the cytoplasm where glycolysis occurs and the mitochondria where the Krebs cycle occurs. Camptothecin and its analogs are known to effect mitochondrial respiration by lowering the expression of subunits of the cytochrome oxidase complex. This results in decreased ATP production (Yu et al., 2007). It has also been reported that apoptosis resulting from DNA damage caused by reactive oxygen species, is followed by an initial glycolytic block, followed by a glycolytic flux that is

necessary for the apoptotic response. Inhibition of this glycolytic flux consequently inhibits apoptosis (Cerella et al., 2009). Therefore, the increase in energy production observed during recovery from camptothecin treatment may be a means whereby the cells compensate for either, the negative effect on mitochondrial respiration or an initial glycolytic block. This may be why camptothecin treatment is seen to increase carbohydrate metabolism in both the cytoplasm and mitochondria.

Glycolysis is largely driven by the activity of the three enzymes hexokinase, phosphofructokinase and pyruvate kinase. These enzymes catalyse the reactions with the largest negative Gibbs free energy. The dramatic increase in the expression levels of pyruvate during recovery from camptothecin treatment (figure 3.10), could be driven by an increase in the amount of glucose entering the glycolytic pathway, increased levels of the substrate phosphoenolpyruvate (PEP) or increased levels of fructose 1,6-bisphosphate, an intermediate in glycolysis.

3.9.3 The link between Dmp53, metabolism and apoptosis.

Disruption of *p53* gene function has been demonstrated to cause the shift to the Warburg Effect (Ma et al., 2007) (Bensaad and Vousden, 2007). Mammalian p53 can inhibit glucose uptake by repressing the expression of glucose transporters such as Glut 1. Another regulator of pyruvate kinase TIGAR (TP53-induced glycolysis and apoptosis regulator) is activated by p53 directly inhibiting the glycolytic pathway. TIGAR functions to decrease the activity of the glycolytic enzyme 6-phosphofructose-1-kinase (PFK1). Thirdly, p53 can directly repress the expression of phosphoglycerate mutase (PGM). Finally p53 plays an important role in influencing

mitochondrial respiration or oxidative phosphorylation through transcriptional control of the *Synthesis of cytochrome c oxidase 2* (*SCO2*) gene (Cheung and Vousden, 2010).

As expected we observed an increase in the transcription levels of *dmp53* following camptothecin treatment. Camptothecin exposure is known to initiate p53 over-expression in mammalian cell lines and the DNA damage repair and apoptosis functions are conserved between mammalian and *Drosophila* p53 (Jassim et al., 2003). Treatment of Hepatocellular Carcinoma Cells with the camptothecin analogue irinotecan resulted in increased p53 expression and activity. The resulting increase in the levels of apoptosis appear to be p53 dependent as decreased p53 expression results in decreased apoptosis (Takeba et al., 2007). This relationship has also been reported for other cancer cell lines (Albihn et al., 2007) (Liu and Zhang, 1998) Significantly the pro-apoptotic effect of camptothecin can be regulated by p53. In p53-defiecient cells the camptothecin analogue topotecan induces apoptosis and DNA damage more effectively than in p53 expressing cells. Topotecan triggers topoisomerase I degradation via the proteasome pathway in a p53 assisted manner. In doing this p53 does not physically interact with topo I but regulates the synthesis of an unknown intermediate that may ubiquitinate topo I or be involved in transcription-coupled repair of damage caused by camptothecin (Tomicic et al., 2005). Our study is the first to show that camptothecin treatment of *Drosophila melanogaster* stimulates *Dmp53* transcription.

An increase in *Dmp53* transcription is accompanied by a decrease in the transcription of *Snama*. During recovery from camptothecin treatment this is followed by an

increase in *Snama* transcription. Methyl pyruvate exposure has a similar effect on the transcription of *Dmp53* and *Snama* as camptothecin exposure. The inverse transcription pattern observed for *Dmp53* and *Snama* following camptothecin treatment seems to indicate that a relationship exists between the two molecules, possibly by controlling each others transcription, either directly or indirectly. This is what would be expected if Snama is a negative regulator of Dmp53, playing a role similar to that of MDM2. The ability of mammalian homologs of Snama such as RBBP6 or PACT to interact with MDM2 and assist in the negative regulation of p53, support these observations as does the DWNN domains structural similarity to ubiquitin (Mather et al., 2005). This relationship may involve Snama directly influencing Dmp53 stability through its function as an E3 ubiquitin ligase.

The lack of any correlation between the expression and transcription data for *Dmp53* implies that Dmp53 activity is largely regulated at the protein level following exposure to camptothecin or methyl pyruvate. The higher levels of the 45kDa protein detected in untreated flies may represent inactive protein that will be activated following DNA damage. Alternatively, some studies have reported that camptothecin induced apoptosis does not solely rely on p53, with no increase in the levels of p53 activity (Schmidt et al., 2001). The decrease in the levels of p53 following exposure to camptothecin or methyl pyruvate is not unheard of. Camptothecin treatment is known to activate both the ATM and ATR pathways. In the absence of ATM, camptothecin treatment leads to the activation of the ATR-Chk-1 pathways, lower levels of p53 and delayed apoptosis (Zuco et al., 2009).

The decrease in the level of the 45 kDa protein may represent a delayed apoptotic response. The lack of any detectable protein in the recovery phase would indicate that most of the Dmp53 was removed from the cell. However, the transcript levels remain high indicating that it may be possible to rapidly return to higher levels of Dmp53.

Since the expression levels of the 30 kDa protein do not change significantly following camptothecin or methyl pyruvate exposure, the increase in the transcription of *Dmp53* could indicate translational control where the level of Dmp53 is kept constant through controlled protein degradation. It is important to note that the PCR based analysis of the *Dmp53* transcripts could not distinguish between the different isoforms of Dmp53 and therefore, the *Dmp53* transcript detected using RT-PCR may not code for the 45 kDa isoform detected in the Western blot. Additionally any differences between the expected and observed sizes may be due to extensive posttranslational modifications that often occur in p53 proteins, which complicates the accurate determination of p53 size (Meek and Anderson, 2009).

One area where the functions of Snama and Dmp53 seem to correspond is in proliferation. Snama is vital for flies to survive embryogenesis, and is required for cells to proliferate. Cells that have differentiated or are postmitoic cells do not die if they lack Snama. Proliferating cells that lack Snama have an excess DNA content which is probably the result of the inability to divide or aberrant DNA synthesis (Jones et al., 2006). Dmp53 does not normally play a role in cell cycle progression but it does seem to be required for compensatory proliferation in tissues that have lost cells to apoptosis. Here Dmp53 is activated by DRONC and is able to control cell progression by inhibiting cdc25 (Wells et al., 2006). Following this process cells

proliferate probably due to signals initiated by Dmp53. However, Dmp53 signalling must be curtailed in order to remove the cell cycle block, allowing proliferation in order to replace the cells killed during camptothecin treatment. Following camptothecin treatment cells that have been killed must be replaced. This would then explain the transcription patterns of *Snama* and *Dmp53* observed in cells recovering from camptothecin treatment.

The anti-Snama antibody is raised against the DWNN domain. Since the anti-Snama antibody is able to detect the recombinant DCM protein, but is unable to detect the full length Snama at 139 kDa, it can only be assumed that the smaller bands represent degraded Snama or DWNNylation (transfer of DWNN groups) of other proteins. This would imply that the bands detected on the western blot represent Snama activity and not Snama itself. Another likely explanation for the detection of no proteins of the correct size is the basic charge of the C' terminus of Snama. This has previously been reported for PACT where the protein needed to be acetylated before it could be detected at the correct size on a Western blot (Simons et al., 1997).

The expression of these proteins do not match the transcription patterns observed previously, with the smallest protein being unaffected by stress caused by methyl pyruvate or camptothecin. The detection of a 50kDa protein in flies that have been exposed to methyl pyruvate is interesting, as a 55 kDa protein is predicted to be produced by a splice variant of the *Snama* gene (Tweedie et al., 2009). This smaller Snama still contains the DCM and would be detected by the antibody.

The expression pattern observed for Snama and Dmp53 seems to reflect a complex interplay between the two molecules. If Snama is maintaining Dmp53 at stable levels in untreated flies, then the decrease in Dmp53 levels following DNA damage seems to indicate an increase in Dmp53 turnover. This implies that initially apoptosis is being prevented. The continued degradation of Dmp53 in the recovery phase may then result in the absence of any Dmp53 protein in these flies, while there are still degradation products.

The absence of any direct relationship between *Reaper* and *Dmp53* transcription seems to imply that Reaper is being regulated through a means other than Dmp53. Reaper can be regulated by both the p53 DNA damage response pathway and the Ecdysone receptor signalling cascade (EcR) (Brodsky et al., 2000). If the normal secretion of this hormone is being disrupted due to camptothecin exposure, it could explain the incongruence in *Reaper* and *Dmp53* transcription patterns.

3.9.4 The effects of bypassing glycolysis.

Due to the fluctuation in metabolic enzymes observed during the recovery phase after camptothecin treatment, methyl pyruvate supplementation was expected to have positive effects on the fecundity, longevity and embryonic development of flies that had been negatively affected by camptothecin exposure. By so bypassing glycolysis it was intended to determine what role the glycolytic flux may play in the stress response to DNA damage.

The negative effects methyl pyruvate had on embryo numbers and recovery rates may be due to an increase in metabolic pathways caused by excess pyruvate. In the absence of glutathione, pyruvate has been reported to decrease cell viability, most likely by generating ROS that damage the mitochondrial membrane (Mari et al., 2002). The viability of embryos produced by flies exposed to methyl pyruvate was decreased compared to embryos from untreated flies. However, when flies were exposed to camptothecin and methyl pyruvate, there was an increase in the viability of embryos compared to embryos from flies that had been exposed to camptothecin. This implies that methyl pyruvate is protecting these embryos from the increased levels of apoptosis that they experience following camptothecin treatment.

Methyl pyruvate exposure increases *Dmp53* mRNA which is followed by the near absence of *reaper* transcripts. Pyruvate prevents the nuclear translocation of p53 as well as affecting the ratios of *Bax/Bcl-2* (Frenzel et al., 2005; Lee et al., 2003) (Kang et al., 2001). This may explain the lack of *Reaper* transcription as cytoplasmic p53 is not able to promote transcription.

The absence of *Dmp53* transcripts is matched by an absence of the larger Dmp53 protein and a decrease in the levels of the smaller protein. Despite this the significantly higher levels of *Reaper* transcription implies that the combination treatment is extremely stressful and the higher levels of *Reaper* transcription would lead to higher levels of apoptosis.

The decrease in p53 transcription and expression may be due increased protection from ROS by methyl pyruvate by increasing substrate availability for the Krebs cycle.

This would result in increased ATP and NADH, which will lead to an increase in the levels of reduced glutathione. Pyruvate is also able to scavenge free radicals and protects DNA from damage caused by high levels of sugar in diabetic rats (Frenzel et al., 2005) (Kang et al., 2001).

3.9.5 Other cellular changes that occur as a result of DNA damage.

The response of *D. melanogaster* to chemotherapeutic agents varies. The generation of reactive oxygen species (ROS), resulting in the increased transcription of a substantial number of phase I (Cytochrome p450) and phase II (GSTs) enzymes, antimicrobial peptides and odorant receptor proteins (Vermeulen and Loeschcke, 2007). Topotecan and camptothecin treatment are known to increase the levels of antioxidant enzymes such as catalase, glutathione peroxidase and superoxide dismutase (Muluk et al., 2005).

One of the stress response genes whose expression was increased in the recovery phase following camptothecin treatment of *Drosophila melanogaster* was GST S1. GST P1 expression is known to increase following camptothecin exposure leading to a decrease in the levels of apoptosis (Haraa et al., 2004). As there is no published evidence that glutathione-camptothecin complexes can form (Gamcsik et al., 2001) GST S1 is most likely up-regulated in this study to protect cells from oxidative damage caused by camptothecin exposure. GST assays showed that the changes in protein expression detected in 2D-PAGE analysis reflect actual changes in protein levels and activity. It may be noted here that GST S1 has a lower affinity for the CNDB substrate used in the GST enzyme assay than most other GST classes

(Agianian et al., 2003) and this may be the cause of the lower than predicted activity observed during the enzyme assay.

There is also a significant increase in the expression of detoxification, and cytoskeletal proteins. Superoxide Dismutase is another antioxidant protein whose expression increases during the recovery from camptothecin treatment. In *Drosophila* mitochondria SOD is regulated by MAPK signalling via AP-1. Mitochondrial SOD has three promoter elements MRE-metal responsive element-, ARE-antioxidant responsive element- and XRE-Xenobiotic responsive element. The activation of one or more of these can initiate SOD transcription (Korsloot et al., 2004). In the case of camptothecin treatment, both the XRE and the ARE may be responding to the drug and initiating SOD transcription. The MRE responds to heavy metal toxins and strong oxidizing agents and camptothecin may be able to induce SOD expression in this way as well.

Components of the 20S proteasome were also found to be expressed at higher levels during recovery from camptothecin treatment (figure 3.10). Increase in the activity of the proteasome has varied effects on apoptosis levels depending on when the action of the proteasome is inhibited. Inhibiting the proteasome before treatment of small cell lung carcinoma cells with camptothecin prevents the pro-apoptotic action of topoisomerase inhibitors. If the proteasome inhibitors are administered after treatment with topoisomerase inhibitors, they enhance their proapoptotic effect (Tabata et al., 2001). The obvious explanation for these observations is that the proteosome is required to allow cells to recover from the effects of camptothecin. This probably involves the removal of damaged or misfolded proteins after camptothecin treatment

and degrading the contents of dead and dying cells during the treatment with camptothecin.

Individual proteins that are responsible for protein folding show increased expression following camptothecin exposure. However, there is no significant overall increase in protein folding protein activity. During the recovery period two proteins that aid in protein refolding, protein disulfide isomerase and chaperonin increase while the expression of a third Erp60, is not significantly affected (figure 3.10B). Erp60 has similar functions to that of protein disulfide isomerase. Erp60 is also required for the correct folding of glycoproteins and can be found outside of the ER, particularly in the nucleus, where it can act to regulate STAT3 signalling by binding to this molecule (Coea and Michalaka, 2010). Since STAT3 is involved in some apoptotic responses (Horvath, 2000), the expression of this protein may not increase, as the levels of STAT3 must remain constant, increasing or decreasing apoptotic signalling.

Another protein whose expression increases during the recovery period is yolk protein. This structural molecule is known to be involved in vitellogenesis; oogenesis and sex differentiation. The protein does possess a lipase domain and is known to act as an antioxidant in queen bees improving their lifespan (Corona et al., 2007) and it may play this passive role in *D. melanogaster* during the stress response to camptothecin. However, stress generally leads to the decrease in yolk level protein due to its regulation, expression and uptake by a balance in the levels of 20H hydroxyecdosyne and juvenile hormone (Gruntenko and Rauschenbach, 2008). It is

likely that the stress response initiated by camptothecin treatment may also lead to hormone fluctuation and the increased levels of yolk protein expression.

Chapter 4

The identification and characterization of Antimicrobial peptides in *Euoniticellus intermedius*.

4.1 Introduction

4.2 The effects of infection and the cost of the immune response

4.3 Antimicrobial activity

4.4 Changes in overall protein expression following infection in *E. intermedius*

4.5 Identification and characterization of Immune related genes

4.6 Discussion

4.1 Introduction.

Despite the order Coleoptera being the largest of the insect orders, the study of the beetle immune system has been confined to a few species, only one of which is a representative of the true dung beetles while most of the work on non antimicrobial signalling components in the coleopteran immune system has been performed in the beetles *A. dichotoma* and *Tribolium castaneum.* The variation amongst beetles due to their varied habitats and diet should result in differences in their immune system.

Insects initially respond to infection via the use of constitutively active responses that involve hemocytes. These responses involve encapsulation and phagocytosis as well as the pro-phenoloxidase response and are largely responsible for the removal of most of the invading pathogen population (Haine et al., 2008). Insects then rely on the induced antimicrobial peptide response to remove remaining bacteria. The first insect antimicrobial peptide cecropin was isolated from the cecropia moth *Hyalophora cecropia* in 1981 (Trenczek, 1997). Numerous peptides have since been isolated from other lepidopteran, dipteran and hymenopteran insects. Antimicrobial peptides have also been isolated from the holometabolous hemipterans (Otvos, 2000).

The only true dung beetle whose immune system has been studied in detail is a beetle from the tribe coprini (black dung beetles) *Coprini tripartitus.* Like *E. intermedius* this is a tunnelling dung beetle. A defensin was isolated from this beetle and named coprisin. It appears in high amounts only four hours after infection and shares 67-71% identity with other beetle defensins. However, it does have a wider range of activity

than most defensins being active against gram negative and gram positive bacteria (Hwang et al., 2009).

In order to study the response of *E. intermedius* to infection, adult beetles were infected with various pathogens. The effectiveness of these infections was confirmed using mortality and fecundity assays, while changes in protein expression were monitored using HPLC and SDS-PAGE. An attempt was made to isolate and identify antimicrobial activity from the beetle following immunization while changes in the proteome of the beetle following infection were also examined. Peptide sequences were used to design primers in order to isolate the genes coding for these proteins. These genes were then used to create cDNA probes to examine the transcript levels following infection.

4.2 The effects of infection and the cost of the immune response.

Beetles were challenged with a variety of different pathogens. *Micrococcus luteus* was used as a representative of gram-negative bacteria and *Escherichia coli* as a representative of gram-positive bacteria. The fungal pathogen *Beauveria bassiana* was used as it is a natural insect pathogen, which is under investigation as a possible bio-control agent. Exposure of the beetles to these pathogens was expected to induce or increase the expression of anti-microbial peptides.

Table 4.1: List of coleopteran families, sub families and tribes where an analysis of the coleopteran immune system has been carried out

Polyphaga			Reference
Scarabaeformia	Scarabaediae		
	Dynastinae		
		Allomyrina dichotoma	(Miyanoshita et al., 1996)
		Oryctes rhinoceros	(Ishibashi et al., 1999)
		Trypoxylus dichotomus	(Miyanoshita et al., 1996)
	Melolonthinae		
		Holotrichia diomphalia	(Lee et al., 1994)
	Rutelinae		
		Anomala cuprea	(Yamaauchi, 2001)
	Scarabaeinae (true dung beetles)		
	Copris		
		Copris tripartitus	(Hwang et al., 2009)
	Scarabaeus		
		Euoniticellus intermedius	This study
Cujiformia			
	Tenebrionidae		
		Zophobas atratus	(Bulet et al., 1991)
		Tribolium castaneum	(Altincicek et al., 2008)
		Tenebrio molitor	(Moon et al., 1994)
	Curculionidae		
		Sitophilus zeamais	(Anselme et al., 2008)
	Chrysomeloidea		
		Acalolepta luxuriosa	(Imamura et al., 1999)
	Elateriformia		
		Pyrocoelia rufa	(Lee et al., 2001)

This table lists those beetle species whose immune systems have been studied. Most studies have centred on the isolation of antimicrobial peptides. Only the tenebroid beetles *T. castaneum* and *T. molitor* have had more in depth studies, involving the study of signalling pathways, following infection.

It was important to ascertain if these infection procedures had been effective. To do this the elution profiles of peptide samples isolated from infected and uninfected beetles were compared using reverse phase HPLC. In the latter stages of this study more emphasis was placed on the use of the fungal pathogen to infect the beetles as indications of infection with *B. bassiana* could be observed as the growth of fungi on dead beetles and a decrease in fecundity. The effects of infection on protein expression were also monitored using the banding patterns observed on a 15% Tris Tricine SDS-PAGE.

The activation of an insect's immune response can have detrimental effects on its normal physiological functioning. This involves a decrease in energy available to the insect to perform other demanding activities such as foraging and mating. For this reason the effect of fungal exposure on the fecundity of *E. intermedius* was also monitored in this study.

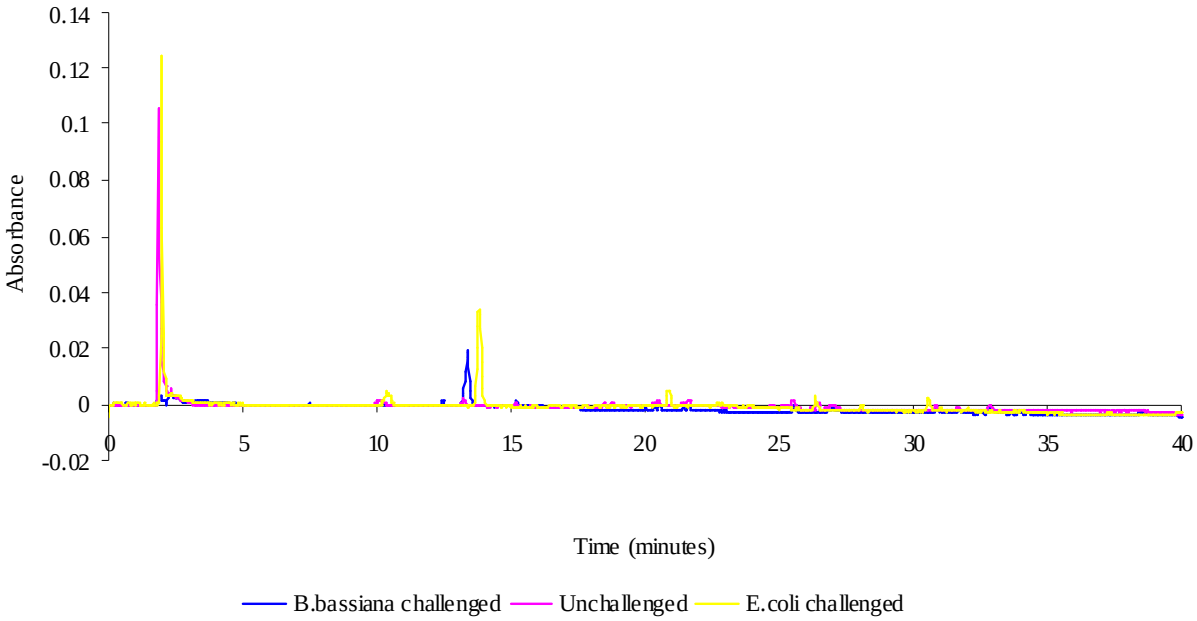
4.2.1 HPLC elution profiles and SDS-PAGE gels show changes in protein expression patterns following bacterial infection.

HPLC analysis was performed on challenged and unchallenged samples. These produced slightly different elution profiles. In figure 4.1 the elution profiles of three different samples were recorded at two different wavelengths and are represented on the same set of axes. All three samples were eluted from a C18 column using 60% acetonitrile, implying that they are hydrophobic and all three samples displayed antibacterial activity. The elution profile produced via detection at 280 nm (figure 4.1 A) shows a protein present only in the bacterial and fungal challenged samples eluting at roughly 13 minutes. The peak is slightly higher in the bacterially infected sample,

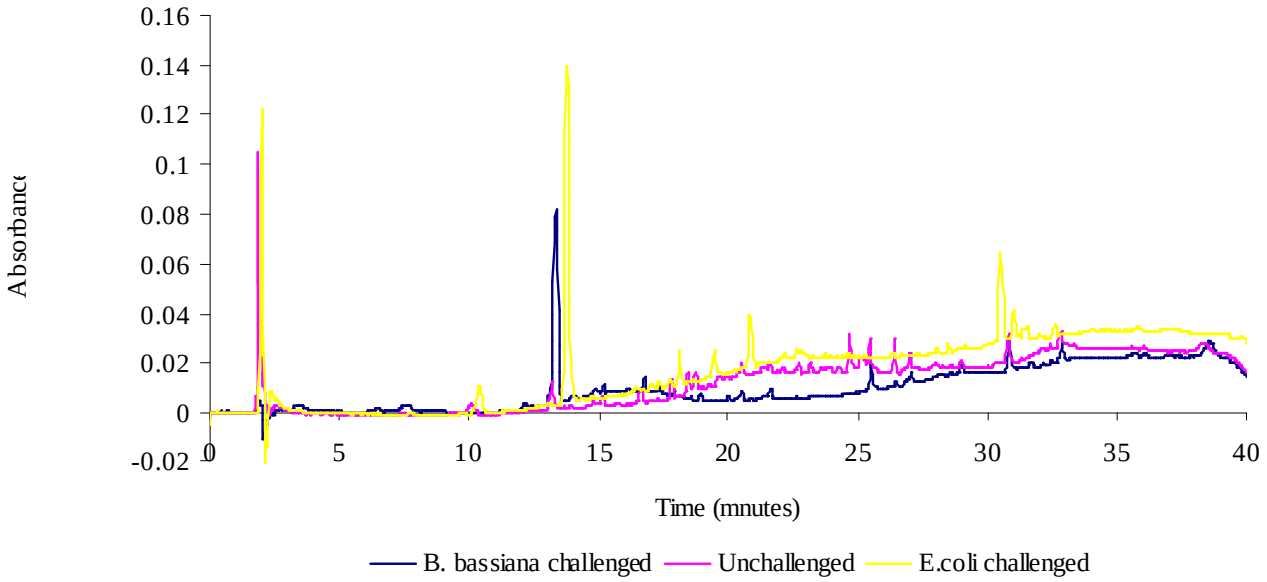
but the peak in the fungal infected sample is still relatively large. This 13 minute peak is visible in the profile recorded at 220 nm (figure 4.1 B) where it follows a similar pattern. This could signify an increase in the expression of a peptide following infection. Although this does not mean that this up-regulated peptide has to be an antimicrobial peptide. Inhibition clearing zone results showed that the peptides in the unchallenged sample (figure 4.2 A) gave rise to a clearing with a larger diameter than that in the challenged samples (figures 4.2 B and 4.2C), suggesting that the observed changes in the elution profiles were not due to changes in antimicrobial peptide expression.

In the latter half of the run the Fungal challenged sample has fewer proteins but there is a protein that elutes at around 39 minutes that is present in this sample and the unchallenged control sample, but is absent from the bacterially challenged sample. The bacterial sample shows two unique proteins at 20 minutes, but a complete absence of at least three large peaks between 25 and 30 minutes.

Differences in protein expression patterns are an indication that the beetles were successfully challenged. This was performed by running the peptides on a 12% tris tricine SDS denaturing polyacrylamide gel. Four different samples were used, each representing a different pathogen treatment of the beetles. These samples were *E.coli* challenged, *M. luteus* challenged, *B. bassiana* challenged and an unchallenged sample. The uninfected sample served as a basis for comparison, with equal amounts of each sample being loaded onto the gels.



A: HPLC elution profiles of different samples at 280nm



B: HPLC elution profile of different samples at 220nm

Figure 4.1: HPLC elution profiles showing changes in peptide levels between challenged and unchallenged beetles: The elution profile produced via detection at 280nm (A) shows very few differences between the peptide samples except for a change brought about by infection at around 13 minutes. The peak is only present in the two infected samples. The profile recorded at 220 nm (B) shows a similar pattern. There are proteins that are unique to individual samples that eluted in the 15-40 minute period.

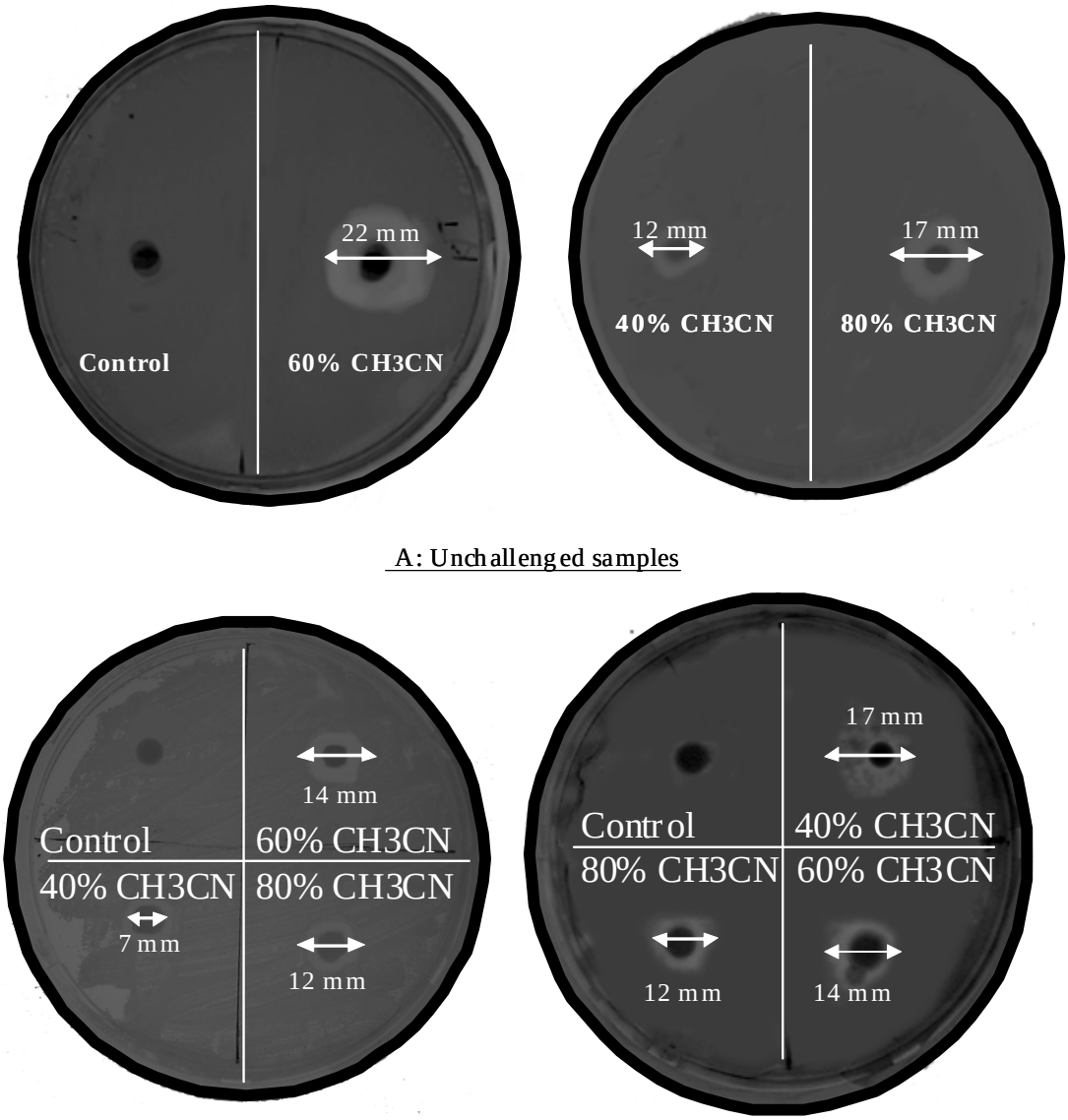


Figure 4.2 Inhibition assays: Photographs of solid media inhibition assays performed against *Micrococcus luteus.* (A) The largest 22 mm diameter clearing is present in a 60% acetonitrile fraction from untreated beetles. Once again the largest clearing is produced by the 60% acetonitrile fractions from *E.coli* challenged beetles (B) and from *B. bassiana* challenged beetles (C). This suggests that the unchallenged beetles are already mounting a powerful immune response, which could be due to their microbe rich environment.

The influence of the time of peptide extraction post infection was also investigated in this way by loading samples from the different immunisation treatments collected at 4 and 24 hours post infection. Additionally all samples were first purified on a Cation exchange column and were therefore cationic.

The result of the electrophoretic analysis of the samples described above is represented in figure 4.3. From the figure it can be concluded that the time of extraction after infection seemed to have very little effect on the banding pattern, with there being little to no change in the expression patterns of the purified peptides between 4 and 24 hours. The bands that appear between 3 and 8 kDa are found in samples that inhibit the growth of *M. luteus.* These bands are present in the unchallenged and *B bassiana* treated samples. This implies that the altered peptide expression following infection probably occurs rapidly and persists for more than 24 hours.

Differences between the protein expression patterns between infected and uninfected beetles are more apparent when the samples are analysed using 2D-tris tricine PAGE. In figure 4.3 B and C proteins that only appear in one gel are marked with a red circle, while those appearing in both are marked with a green circle. There are obviously more proteins in the whole body extracts, with the untreated sample showing a greater number of small positively charged proteins. There are fewer obvious differences between the infected and uninfected hemolymph samples, with new proteins around 17 kDa appearing following infection.

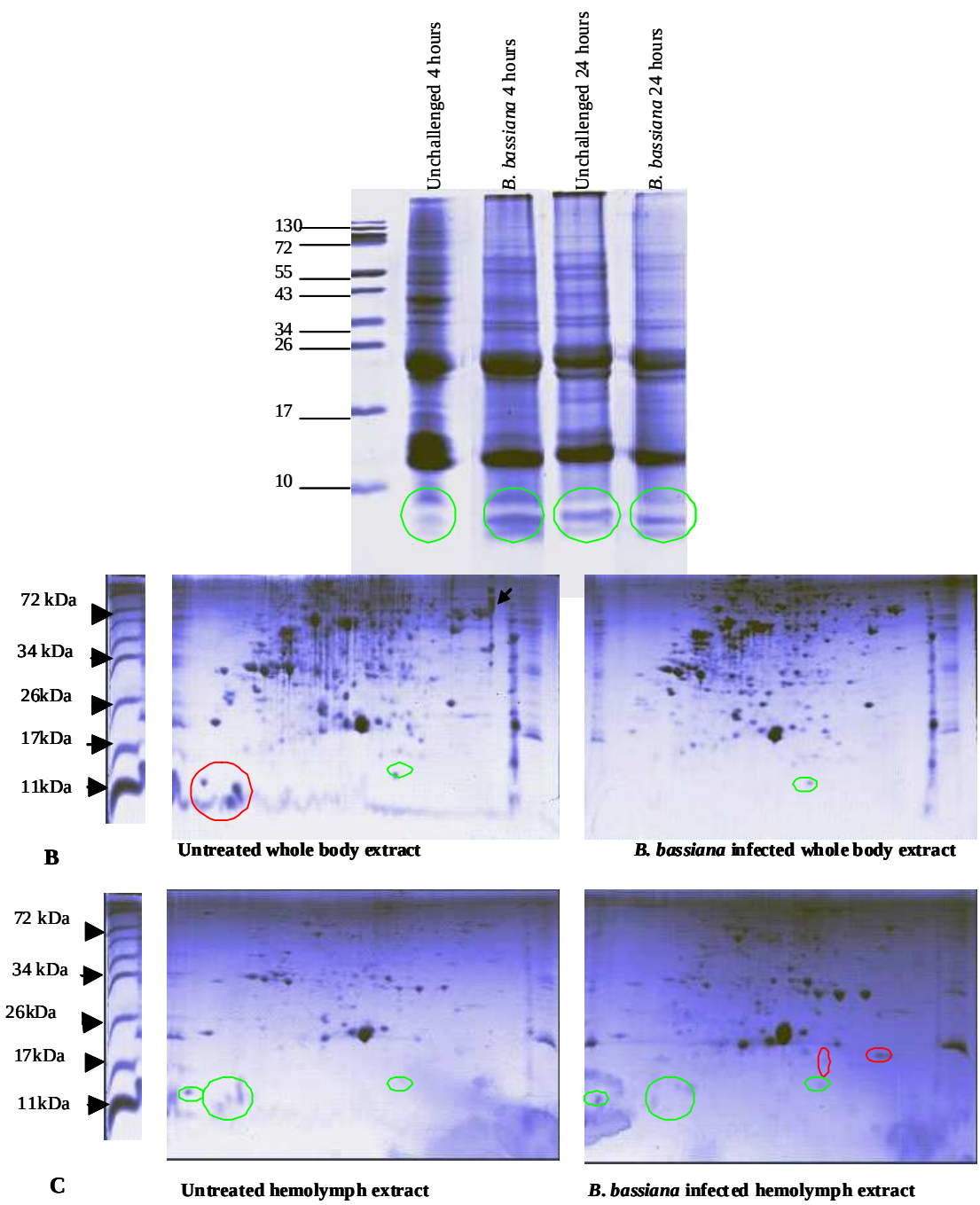


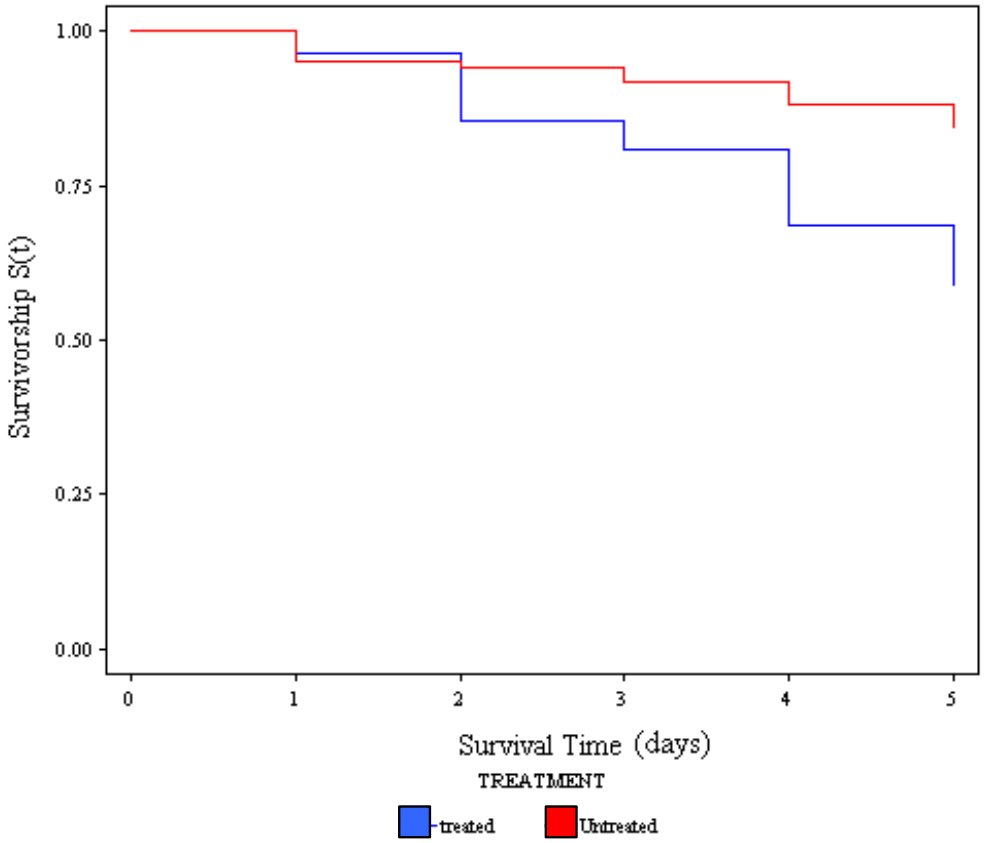
Figure 4.3: Expression patterns on Tris Tricine PAGE gels as an indicator of peptide expression pattern change after immuno-challenge. (A) All samples run on the gel were of equal total protein concentration and all were positively charged peptides that eluted after the crude extracts were sent through the Cation exchange column. A 2D PAGE comparison of infected and uninfected beetle protein extracts (B and C). These protein samples were extracted from the whole body (B) and the hemolymph of the beetles (C). The same small peptides that may represent the antimicrobial peptides are present in all gels (A) (B) and (C) and are marked with a green circle. Those peptides unique one set of gels (B) and (C) are marked with red circles.

In figure 4.3 B it can be seen that there are a large amount of low molecular weight proteins that seem to disappear following antimicrobial infection (marked with red circles). This was unexpected and it seems unlikely that any of these could represent the antimicrobial peptide as both samples show similar antimicrobial activity.

Following fungal infection, a large number of small peptides appear in the hemolymph (figure 4.3 C). There are also small peptides that seem common to both samples that are clearly visible on the 2D PAGE gels representing the hemolymph samples and whole body extracts.

4.2.2 Mortality assays show increased mortality rates and lower survival probabilities.

Mortality assays were performed by leaving the beetles for up to 5 days post infection, and recording the number and time of any beetle deaths. A Kaplan Meier survival analysis of beetles (figure 4.4 A), shows a decrease in the beetle's survival probability compared to those beetles that had not been exposed to fungus. This increase in mortality took the form of a sharp rise in the number of deaths occurring in the beetles treated with fungus on the second and fourth days post infection. Although the mortality rate was higher in those beetles infected with fungus, the survivorship probability never dropped below 50% in these infected beetles.



Kaplan Meier Survivor probability plot

Figure 4.4: Mortality Assays. A Kaplan-Meier product limit estimate of the survival functions of each individual beetle grouped according to treatment was performed at a 95% confidence interval. The resulting survivorship plot shows that the survival probability of the beetles was much lower following exposure to the fungus. There was a sharp decrease in the survival probability of the beetles exposed to fungus on day two followed by another sharp decline on day 4.

4.2.3. Fungal exposure lowers beetle fitness.

In order to further establish if the fungal exposure was having any effect on the beetles, the effect that infection had on reproduction was examined. Breeding pairs of mature *E. intermedius* were infected with *B. bassiana.* After a four day period the number of broodballs produced were counted and divided by the number of surviving females. This was to account for any decrease in broodball number due to higher mortality rates.

Figure 4.5 shows that fungal exposure has a significant effect on broodball production ($p = 0.0152$), with infected beetles producing nearly 4 times less broodballs than uninfected beetles. This large decrease in the number of broodballs produced is therefore most likely due to an effect the fungus is having on the beetle's fitness due to the increased energy expended on the immune response.

4.3 Antimicrobial activity.

An important aspect of this project was the isolation of antimicrobial peptides from *E. intermedius.* Members of most of the four classes of antimicrobial peptides have been isolated from the order Coleoptera. These include the linear amphipathic peptides in the form of cecropin; the insect defensins, the cyclic antifungal peptides and the glycine rich peptides. As yet no member of the proline rich peptide family has been isolated from the Coleopteran order.

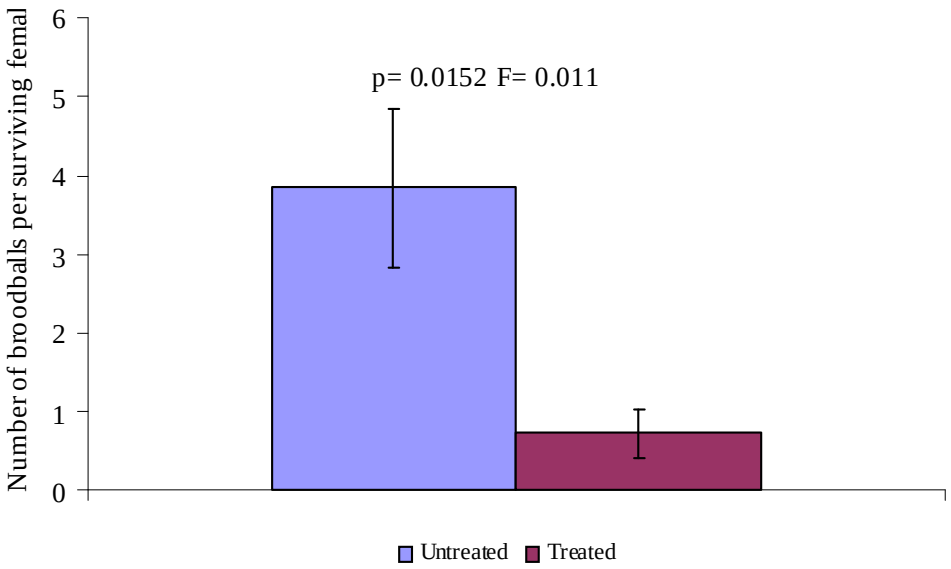


Figure 4.5: The effect of fungal treatment on broodball production. Infection of mature breeding pairs of *E. intermedius* with *B. bassiana* results in a decrease in the number of broodballs produced by the beetles. The data in this figure represents broodballs produced per female surviving at the end of a four day period. Therefore the number of broodballs produced is not solely dependent on the number of living adults. The large decrease in the number of broodballs produced is therefore most likely due to there being less energy available for the beetles to produce broodballs following an increase in immune costs. The error bars represent the standard error of the means of biological replicates.

The novel peptides Alo-3 isolated from *Acrocinus longimanus* (Barbault et al., 2003), Scarabaecin isolated from *Oryctes rhinoceros* (Tomie et al., 2003), luxuriosin isolated from *Acolalepta luxuriosa* (Kenjiro Uedaa, 2005) and Thaumatin isolated from *Tribolium castaneum* (Altincicek et al., 2008). All serve an antifungal function in addition to various anti-bacterial activities. It would make sense that homologs of these proteins would be present in *E. intermedius* and perform similar antifungal activities.

Finally, the occurrence of the glycine rich antimicrobial peptides throughout the order of coleoptera, also suggests that *E. intermedius* would possess a glycine rich peptide similar to coleoptericin (Bulet et al., 1991), holotricin (Lee et al., 1989), protaetin 1 (Yoon et al., 2003) or acoleptin (Imamura et al., 1999).

4.3.1 Solid media inhibitions indicated the presence of an antimicrobial substance in cationic hydrophobic samples.

Solid media inhibitions such as those in figure 4.2 indicated that all crude extracts consistently displayed inhibitory activity towards *M. luteus*, but not against the gram negative *E.coli*, with there being no significant difference between the activity of the untreated and challenged samples and no significant pairwise differences among the mean diameter clearings for any of the samples ($p = 0.2352$). This implies that the antimicrobial activity is constitutively active in the beetles, probably due to the environment in which they live.

The negatively charged peptides showed significantly lower activity against *M. luteus* regardless of treatment ($p = 0.0001 - 0.0009$). The positively charged peptide fraction from the fungal infected and gram negative challenged beetles showed significantly lower activity than the crude extracts ($p = 0.025$, $p = 0.0342$), while those from uninfected beetles and gram positive challenged beetles showed comparable activity to the crude extracts ($p = 0.6439$, $p = 0.3568$). The inhibitory molecules would be in a very low concentration in these samples and the high concentration of salt may also interfere with the peptides activity.

The purification step that consistently showed the highest activity was the 40-60% acetonitrile fraction ($p= 0.0274$ $F =0.1712$), closely followed by the 60-80% acetonitrile concentration ($p = 0.015$ $F = 0.2348$). This shows that the antimicrobial molecules are hydrophobic and positively charged, which follows the characteristic of most known antimicrobial peptides being hydrophobic. Defensins typically elute with solutions having between 40 and 60% acetonitrile (Lowenberger 1995), while cecropins typically elute from C18 columns when 40% acetonitrile solutions are added to the columns (Louwenberger, 1999).

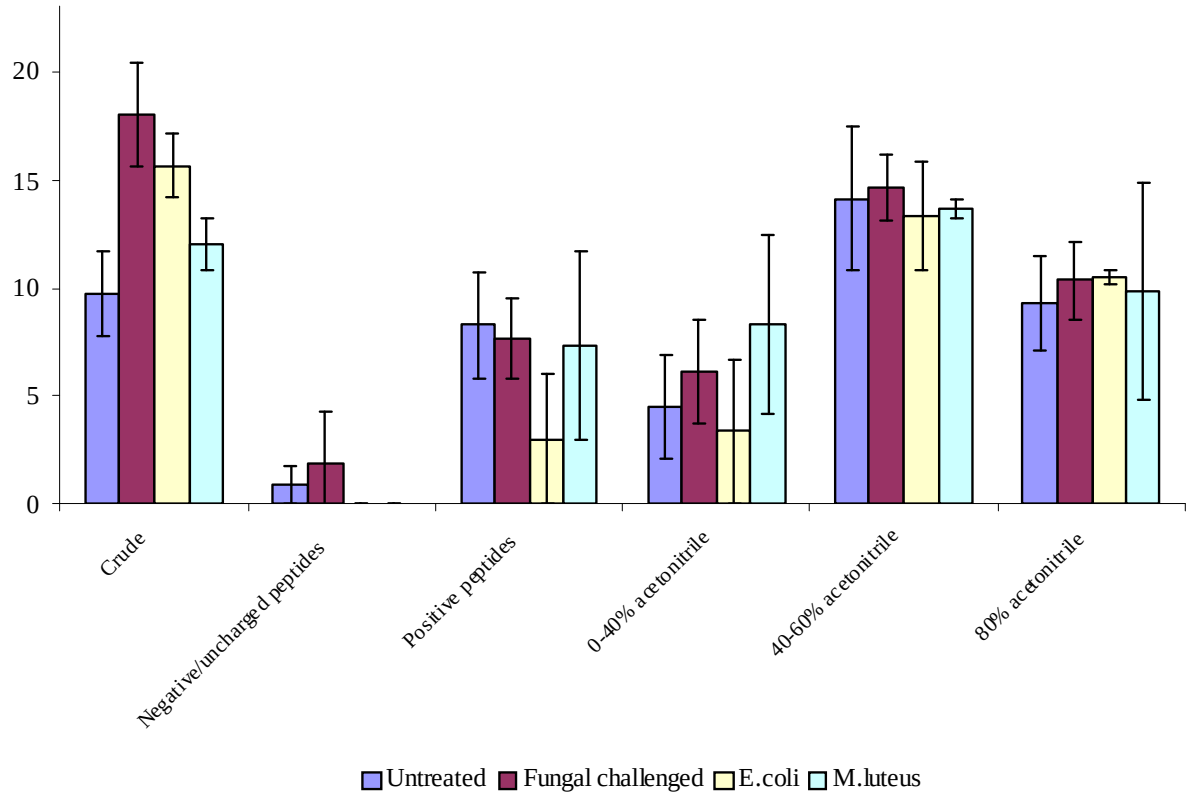


Figure 4.6: Activity of extractions against *M. luteus* recorded by solid media inhibition assays. The crude extracts consistently show antimicrobial activity as do the positively charged peptides that eluted from the cation exchange columns. The negatively charged peptides showed significantly lower antimicrobial activity. Most of the inhibitory activity in the purified samples is found in the 40-60% acetonitrile fraction, making them hydrophobic. Immune challenge of the beetles had a lower effect on the potency of the antimicrobial activity than was expected. The error bars represent the standard error of the means of technical replicates of biological replicates.

4.3.2 The Antimicrobial agent is a heat stable protein.

In order to positively identify the inhibitory agent isolated from the beetles as a protein, an inhibition assay was performed with the antimicrobial sample being treated with proteinase K. Figures 4.7 and 4.8 A represent the solid media inhibition assay and the liquid inhibition assay respectively, both indicate that treatment with proteinase K decreases or abolishes antimicrobial activity totally. They also indicate that heat inactivation of proteinase K at 65 degrees Celsius for 20 minutes is enough to abolish its activity and not interfere with the assay.

These results show that the antimicrobial activity in the samples is indeed due to the presence of a protein. Once this protein is cleaved by proteinsae K the antimicrobial activity disappears. When the same sample is left untreated by proteinase K it is still able to inhibit bacterial growth. This was confirmed using liquid inhibition assays which relied on spectrophotometric measurements and colony counts. Both these methods indicated that there was a significant decrease in the inhibitory activity of the extracts following proteinase K treatment ($p = 0.0114$ for the spectrophotometric readings and $p = 0.0021$ for the colony counts). The addition of the proteinase K enzyme to the bacteria alone had no significant effect on the growth of the bacteria and there was no significant difference between the inhibitory activity of the proteinase K alone or the proteinase k treated extract according to the spectrophotometric analysis ($p = 0.1373$). However, the colony count data indicated that the inhibitory activity of the proteinase K treated sample was still significantly lower than that of the proteinase K alone ($p =0.001$). This indicates that the antimicrobial activity is due to the presence of antimicrobial peptides in *E. intermedius*.

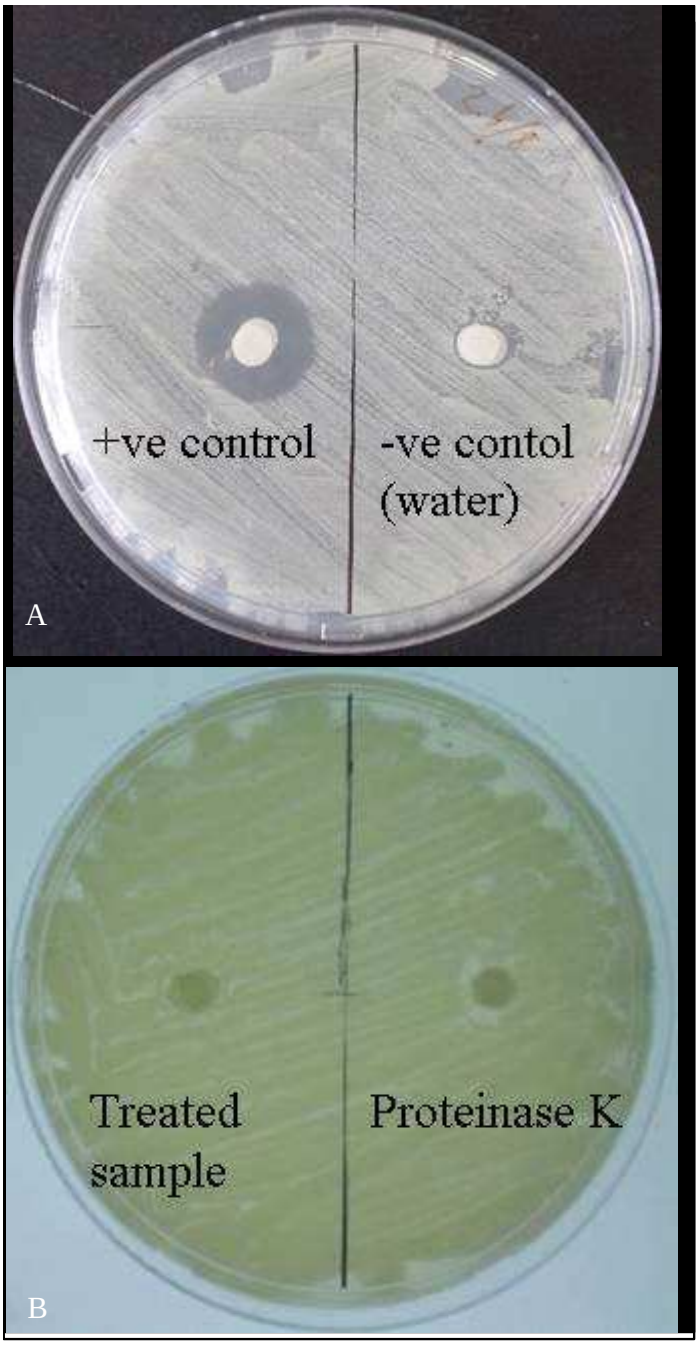


Figure 4.7: Identification of the nature of the inhibitory factor. The sample responsible for inhibiting the growth of *Micrococcus luteus* (A) was used as the positive control. This sample was then treated with proteinase K (B). This decreased the inhibitory activity of the sample so that it was comparable to the control consisting of proteinase K on its own (B).

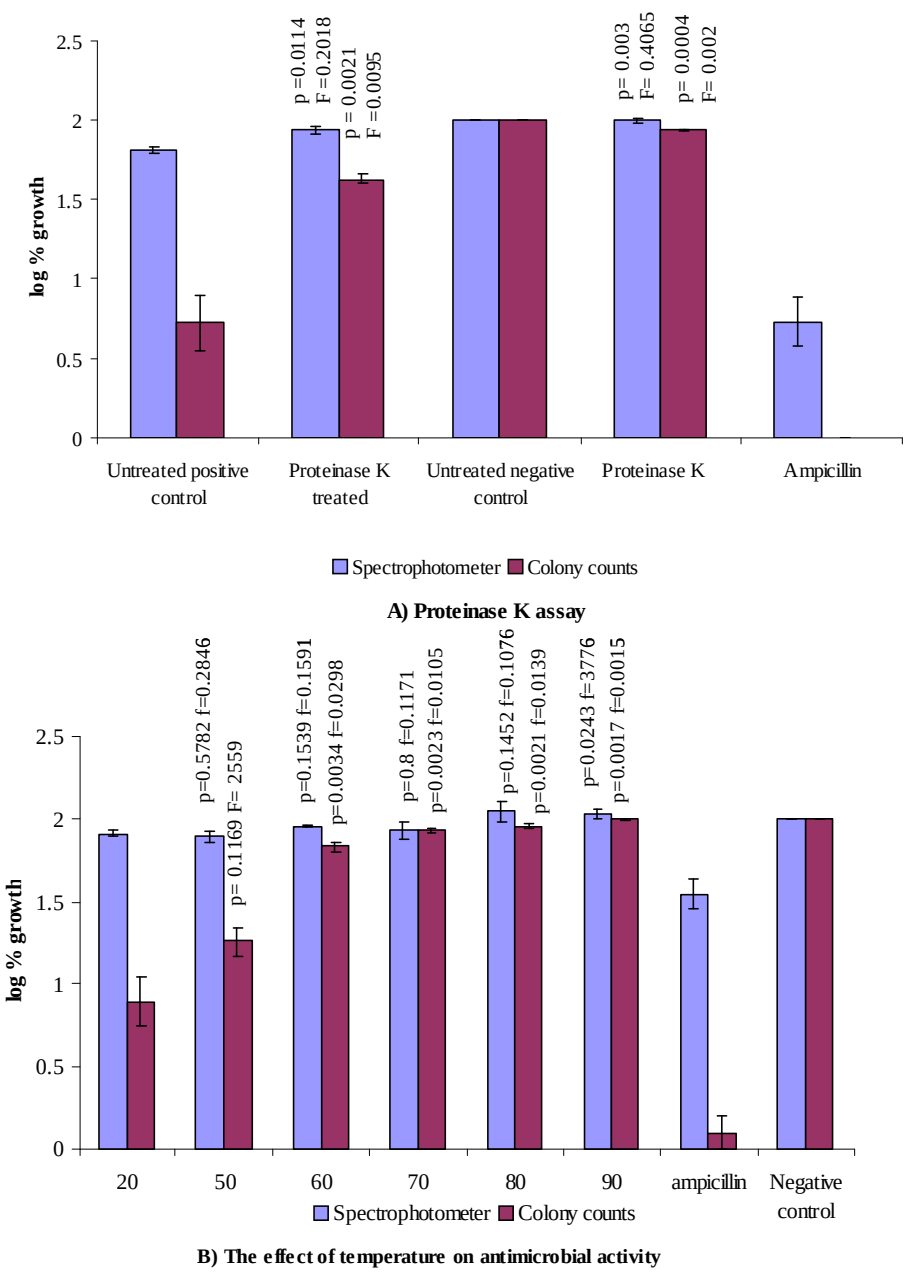


Figure 4.8: Liquid inhibition assays performed on *Micrococcus luteus* demonstrate that the antimicrobial agent is a heat stable protein. (A) Treatment of the sample with proteinase K decreases the antimicrobial activity. The p values indicate the statistical significance between the activity of the treated samples and the untreated positive control. (B) A liquid antimicrobial assay was performed to test the effect of heat on the antimicrobial activity of the samples and suggests that the peptide performs similarly at 20, 50 and 60 degrees. At 70 degrees the activity is still present but is significantly decreased. Antimicrobial activity virtually vanishes at 80 and 90 degrees. The p values indicate the statistical significance of the differences between the activity of the various temperatures and the activity at room temperature (20 ^{0}C). The error bars represent the standard error of the means of biological replicates.

In order to confirm that the inactivation of the antimicrobial activity was due to the action of the proteinase K and not the high temperature treatment, a heat activity assay was performed. This involved performing liquid assays using an antimicrobial sample following treatment at different temperatures. Figure 4.8 B depicts the results of this assay. Colony counts show there is no significant difference between the antimicrobial activity of the sample at room temperature and 50 degrees Celsius ($p = 0.1169$) while spectrophotemetric analysis indicated that band shows that the antimicrobial molecule is heat stable up to temperatures as high as 70 possibly 80 degrees ($p = 0.1452$). The difference between the colony count and the spectrophotometric results may be the result of cellular debris interfering with the spectrophotometric readings. Together these results indicate that a heat stable protein is responsible for the antimicrobial activity of the beetle protein extracts.

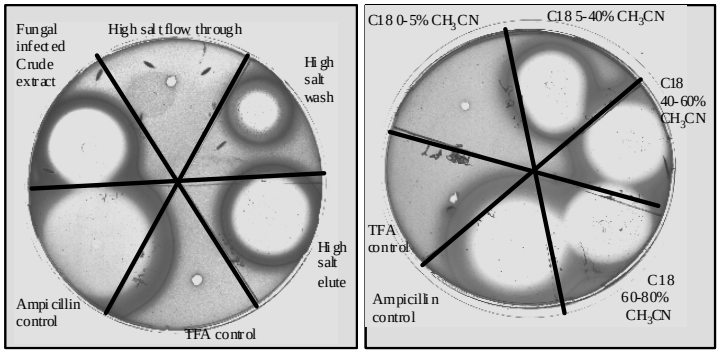
4.3.3. Radial diffusion assays were adopted due to their increased sensitivity and reproducibility.

In order to improve sensitivity and reproducibility of the inhibition assays the radial diffusion technique was adapted. This technique is similar to the solid media inhibition assay but is more sensitive due to the bacteria being exposed to the samples in an initially nutrient deficient environment. The bacteria are then exposed to a nutrient rich environment but will only grow in this second media where they come into contact with bacteria that have survived on the initial nutrient poor media. The antimicrobial substance diffuses through the nutrient poor media creating a zone of clearing based on its effective concentration. In this way the clearing could reflect the

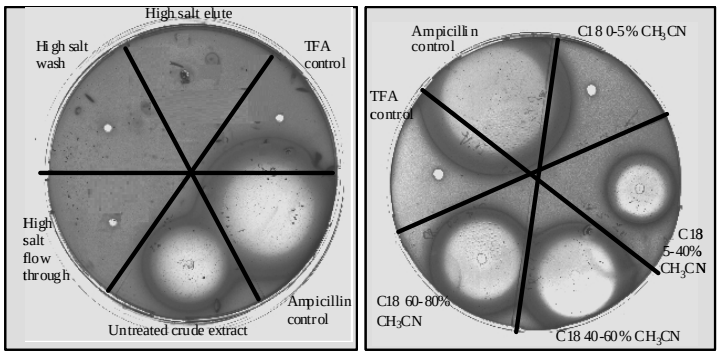
effectiveness of the peptide as well as its concentration. Figure 4.9 shows a typical example of this inhibition assay.

Various dilutions of a 100 mg/ml stock of ampicillin were used as a positive control for the radial inhibition assays. Figure 4.10 shows that the relationship between the amount of ampicillin added and the diameter of the clearings is only linear at higher concentrations. When lower concentrations of ampicillin are used, small increases in the antibiotic concentration only have a slight effect on clearing diameters. This may be due to the nature of ampicillin as a time-dependent antibiotic that is optimally effective when the concentration is maintained at 2 to 4 times above the minimum inhibitory concentration. Therefore, at lower concentrations, increasing the concentration of ampicillin should not result in a higher bactericidal effect (Levison and Levison, 2009). The activity of the antimicrobial peptides as established by the radial diffusion assays is represented in figure 4.11.

Unsurprisingly the largest clearings were produced by the ampicillin control and in all cases these clearings were significantly larger than those produced by the rude extracts ($p=0.0001$). There was no statistically significant differences between the antimicrobial activity of the crude extracts from the fungal infected or uninfected beetles, regardless of the test organism used (*M. luteus* $p = 0.9178$ $F = 0.815$ and *E. coli* $p = 0.6752$ $F = 0.4042$). The same is true of the hemolymph extracts from both fungal treated and untreated beetles (*M. luteus* $p = 0.4647$ $F = 0.1364$ and *E. coli* $p = 0.6213$ $F = 0.5$).

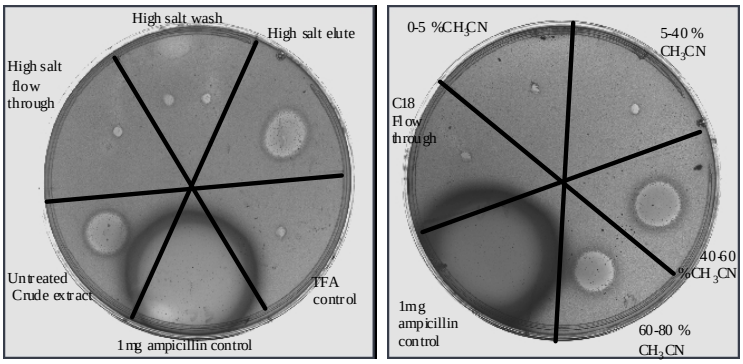


Extracts from fungal infected beetles

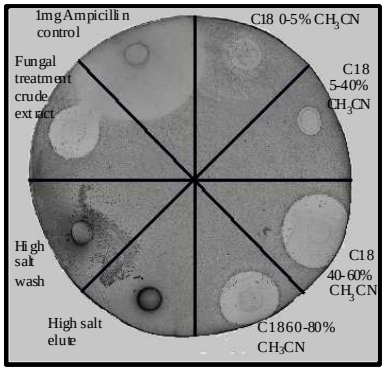


Extracts from uninfected beetles

A) Inhibition assays against *M. luteus*



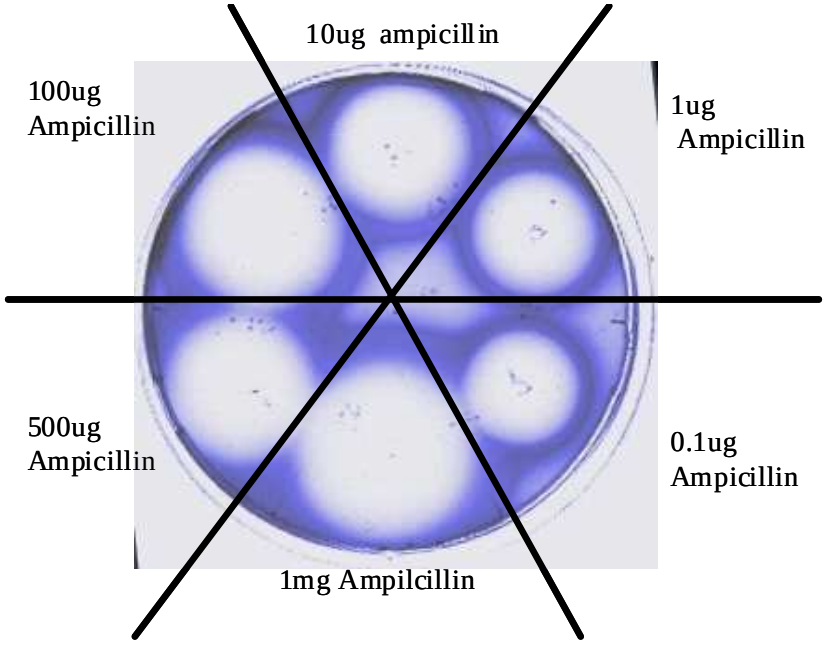
Extracts from uninfected beetles



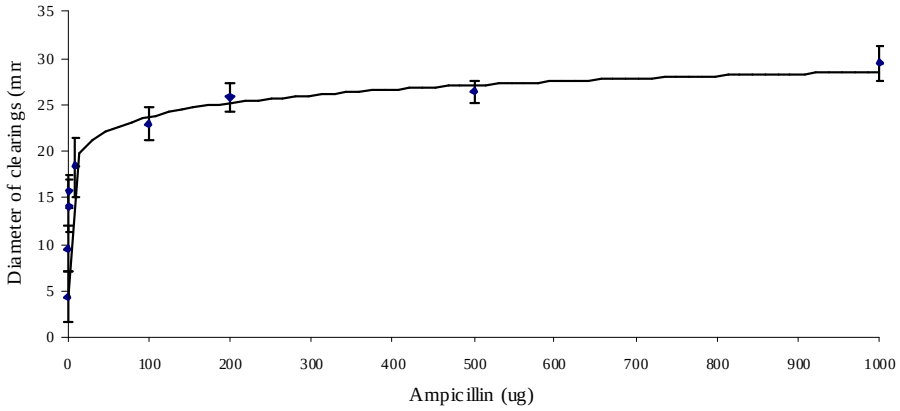
Extracts from fungal infected beetles

B: Antibacterial activity against *E. coli*

Figure 4.9: Examples of radial diffusion assays: (A) Inhibition assays using the gram positive *M. luteus* with protein extracts from untreated and fungal infected beetles. (B) Inhibition assay using the gram negative bacteria *E. coli* with protein extracted from untreated and infected beetles.



A: Inhibition assays using antibiotic controls

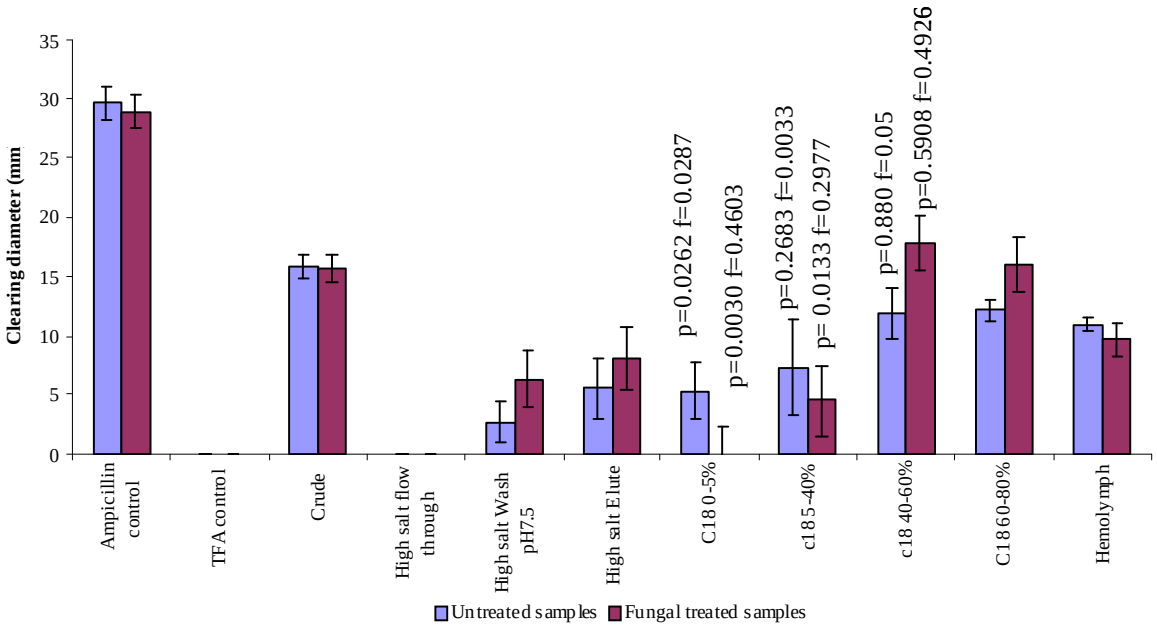


B: Ampicillin dilution series

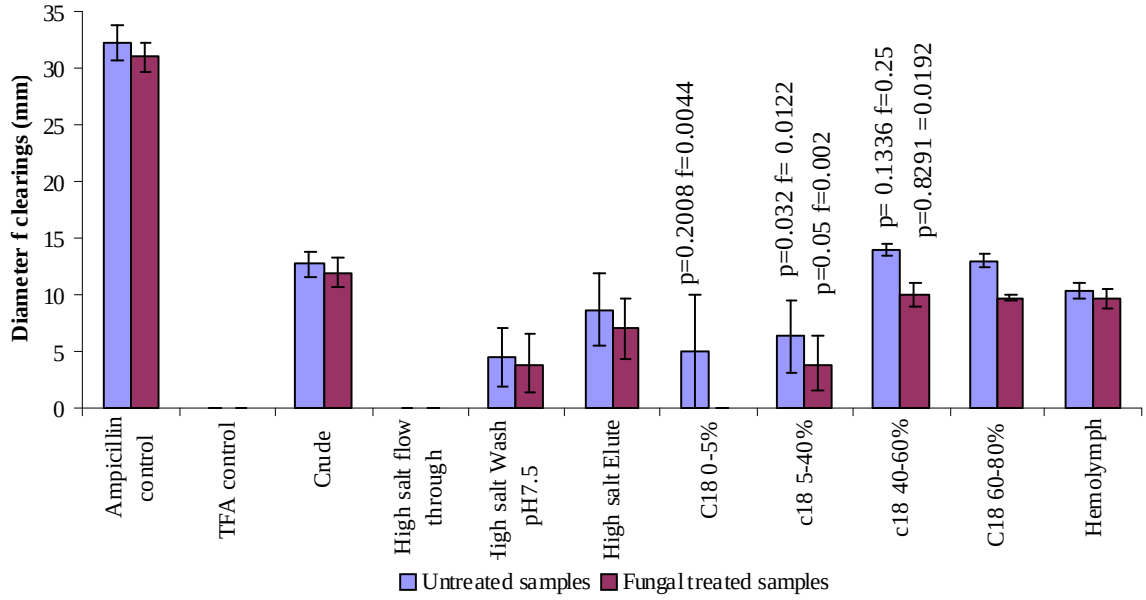
Figure 4.10: Radial diffusion assays with varying concentrations of an Ampicillin control. Radial diffusion assays were adopted as the most sensitive and reliable method of detecting antimicrobial activity. Control inhibitions were set up using varying concentrations of Ampicillin as a control (A). This also demonstrated the relationship between the diameter of the clearings and the amount of antimicrobial substance in each well (B). This is not strictly a linear relationship showing that the assay is insensitive to small changes in the concentration of the antimicrobial agent.

Unexpectedly there was no statistically significant differences between the antimicrobial activities of the Cation exchange wash and elute fractions from the fungal infected or uninfected beetles. This was true when the fractions were tested against *M. luteus* (fungal treatment p= 0.6469 F = 0.3974 and untreated treatment p= 0.3809 F= 0.1986) and against *E. coli* (Fungal treatment p = 0.4507 F = 0.4923 and untreated p = 0.3502 F = 0.36820). The presence of antimicrobial activity in the wash fractions may be due to the pH of the wash solution being 7.5. Some of the smaller glycine rich peptides isolated from other coleopteran species have pI values lower than 7.5 when the peptide sequence is entered into the ExPASy compute pI tool. This implies that at this pH they would have a negative charge and would not bind to the Cation exchange matrix and would be present in the wash fraction. It is the presence of these peptides which may give rise to the activity observed. Additionally the high salt concentration may lower the activity of the antimicrobial peptides present in the elution fraction.

Regardless of the state of infection, the highest activity amongst the purified samples is in the 40-60 and 60-80% acetonitrile fractions. This was observed when the fractions were tested against *M. luteus* and *E. coli.* The p values comparing the activity of the 0-5, 5-40 and 40-60 % fractions against the 60-80% fractions are shown in figure 4.11. The antimicrobial spectrum of the extracted peptides is shown in table 4.2. The more sensitive liquid and radial diffusion assays showed that the antimicrobial peptide inhibited the growth of the gram negative bacteria *E. coli.* However, the peptide does not seem effective against all bacteria with *S. aureus* and *M. morganii* representing gram positive and gram negative bacteria respectively.



A) The antimicrobial activity of differently purified fractions from fungal treated and untreated beetles against *Micrococcus luteus*



B)The antimicrobial activity of differently purified fractions from fungal treated and untreated beetles against *Escherichia coli*

Figure 4.11: The inhibitory effectiveness of different peptide samples was established based on, the diameters of the clearings they produced in radial diffusion assays. Like the solid media and liquid assays the more hydrophobic positive fractions were the most reliable inhibitors of microbial growth. The increased sensitivity of the radial diffusion assays allowed the detection of activity against the gram negative pathogen *E.coli*. These samples include the high salt wash and the lower acetonitrile concentrations. Once again as in other inhibition assays the immune challenge of the beetles seemed to have no effect on the potency of the antimicrobial peptide. The error bars represent the standard error of the means of biological replicates.

Table 4.2: The spectrum of antimicrobial activity of protein extracts from fungal challenged beetles

Gram negative	Type of bacterial inhibition assay		
Test organism	Solid media	Liquid	Radial diffusion
Escherichia coli	No inhibition	Inhibition	Inhibition
Morganella morganii	No inhibition	No inhibition	No inhibition
Serratia marcesens	No inhibition	Not tested	Not tested
Gram positive	Type of bacterial inhibition assay		
Test organism	Solid media	Liquid	Radial diffusion
Micrococcus luteus	Inhibition	Inhibition	Inhibition
Bacillus subtilis	No inhibition	Inhibition	Inhibition
Staphylococcus aureus	No inhibition	No inhibition	No inhibition
Fungi	Type of bacterial inhibition assay		
Test organism	Solid media	Liquid	Radial diffusion
Candida albicans	No inhibition	Not tested	Not tested
Sacchromyces cerevisae	No inhibition	Not tested	Not tested
Beauveria bassiana	No inhibition	Not tested	Not tested

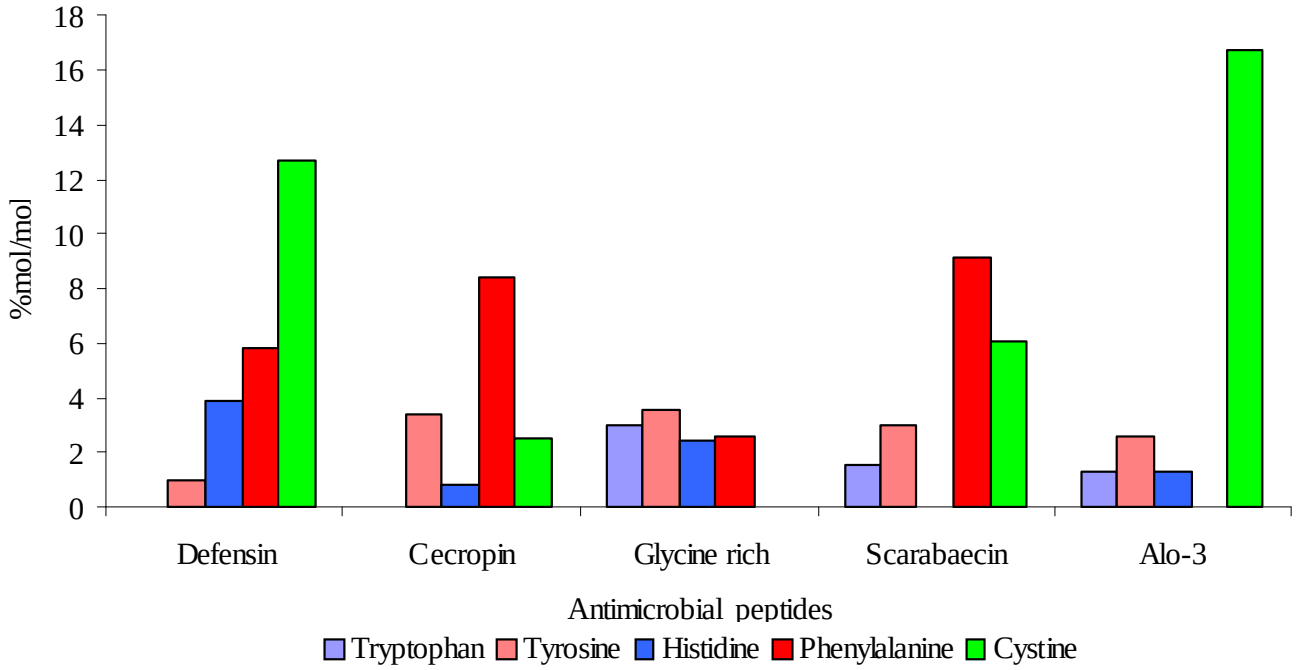
All inhibition assays showed that the protein extracts showed activity against the gram positive bacteria *M. luteus.* However, only the more sensitive liquid assays and radial diffusion assays detected any activity against gram negative pathogens such as *E. coli.* These more sensitive assays also detected activity against another gram positive bacteria *B. subtilus.* No antifungal activity has been observed.

The peptide seems to have no anti-fungal ability despite being isolated from beetles that had been challenged with fungus.

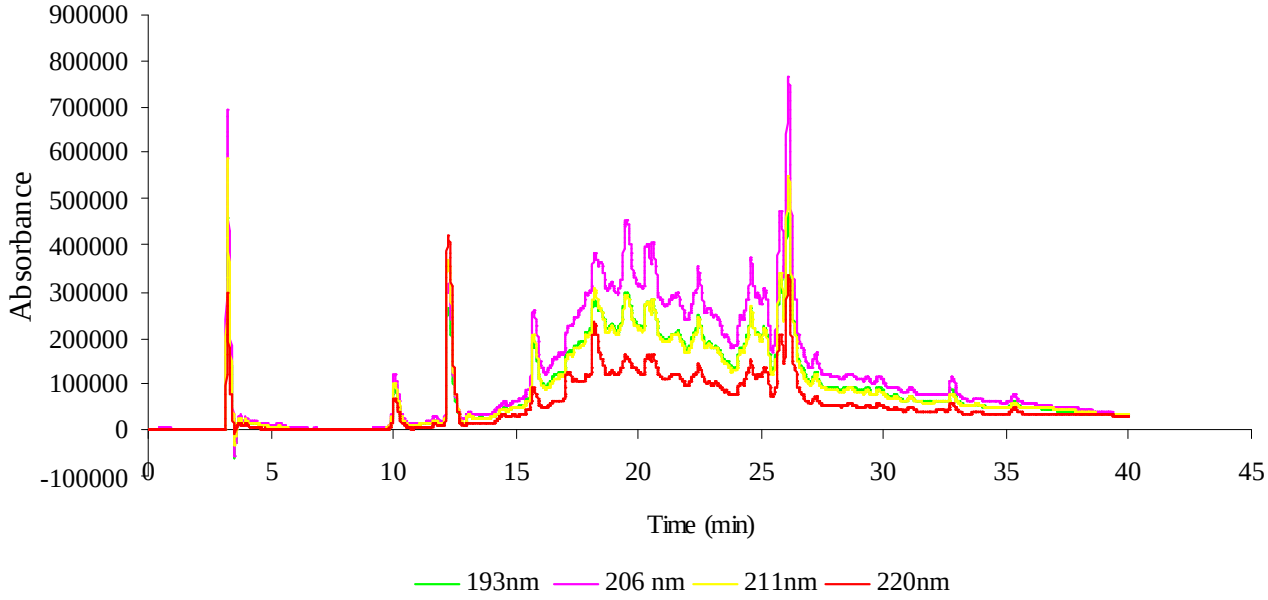
4.3.4 Wavelength absorption maxima were used to identify peaks of interest.

To further isolate peaks of interest an absorbance spectrum from 190 to 280 nm was performed using HPLC. The data sets of certain wavelengths were used to plot the profiles in figure 4.12. These wavelengths are those where chromophore amino acids have their maximum absorbance. This data combined with sequence data on antimicrobial peptides isolated from coleoptera (figure 4.12) were used to identify peaks with a similar absorbance profile to these antimicrobial peptides.

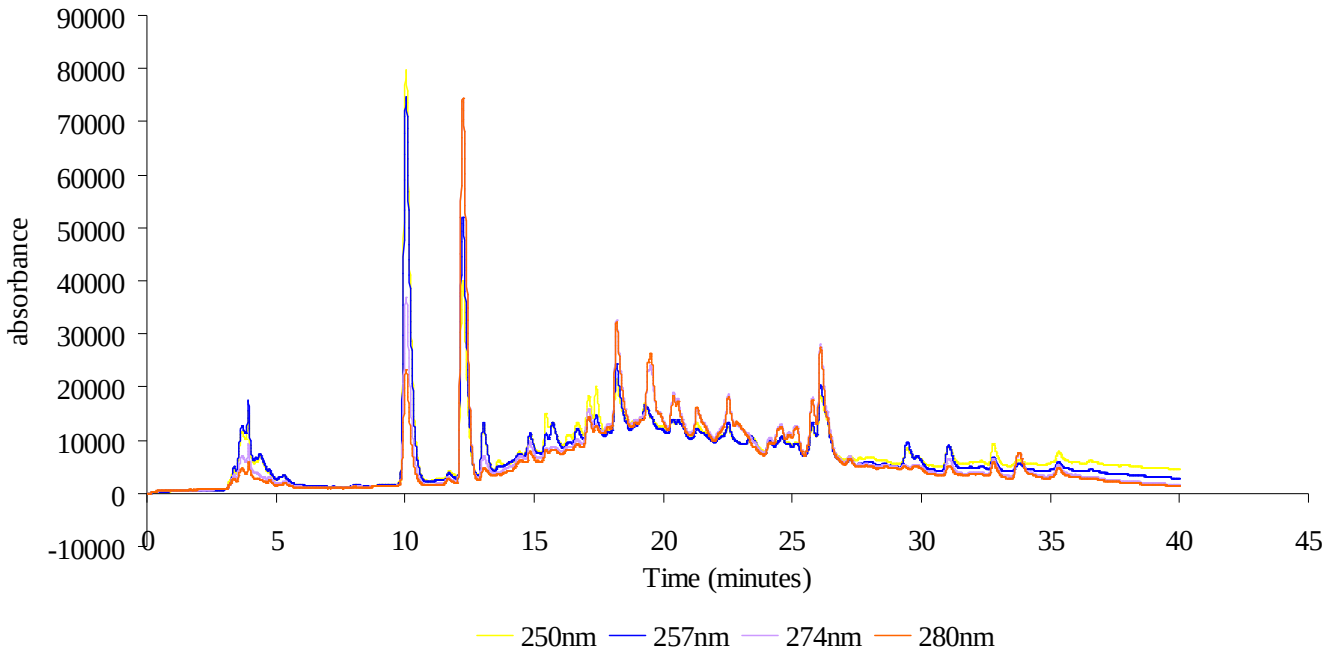
Peaks at 10, 17, 32 and 35 minutes match the expected absorption peak pattern for defensin. Specifically the highest absorbance at 250 nm followed by relatively high peaks at 206 and 257 nm, a medium peak at 211 nm and finally relatively low peaks at 193, 220 and 274 nm (figure 4.12). The peak at 13 minutes matches the absorbance peak pattern for cecropin with the highest peaks at 206 and 257 nm, followed by lower peaks at 274, 220 and 193 nm. These are then followed by a slightly lower peak at 250 nm and finally by a low peak at 211 nm. The final peak to match any of these absorbance peak patterns is the peak at 3 minutes. This pattern has the highest peaks at 257 and 206 nm. This is followed by a slightly lower peak at 250 nm and then smaller peaks at 274, 220 and 193 nm. Finally there should be low peaks at 220 and 280 nm.



A.Amino Acid makeup of Antimicrobial peptides



B:Absorbance spectra of untreated hydrophobic sample (190-220nm)



C:Absorbance spectra of untreated hydrophobic sample (250-280nm)

Figure 4.12: Wavelength absorption maxima correlated with the percentage of certain amino acids in coleopteran antimicrobial peptides. (A)The amount of certain amino acids in % mol/mol present in different antimicrobial peptides of coleopteran was calculated. (B and C) Elution profiles of the same sample monitored with many wavelengths. These wavelengths are those where optically active amino acids have absorbance maxima. By comparing these profiles and the amino acid content of known coleopteran antimicrobial peptides, peaks of interest can be identified.

From these observations the proteins that eluted out at 3, 10, 13, 17, 32 and 35 minutes were used in the antimicrobial assays first. Interestingly when comparing these results to those based on proteins that showed up-regulation following infection, the proteins that eluted at 3, 10, 13 and 32 minutes was in both groups.

It must be pointed out that this is only a rough estimation to help prioritize peaks for further analysis. The amino acid chromophores spectrum shifts with increasing or decreasing polarity of their environment, which results in changes in wavelength of maximum absorbance. Additionally there may be many other proteins with a similar percentage composition of these amino acids.

4.3.6 Only one of the isolated peaks shows any antimicrobial activity

In order to identify the peaks (proteins) responsible for the antimicrobial activity of the sample, the eluted peptides were collected individually and used in a solid media antimicrobial assay. Figure 4.13 represents the results of one of these antimicrobial assays.

Only one of the proteins isolated with HPLC ever inhibited bacterial growth. This was the protein represented by the peak at 10 minutes (figures 4.1 and 4.12). However protein concentration assays showed that there was very little protein present. The entire isolated sample was used in the inhibition assays and this low amount of peptide may also be the cause of the lack of inhibition by many of the other samples.

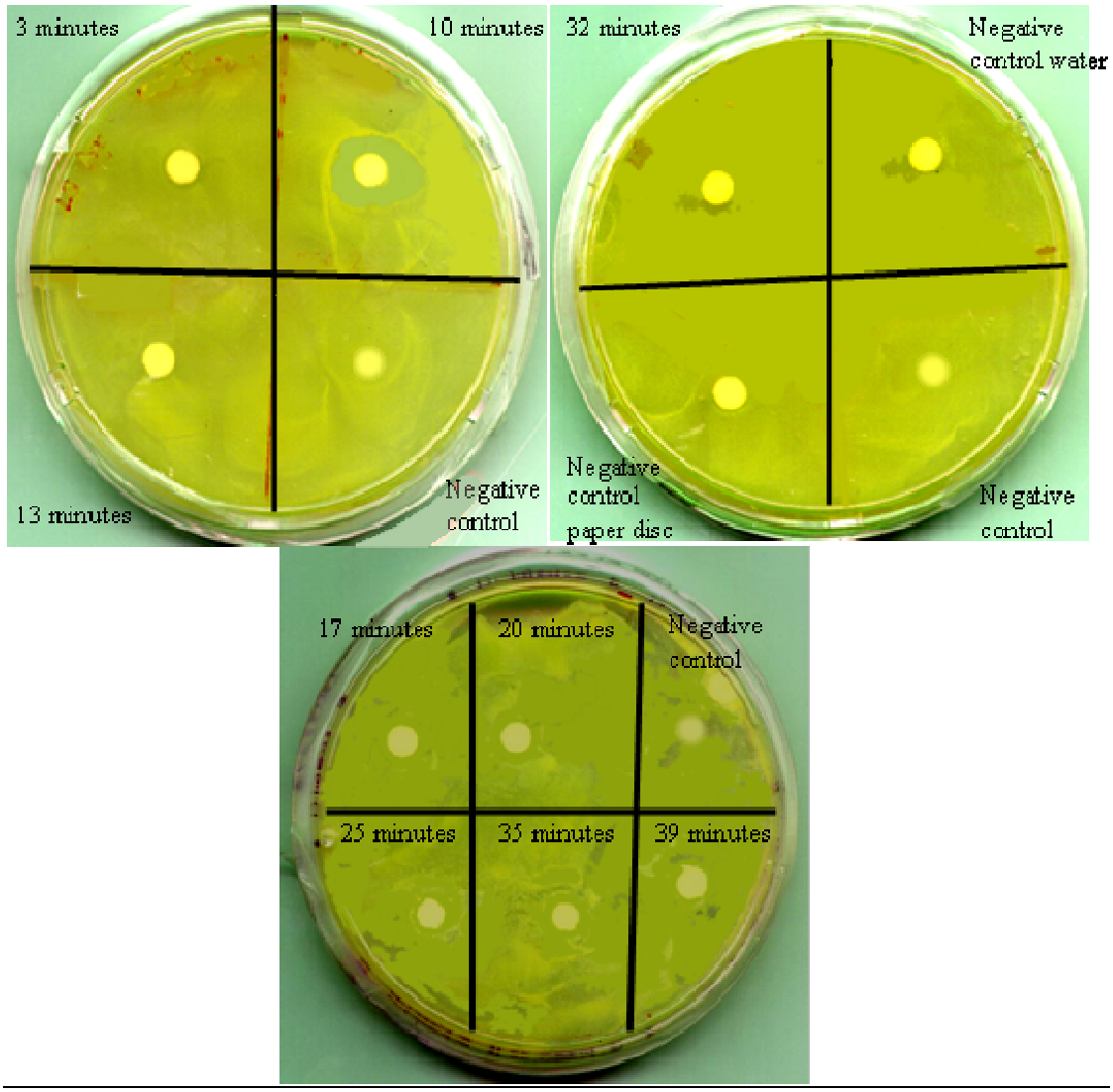


Figure 4.13: Inhibition of *M. luteus* growth with HPLC purified samples. The peptide that elutes at 10 minutes is the only sample that produced any sign of inhibition. However, the whole sample obtained via HPLC had to be used in the inhibition to obtain this result.

4.4 Changes in overall protein expression following infection in *E. Intermedius.*

In order to establish how the protein expression patterns of *E. intermedius* changes following infection, whole protein extracts and hemolymph extracts from infected and uninfected beetles were analysed and compared using tris-tricine gels as well as 2D-PAGE tris tricine gels. Proteins were then excised from these gels and analysed by MALDI-TOF mass spectrophotometry.

4.4.1 Small peptides are present in samples that inhibit bacterial growth.

In order to identify and isolate proteins whose expression levels change following infection tris tricine SDS-PAGE was used to separate samples from infected and uninfected beetles. Bands of interest could then be excised and analysed using MALDI-TOF mass spetrophotometry. The analysis of these samples with SDS-PAGE and 2D PAGE also allowed comparisons between differently purified inhibitory samples and fractions (figure 4.14 and 4.15).

In figure 4.14 the banding pattern of all the fractions from each sample, were examined using 12% tris tricine gels. The banding patterns of those fractions that inhibited the growth of *Micrococcus luteus* were compared to those that did not have any inhibitory activity. The small proteins that can be seen on the gels in figure 4.14 in those sat display antibacterial activity may represent the antimicrobial peptides. These small bands are present in the crude and high acetonitrile concentration purified samples as expected. However, small peptides are also present in those samples that do not inhibit bacterial growth, such as the negatively charged peptide samples. The

banding patterns are similar between the differently challenged samples with respect to the low molecular weight samples. Higher molecular weight proteins are not clear on the tris tricine gels (figure 4.14). The green and red circles in figure 4.14 indicate small peptides. The green circles are possible inhibitory antimicrobial peptides, while the red circles are in samples where there is no antimicrobial activity.

4.4.2 Common proteins are found in both the 40-60 and 60-80% acetonitrile fractions.

In many of the lanes in figure 4.15 there are bands that seem to be present in more than one lane. This is especially true in those fractions purified by the c18 columns and the most common samples where this seems to occur are the antibacterial 40-60 and 60-80 % acetonitrile fractions. Therefore, the antimicrobial activity detected in these two samples may not be due to two distinct antimicrobial peptides, but rather due to the same peptide appearing in both fractions.

To confirm this, the purified high percentage acetonitrile fractions were analysed on a 2D-PAGE Tris Tricine gel (Figure 4.15). The expected position of the pH 7 area on the gel is indicated (figure 4.15) and not many spots are found beyond this point. This is as expected as the Cation exchange column was washed with a pH 7.0 Buffer which would have altered the charge of all proteins with a pI greater than or equal to this to negative or neutral, resulting in their elution. Figure 4.15 also shows that many of the spots found in the 40-60% acetonitrile sample are also found in the 60-80% acetonitrile sample.

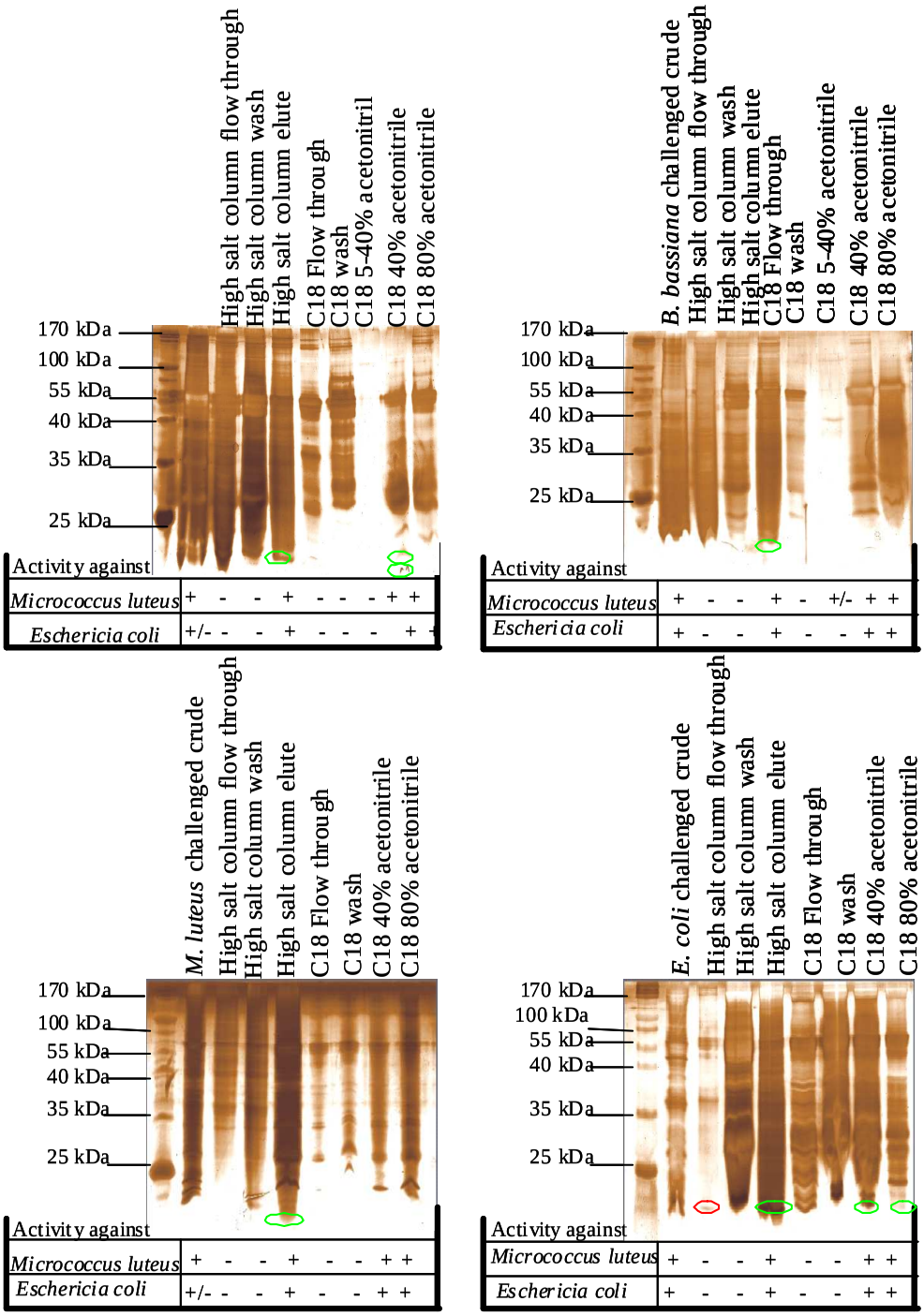


Figure 4.14: Tris Tricine SDS-Gel patterns of Inhibitory peptide samples. Samples with inhibitory activity against *M. luteus* or *E. coli* are marked as positive (+) negative (-) or only inhibiting growth occasionally (+/-). Samples that are similarly purified show similar patterns regardless of pathogen challenge. Smaller bands were visible in the 40-60 % acetonitrile samples. However, many bands seem common to both samples and the smaller bands in the *M. luteus* and *E. coli* challenged samples are found in both.

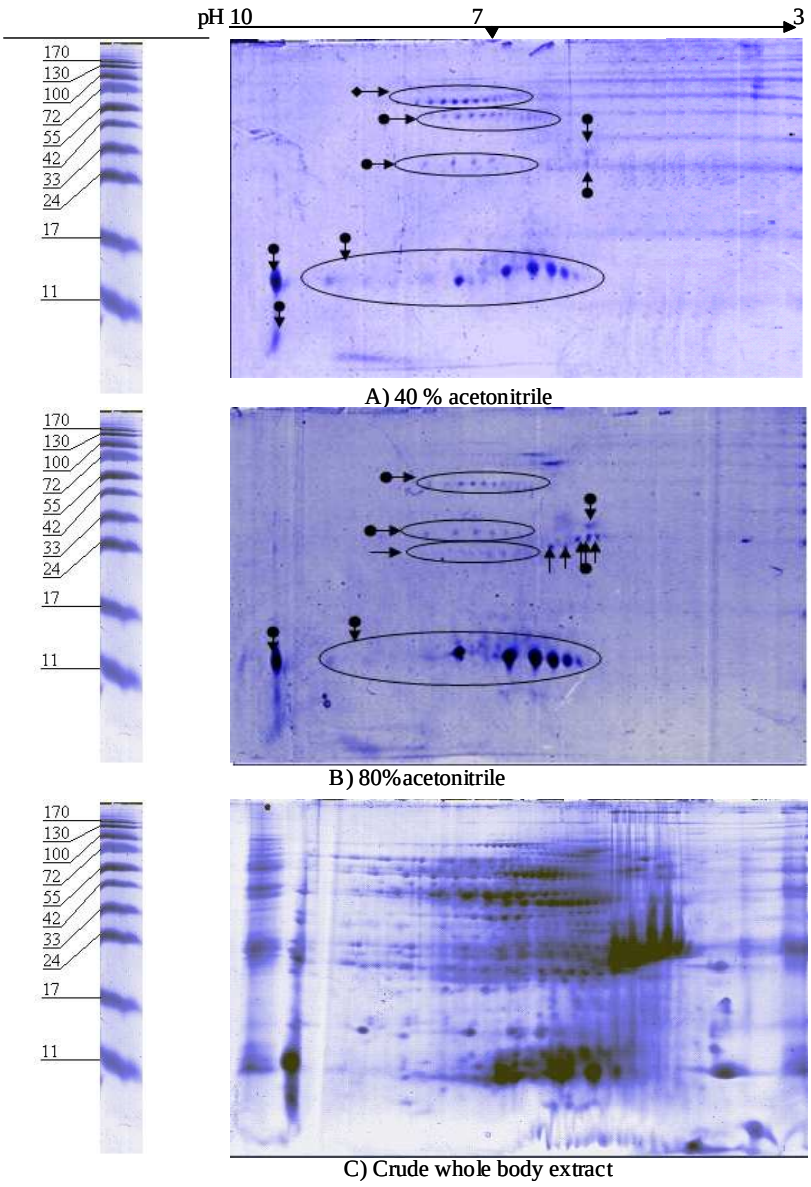


Figure 4.15: 2D PAGE analysis of hydrophobic antibacterial samples. The 40 and 80% acetonitrile concentrations were the samples that most commonly inhibited bacterial growth. As expected the spots are on the more acidic positively charged region of the gel, with no spots appearing past the pI 7.5 mark on the gel. Spots common to both the 40-60 % A and 60-80 % acetonitrile B make up the vast majority of the sample. Spots common to both gels are marked with an arrow-circle while those specific to a single gel are marked with just an arrow. The smallest peptides are common to both gels and these may represent the antimicrobial peptides.

It can be seen in the gel, with only a small number unique to one gel and not the other. However, these unique spots are large and not the small molecular weight peptides that we expected. The smallest molecular weight proteins are smaller than 10 kDa, found in both gels and have a pI around 3. This shows that the 60 % and 80 % acetonitrile concentrations are not all or nothing elution boundary for many of these peptides. And there is carryover from one sample to another. This is especially noticeable in the *E. coli* samples (figure 4.14).

4.4.3 2D gel electrophoresis analysis of differently infected and purified samples revealed changes in protein expression patterns.

Hemolymph proteins from beetles that were infected with the fungus *B. bassiana* and from uninfected beetles were separated using 2D gel electrophoresis and analysed with the PDQuest analysis software. Proteins whose expression pattern changed dramatically were selected, excised and sent for mass spectrophotometric analysis. These spots are labelled in figure 4.16 and the optical density readings and therefore, their relative quantities are represented in figure 4.17. Most of the spots in the gels were present in the M_r range 10 – 100 kDa and between the pI ranges of 4 -7 (Fig. 4.25). Proteins whose appearance or disappearance in the samples before and after infection, are marked in figure 4.16 by the relevant arrows. The levels of these changes in expression are represented in figure 4.17.

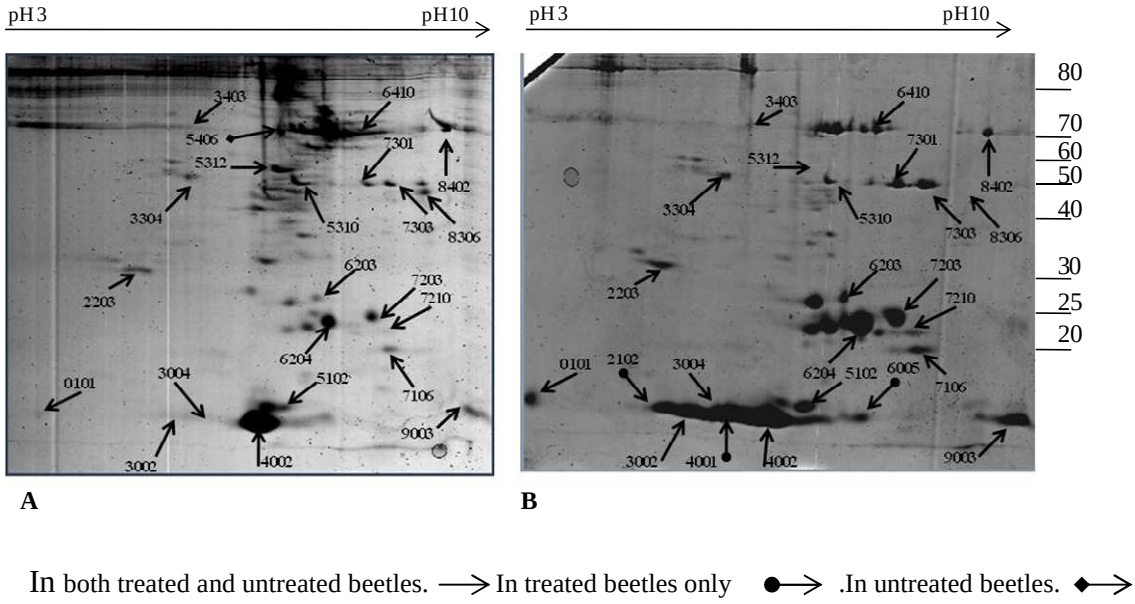


Figure 4.16: 2D gel electrophoresis of *E. intermedius* hemolymph proteins. The gel shows proteins in infected beetles (A) and in unchallenged beetles (B). Identified proteins were found in treated beetles only, in both treated and untreated beetles and in untreated ones only.

4.4.4 Tandem Mass spectrometry and bio-informatic analysis identified partial protein sequences of proteins that may be involved in immunity.

The spots of interest indicated in figure 4.16 and 4.17 were analysed by tandem (MS/MS) spectrometry. The MS/MS spectra of peptides obtained from each spot were used to search the National Centre for Biotechnology Information (NCBI) database using the Mascot search engine. At the same time the partial peptide sequences were used to search the NCBI protein databases using BLAST and the Flylab *E. intermedius* Genome base using tBLASTx.

The peptide fragments shared sequence identity with proteins involved in biological processes such as protein modification and degradation, development, chemical and light detection, mRNA modification and processing, metabolism, stress and immune responses, membrane transport and cytoskeletal elements (figure 4.17).

Many of these sequences were identified as sharing sequence identity with different proteins with different functions. For example the peptide fragment from the spot 5202 identified two proteins that both contain ankyrin repeats. However, these proteins differ vastly in function. The second peptide shows sequence identity with an RNA helicase from the human louse *Pediculis humanis copris.* The peptide list and identification by MS/MS is shown in Table 6.1 Generally these three different methods of identifying the peptide resulted in different conclusions.

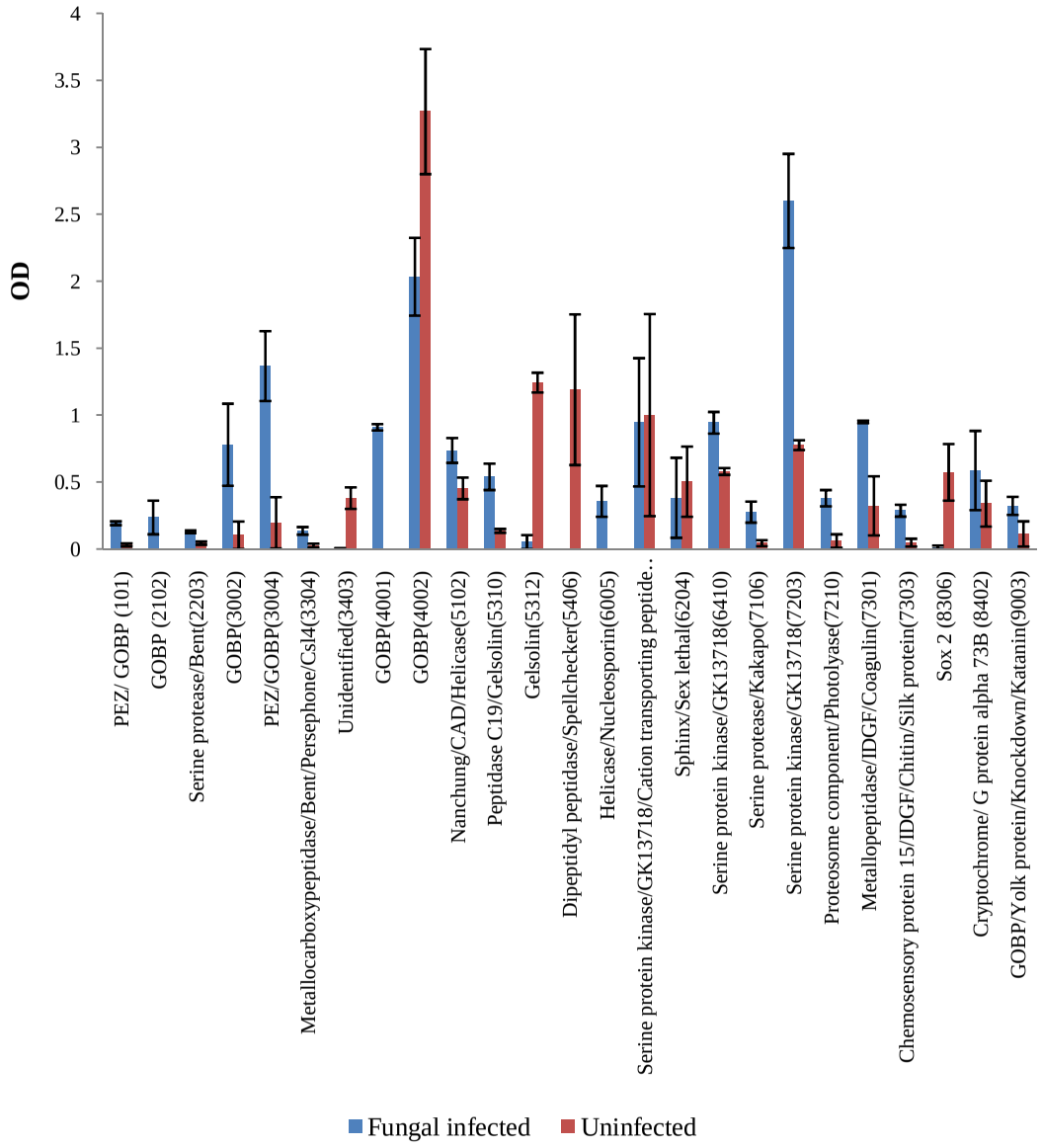


Figure 4.17: Relative quantity of proteins in fungal infected and uninfected hemolymph samples as determined by 2D PAGE. The identities of the proteins were determined using bio-informatic analysis of fragment sequences as obtained by MALDI-TOF analysis. Some proteins gave rise to multiple fragments which showed sequence similarity to different proteins this is why some spots have multiple proteins assigned to them. The relative quantity of each protein is based on optical density readings as determined by the PD quest software.

4.4.5 Peptide fragments may represent proteins that modulate protein stability.

A large number of the peptide sequences were identified as sharing sequence identity with proteins involved in protein modification (figure 4.17). At least three of these are involved in the phosphorylation or dephosphorylation of amino acids. Spots (0101, 3004, and 9003) are identified as similar to Pez (phosphatase with ezrin domain) and are highly up-regulated after fungal infection. Pez like sequences have been identified in the honeybee *Apis mellifera* and the fruitfly and are involved in amino acid dephosphorylation and may be responsible for activating or deactivating downstream signaling pathways.

Convincingly all the databases identify the five peptide fragments from protein 2203 as sharing sequence identity with some types of trypsin proteinase. These fragments were expressed at higher levels in the hemolymph from infected beetles and showed highest sequence similarity with trypsin proteinases from different insect species including beetles and moths. The Flylab genomebase also identifies a protein that shares sequence identity with a *Drosophila melanogaster* serine endopeptidase Jonah 65Aiv. Other proteases identified from the peptide fragments include a proteosome component from *Culex quinquefasciatus*, a peptidase C19 which contains an ubiquitin hydrolase domain, a zinc metallopeptidase from *A. pisum* and a Serine/Threonine protein kinases from *D. psuedoobscura.*

.

4.4.6 Many of the peptide sequences share identity with sensory proteins.

Apart from olfactory binding proteins, which are commonly up-regulated during stress responses in insects, other peptide fragments sharing sequence identity with sensory peptides were identified. The expression levels of all these peptides increases following fungal exposure. These include a homolog to the *D. melanogaster* peptide nanchung which is an environmental response peptide detecting odours and changes in humidity. Another is a homolog of a cryptochrome from the fly *Sarcophaga bullata.* This photoreceptor plays a role in response to light stimuli, circadian rhythm regulation, light perception, locomotory behavior; and the negative regulation of cellular biosynthetic processes.

4.4.7 Many of the peptide fragments share sequence similarity with proteins that play a role in insect development.

A high proportion of the peptide sequences share identity with proteins that are involved in development, only one of which is down regulated following fungal exposure. This fragment shares identity with the sex lethal protein from *T. castaneum*. This protein is involved in signaling pathways that control oocyte differentiation, imaginal disc growth and the negative regulation of translation.

Fragments from up regulated proteins share sequence identity with proteins such as the *Aedes aegypti* chaoptin, a 160 kDa glycoprotein which is involved in the development of photoreceptor cells. Three other up-regulated fragments share sequence identity with *Drosophila melanogaster* yolk protein, a structural molecule that is involved in vitellogenesis; oogenesis; sex differentiation. Another peptide sequence was identified as being similar to the *Bombyx mori* protein neverland. This

protein has oxidoreductase activity and is involved in larval development, growth and ecdysteroid synthesis. Finally one fragment was identified as sharing sequence identity with Imaginal Disc Growth Factor proteins from various species including *T. castaneum* and *B. mori.*

4.4.8 Fragments which share sequence identity with proteins that have an immune function are both up and down- regulated.

Two of the peptide sequences which share identity with proteins with immune function are down regulated following fungal exposure. This includes a fragment that shares identity with the Sox21a transcription factor from the louse *P.humanis*. This transcription factor is thought to play a role in phagocytosis and engulfment. The second fragment shares sequence identity with a serine protease.

Those fragments that are up regulated following fungal challenge share identity with a metallocarboxypeptidase, a chitinase (Cht6) from *D. melanogaster* and a coagulin from the horse shoe crap *Tachypleus tridentalus*. Many fragments shared identity with transferrins from *T. castaneum, D. melanogaster* and *Protaetia brevitarsis.* Transferrin can act as an antimicrobial peptide by sequestering iron and impeding bacterial survival. The last peptide sequence was identified as having sequence similarity with a leucine rich transmembrane protein from *T. castaneum*, this (LRR) is shared by the transmembrane receptor Toll.

4.4.9 Only 5.5 percent of the peptide fragments share identity with Stress response proteins.

One fragment that shared sequence identity with stress response proteins was down regulated. This fragment shared sequence identity with an *Anopheles gambiae* protein containing a P-loop containing Nucleoside Triphosphate Hydrolase domain, as well as a MutS domain III (DNA-binding domain found in the DNA mismatch repair MUTS family). Those fragments whose expression increased following fungal treatment shared similarity with a metallocarboxypeptidase and a multidrug-resistance like protein from *D. melanogaster*, which is involved in ATPase dependent movement of molecules across membranes. Finally one of the fragments shared sequence similarity with a photolase from *Anoheles gambaie* which may play a role in DNA damage repair.

4.4.10 Fragments were identified that were Cytoskeletal proteins or played a role in cytoskeletal modification.

Gelsolin is an actin binding protein and many of the peptide sequences were shown to be similar to it. However there was conflicting information as to whether it was up or down regulated following fungal exposure. The fragment that shared sequence homology with the kakapo protein from *T. castaneum* was down regulated following fungal treatment. This protein is involved in the negative regulation of microtubule depolymerization, cytoskeletal organization and the development of the tracheal system in *D. melanogaster*. The final peptide sequence shared sequence identity with

the *Aedes aegypti* katanin 60 protein, which is involved in microtubule processing. The expression of this protein increased following fungal treatment.

4.4.11 Treatment of beetles with fungus increased the expression of many proteins that share sequence identity with proteins involved in metabolic pathways.

Three proteins involved in the pathways for normal aerobic energy production are lipase domain. The second sequence with a common identity is identified as sharing sequence identity with a Glyceraldehyde 3-phosphate dehydrogenase from *Tribolium castaneum*, knockdown (citrate (Si)-synthase in the tricarboxylic acid cycle) from *Drosophila melanogaster* and NADH dehydrogenase. Another fragment shares sequence identity with a phosphoglycolate phosphohydrolase from *D. melanogaster*, which is involved in glyoxylate and dicarboxylate metabolism.

Two other proteins that are indirectly involved in metabolism that these fragments share sequence identity with are a *D. melanogaster* alcohol dehydrogenase transcription factor. A second sequence was similar to the trifunctional enzyme CAD. This enzyme consists of functional domains that perform a carbamoylphosphate synthetase-aspartate transcarbamoylase-dihydroorotase. Carbomyl phosphate synthesis is required for the initiation of the biosythesis of pyrimidines, CP is coupled to aspartate and its carbon and nitrogen nuclei are incorporated into the aromatic rings of pyrimidine nucleotides. Cabamoyl phosphate is condensed with ornithine at the start of the urea cycle and is utilized for the detoxification of ammonia and biosynthesis of arginine.

4.5. Identification and characterization of Immune related genes.

Other than antimicrobial peptides, homologs of many of the proteins identified as having immune function in other insects, have been identified in members of the order coleoptera. These include a toll homolog from the beetle *Tribolium castaneum* (Maxton-Kuchenmeister et al., 1999), transcription factors resembling Rel/NF- κB transcription factors have been identified in the beetle *A. dichotoma* (Sagisaka et al., 2004). Additionally following septic injury *Tribolium castaneum* shows an increase in the transcription of genes encoding Toll, G protein receptors, serine threonine kinases a PGRP-SC, serine proteases, prophenoloxidase, serpins and defensins. The immune challenge also results in an increase in the transcription of stress response and detoxification enzymes.

4.5.1. The identification and cloning of a serine protease.

Primers were designed based on one of the peptide fragments obtained for each of the spots. There is no consensus codon usage table for coleopteran so the *Drosophila melanogaster* codon usage was used to design the primers. These primers were then use with an oligo-dt primer to perform reverse transcription on a RNA extract from fungal infected *E. intermedius*. The results of these RT-PCR reactions is seen in figure 4.18. Attempts were made to optimize the reactions so only one product was obtained. This was not always successful with many primers constantly giving multiple products despite changes made to the annealing temperature and magnesium chloride concentration.

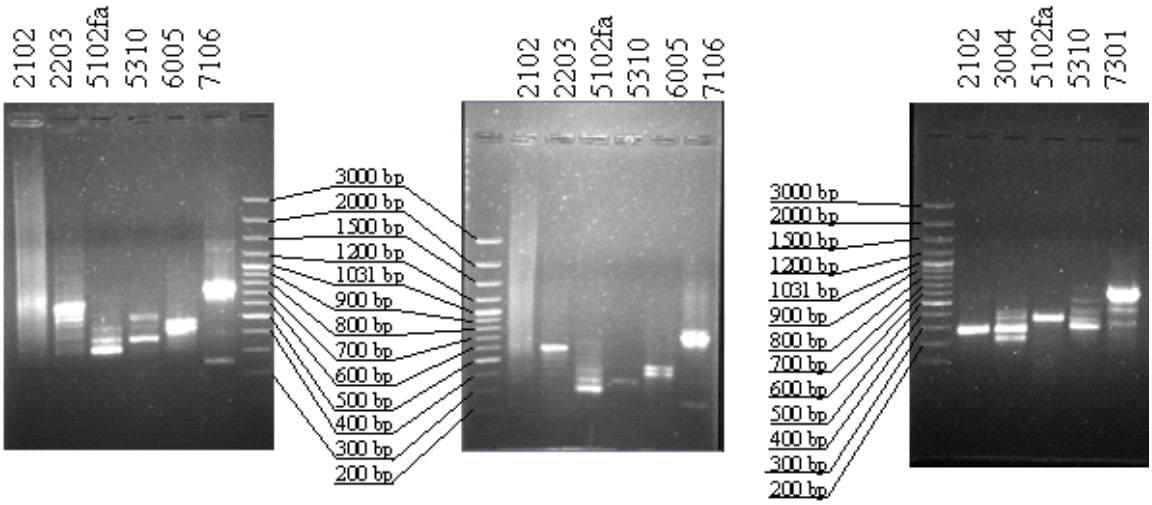
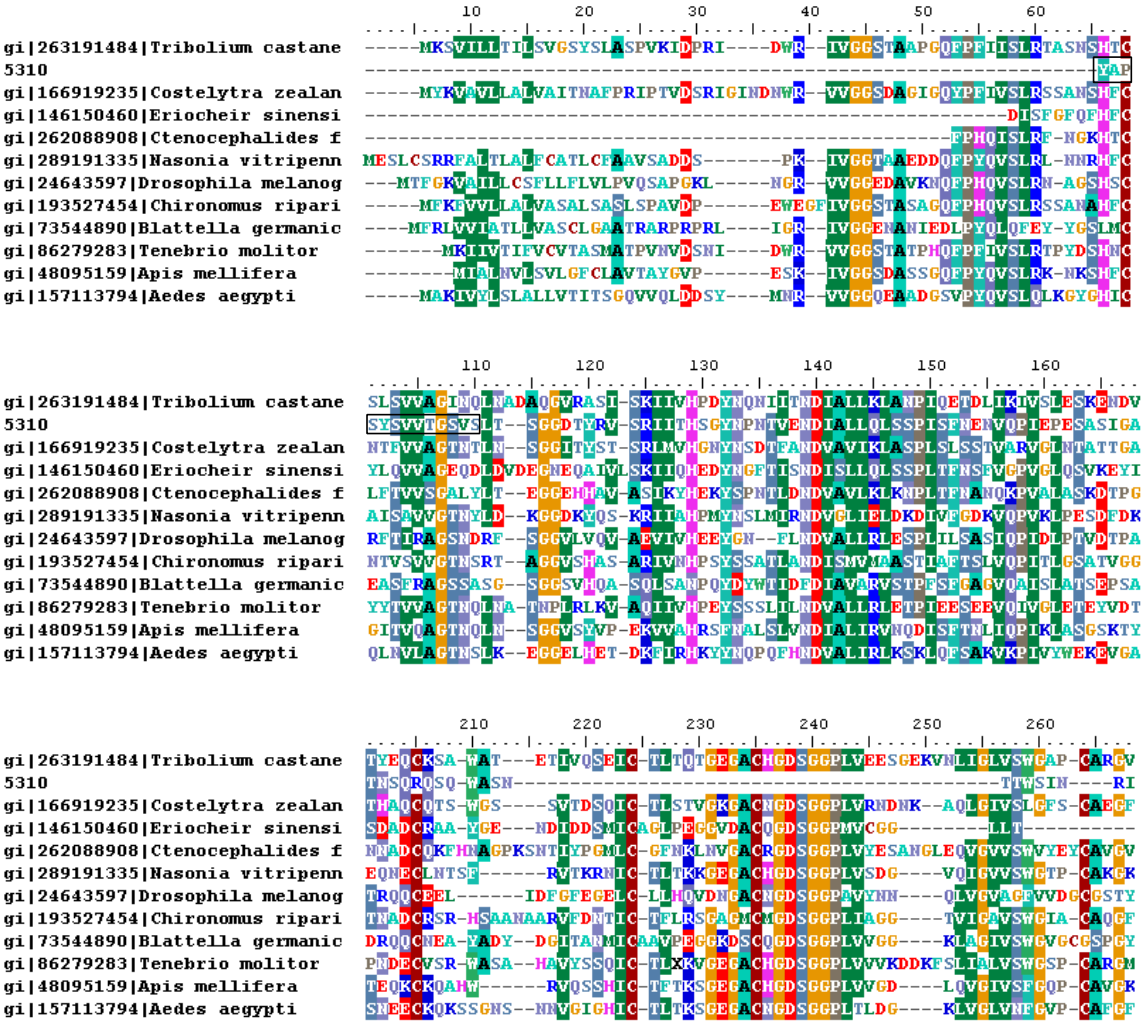


Figure 4.18. RT-PCR amplification of target mRNA using primers designed using peptide sequences. When more than one band was produced, attempts were made to optimise the reaction.

The cloned 5310 sequence was obtained via automated DNA sequencing and used to perform a BLAST search using tBlastn. This resulted in the sequence being identified as a serine protease (figure 4.19). It was also used to perform a tBLASTp search in the Flylab genomebase, where it also aligned with a serine protease (cl15 contig1). As expected the sequence showed highest identity with coleopteran serine proteases, with all these sequences grouping together. However, the 5310 sequence was on a separate branch to the other two beetle sequences, both of which are tenebroid beetles.

4.4.2 The serine protease (5310) appears as two transcripts one of which is down-regulated following infection.

Radioactive probes produced from the cloned serine protease sequence were used to probe RNA isolated from female beetles that had been infected with *B. bassiana* and beetles of both sexes that had not been infected. The results of these blots can be seen in figure 4.20 A. Additionally the Flylab genome base was used to identify the sequence for the ribosomal protein Rps9 which was used as a loading control, a sequence for actin was identified and used to produce a probe which served as a positive control. Finally, the sequence for a serine protease inhibitor was also identified in the database and this used to produce a probe for the transcription of a gene whose product may act as an inhibitor of serine protease activity. These sequences were used to design primers and these primers were then used to amplify the sequences from total RNA extracts from *E. intermedius*. These PCR products were then used to produce p^{32} labelled probes.



Accession	Description	Species	Score	Coverage	E value	Taxon
						Insecta : Endopterygota
NP_001161087.1	chymotrypsin- 5A	*Tribolium castaneum*	91.7	86%	6e-21	Coleoptera
ABZ04015.1	serine protease 7	*Costelytra zealandica*	90.9	84%	1e-20	Coleoptera
ABC88737.1	Serine proteinase	*Tenebrio molitor*	85.5	90%	9e-19	Coleoptera
ACY24346.1	chymotrypsin	*Ctenocephalides felis*	82.4	81%	9e-18	Siphonoptera
NP_001166054.1	serine protease	*Nasonia vitripennis*	78.2	79%	5e-16	Hymenoptera
XP_001657905.1	serine-type enodpeptidase	*Aedes aegypti*	79.7	84%	2e-16	Diptera
ACF19792.1	serine-type enodpeptidase	*Chironomus riparius*	77.8	84%	9e-16	Diptera
XP_394370	chymotrypsin-1	*Apis mellifera*	79.0	79%	2e-15	Diptera
NP_523426	serine protease 6	*Drosophila melanogaster*	72.4	82%	1e-13	Diptera
						Insecta:Orthopteroidea
AAZ78212.1	Trypsin	*Blattella germanica*	48	51%	8e-8	Dictyoptera
						Crustacean
ABQ02519.1	trypsin	*Eriocheir sinensis*	84.0	69%	9e-19	Crustacean

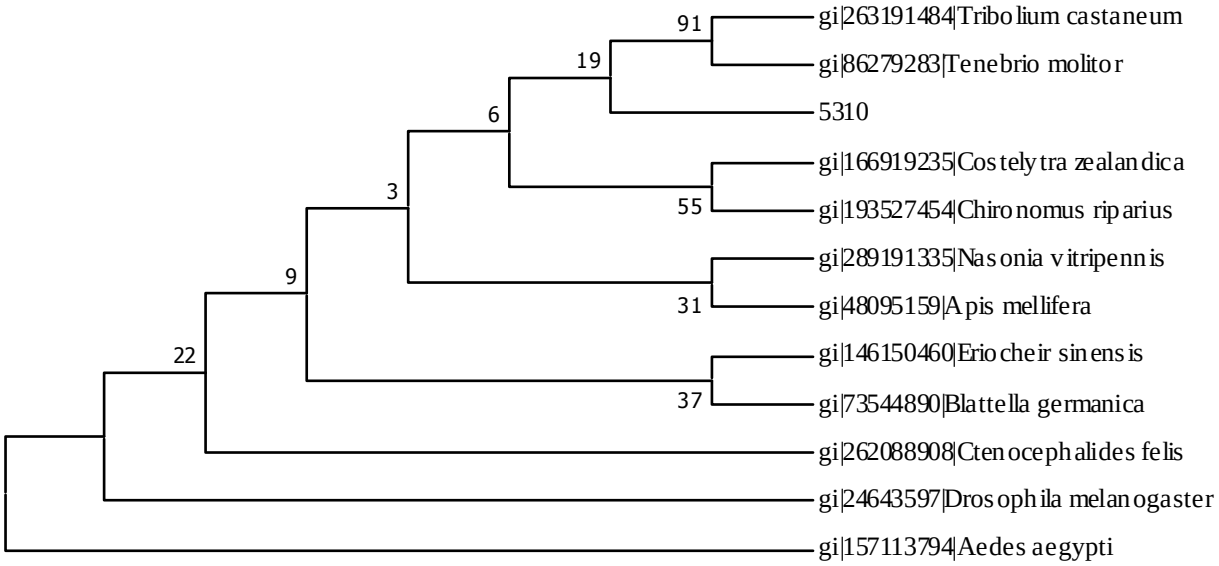


Figure 4.19: Alignment of the 5310 translated amino acid sequence with serine proteases amino acid sequences from insects across different insect orders. (A) The alignments include other coleoptera and closely related orders such as diptera and hymenoptera as well as the distantly related order dictyoptera, which serves as an out group. Regions of high conservation are present in the 5310 sequence. This includes a glutamine which is found in all the other sequences. (B) The proteins with the highest degree of homology are those from the coleopterans *T. castaneum* and *T. molitor*. The phylogenetic tree constructed from these alignments shows the *E. intermedius* serine protease (5310) is grouped with the other coleopteran proteins. The location of the peptide sequence upon which the primer sequence was based, is shown by the box on the alignment. The alignment was performed using the muscle algorithm by the MEGA5 analysis software. The tree was constructed using the neighbour joining method with a bootstrap analysis of 500. This was also performed using the MEGA 5 software.

RNA extracted from uninfected beetles of both sexes as well as male beetles infected with *B. bassiana* was used as a target for these probes. A duplicate of the denaturing agarose gel used to perform the blot can be seen in figure 4.20 B showing that the RNA was intact. The result of the northern blots can be seen in figure 4.20 A and the relative quantity detected of each transcript is depicted in figure 4.20 B. Both figure 4.20 A and B show that the levels of RpS9 detected in all three of the samples matches closely, implying that each lane contains similar amounts of RNA which allows a direct comparison of the intensity for each of the other transcripts.

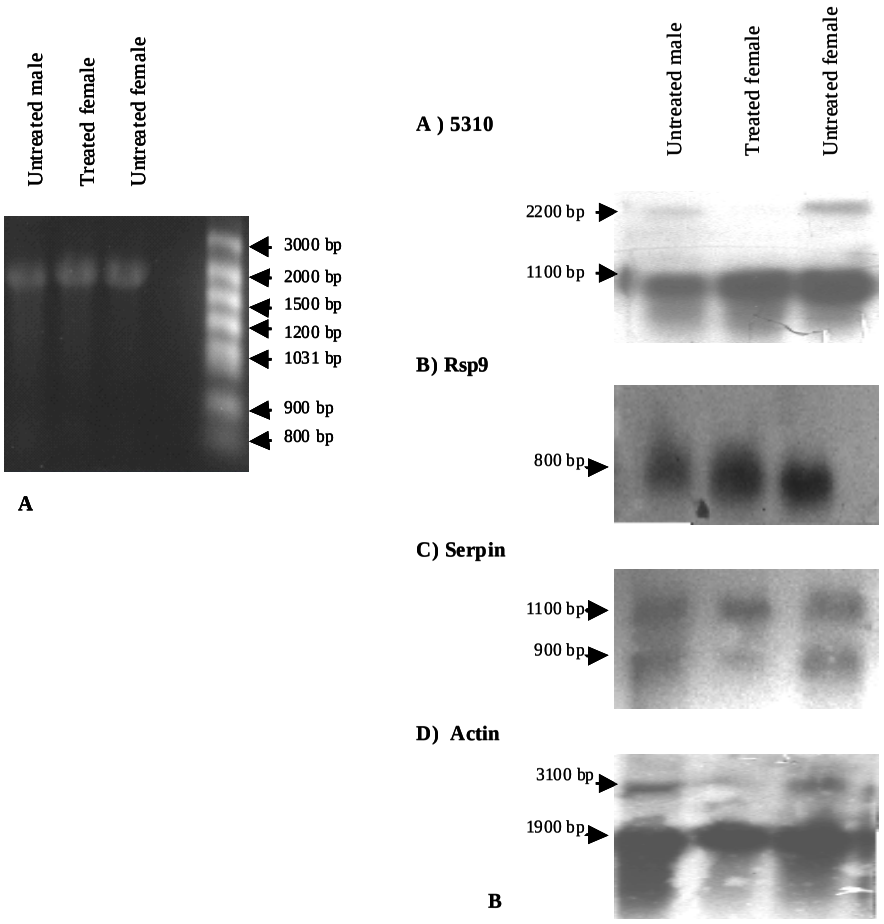
The serine protease 5310 is detected as two different transcripts, one at 2200 bp the other at 1100 bp. This is much larger than the expected size of the transcript based on other insect homologs and the sequence available in the Flylab genomebase. These databases give an expected size of around 866-900 base pairs. The much larger transcript is only detected in the uninfected samples and is absent from the fungal infected female sample. The smaller transcript is detected as having higher levels in female beetles ($p = 0.05$ $F = 0.3846$) and these levels are not significantly altered in the infected females ($p = 0.3426$ $F = 0.4319$). The 2D-PAGE analysis of the infected and uninfected beetles (figure 4.16 and 4.17) shows that the spot 5310 is only detected in infected beetles. This is not supported by the mRNA levels, as these are equal for the 1100bp sample and show that the 2200 bp transcript is absent from infected beetles.

4.5.3 The Serpin probe detects only two of eleven expected transcripts, and transcript levels seem to be independent of sex or infection status.

When the serpin probe was used to probe the northern blot, only two transcripts of around 1100 and 900 bp were identified. This is smaller than the expected transcript size as determined from *Drosophila melanogaster* homologs which range from 1400-2200 base pairs. Additionally, there are 11 isoforms of this gene expressed in *D. melanogaster*. Using this probe we only detect two transcripts in *E. intermedius*, both of which are smaller than expected. When the levels of transcription are examined (figure 4.20 C), the sex or infection status of the beetles seem to have no significant effect on the levels of serpin mRNA.

4.6 Discussion.

Treatment of the beetles with different pathogens led to an increase in mortality and a decrease in fecundity. The HPLC and SDS results show a difference in protein expression patterns and elution profiles between infected and uninfected samples. However, it is not clear what these differences are and if they can be clearly attributed to the infections or merely due to the wounding of the insects. Tris-tricine gels also indicate that the altered peptide expression following infection probably occurs rapidly and persists for more than 24 hours. The decrease in the ability of the beetles to produce broodballs following fungal infection could be the result of energy restrictions placed on the beetle following fungal exposure due to an increase in immune function.


Untreated male
Treated female
Untreated female
3000 bp
2000 bp
1500 bp
1200 bp
1031 bp
900 bp
800 bp
A
A) 5310
2200 bp
1100 bp
B) Rsp9
800 bp
C) Serpin
1100 bp
900 bp
D) Actin
3100 bp
1900 bp
B

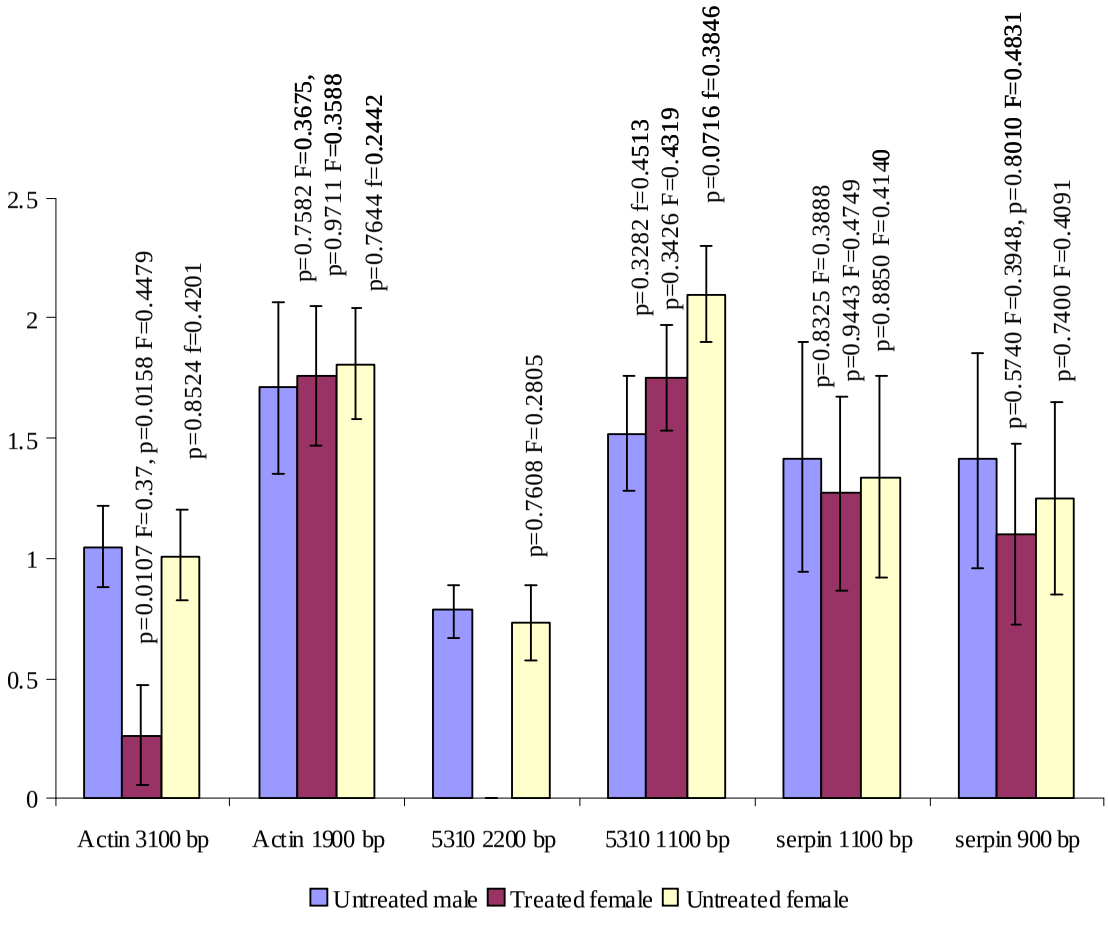

2.5
2
1.5
1
0.5
0
p=0.0107 F=0.37, p=0.0158 F=0.4479
p=0.8524 f=0.4201
p=0.7582 F=0.3675,
p=0.9711 F=0.3588
p=0.7644 f=0.2442
p=0.7608 F=0.2805
p=0.3282 f=0.4513
p=0.3426 F=0.4319
p=0.0716 f=0.3846
p=0.8325 F=0.3888
p=0.9443 F=0.4749
p=0.8850 F=0.4140
p=0.5740 F=0.3948, p=0.8010 F=0.4831
p=0.7400 F=0.4091
Actin 3100 bp
Actin 1900 bp
5310 2200 bp
5310 1100 bp
serpin 1100 bp
serpin 900 bp
Untreated male
Treated female
Untreated female

Figure 4.20: Northern blot analysis of the transcript levels of the serine protease 5310, following fungal challenge. A duplicate of the gel used to perform the blot is depicted in (A). The results of the Northern blot are depicted in (B). Rps9 is detected in all samples at similar amounts showing that equal amounts of total RNA were added to the gel. (D)Actin is detected as two differently sized transcripts. (c) Two serpin transcripts are detected in *E. intermedius*, both are smaller than expected. (A) The 5310 probe detects two differently sized transcripts in the untreated samples and only one transcript in the treated female samples. Only the smaller transcript detected in all three samples is of the expected size of around 1100 bp. The relative quantity of the bands detected using was calculated using the optical density readings of the bands seen in (B). Both forms of actin detected are present in similar levels across all samples, while the transcription of both serpins seems to be more dependent on sex than state of infection. Finally the transcript levels of the smaller 5310 transcript are the same in the treated and untreated female samples. However, the larger transcript is only present in the uninfected beetles.

4.6.1. Antimicrobial activity is present in the hemolymph as a heat stable, positively charged, hydrophobic protein.

Due to the antibacterial protein whose activity was detected in the hemolymph and whole body protein extracts being heat resistant, having a pI above 7.4 and eluting from the C18 column with 40-80 % acetonitrile. The peptide must be a positively charged hydrophobic protein with a pI in the range for the defensins cecropins and glycine rich peptides. This excludes antifungal peptides like rhinocerosin and thaumatin. The appearance of the antibacterial peptide in the 40-80 % acetonitrile fractions is similar to patterns seen for defensins, scarabaecin and Alo-3. The glycine rich peptides elute with lower concentrations of acetonitrile as does cecropin (see table 5.1). The antibacterial activity against both gram negative and gram positive bacteria, with a higher activity against gram positive bacteria, matches the activity spectrum of both the defensins and cecropins. Cecropins typically inhibit all bacteria and sometimes fungi (Bullet 2004). Insect defensins typically only inhibit gram positive bacteria (Bulet *et al*, 1991). Finally the absorbance spectrum of the antibacterial peptide closely matched that of a defensin. Therefore, it appears as if the antimicrobial peptide we have isolated from *E. intermedius* is most likely an insect defensin (see table 4.3). Tris-tricine SDS-PAGE, HPLC and 2D-PAGE analysis demonstrated that the inhibitory sample consists of multiple proteins, some of which were small enough to be the active antimicrobial peptide(s).

4.6.2 Fungal infection of *E. intermedius* results in the induction of defence as well as stress response proteins.

Analyses of protein extracts using HPLC and Tris-Tricine SDS-PAGE (figures 4.1-4.3), clearly shows that infection with fungi or bacteria altered protein expression patterns. However, it is not clear what these differences are and if they can be clearly attributed to the infections or merely due to the wounding of the insects.

The protein profile of *Drosophila* following infection changes with time as some proteins are rapidly up-regulated following infection (Vierstraete et al., 2004). These rapid responses may represent the secretion of proteins into new compartments, or changes in protein processing (Engstro¨m et al., 2004). Twenty four hours after infection most of these proteins had returned to their original levels indicating a partial recuperation (Guedesa et al., 2005).

Previous studies on the immune response of *D. melanogaster* adults and larvae show an increased expression of a variety of immune response proteins. These include serine proteases, serpin, prophenoloxidase activating enzymes and pathogen recognition molecules. Stress response and partial antimicrobial peptides transferrin and ferritin were also up-regulated as well as enzymes involved in post-translational processing of antimicrobial peptides (Guedesa et al., 2005; Levy et al., 2004) (Vierstraete et al., 2004).

Similar immune responses were detected in *E. intermedius* following immune challenge. Peptide fragments from proteins whose expression had increased following

infection, showed sequence similarity with proteins involved in pathogen recognition, haemocyte response and wound healing. One of the up-regulated proteins (spot number 8306 table 6.1) shares identity with the Sox21a transcription factor from the louse *P. humanis* copris, which is thought to play a role in phagocytosis and engulfment. Another (spot number 7301F table 6.1) showed sequence similarity to a coagulin from the horse shoe crab *Tachypleus tridentalus* while another (spot number 7303 table 6.1) was similar to chitinase (Cht6) from *D. melanogaster*. Others share sequence similarity with proteins required for phagocytosis such as Thiolester-containing protein (TEP) complement like molecules. Furthermore proteins similar to the stress response and partial antimicrobial peptides transferrin and ferritin were also up-regulated.

Lipophorin is a lipid transport protein in the hemolymph and contains a 18.5 kDa subunit called apolipophorinIII. Apolipophorin III also binds to fungal β-1,3-glucan and acts as a pattern recognition molecule (Whitten et al., 2004). The spots 6203, 7101 and 7203 share sequence identity with Apolipophorin III and Cl123Contig1 in the FlylabGenomebase (Khanyile et al., 2008). These spots are also the correct size for ApolipophorinIII (figure 4.9) of about 18.5 kDa.

Many of the proteins whose expression is increased following infection are similar to serine proteases which are implicated in the activation of the immune system. Proteins were identified as sharing sequence identity with proteinases from different insect species including beetles and moths. Some of these spots (2203 and 3304) aligned with clones in the *E. intermedius* cDNA database (CL20 contig1, CL23 contig1 and CL8contig1). These three contigs share a high sequence identity with the Persephone

serine protease. The spot 3304 is also the correct size (figure 4.9) for Persephone (43 – 50 kDa). The identification of Persephone is consistent with response the fungal response observed in *Drosophila melanogaster* where the proteases Grass and Persephone are known to transfer fungal recognition signals to pro-Spatzle during activation of the Toll pathway (Roh et al., 2009). Homologs of Persephone have been identified in *Tribolium,* these were given the name *Tc*-SP44 or SP66 (Zou et al., 2007). In *Drosophila* there are at least five separate serine proteases that play a role in Toll activation in the immune response (Kambris et al., 2006).

The spot 6410 is identified as sharing sequence identity with a *Tribolium* Toll. Four Toll proteins have been identified in *Tribolium* (Zou et al., 2007).

The Serpin identified in the database was identified as sharing the highest sequence homology with Serpin4 from *Drosophila.* This Serpin was initially involved in the regulation of peptide maturation (Richer et al., 2004). This may explain why fungal infection had no significant effect on the levels of transcription of this *serpin*. However, Spn4 is able to inhibit members of multiple protease families and these could include members of the serine protease cascade or pathogen proteases. They are also likely to play a role in the processing of immature antimicrobial peptides into mature peptides (Bruning et al., 2007).

The serine protease that was represented by spot 5310 showed highest sequence homology with those of two tenebroid beetles (figure 4.19). It also shares many similar features with a serine protease isolated from the firefly *Pyrocoelia rufa* which is a 274 amino acid protein found in the gut of the firefly which may help to process

food (Li et al., 2005). It shows highest sequence similarity with the *Drosophila* CG8213 serine protease which clusters into the subfamily D of CLIP serine proteases and has trypsin like activity (Rossa et al., 2003). There is however no evidence that it plays a role in the immune system. It also shares sequence similarity with Peptidase C19 a serine protease that function in the *Anopheles gambiae* digestive system. The protein is only detected on the 2D PAGE in the fungal infected beetles (figure 4.9), while the larger transcript is detected only in the uninfected beetles. The smaller transcript is detected at equal levels in the infected and uninfected beetles. Additionally, the 1100bp fragment is more likely to represent the 40 kDa protein detected on the gel corresponding to spot 5310. Therefore, the smaller transcript most likely represents the spot 5310 and posttranscriptional processing may be required before an active protein is produced.

4.6.3. Other protein expression changes that accompany infection.

The proteomic analysis of *D. melanogaster* larvae hemolymph proteins following infection, indicated the increase in the expression of proteins involved in metabolism, antioxidant and detoxification (Guedesa et al., 2005). An analysis of adult *D. melanogaster* hemolymph proteins following infection, show a similar increase (Levy et al., 2004) (Vierstraete et al., 2004). Similar results were also obtained when the proteomic response of the flesh fly *Sarcophaga bullata* was examined post infection (Masova et al., 2010).

Table 4.3 Characteristics of antimicrobial peptides isolated form coleoptera

Peptide	Antimicrobial activity	Size	% acetonitrile at which elution occurs	pI	Heat resistant	Amino Acid Composition	Reference
cecropin	Bacteria some fungi	4kDa	30%	9.2-10.4	yes	Phenylalanine Tyrosine	(Saito et al., 2005)
defensins	Gram positive bacteria	4kDa	40-60%	8.2 -9.3	yes	Cystine Phenylalanine	
scarabaecin	Fungi, bacteria	4kDa	40%	9.21	yes	Phenylalanine Cystine	(Tomie et al., 2003)
alo-3	Fungi, bacteria	3.8 kDa	60%	9.13	yes	Cystine tyrosine	(Barbault et al., 2003)
Glycine rich	Bacteria	14 kDa	30%	9.5-10.1	No	Tyrosine Tryptophan	(Lee et al., 1994) (Bulet et al., 1991) (Imamura et al., 1999)
rhinocerin	Bacteria	14 kDa	30%	5.03	No	Tyrosine Tryptophan	
Unidentified *E. intermedius* antimicrobial peptide	Gram positive bacteria	4kDa, 12kDa suspected	40-80%		yes	Cystine Phenylalanine Tyrosine	

This table lists the characteristics of antimicrobial peptides isolated from beetles at this time. It also includes the characteristics identified for the *E. intermedius* peptide.

Following infection of *E. intermedius* with fungal pathogens, the expression of metabolic, antioxidant and detoxification proteins are all found to increase significantly. Additionally 10% of the peptides with increased expression are similar to proteins that have an immune function.

The increased expression of enzymes involved in glycolysis and the Krebbs cycle ($p = 0.001$) suggests an increase in the energy required to mount an immune response.
This has been observed in bumblebees where an increase in food intake must occur in order to support an increased immune response (Moret and Schmid-Hempel, 2000) and in *D. melanogaster*, where it was suggested that this was due to increased ATP production required for an effective immune response (Guedesa et al., 2005). Following bacterial treatment proteins that are involved in glycerol metabolism decreased, indicating a switch to ATP production and metabolism (Guedesa et al., 2005).

The up-regulation of cytoskeletal proteins in *E. intermedius* following infection was not significant overall ($p = 0.176$). However, one of the cytoskeletal proteins whose expression is up-regulated is the actin binding protein gelsolin. This protein is also observed to be up-regulated in the hemolymph of the mosquito *Aedes aegypti* following parasitic infection (Bartholomay et al., 2004) and may be required to modify actin filaments during phagocytosis or other cell movements. The increase in the expression of proteins that share identity with the microtubule regulating proteins katanin 60 from *Aedes aegypti* and kakapo protein from *T. castaneum* is also observed following infection in *E. intermedius*. Microtubules have been implicated in viral infection as well as in endoparasitic bacterial infection where they use the hosts own

microtubule system to infect oocytes (Serbus and Sullivan, 2007). However, *Beauveria bassiana*, the entomopathogenic fungus used in this study has been reported to produce a MAP kinase whose downstream targets include proteins involved in regulating microtubule dynamics. This seems to be required for the penetration of the insect cuticle (Zhang et al., 2010). As both kakapo and katanin control microtubule polymerisation it could imply that these proteins are being utilised by the pathogen to aid in the infection of *E. intermedius.*

The majority of proteins whose expression increased in *E. intermedius* following infection shared identity with proteins that were involved in development ($p = 0.001$). These included a protein that shared sequence similarity with the *Bombyx mori* protein Neverland. This protein has oxidoreductase activity and therefore may serve as a stress response protein. It is also involved in larval development, growth and ecdysteroid synthesis. It contains a 2 iron, 2 sulphur binding Rieske domain (Yoshiyama et al., 2006). It may also play a more active immune defence role by implicating hormone level changes following immune challenge. The levels of 20E and (JH) can influence the immune response. In this instance JH will lead to a decrease in immune response, while 20E increases it (Flatt et al., 2005) (Gruntenko and Rauschenbach, 2008). This is reinforced by another protein fragment whose expression increases following immune challenge, sharing sequence with yolk protein. Another up-regulated protein shares sequence similarity with Imaginal Disc Growth Factor which controls the proliferation of imaginal disc cells. However, its structure implies that it is also involved in chitin binding, and therefore may play a role in pathogen recognition and immune response (Varela et al., 2002).

At least 7% of the proteins, whose expression is increased following immune challenge of *E. intermedius,* resemble proteins involved in chemosensory functions. However, there was no statistically significant increase in the overall expression of sensory proteins ($p = 0.198$). This was due to the increase of the expression of the Odorant Binding Proteins represented by spots 4001 and 4002. There are instances in which odorant receptor molecules are involved in innate immune signalling systems (Levy et al., 2004). While the expression of these odorant proteins may be in response to oxidative stress, their role in the transport of hydrophobic odorants to and from odorant receptors may be a defensive response that has evolved to protect the beetle from environmental toxins and pathogens. There are reports of immune activated proteins leading to behavioural changes. These include the Toll isoform Tol-1 from the nematode *C. elegans*. Following activation by PAMP recognition this protein initiates avoidance behaviour by the nematode (Pujol et al., 2001). One of the chemosensory peptides up-regulated following the infection of *E. intermedius* is the protein Nanchung which is an environmental response peptide detecting odours and changes in humidity. There is no evidence that this protein plays a role in responding to oxidative stress and it is most likely playing a role in detecting potential pathogens.

Following infection of *E. intermedius* with fungus, Pez sequence containing proteins were found to be up-regulated. Pez-like sequences have been identified in the honeybee *Apis mellifera* and the fruit fly. They are protein tyrosine phosphatases that are able to bind cytoskeletal elements and are involved in amino acid dephosphorylation (Edwards et al., 2001). There is no evidence that they play a direct role in the immune system or stress response. They do, however, modulate phosphorylation they may be responsible for activating or deactivating downstream

signalling pathways. Also up-regulated following infection in *E. intermedius* is a protein that resembles an ubiquitin carboxy terminal hydrolase. Ubiquitin is an important protein for stress and immune response in insects. Therefore, any protein responsible for modulating the ubiquitination of proteins may serve an important role in the immune system.

Chapter 5

Discussion

5.1 DNA damage in *Drosophila melanogaster*

5.2 The immune response of *E. intermedius*

5.3 Stress responses common to both DNA damage and infection

5.4 Future studies

Chapter 5: Discussion

The exposure of these model insects to stressful conditions resulted in changes in gene expression. The DNA damaging drug camptothecin was used as the stressor in *D. melanogaster* and infection with pathogens served as an immune challenge in the dung beetle *Euoniticellus intermedius*. The changes in gene expression were assessed using a proteomic analysis of protein extracts or hemolymph before and after infection or DNA damage.

Notably; DNA damage by the topoisomerase 1 inhibitor camptothecin, resulted in changes in the expression levels of enzymes involved in carbohydrate metabolism in *D. melanogaster* recovering from camptothecin treatment. This metabolic shift is characterized by the appearance of a glycolytic flux in recovering cells. This flux is also accompanied by an increase in apoptosis. The pro-apoptotic tumour suppressor p53 is known to regulate metabolism directly via its transcriptional targets. In this thesis I investigated the role of Dmp53 and its putative regulator Snama in this apoptosis-linked glycolytic flux. This was done by monitoring changes in the expression and transcription levels of *Dmp53*, *Snama* and the pro-apoptotic *Reaper*. This revealed interesting trends in the levels of expression and transcription of these genes during and after DNA damage.

Immune challenge of the dung beetle *E. intermedius* was carried out using bacteria and fungi. This study focused mainly on fungal infection as it produced clear signs of infection and immune response. Proteomic analysis showed the increased expression of proteins that shared a high sequence identity with proteins from other organisms

that have an immune function. These beetles displayed an antimicrobial activity towards both gram-positive and gram negative bacteria. This activity was present before infection, but was detected at higher levels, following infection.

5.1. DNA damage in *Drosophila melanogaster.*

Exposure of *Drosophila melanogaster* to camptothecin led to a Dmp53 dependent apoptotic response resulting in increased mortality and developmental defects in the offspring of the treated fly. This increase in Dmp53 dependent apoptosis was matched by an increase in *dmp53* transcription but a decrease in the levels of the largest protein was detected. The levels of the smaller protein remained unchanged. This seems to indicate a decrease in dmp53 expression following exposure to stress. If Snama is a negative regulator of Dmp53 as the transcription patterns suggest. Then the expression patterns suggest a complex relationship between the two. Stable levels of the protein in untreated flies may be maintained at lower levels by high levels of Snama activity. Following camptothecin treatment the apoptotic role may be played by the stable smaller transcript as steady Snama activity degrades the larger transcript.

During recovery from camptothecin exposure the levels of Dmp53 decrease which may allow cells to recover from camptothecin induced damage. Additionally cells lost due to apoptosis must be replaced, leading to an increase in *snama* mRNA levels. Transcription patterns display decreasing *dmp53* mRNA levels being matched by increasing *snama* mRNA levels and vice versa. This indicates that there is a relationship between the two.

Following camptothecin exposure flies undergo a glycolytic flux that involves a metabolic shift that is different to that observed in cancer cells. The increase in the levels of arginine kinase provides a means whereby this flux can be generated. This flux appears to be generated by the activity of the enzyme arginine kinase (Hull and Ntwasa, 2010). This implies that recovery from camptothecin treatment requires an increase in energy output or involves signalling pathways that perturb glycolysis (Hull and Ntwasa, 2010). This may present a target for future cancer therapies.

The ability of cells to recover from DNA damage seems reliant on an increase in energy production and utilisation. This investigation highlights the importance of energy production mechanisms in cells that recover from chemotherapy and the important differences between the metabolic programmes employed by the recovering cells and those adopted by cancer cells.

Embryos from camptothecin treated adults show abnormal development and incorrect levels of apoptosis at inappropriate times. There was some evidence that this may be due to loss of maternally supplied topoisomerase I, alternatively, the ovary damage resulting from camptothecin treatment may result in hormone levels changing, negatively influence embryo viability as well as influencing *reaper* expression which is controlled via ecdysone receptor activation. Further evidence for changes in hormone levels includes the altered expression of yolk proteins.

Surprisingly, not only did methyl pyruvate fail to increase fecundity but on its own it had a negative effect on embryo numbers, it also seemed to retard recovery rates compared to those flies that were exposed to camptothecin only. The negative effects

methyl pyruvate had on embryo numbers and recovery rates may be due to an increase in metabolic pathways generating resulting in more reactive oxygen species. The viability of embryos produced by flies exposed to camptothecin and methyl pyruvate was increased, implying that pyruvate is able to partially alleviate apoptosis induced by camptothecin treatment. Methyl pyruvate may be protecting cells by preventing the nuclear translocation of p53 resulting in a decrease in the transcription of *reaper*. Alternatively methyl pyruvate may provide increased protection from ROS.

Metabolic pathways play a role in the apoptotic response of bacteria to antibiotics. This pathway occurs in all bacteria regardless of the antibiotics target or mode of action. Following the primary drug target interactions there is an alteration in NADPH consumption that is the result of increased TCA and respiratory activity. This leads to the generation of superoxide groups which then damage the cell via ROS generation, leading to cell death (Kohanski et al., 2010). This is very similar to the situation observed in this study, where the increase in metabolism brought on by the presence of more substrate for the TCA pathway, leads to increased apoptosis. At the same time in the recovery of embryos from adults treated with camptothecin and methyl pyruvate. The presence of pyruvate can "soak" up excess ROS molecules and protect cells from apoptosis. Our results imply that pyruvate is able to partially alleviate apoptosis induced by camptothecin treatment.

The combination of methyl pyruvate and camptothecin also led to an increase in *reaper* transcription, implying active pro-apoptotic signalling despite the decrease in Dmp53 expression. Due to the changes that this treatment had on Snama and Dmp53 expression and transcription we can conclude that methyl pyruvate is protecting these

embryos from the increased levels of apoptosis in a specific manner and not by merely absorbing ROS.

As the effect of camptothecin exposure in mammals is closely replicated in flies, they may be useful model organisms to elucidate the exact pathways that lead to these side effects of camptothecin exposure. *D. melanogaster* has recently proved a useful model organism for the study of epilepsy, with sodium channel and gap junction mutants suppressing seizure activity. Of special interest with reference to camptothecin is the discovery that mutations in topoisomerase 1 act as seizure suppressors. This fact led to camptothecin trials in *D. melanogaster*, which showed successful seizure suppression through the inactivation of topisomerase1 (Song and Tanouye, 2008).

5.2 The immune response of *E intermedius.*

Inhibition assays detected what appears to be an insect defensin in the hemolymph and whole body protein extracts from infected, with fungi and bacteria, as well as uninfected *E. intermedius.* Despite the fact that infection does seem to have an effect on protein expression patterns and mortality rates, it has little noticeable effects on the activity of the defensin of this beetles. This suggests that the defensin expressed constitutively. The peptide expression gels indicated that changes following infection in *E. intermedius* were present at least 4 hours after infection and persisted at a constant level for 24 hours. A study of a closely related species of dung beetle *Copris tripartitus* indicated that the mRNA for the defensin is up-regulated 4 hours after infection and reaches a peak after 16 hours (Hwang et al., 2009). It is likely that the

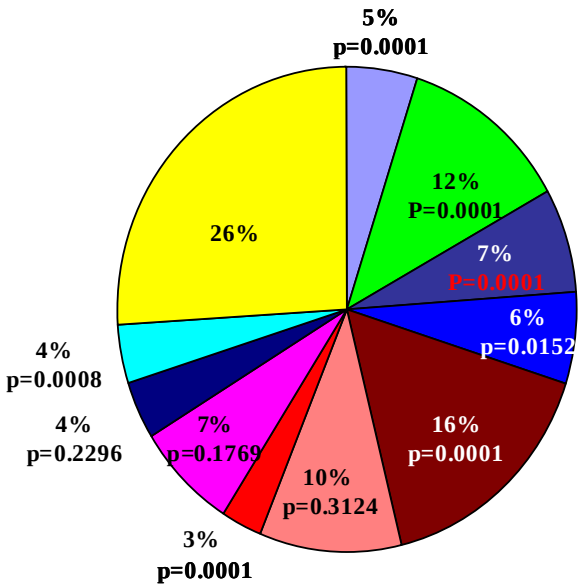
beetles in our culture could have been exposed to a low level of infection in the culture resulting in the near continues presence of the defensin.

Fungal infection led to the up-regulation of several serine proteases, some of which share sequence identity with translated sequences in the *E. intermedius* database that are homologous to the serine protease Persephone. Persephone plays a role in the serine protease cascade that activates the toll pathway. Additionally spot 6410 may represent a toll like protein. Therefore this proteomic study has identified at least two probable homologs of proteins involved in the response of insects to fungal infection.

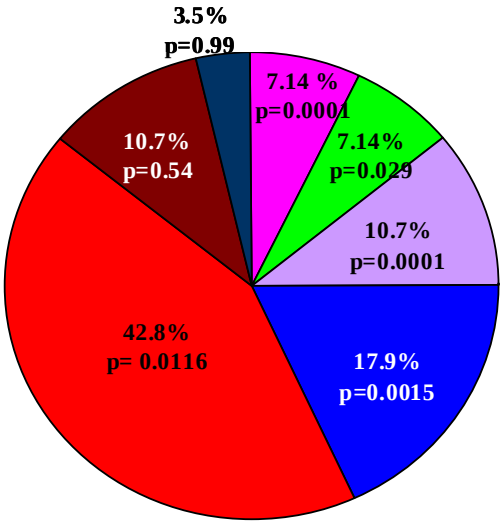
5.3. Stress responses common to both DNA damage and infection.

Regardless of whether the stressor used in this study was the initiation of DNA damage or immune challenge, there were certain proteomic responses that were common to both. Chief among these were the increase in proteins involved in metabolism, development, protein degradation and mRNA processing cytoskeletal elements. Both insect models displayed an increase in the expression of proteins involved in oxidative metabolism.

The percentage of metabolic proteins up-regulated in *E. intermedius* following infection is relatively low (3%) compared to that seen in *D. melanogaster* following camptothecin treatment (42%). The response to immune challenge would very likely require an increase in the energy requirements of the cell. This is different to the metabolic changes following DNA damage which are due to direct alteration in the metabolic pathways by molecules such as Dmp53.



A Functional classification of proteins that share identity with those proteins whose expression increases following fungal infection in the hemolymph of E. intermedius adults



B: Functional classification of proteins whose expression change s following camptothecin treatment of Drosophila melanogaster adults (Hull and Ntwasa, 2010)

Protein modification
Protein degredation
Sensory proteins
Stress response
Development
Immune function
Metabolism
Cytoskeletal
mRNA processing
Membrane transport and signaling
Unidentified

Figure 5.1 Comparison between the proteomic response of *D. melanogaster* to DNA damage and *E. intermedius* to infection. The pie charts predict the percentage that each functional class makes up of the protein fragments identified in this study, and demonstrates the similarities and differences between the two stress responses. The p values show the significance of the functional enrichment of these protein classes following treatment. Red p values depict a significant decrease in expression following exposure to stress.

Yolk protein is a developmentally associated protein that is up-regulated in both stress responses. As previously stated, the expression of this protein is under hormonal control. The two main hormones JH and 20E respond to and influence the stress response. Following stress JH leads to a decrease in the levels of apoptosis as well as a decrease in stress response and immunity, while 20E increase apoptosis and the intensity of the stress and immune responses (Flatt et al., 2005) (Gruntenko and Rauschenbach, 2008).

It has been noted in previous studies that immune challenge leads to the release of stress hormones. In insects the neurohormone octopamine is released following stress. Octopamine is chemically similar to norepinephrine and induces the liberation of energy from the fat body (Adamo, 2010). We have seen evidence of hormone changes following both the immune and DNA damage response. These changes may then provide the means for the increase in metabolic pathways as the lipid content of the hemolymph increases providing raw materials for increased energy production. Further evidence supporting this hypothesis is given by the increase in the expression of a protein resembling Apolipophorin III. This protein is involved in both immune surveillance and lipid transport (Adamo, 2010).

In both the DNA damage and immune stress responses components of the 20S proteasome were identified as being up-regulated. This may be due to un-folded proteins or increased protein turnover during the stress response. Damage to proteins is an expected consequence of camptothecin exposure and the activation of the pro-phenoloxidase system, due to ROS production. However, this also implies that the

levels of the proteasome are increased in order to regulate stress responses by increasing protein turnover.

Not surprisingly both camptothecin and pathogen initiated stress responses induced changes in the expression patterns of cytoskeletal proteins. A component of the immune response includes phagocytosis, which would involve the remodelling of the cytoskeleton (Greenberg and Grinstein, 2002) while apoptosis is associated with changes in the cell shape and membrane blebbing, which would involve cytoskeletal changes. This has implications for the use of cytoskeletal genes as reference genes.

Equally as important as the use of stress response genes to deal with stress is the use of avoidance behaviour. In order to escape the negative effects of a stressful situation the insect must avoid the stress, in order for this to occur the insect must be able to detect and recognise a potential toxin or pathogen. Odorant binding proteins and other chemosensory proteins were found to be up-regulated following immune challenge or DNA damage. The expression of these molecules may increase following the initial detection of foreign toxins such as camptothecin or pathogens. This may serve the function of detecting pathogens to allow the beetle to avoid exposure to pathogens.

5.4 Future Studies

The role played by the glycolytic flux needs to be confirmed in order to establish if this is a viable target for anticancer therapies. Additionally the means whereby methyl pyruvate is able to alleviate the effects of camptothecin treatment also needs to be

established. Currently it is not known how camptothecin treatment of adults results in increased apoptosis and decreased survivability in embryos.

Snama protein expression patterns following camptothecin treatment still need to be established. While there is evidence of a relationship between Snama and Dmp53, the exact nature of this relationship is still not known.

The antimicrobial peptides detected in this study still need to be isolated and further characterized. The establishment of an *E. intermedius* cell line during the course of this study (SAEIE cells) would allow for easier infections and protein isolation.

The full length sequence of the serine protease represented by spot 5310 still needs to be obtained in order to fully characterise the gene and therefore establish what role the protein may play in immune defence.

Chapter 6

Appendix

6.1 Vectors

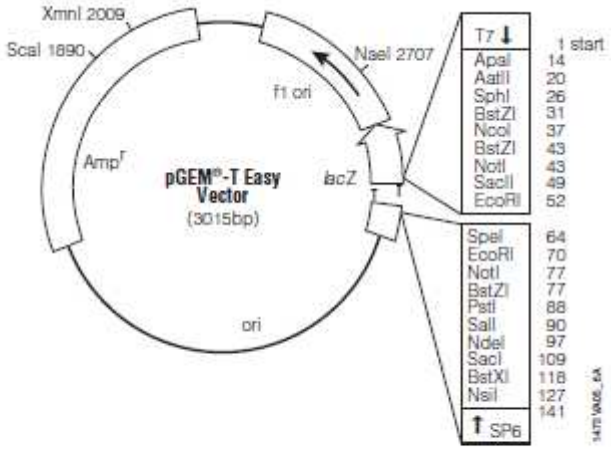
6.2 *E. intermedius* gene sequences

6.3 *Drosophila melanogaster* gene sequences

6.4 Markers

Chapter 6: Appendix

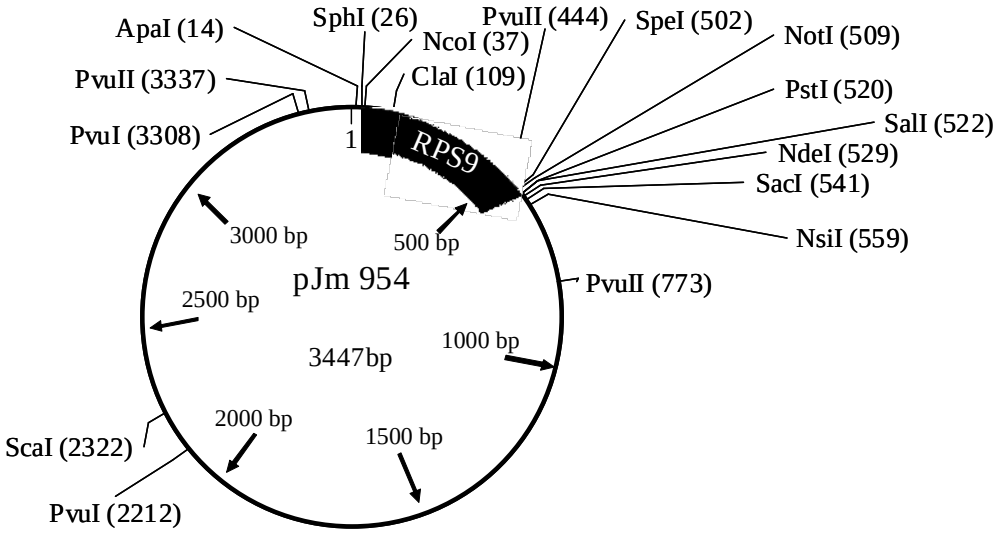
6.1 Vectors



pGEM®-T Easy Vector sequence reference points:

T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–128
SP6 RNA polymerase promoter (–17 to +3)	139–158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176–197
lacZ start codon	180
lac operator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
lac operon sequences	2836–2996, 166–395
pUC/M13 Forward Sequencing Primer binding site	2949–2972
T7 RNA polymerase promoter (–17 to +3)	2999–3

Figure 6.1 pGEM-T-Easy Cloning vector (Promega cat# A1360)




```
ATGGATACTGCCCAAGAAGCTAGCCCAACCAGCTTCAAGATGACCATCCGCCCAGCATACAGGCCCAAG
  M  D  T  A  Q  E  A  S  P  T  S  F  K  M  T  I  R  P  A  Y  R  P  K
ATCGTGAAGAAGCGCACCAAGCACTTCATCCGCCACCAGTCGGATCGATATGCTAAGCTGTCGGTGTCC
  I  V  K  K  R  T  K  H  F  I  R  H  Q  S  D  R  Y  A  K  L  S
TTCCGTGAGTGCCAACGAGGATTGTGCCAAATTGTACCCGTGTTTAATCAACATGTCTCCTTGCAGCAC
                          INTRON                                      H
AAATGGCGCAAGCCCAAGGGTATCGACAACAGAGTGCGTCGCCGCTTCAAGGGACAGTATCTGATGCCC
  K  W  R  K  P  K  G  I  D  N  R  V  R  R  R  F  K  G  Q  Y  L  M  P
AACATCGGTTACGGATCGAACAAGCGCACCCGCCACATGCTGCCCACCGGATTCAAGAAGTTCCTGGTG
  N  I  G  Y  G  S  N  K  R  T  R  H  M  L  P  T  G  F  K  K  F  L  V
CACAACGTGCGCGAGCTGGAGGTCCTGCTCATGCAGAACCCGCGTTTACTGCGCGAGATCGCCACGGCG
  H  N  V  R  E  L  E  V  L  L  M  Q  N  P  R  L  L  R  E  M  P  T  A
TCTCCTCCAAGAAGCAAGGAGATTGTCGAGCGCGCCAAGCAGCTGTCGGTCCGCTCACCAACCCCAACG
  S  P  P  R  S  K  E  I  V  E  R  A  K  Q  L  S  V  R  S  P  T  P  T
GTCGCCTGCGTCTCAAGAAGAACGAG
  V  A  C  V  S  R  R  T  R
```

Figure 6.2 (A) The pJM 954 vector containing the *rp49* gene was a gift from Professor Jean-Marc Reichhart of the Institut de Biologie Moléculaire et Cellulaire, Strasbourg, France. (B) The sequence of *rp49* highlighted in red was present in the vector with the first 57 bp being absent (marked in green). Also included is an intron.

6.2 *E. intermedius* gene sequences

Actin (Flylab Genomebase ID Cl3contig1)

```
  1     CGTGTCTTAG CCGGTGACTG CGAGTCCCCT TTCGTTGCCG CCTCTTTTCC GAACTTAGAA
 61     CGTACACACC ACAAACCCAG TCAAAATGTG TGACGATGAT GTTGCCGCGT TGGTCGTTGA
121     CAATGGATCC GGTATGTGCA AGGCCGGTTT CGCCGGAGAC GACGCGCCCC GCGCCGTCTT
181     CCCGTCGATC GTGGGTCGCC CCCGTCATCA GGGCGTAATG GTCGGTATGG GTCAGAAAGA
241     CTCGTACGTA GGCGACGAGG CCCAAAGCAA GAGAGGTATC CTCACCTTGA AATACCCAAT
301     TGAACATGGT ATCATCACCA ACTGGGACGA TATGGAAAAG ATCTGGCATC ACACCTTCTA
361     CAACGAACTT CGTGTTGCCC CAGAAGAACA CCCGATCCTC CTTACCGAAG CCCCGTTGAA
421     CCCGAAAGCC AACAGGGAAA AGATGACCCA AATCATGTTT GAAACCTTCA ACGCCCCGGC
481     CATGTACGTC GCCATCCAAG CCGTACTCTC CTTGTACGCT TCCGGTCGTA CCACCGGTAT
541     CGTCTTGGAC TCCGGAGATG GTGTATCCCA CACCGTACCC ATCTACGAAG GTTACGCTCT
601     TCCCCATGCC ATCCTTCGTC TTGACTTGGC CGGTCGCGAT TTGACCGACT ACTTGATGAA
661     GATCCTCACC GAAAGAGGTT ACTCATTCAC CACCACGGCT GAACGTGAAA TCGTTAGGGA
721     CATCAAGGAA AAACTTTGCT ATGTCGCCCT GGACTTCGAA CAGGAAATGG CCACCGCCGC
781     CGCCTCCACC TCATTGGAAA AGAGCTACGA ATTGCCTGAT GGTCAAGTCA TCACCATCGG
841     TAACGAGAGA TTCCGTTGCC CAGAGGCCCT CTTCCAACCT TCCTTCTTGG GTATGGAATC
901     TTGCGGTATC CATGAAACCG TATACAACTC CATCATGAAG TGCGACGTTG ATATCCGTAA
961     GGACCTCTAC GCCAACAACG TACTCTCCGG TGGTACCACC ATGTACCCCG GTATTGCTGA
1021    CCGTATGCAA AAGGAAATTA CCGCTTTGGC CCCATCGACC ATCAAGATCA AGATCATCGC
1081    TCCCCCAGAA AGGAAATACT CCGTATGGAT CGGTGGATCC ATCTTGGCTT CCTTGTCCAC
1141    CTTCCAACAG ATGTGGATCT CCAAACAAGA ATACGACGAA TCCGGCCCAG GCATTGTCCA
1201    CCGCAAGTGC TTCTAA
```

RPS9 (Flylab Genomebase ID CL160 contig1)

```
1       ATGGTAAACG GACGGATACC GTCGGTCTGC TCGAAGACCT ACGTCACCCC ACGTAGGCCG
61      TATGAAAAGG CACGTTTAGA TCAAGAATTG AAAATCATCG GAGAGTATGG TCTCCGTAAC
121     AAACGTGAAG TGTGGAGGGT CAAGTACACG CTCGCTAAAA TTCGTAAAGC TGCTCGTGAG
181     TTATTGACGC TTGAAGAAAA GGACCCTAAA CGTCTATTTG AAGGTAATGC CCTTTTACGT
241     CGTTTGGTGC GTATCGGCGT CTTGGACGAA GGCAGGATGA AGCTCGATTA CGTGTTGGGT
301     TTGAAAATCG AGGACTTTTT AGAGAGACGC TTACAAACGC AAGTTTTCAA ATTGGGATTG
361     GCCAAGTCGA TTCATCACGC GCGCGTTTTG ATCCGTCAGC GACACATTCG GGXX
```

Serpin (Flylab Genomebase ID CL111 contig1)

```
1       CCGGCATTAA ATTCCGGTTT AGGCCGGNTA AAATGTGCGG TGCGCATCAT TACAACGAAA
61      CCGGTGGCCG CGGCAGCTTC GGCGCCTTCC TCGTTTACTT CGATAAATGC TTTTTGTATA
121     GCTTTCGATA CCGTTAGTTG TTCTTTTCCT TTAATTAAAT TGGGAAAATC AGCGGAATCG
181     CTGAATATTA CGCCCATGCC CAACTTAACG AGAGACTCTT TCAAGTCGAT AGTGGTTTCA
241     ATCTTGAATT TAGGCAGAGA AACGATAACT TCACGATTTA CAAGCGAATT GGTTAAAGTG
301     CTTAAATCTC TATTAGCCAG TTTTTGTTCA AGTGCGTAAA TACCAGTTTT GGTGTTTGGC
361     AAAACGATTA CCATACTGAC GTCCTTATTT TTGTATGGCA TTCTTAAAAT CTTAGCATCC
421     AAATCGGCAT CTTCGGCATA TCCAAAGGTT TCGGTAATGT GCA
```

Serine protease (Flylab Genomebase ID cl15contig1)

```
1       ACGGGTATTT TGATTGATCC AATCGATGAA GGAGGAAACC CTAGTGAAAA CGTCTGGGTA
61      TCCGTTGGCG CATGGGTTAC CCCACGATAC AATACCAATT TGTTTTCCGC CACTGGCCAA
121     TGGACCGCCA GAGTCACCAT GGCAGACACC TTCACCGGCG GTGGTCAAAG TGCAAATGTT
181     GCTGTCGAAA ACGGTGTTGC TGCCCCATTG GGATTGGCAC TGGGAGTTGG TGATGGTTTT
241     CAACGCGAGG TATTGCAATC TGTCGGGAAT GTTGCCCGGG TAGCTGGTCC TTCCCCAACC
301     GGAGATGACG CAATCACCGG CTCCGATGGA CGCGCTTTCC GGTTCGATCG GTTGTACGTT
361     CTCGTTGAAA GAGATCGGAC TGGACAATTG AAGAAGGGCA ATGTCGTTTT CAACGGTGTT
421     GGGGTTGTAA CCGCTGTGGG TGATGATCCT GCTGACTCTG TAAGTGTCTC CGCCGCTGGA
481     CAAACTTACG GATCCGGTGA CGACGCTGTA CGAGCTAGCG ACGCCGCCGT ACACGCAATG
541     GGAAGCGGTC AAAATCCAGT TTTCGTTGAG GATGGATCCG CCGCAAGTGT GACTTCCGGA
601     GGAACCTCTC AAAGAGATTT GATAGGGGAA TTGACCCAAG GAGGCATCGG CACCGCCGAC
661     GATACGCCAG TTGGCGGTGG GGTCGATTTT CGGCTCGAAA GCAGCGCCGA AAGCGACTGC
721     GAGAACAGCT GA
```

6.3 *Drosophila melanogaster* sequences

Snama

```
GCATTTCACATCTCTGGGGCTTTGGCGTCACGTTCGCATCTCTAGTAAATTGGAAAAAAA
TAAAATCGTCGGAGTTTTTATGTGCTTGCAGAGTAGTATTTCTTTCATATGCAACTATGT
                                                  M   S
CGGTACACTATAAATTTAAGAGTACACTCAACTTTGATACAATTACTTTTGATGGACTTC
  V   H   Y   K   F   K   S   T   L   N   F   D   T   I   T   F   D   G   L   H
ACATTTCTGTCGGGGACTTAAAAAGGGAGATTGTGCAGCAGAAGCGACTGGGCAAAATCA
  I   S   V   G   D   L   K   R   E   I   V   Q   Q   K   R   L   G   K   I   I
TCGACTTTGATCTCCAAATAACAAATGCGCAGAGTAAAGAAGAATACAAGGACGATGGGT
  D   F   D   L   Q   I   T   N   A   Q   S   K   E   E   Y   K   D   D   G   F
TCCTTATTCCCAAAAACACAACGCTGATCATATCGCGCATCCCCATCGCCCATCCCACAA
  L   I   P   K   N   T   T   L   I   I   S   R   I   P   I   A   H   P   T   K
AAAAGGGCTGGGAGCCACCAGCAGCAGAAAATGCCTTTTCGGCGGCGCCTGCCAAGCAGG
  K   G   W   E   P   P   A   A   E   N   A   F   S   A   A   P   A   K   Q   D
CAACTTCAACATGGACCTGTCCAAAATGCAAGGCACGGAGGAGGACAAAATCCAGGCCA
  N   F   N   M   D   L   S   K   M   Q   G   T   E   E   D   K   I   Q   A   M
TGATGATGCAGAGCACAGTCGACTATGATCCTAAGACGTACCATCGTATTAAAGGACAAT
  M   M   Q   S   T   V   D   Y   D   P   K   T   Y   H   R   I   K   G   Q   S
CGCAAGTGGGAGAAGTTCCCGCATCCTACCGATGCAACAAATGCAAGAAAAGCGGACACT
  Q   V   G   E   V   P   A   S   Y   R   C   N   K   C   K   K   S   G   H   W
GGATCAAAAACTGTCCCTTTGTGGGGGGAAAGGACCAGCAAGAGGTCAAACGGAATACTG
  I   K   N   C   P   F   V   G   G   K   D   Q   Q   E   V   K   R   N   T   G
GTATTCCGCGGTCTTTCCGCGACAAGCCAGATGCGGCTGAGAACGAATCAGCCGATTTTG
  I   P   R   S   F   R   D   K   P   D   A   A   E   N   E   S   A   D   F   V
TGCTGCCTGCTGTACAAAACCAAGAGATACCGGAGGATCTGATATGCGGCATATGCCGAG
  L   P   A   V   Q   N   Q   E   I   P   E   D   L   I   C   G   I   C   R   D
ATATATTCGTCGATGCTGTCATGATACCCTGCTGCGGAAGTTCCTTTTGTGACGACTGTG
  I   F   V   D   A   V   M   I   P   C   C   G   S   S   F   C   D   D   C   V
TGCGAACCTCCTTATTGGAGTCAGAGGATAGTGAGTGCCCCGATTGCAAGGAGAAGAACT
  R   T   S   L   L   E   S   E   D   S   E   C   P   D   C   K   E   K   N   C
GTTCGCCTGGCTCCCTGATACCTAATCGGTTCTTGAGGAATTCGGTGAACGCCTTTAAAA
  S   P   G   S   L   I   P   N   R   F   L   R   N   S   V   N   A   F   K   N
ATGAGACTGGGTATAACAAAAGCGCGGCTAAGCCAGCTGCAGTAAAAAATGAGGAAAAAC
  E   T   G   Y   N   K   S   A   A   K   P   A   A   V   K   N   E   E   K   P
CTCCTGTTGAAAAAGAAGTGGAGAAAAAGCCAGTCGCGGAGGTGGAACCCGAAGAGACTG
  P   V   E   K   E   V   E   K   K   P   V   A   E   V   E   P   E   E   T   E
AGGTGAAACCTGAAAAGCAAAAAGAATCCGAAACCAATGGCAGTAATCCGCCAAAATCGG
  V   K   P   E   K   Q   K   E   S   E   T   N   G   S   N   P   P   K   S   E
AATCTCCAGAGCCTCCCGCAACCACAGAACCATCACAGAAGGAGAAAGATAAATATGATT
  S   P   E   P   P   A   T   T   E   P   S   Q   K   E   K   D   K   Y   D   S
CAGACTACGAGGATAACATTACCATAAAAATGCCCCAGCCTGCAGCTGATTCTACAACAG
  D   Y   E   D   N   I   T   I   K   M   P   Q   P   A   A   D   S   T   T   V
TGCCCAGCAAAAGATCCCCCAGTTATTCCCACAGAAGTGAATCCTCTCATCGACGGGACA
  P   S   K   R   S   P   S   Y   S   H   R   S   E   S   S   H   R   R   D   R
GGTCGGATTATGTTTCCGATCACGATCACAAGCACCAACGTCCATCAAAATCGGAGTCTG
  S   D   Y   V   S   D   H   D   H   K   H   Q   R   P   S   K   S   E   S   V
TTAACAAGGATCGCAGTCTCCTGCCCTTGCCCATTGGCACCCTGCCTAGCTACCAGGGCC
  N   K   D   R   S   L   L   P   L   P   I   G   T   L   P   S   Y   Q   G   H
ACATGATGGCCGAATCAGAAGAAGCTCGTCGATCGAGTGCCTATAAGCCCCCTTATATGC
  M   M   A   E   S   E   E   A   R   R   S   S   A   Y   K   P   P   Y   M   Q
AAATGCAGCGGGGCCCACCTCCTATGCACATGATGAGTCACCACATGCCAGCCTACAACA
  M   Q   R   G   P   P   P   M   H   M   M   S   H   H   M   P   A   Y   N   N
ACGGGTTTAACAACATGGGACAGAGGCCTCCCCTCAGCTATGTGCCGTATCAAAACCAAT
  G   F   N   N   M   G   Q   R   P   P   L   S   Y   V   P   Y   Q   N   Q   S
CCGTACACCCAATGCGTGCGCCGTACGGATCTGCAGGCGGAGGTATGAATATGAATATGT
  V   H   P   M   R   A   P   Y   G   S   A   G   G   G   M   N   M   N   M   S
```

```
CACAACCATTTCAGTCCCCAAATTTAGCCTCGATATACCAAGGGGTGGCAGCGAAGGTCG
  Q  P  F  Q  S  P  N  L  A  S  I  Y  Q  G  V  A  A  K  V  G
TTCCGGTCCCATTGACGATCCGTTGGAGGCCTTCAATCGCATCATGAAGGAGAAGGAGC
  S  G  P  I  D  D  P  L  E  A  F  N  R  I  M  K  E  K  E  R
GAAGAAGGTGGACCGCTTTCGAAGCTCTGACCGCCACAGGTCAAGGTCCCCGGATAGAC
  K  K  V  D  R  F  R  S  S  D  R  H  R  S  R  S  P  D  R  Q
AAGGCACCGCTTTAAGTCTCCCATGTACGAAAAGGACAACTCCAGGGATAATCTCAAGG
  R  H  R  F  K  S  P  M  Y  E  K  D  N  S  R  D  N  L  K  D
ACAAAAGACCGCGATCCCGGGAAAGGAAGCGAGAACATAGCTACGAACGGCATATACGCC
  K  R  P  R  S  R  E  R  K  R  E  H  S  Y  E  R  H  I  R  H
ACCCTCGTTCTAGTCGCCAGCCGAATGATGGCTCTAAGTCCCCAGGTGGCAGAATCAAAA
  P  R  S  S  R  Q  P  N  D  G  S  K  S  P  G  G  R  I  K  R
GATCTGGACATCGTCGCTCTGCATCTCCAAAGCCGGGCTACAAGAGTGATTACAGAGACA
  S  G  H  R  R  S  A  S  P  K  P  G  Y  K  S  D  Y  R  D  K
AGCCGTACAACAAGCCTAGTGCTCCCAAAACGGAGGCAGTTGAGCCTCCTCCCCCCGGAT
  P  Y  N  K  P  S  A  P  K  T  E  A  V  E  P  P  P  P  G  F
TCGAGCCGTTGCAGCTGACGGATGAAGACGGCTACAGGAACAAGCACCCGACCAGTTCGG
  E  P  L  Q  L  T  D  E  D  G  Y  R  N  K  H  P  T  S  S  E
AAGCATCACAAAGCAGCAAGGGTGATAGCAGCAAGAAGAGAGGGGAAAACAGGCACGAAG
  A  S  Q  S  S  K  G  D  S  S  K  K  R  G  E  N  R  H  E  E
AGGCGCCACGAAAGAGGCACAGGTCTCGCAGCATTAGCAAGGAACCGAAGCCGAATGACA
  A  P  R  K  R  H  R  S  R  S  I  S  K  E  P  K  P  N  D  S
GCAACTACAGGAGCCTGACTCCACCAGCAAAGATCACCACACCGAAAATGACTGCTGCCC
  N  Y  R  S  L  T  P  P  A  K  I  T  T  P  K  M  T  A  A  Q
AGTTGAGGCAACGCGAAAGTTCACCGAAGACGCCGGAAAAGAGTCACGACGATTATCTGA
  L  R  Q  R  E  S  S  P  K  T  P  E  K  S  H  D  D  Y  L  T
CCGCGAAGGCCAGAATTATGGCCTCCCAGCCCGTCATCAACGACACGGAAATGGAGACCA
  A  K  A  R  I  M  A  S  Q  P  V  I  N  D  T  E  M  E  T  N
ATGTGGGCAAGGAGAACAAGGCCAAGAGTCCGTTGTCAAAAGATCGCAAGAAGAAGAAGA
  V  G  K  E  N  K  A  K  S  P  L  S  K  D  R  K  K  K  K  K
AGGACAAGGACAAGGCTGAGCGCAAGAAAAACAAGAAGGACAAGCGCGCTAAGAAGGAGA
  D  K  D  K  A  E  R  K  K  N  K  K  D  K  R  A  K  K  E  K
AAGGGGATCGCCAGAAGAAGAGCTCCTCAGTTAATCGATCTGACTCGGATATTAACAACA
  G  D  R  Q  K  K  S  S  S  V  N  R  S  D  S  D  I  N  N  S
GCTCACTAATGAACGAGTCAAATTATAAAGTATTGTCTCCCAGGGCTCAAAGTCCCAGCA
  S  L  M  N  E  S  N  Y  K  V  L  S  P  R  A  Q  S  P  S  I
TTGAGATCAATGCGGCTCAACTTTCCCCTACTCACAACGCTACTGAAAACGTTAATCCGA
  E  I  N  A  A  Q  L  S  P  T  H  N  A  T  E  N  V  N  P  K
AGAGTCATTCCATCCTTACTGTGGGTGCTGCTAGCGACGATAATCTTGGCCCAAGAAGCA
  S  H  S  I  L  T  V  G  A  A  S  D  D  N  L  G  P  R  S  K
AACTCAGCGAGGCTAATTCTGTCAATCTATCCAAATGGGAAATCGACGAGAATATCTTAG
  L  S  E  A  N  S  V  N  L  S  K  W  E  I  D  E  N  I  L  G
GTTTGGAGGATTCCTCCAAAAAAGCTGCCGGGGCCTCCGACGATCCGTCGGAAATAACTT
  L  E  D  S  S  K  K  A  A  G  A  S  D  D  P  S  E  I  T  S
CAGACGTCCTGCGAAAGGCTGAGAACGCAATATTTGCAAAGGCTATTAATGCCATCAGGC
  D  V  L  R  K  A  E  N  A  I  F  A  K  A  I  N  A  I  R  P
CTATGGAGTTTCAAGTTATTATCAATTCCAAGGACAACAGCAAGGACCGCTCCGTAGTTC
  M  E  F  Q  V  I  I  N  S  K  D  N  S  K  D  R  S  V  V  R
GAAGTGACAAGGATCGCTCCTCCTCACCCAGGCGTAACAACAGCAGCAGGTCGGTAAAGG
  S  D  K  D  R  S  S  S  P  R  R  N  N  S  S  R  S  V  K  D
ATAGGCTGGGCACCAAGATTTCCAATGATAGAAGCCGTTCGCGAGACAAGTCGAAGGGCA
  R  L  G  T  K  I  S  N  D  R  S  R  S  R  D  K  S  K  G  R
GGCGCCGGGCCGCAAGGAGCTCCGACGACGATGCGAACCGCGGCAGGTCGGATCGTCATG
  R  R  A  A  R  S  S  D  D  D  A  N  R  G  R  S  D  R  H  G
GCAGCCGGAAGAGGGACAACAGATCCCGCGACAGGGCGGCGCCTTCAGAGAAGAGGCAGG
  S  R  K  R  D  N  R  S  R  D  R  A  A  P  S  E  K  R  Q  E
AGCGTTCGTACAAGCGAAGCTCGCCGGAGGACGACAAGCTGAGGCGCCAGAACAAGGAAC
  R  S  Y  K  R  S  S  P  E  D  D  K  L  R  R  Q  N  K  E  Q
AGTCCGAATCCAAGCACGGAAAGCATGATCAAAACAATAGCGACGACTCGGATCGGAGGG
  S  E  S  K  H  G  K  H  D  Q  N  N  S  D  D  S  D  R  R  A
CGGCCAAAAACACCAAGTCCAGCGACAGCCGAGTGGTCTCCTCTGTAACAGCCGTGGTTG
```

```
  A  K  N  T  K  S  S  D  S  R  V  V  S  S  V  T  A  V  V  A
CTCCTCCCAAACCCTGTCGTCCAGACAACCCGTTCCGCAAGTTCGTCGACACCAGTTCGT
  P  P  K  P  C  R  P  D  N  P  F  R  K  F  V  D  T  S  S  S
CGAGCAGCTTAGTTGTAAAATATGATAACACGATACAGAAGGAGGGCGCGTCCTCGGACA
  S  S  L  V  V  K  Y  D  N  T  I  Q  K  E  G  A  S  S  D  N
ACGGCATGGAGCACAGGAAGCAGAGGGATAAGAAGCTGAAGAAACATTCAAAATATTCGT
  G  M  E  H  R  K  Q  R  D  K  K  L  K  K  H  S  K  Y  S  S
CAACCGATTCGTTGAAGAGCGAGAAGCGCAAGGATCCGAAGAGCAAAAAGAAGAGCAAGA
  T  D  S  L  K  S  E  K  R  K  D  P  K  S  K  K  K  S  K  I
TTTTGAAGAAGAAGAAAAAATCAAAGAA
  L  K  K  K  K  K  S  K  K
```

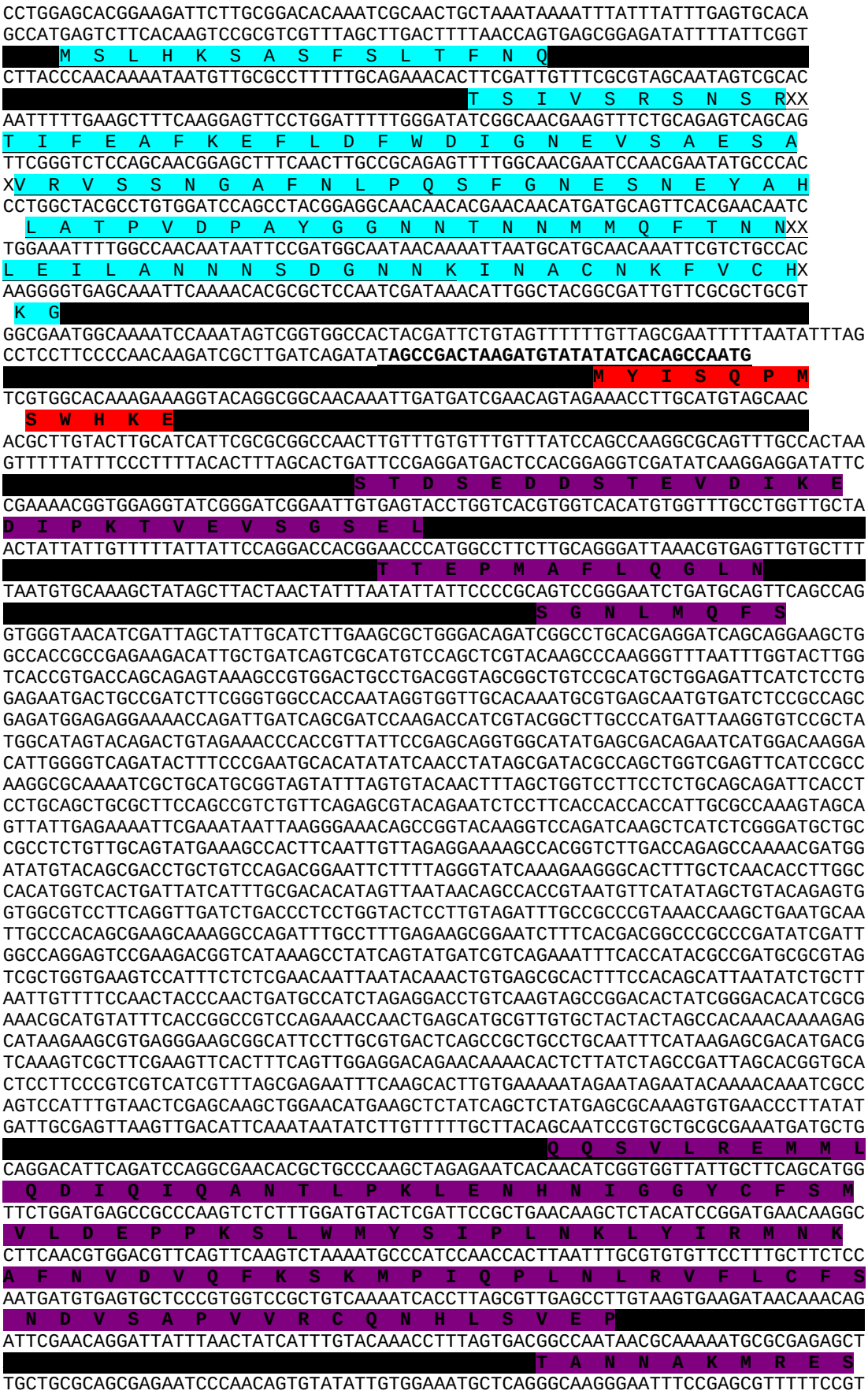
cDNA and protein sequence for Snama (FBtr0072249). The DWNN domain is in red while the DCM is underlined

Reaper (FBtr0075120)

```
TGAATAAGAGAGACACCAGAACAAAGTGAACGAACTCGAAAATACGAAAGAAAAGTGTGTGCGCCAGCA
ACAAAGAACTAACTCGATAAATATTCATTGTGCAGAAGAGAAAGTTATTGAGTCACTACCAGTTGTGTA
ATTCCGAACGAGAAGAAAGATAAACCAACAATGGCAGTGGCATTCTACATACCCGATCAGGCGACTCTG
                                M  A  V  A  F  Y  I  P  D  Q  A  T  L
TTGCGGGAGGCGGAGCAGAAGGAGCAGCAGATCCTTCGCTTGCGGGAGTCACAGTGGAGATTCCTGGC
 L  R  E  A  E  Q  K  E  Q  Q  I  L  R  L  R  E  S  Q  W  R  F  L  A
CACCGTCGTCCTGGAAACCCTGCGCCAGTACACTTCATGTCATCCGAAGACCGGAAGAAAGTCCGGCAA
  T  V  V  L  E  T  L  R  Q  Y  T  S  C  H  P  K  T  G  R  K  S  G  K
ATATCGCAAGCCATCGCAATGAGGATTCGAGTAACTAACAAATACGGGGAAAACCAATAGTCCAGTCCA
  Y  R  K  P  S  Q
AAATCCAGAGTACAAAGGAAATAAGCATGAGCCAACCCAAAACCCAAACACAGTCACCACTCATCAGCC
GACGGCACTCGATTTCTACTGCAGTCAAGGACACAGAGCCACAACACCCACCCAATTTTAGTTTACTCA
TCAAAGCGATTGTGATAATGGTTTTGTTTCTACAAAAAAGCGGAGGAAAAATTTGAAAAAATAACGTTT
TTATAAAGTCCCCAATTTTTTACAAAAATGTTTCAATGATATAAATCAACTTTTTTAGAAATAATTTAC
TCTTAAAGCCTATTTAAATGAATTACTACTGTAATAGTTTGTAAGTTCTTTTGTAAGACGAGTTTTTCT
AAGTTTTTTTTAAGAAGAAACCCCAGAAAAAAACGAAGATGAGTCGAAGTTGGCTAAAAATTGCATCAA
TTTTTTGTCAAACAAAAAGTCAAT
```

The position of the forward and reverse primers is underlined.

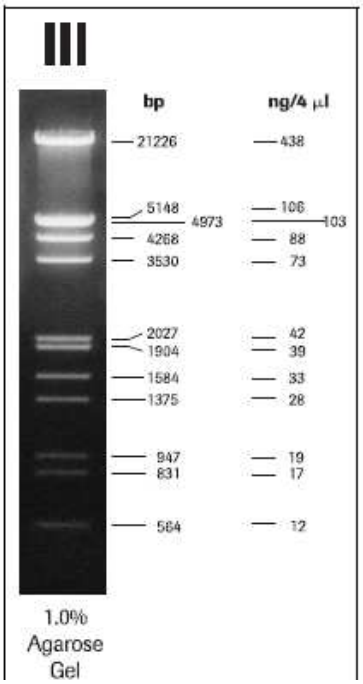
Dmp53


CCTGGAGCACGGAAGATTCTTGCGGACACAAATCGCAACTGCTAAATAAAATTTATTTATTTGAGTGCACA
GCCATGAGTCTTCACAAGTCCGCGTCGTTTAGCTTGACTTTTAACCAGTGAGCGGAGATATTTTATTCGGT
M S L H K S A S F S L T F N Q
CTTACCCAACAAAATAATGTTGCGCCTTTTTGCAGAAACACTTCGATTGTTTCGCGTAGCAATAGTCGCAC
T S I V S R S N S RXX
AATTTTTGAAGCTTTCAAGGAGTTCCTGGATTTTTGGGATATCGGCAACGAAGTTTCTGCAGAGTCAGCAG
T I F E A F K E F L D F W D I G N E V S A E S A
TTCGGGTCTCCAGCAACGGAGCTTTCAACTTGCCGCAGAGTTTTGGCAACGAATCCAACGAATATGCCCAC
XV R V S S N G A F N L P Q S F G N E S N E Y A H
CCTGGCTACGCCTGTGGATCCAGCCTACGGAGGCAACAACACGAACAACATGATGCAGTTCACGAACAATC
L A T P V D P A Y G G N N T N N M M Q F T N NXX
TGGAAATTTTGGCCAACAATAATTCCGATGGCAATAACAAAATTAATGCATGCAACAAATTCGTCTGCCAC
L E I L A N N N S D G N N K I N A C N K F V C HX
AAGGGGTGAGCAAATTCAAAACACGCGCTCCAATCGATAAACATTGGCTACGGCGATTGTTCGCGCTGCGT
K G
GGCGAATGGCAAAATCCAAATAGTCGGTGGCCACTACGATTCTGTAGTTTTTTGTTAGCGAATTTTTAATATTTAG
CCTCCTTCCCCAACAAGATCGCTTGATCAGATAT**AGCCGACTAAGATGTATATATCACAGCCAATG**
M Y I S Q P M
TCGTGGCACAAAGAAAGGTACAGGCGGCAACAAATTGATGATCGAACAGTAGAAACCTTGCATGTAGCAAC
S W H K E
ACGCTTGTACTTGCATCATTCGCGCGGCCAACTTGTTTGTGTTTGTTTATCCAGCCAAGGCGCAGTTTGCCACTAA
GTTTTTATTTCCCTTTTACACTTTAGCACTGATTCCGAGGATGACTCCACGGAGGTCGATATCAAGGAGGATATTC
S T D S E D D S T E V D I K E
CGAAAACGGTGGAGGTATCGGGATCGGAATTGTGAGTACCTGGTCACGTGGTCACATGTGGTTTGCCTGGTTGCTA
D I P K T V E V S G S E L
ACTATTATTGTTTTTATTATTCCAGGACCACGGAACCCATGGCCTTCTTGCAGGGATTAAACGTGAGTTGTGCTTT
T T E P M A F L Q G L N
TAATGTGCAAAGCTATAGCTTACTAACTATTTAATATTATTCCCCGCAGTCCGGGAATCTGATGCAGTTCAGCCAG
S G N L M Q F S
GTGGGTAACATCGATTAGCTATTGCATCTTGAAGCGCTGGGACAGATCGGCCTGCACGAGGATCAGCAGGAAGCTG
GCCACCGCCGAGAAGACATTGCTGATCAGTCGCATGTCCAGCTCGTACAAGCCCAAGGGTTTAATTTGGTACTTGG
TCACCGTGACCAGCAGAGTAAAGCCGTGGACTGCCTGACGGTAGCGGCTGTCCGCATGCTGGAGATTCATCTCCTG
GAGAATGACTGCCGATCTTCGGGTGGCCACCAATAGGTGGTTGCACAAATGCGTGAGCAATGTGATCTCCGCCAGC
GAGATGGAGAGGAAAACCAGATTGATCAGCGATCCAAGACCATCGTACGGCTTGCCCATGATTAAGGTGTCCGCTA
TGGCATAGTACAGACTGTAGAAACCCACCGTTATTCCGAGCAGGTGGCATATGAGCGACAGAATCATGGACAAGGA
CATTGGGGTCAGATACTTTCCCGAATGCACATATATCAACCTATAGCGATACGCCAGCTGGTCGAGTTCATCCGCC
AAGGCGCAAAATCGCTGCATGCGGTAGTATTTAGTGTACAACTTTAGCTGGTCCTTCCTCTGCAGCAGATTCACCT
CCTGCAGCTGCGCTTCCAGCCGTCTGTTCAGAGCGTACAGAATCTCCTTCACCACCACCATTGCGCCAAAGTAGCA
GTTATTGAGAAAATTCGAAATAATTAAGGGAAACAGCCGGTACAAGGTCCAGATCAAGCTCATCTCGGGATGCTGC
CGCCTCTGTTGCAGTATGAAAGCCACTTCAATTGTTAGAGGAAAAGCCACGGTCTTGACCAGAGCCAAAACGATGG
ATATGTACAGCGACCTGCTGTCCAGACGGAATTCTTTTAGGGTATCAAAGAAGGGCACTTTGCTCAACACCTTGGC
CACATGGTCACTGATTATCATTTGCGACACATAGTTAATAACAGCCACCGTAATGTTCATATAGCTGTACAGAGTG
GTGGCGTCCTTCAGGTTGATCTGACCCTCCTGGTACTCCTTGTAGATTTGCCGCCCGTAAACCAAGCTGAATGCAA
TTGCCCACAGCGAAGCAAAGGCCAGATTTGCCTTTGAGAAGCGGAATCTTTCACGACGGCCCGCCCGATATCGATT
GGCCAGGAGTCCGAAGACGGTCATAAAGCCTATCAGTATGATCGTCAGAAATTTCACCATACGCCGATGCGCGTAG
TCGCTGGTGAAGTCCATTTCTCTCGAACAATTAATACAAACTGTGAGCGCACTTTCCACAGCATTAATATCTGCTT
AATTGTTTTCCAACTACCCAACTGATGCCATCTAGAGGACCTGTCAAGTAGCCGGACACTATCGGGACACATCGCG
AAACGCATGTATTTCACCGGCCGTCCAGAAACCAACTGAGCATGCGTTGTGCTACTACTAGCCACAAACAAAAGAG
CATAAGAAGCGTGAGGGAAGCGGCATTCCTTGCGTGACTCAGCCGCTGCCTGCAATTTCATAAGAGCGACATGACG
TCAAAGTCGCTTCGAAGTTCACTTTCAGTTGGAGGACAGAACAAAACACTCTTATCTAGCCGATTAGCACGGTGCA
CTCCTTCCCGTCGTCATCGTTTAGCGAGAATTTCAAGCACTTGTGAAAAATAGAATAGAATACAAAACAAATCGCC
AGTCCATTTGTAACTCGAGCAAGCTGGAACATGAAGCTCTATCAGCTCTATGAGCGCAAAGTGTGAACCCTTATAT
GATTGCGAGTTAAGTTGACATTCAAATAATATCTTGTTTTTGCTTACAGCAATCCGTGCTGCGCGAAATGATGCTG
Q Q S V L R E M M L
CAGGACATTCAGATCCAGGCGAACACGCTGCCCAAGCTAGAGAATCACAACATCGGTGGTTATTGCTTCAGCATGG
Q D I Q I Q A N T L P K L E N H N I G G Y C F S M
TTCTGGATGAGCCGCCCAAGTCTCTTTGGATGTACTCGATTCCGCTGAACAAGCTCTACATCCGGATGAACAAGGC
V L D E P P K S L W M Y S I P L N K L Y I R M N K
CTTCAACGTGGACGTTCAGTTCAAGTCTAAAATGCCCATCCAACCACTTAATTTGCGTGTGTTCCTTTGCTTCTCC
A F N V D V Q F K S K M P I Q P L N L R V F L C F S
AATGATGTGAGTGCTCCCGTGGTCCGCTGTCAAAATCACCTTAGCGTTGAGCCTTGTAAGTGAAGATAACAAACAG
N D V S A P V V R C Q N H L S V E P
ATTCGAACAGGATTATTTAACTATCATTTGTACAAACCTTTAGTGACGGCCAATAACGCAAAAATGCGCGAGAGCT
T A N N A K M R E S
TGCTGCGCAGCGAGAATCCCAACAGTGTATATTGTGGAAATGCTCAGGGCAAGGGAATTTCCGAGCGTTTTTCCGT

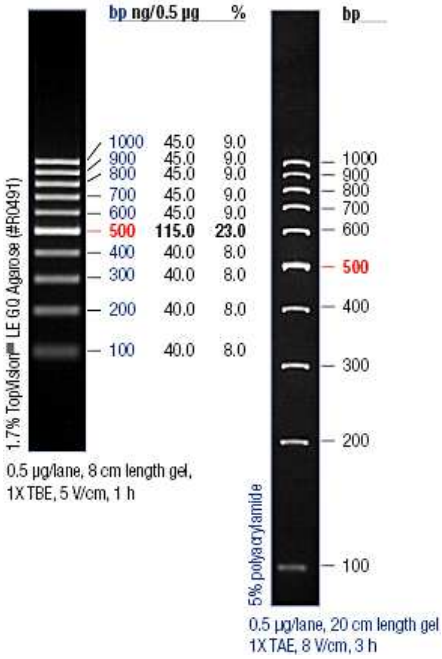
L L R S E N P N S V Y C G N A Q G K G I S E R F S
TGTAGTCCCCCTGAACATGAGCCGGTCTGTAACCCGCAGTGGGCTCACGCGCCAGACCCTGGCCTTCAAGTTCGTC
V V V P L N M S R S V T R S G L T R Q T L A F K F V
TGCCAAAACTCGTGTATCGGGCGAAAAGAAACTTCCTTAGTCTTCTGCCTGGAGAAAGCATGGTAAGGTGACACAA
C Q N S C I G R K E T S L V F C L E K A W
GAACTCTAGATGGCTAGAACAAAGCTTAACGTGTTTTCTTTCTTGCAGCGGCGATATCGTGGGACAGCATGTTATA
G D I V G Q H V I
CATGTTAAAATATGTACGTGCCCCAAGCGGGATCGCATCCAAGACGAACGCCAGCTCAATAGCAAGAAGCGCAAGT
H V K I C T C P K R D R I Q D E R Q L N S K K R K
CCGTGCCGGAAGCCGCCGAAGAAGATGAGCCGTCCAAGGTGCGTCGGTGCATTGCTATAAAGACGGAGGACACGGA
S V P E A A E E D E P S K V R R C I A I K T E D T
GAGCAATGATAGCCGAGACTGCGACGACTCCGCCGCAGAGTGGAACGTGTCGCGGACACCGGATGGCGATTACCGT
E S N D S R D C D D S A A E W N V S R T P D G D Y R
CTGGCTATTACGTGCCCCAATAAGGAATGGCTGCTGCAGAGCATCGAGGGCATGATTAAGGAGGCGGCGGCTGAAG
L A I T C P N K E W L L Q S I E G M I K E A A A E
TCCTGCGCAATCCCAACCAAGAGAATCTACGTCGCCATGCCAACAAATTGCTGAGCCTTAAGAGTAAGCAGTGA
V L R N P N Q E N L R R H A N K L L S L K
ATCGGAGGACAAAGAGATTAAGCTTTACTTACCGAACTTTCCTTTCAGAACGTGCC**TACGAGCTGCCATGA**
R A Y E L P
CTTCTGATCTGGTCGACAATCT

The gene sequence of *dmp53* showing the different splicing of the gene that gives rise to different isoforms. The longer isoform is marked in light blue. The shorter isoform is marked in red and the short N terminal 110 amino acid long isoform is underlined. The primer binding regions are in bold.

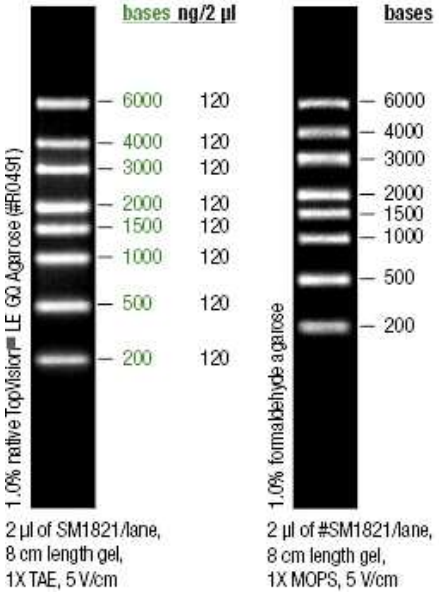
6.4 Markers



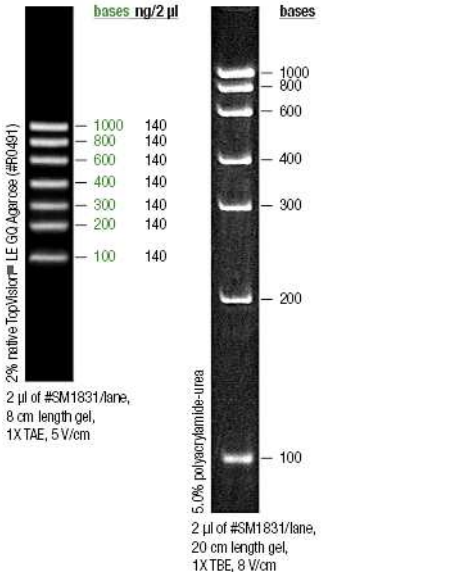
DNA marker III (Roche cat# 0 528 552 001)



Generuler 100bp (Fermentas SM0421)

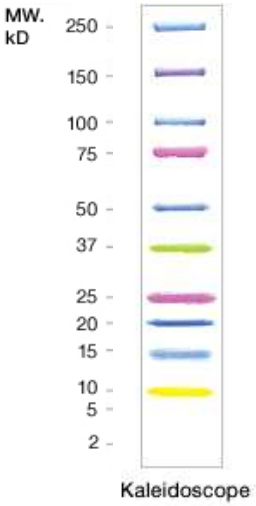


Riboruler High range (Fermentas cat# SM1821)



Riboruler Low range (Fermentas cat# SM1831)

Figure 6.3 DNA and RNA markers.

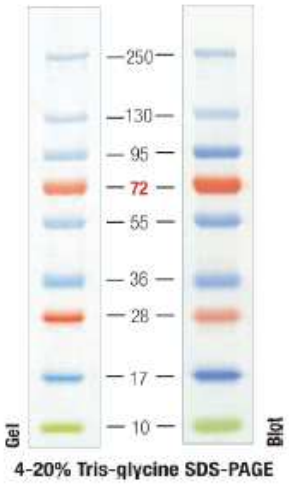


Kaleidoscope marker (Bio-Rad cat# 161-0324)



10-20% Tris-Tricine gel

Ultra-Low Range: (Sigma Cat# C6210)



Pageruler Plus prestained (Fermentas cat# SM111)

Figure 6.4 Protein markers.

Table 6.1 Identification of peptide fragments based on sequence similarity with proteins in different databases.

Spot	Peptide	Identity	Organism	NCBI Accession	E value
Protein Modification/ Protein Processing					
0101	LDALGQECLK	Similar to Pez	*Apis mellifera*	XP_392377,2	50
3004					
2203F	TSYPGNLPDR	to chymotrypsin-like	*Tribolium castaneum*	XP_967741,1	
2203F	SNAACAAAYG	Putative chymotrypsin	*Antheraea assama*	AAF71515,1	8.8
2203F	FTSPLQYTDTLQLPR	CG32019-PA	*T. castaneum*	XP_971502,1	16
2203F	PAYPGVYSSV	trypsin-like proteinase	*T. castaneum*	EAL25738,1	
2203U	VSSAGCASGA	serine type endopeptidase, putative	*Aedes aegypti*	EAT41458,1	8.7
3004	STLAEFR	ubiquitin carboxyl-terminal hydrolase isozyme L5	*Scylla serrata*	AAF08281,1	73
5310	YAPGGVASGFNQVNR	Peptidase C19	*Anopheles gambiae*	XP_311192	153
5406	NSPSEPLNVGLLYR	Dipeptidyl peptidase	*T.castaneum*	XP_972016,1	2
6203	TFLDSFLEFAN	ATP binding; protein serine/threonine kinase	*D. pseudoobscura pseudoobscura*	XP_001357809	27
7210	SQLETLAGGANK	Proteasome component	*Culex quinquefasciatus*	XP_001851301	106
6204F	AWNVQAFTK	Serine/ threonine	*Roseiflexus castenholzii*	ZP_01531942,1	47
7203U		protein kinase with FHA domain	DSM 13941		
7303	HWLETKPSNK	Endoplasmic reticulum metallopeptidase 1	*Acyrthosiphon pisum*	XP_001947171	71
Sensory proteins					
4002U	QEALDALGQECLK	GA26235	*D. pseudoobscura*	XP_002137938.1|	45
5102	SQEECFK	nanchung	*D. melanogaster*	CG5842-PA	146
7301f	VQMYYDSR	chemosensory protein	*Bombyx mori*	NP_001091781	133
8402	LLTQQAECL	cryptochrome	*Sarcophaga bullata*	ACJ08741	40
Development					
2203U	SPLQYTDTLQ	Bent	*T.castaneum*		202
3304	FSLCAGGEQK	Bent	*T. castaneum*		143
3304	YPTNALPSFDK	Chaoptin	*A. aegypti*	XP_001653633	123
6204f	ADLDGNLLEDK	Sex lethal	*T. castaneum*	NP_001139415	15
7301f	FDVTQDNYR	Imaginal disc growth factor 4 (IDGF)	*T. castenium*	NP_001038091,1	1.7
7301f	GLLSYPEVCGSK	IDGF	*B. mori*	BAF73623	0.049
7301f	LAFVNSAHTLVK	imaginal disc growth factor 2	*T. castaneum*	NP_001038092	0.070
7301f	NYPAPLYSL	imaginal disc growth factor 4	*T. castaneum*	NP_001038091	40
7303	TGPLLSYPEV	IDGF Protein	*B. mori*	BAF73623	71
7303	DYPAPLY	imaginal disc growth factor 4	*T. castaneum*		9
9003	NDALGQELCK	neverland	*B. mori*	NP_001037626	203
9003	LNEKPLEKGAK	yolk protein 3	*D. melanogaster Musca domestica*		61
Immune function					
3304	VVLTAAHAVLQ	Serine protease	*A. aegypti*	EAT46512,1	21
6410	HVTVPNTDGK	transferrin	*Protaetia brevitarsis*	ABI31834,1	17
6410	CFLSQAPPHFVVT	transferrin	*T. castaneum*	XP_001808066	11
6410	CLVEGDGDVAFVK	transferrin	*T. castaneum*	XP_001808066	0.002
6410	VLELSENNVAQPNK	leucine-rich transmembrane protein.	*T. castaneum*		4.7
7301F	ASVLNADTNK	Coagulogen type II	*Tachypleus tridentalus*	CAA27780,1	35
7303	LVLGLPTYGR	Probable chitin protein	*D. melanogaster*	CG2989-PA	0.75
8306	VYQLPN	transcription factor Sox-2, putative	*Pediculus humanus corporis*	XP_002430102	162

Metabolism					
5102F	PAETAYLYQK	CAD	*Atelestus pulicarius*	AAQ67196,1	100
9003	GTVAAEGGFLVVN	Glyceraldehyde 3 –P-dehydrogenase	*T. casteum*	XP_974181,1	2.7
9003	GNNPSEEQLK	knockdown, isoform B	*D. melanogaster*	NP_727091	0.047
Stress response					
5406	NSPSEPLNVGLLYR	DNA mismatch repair MutS core	*A. gambiae*	EAA07673,3	215
6203 7203u	RAESFDQLVK	GK13718	*D. willistoni*	XP_002072653	71
7210	AGENVQAFTK	DNA photolyase	*A. gambiae*	XP_313925,1	143
mRNA processing					
3304	TQGSPLVCLP	Similar to CSl4	*T. castaneum*	XP_971155,1	71
3304	LCAEETSATQ	Similar to xmas-2	*A. mellifera*	XP_395462,3	187
5102 6005	NFVSSATMDK	ATP-dependent RNA helicase, putative	*Pediculus humanus corporis*	XP_002428983	17
Membrane transport and signaling					
6005	VVGMWNVK	SEC13-like protein 1 (Nuclear Pore complex)	*A. pisum*	XP_001949105	187
6203	SQTLETLAN	Organic cation transporting polypeptide 58Dc	*D.melanogaster*	NP_611659,2	163
8402	TEEKLPPRALK	similar to G protein alpha 73B	*T. castaneum*	- AAF49429	5.3
8306	YPTNALPSFDK	similar to retinal degeneration B	*A. mellifera*	XP_393355	175
	Unidentified				
3304	NGEYLNFG	GL11620	*D. persimilis*		
4001	EASTLAEFR	conserved hypothetical protein	Culex quinquefasciatus		
4001	SLSEPESCLT	conserved hypothetical protein	*P. humanus*		
4002f	VSSATFVK	GE21211	*D. yakuba*	XP_001359628	
5102U	LYTAEQLEK	SD02665p	*D melanogaster*	AAR31113,1	
5310 5312	TPTLGGTGSTYSTGGK	GD17305	*D. simulans*	XP_002107154	
6005	LSQFDDDVLLK	Hypothetical protein	*A. aegypti*	EAT45043,1	
6204f	QGVDADLNLGR	GJ23342	*D. virilis*	XP_002053429	
6203 6204f 7210	NQLLQLEQK	GH13043	*D. grimshawi*	XP_001993479	
6204U	SQLELETLA	GK17193	*D. willistoni*	XP_002061796	
8402	LQNLQLAGDQK	GE23832	*D. yakuba*	XP_002098633	
8402	GGVVPQTFK	TPA_inf: HDC19375	*D. melanogaster*	DAA03371	
7203u	LETLANNAK	GJ10621	*D. virilis*	XP_002055870	
8306	KPNESVETKLASR	Zinc finger, MYM domain containing 1	*A. pisum*	XP_001943430	

Identifications made using the *E. intermedius* EST database

Protein Modification/ Protein Processing

Spot	**Peptide**	Identity	**Homolog**	**NCBI Accession**	E value
2203F	TSYPGNLPDR	CL15Contig1-	trypsin-like serine protease		0.046
2203F	SNAACAAAYG	CL8Contig3-	Trypsin 29F		0.1
2203U	VSSAGCASGA	006991_1682_0418_c_s	Jonah 65 Aiv		0.23
2203F	FTSPLQYTDTLQLPR	CL20Contig1	Jonah 65Aiv		0.12
2203F	PAYPGVYSSV	CL8Contig1	Trypsin 29F		0.060
2203U	CADGA	CL8Contig3	Trypsin 29F		0.01

2203U	SPLQYTDTLQ	CL20Contig1	Jonah 65Aiv		0.30
6204F 7203U	AWNVQAFTK	CL123Contig1	Apolipophorin-III isoform 2	XP_975784	8.7
Sensory proteins					
0101 3004 4001	LDALGQECLK	CL32Contig1	PBP/GOBP family		0.30
2102 3002 4002F 9003	TGGMNADGTFNK	CL32Contig1-	PBP/GOBP family		0.003
4002U	QEALDALGQECLK	CL32Contig1	PBP/GOBP family		0.39
5102F	GLTPEQLEQLK	012264_1557_3398_c_s	Odorant-binding protein 56d	CG11218,	8.1
Immune function					
7101	SLLDSYPTAYTNVK	CL351Contig	Apolipophorin-III isoform 2	XP_975784	7.9
Stress response					
3304	TQGSPLVCLP	CL339Contig1	Metallocarboxypeptidase	CG14820	9.7
3304	FSLCAGGEQK	009895_1663_3195_c_s	Mutagen-sensitive 312	CG8601	7.4
6203 7203u	RAESFDQLVK	CL123Contig1	Apolipophorin-III isoform 2	XP_975784	1.1
7210	AGENVQAFTK	CL123Contig1	Apolipophorin-III isoform 2	XP_975784	0.18
6203 6204f 7210	NQLLQLEQK	011178_1666_3502_c_s	Apolipophorin-III isoform 2	XP_975784	1.0
6204f	LETLANNAK	011224_1655_1295_c_s	Apolipophorin-III isoform 2	XP_975784	1.0
7203u	LETLANNAK	011224_1655_1295_c_s	Apolipophorin-III isoform 2	XP_975784	1.0
6410	VLELSENNVAQPNK	008196_1603_1902_c_s	Eukaryotic initiation factor 4a	NP_001177648.1|	7.9
Unidentified					
6410	CFLSQAPPHFVVT	007076_1596_1424_c_s			7.4
Identifications made using Mascot					
Protein Modification/ Protein Processing					
Spot	**Peptide**	Identity	**Organism**	**NCBI Accession**	**E value**
2203F	TSYPGNLPDR	Trypsin Like serine protease			
2203U	VSSAGCASGA		*D.melanogaster*	CG10745	
Development					
7301f	FDVTQDNYR	IDGF Protein			
7301f	GLLSYPEVCGSK	IDGF Protein			
7301f	LAFVNSAHTLVK	IDGF Protein			
7301f	NYPAPLYSL	IDGF Protein			
7301U	TPGLLSYPEV	IDGF Protein			
9003	LNEKPLEKGAK	Yolk protein			
Immune function					
3304	VVLTAAHAVLQ				
6410	HVTVPNTDGK	Transferrin			
6410	TQEEPEAEFR	Transferrin			
6410	CFLSQAPPHFVVT	Transferrin			

6410	CLVEGDGDVAFVK	Transferrin		
6410	VLELSENNVAQPNK			
7101	SLLDSYPTAYTNVK	Serine protease		
7301F	ASVLNADTNK	Coagulin		
7303	LVLGLPTYGR	Probable chitin protein		
Metabolism				
5102F	GLTPEQLEQLK	phosphoglycolate phosphatase	*D. melanogaster*	
6410	VLELSENNVAQPNK	NADH dehydrogenase		
9003	GTVAAEGGFLVVN	Glyceraldehyde 3–P-dehydrogenase		
Stress response				
6203 7203u	RAESFDQLVK	Multidrug-Resistance like Protein 1	*D.melanogaster*	CG6214
Cytoskeletal				
5310 5312	YAPGGVASGFNQVNR	Gelsolin precursor	*A. gambaie*	
Unidentified				
5102U	LYTAEQLEK	SD02665p	*D. melanogaster*	

The table contains the peptide sequences obtained via MS/MS of the individual spots isolated in the 2D PAGE analysis of the hemolymph from *E. intermedius* infected with *B. bassiana* and uninfected beetles. Putative identities have been assigned to these peptides via bioinformatic analysis from three different sources. The National Centre for Biotechnology Information (NCBI) database was searched using the Mascot search engine. Secondly the partial peptide sequences were used to search the NCBI protein databases using pBLAST. Thirdly the peptide sequences were used to perform tBLASTn searches in the *E. intermedius* cDNA sequence database *Flylab* Genomebase using tBLAST.

Chapter 7: References

Abdelwahid, E., Yokokura, T., Krieser, R. J., Balasundaram, S., Fowle, W. H. and White, K. (2007). Mitochondrial Disruption in Drosophila Apoptosis. *Dev Cell.* 12, 793-806.

Abrams, J. M., White, K., Fessler, L. I. and Steller, H. (1993). Programmed cell death during Drosophila embryogenesis. *Development* 117, 29-43.

Adamo, S. A. (2010). Why should an immune response activate the stress response? Insights from the insects (the cricket Gryllus texensis). *Brain, Behavior, and Immunity* 24 194-200.

Aggarwal, K. and Silverman, N. (2008). Positive and negative regulation of the Drosophila immune response. *BMB reports* 41 267-277.

Agianian, B., Tucker, P. A., Schouten, A., Leonard, K., Bullard, B. and Gros, P. (2003). Structure of a Drosophila Sigma Class Glutathione S-transferase Reveals a Novel Active Site Topography Suited for Lipid Peroxidation Products. *J. Mol. Biol.* 326, 151-165.

Albihn, A., Mo, H., Yang, Y. and Henriksson, M. (2007). Camptothecin-induced apoptosis is enhanced by Myc and involves PKC delta signaling. *Int. J. Cancer* 121, 1821-1829.

Altincicek, B., Knorra, E. and Vilcinskasa, A. (2008). Beetle immunity: Identification of immune-inducible genes from the model insect Tribolium castaneumstar, open. *Developmental & Comparative Immunology* 32, 585-95.

Anderson, K. V., Bokla, L. and Nüsslein-Volhard, C. (1985). Establishment of dorsal-ventral polarity in the Drosophila embryo: the induction of polarity by the Toll gene product. *Cell Mol Life Sci* 42, 791-8.

Anderson, L. and Seilhamer, J. (1997). A comparison of selected mRNA and protein abundances in human liver. *Electrophoresis* 1, 533-537.

Anselme, C., Pérez-Brocal, V., Vallier, A., Vincent-Monegat, C., Charif, D., Latorre, A., Moya, A. and Heddi, A. (2008). Identification of the weevil immune genes and their expression in the bacteriome tissue. *BMC Biol* 6.

Antunes, R. J. (2008). Characterization of the DWNN domain and RING finger-like motif of the *Drosophila melanogaster* SNAMA Protein. In *Molecular And Cell Biology*, vol. MSc (ed., pp. 143. Johannesburg: University of The Witwatersrand.

Bangham, J., Jiggins, F. and Lamaitre, B. (2006). Insect Immunity: The Post-Genomic Era. *Immunity* 25, 1-5.

Bangs, P. and White, K. (2000). Regulation and Execution of Apoptosis During Drosophila Development. *Developmental Dynamics* 218, 68-79.

Bao, Q. and Shi, Y. (2007). Apoptosome: a platform for the activation of initiator caspases. *Cell Death Differ* 14, 56-65.

Barbault, F., Landon, C., Guenneugues, M., Meyer, J. P., Schott, V., Dimarcq, J. L. and Vovelle., F. (2003). Solution structure of Alo-3: a new knottin-type antifungal peptide from the insect *Acrocinus longimanus. Biochemistry* 42, 14434-42.

Barillas-Mury, C., Wizel, B. and Han, Y. S. (2000). Mosquito immune responses and malaria transmission: lessons from insect model systems and implications for vertebrat innate immunity and vacine development. *Insect Bioc Mol Biol* 30, 429-442.

Barkhouse, J. and Ridsill-Smith, T. J. (1986). Effect of soil moisture on brood ball production by *Onthophagus binodis thenberg* and *Euoniticellus intermedius* (Reiche)(Coleoptera: Scarabaeinae). *J . A ust. en t. Soc.*. 25, 75-78.

Barnes, P. J. (1991). Molecular biology. Inflammatory activities. *Nature* 349, 284-5.

Barra, D., Simmaco, M. and Boman, H. G. (1998). Gene-encoded peptide antibiotics and innate immunity. Do 'animalcules' have defence budgets? *FEBS Lett.* 430, 130-4.

Bartholomay, L. C., Cho, W.-L., Rocheleau, T. A., Boyle, J. P., Beck, E. T., Fuchs, J. F., Liss, P., Rusch, M., Butler, K. M., Wu, R. C.-C. et al. (2004). Description of the Transcriptomes of Immune Response-Activated Hemocytes from the Mosquito Vectors Aedes aegypti and Armigeres subalbatus. *Infection and Immunity* 72, 4114-4126.

Bauer, J. H., Chang, C., Morris, S. N. S., Hozier, S., Andersen, S., Waitzman, J. S. and Helfand, S. L. (2007). Expression of dominant-negative Dmp53 in the adult fly brain inhibits insulin signaling. *Proc Natl Acad Sci U S A* 104, 13355-60.

Bauer, J. H., Poon, P. C., Glatt-Deeley, H., Abrams, J. M. and Helfand, S. L. (2005). Neuronal expression of p53 dominant-negative proteins in adult Drosophila melanogaster extends life span. *Curr Biol.* 15, 2063-8.

Bensaad, K., Tsuruta, A., Selak, M. A., Nieves, C. V. M., Nakano, K., Bartrons, R., Gottlieb, E. and Vousden, K. H. (2006). TIGAR, a p53-Inducible Regulator of Glycolysis and Apoptosis. *Cell* 126, 107-120.

Bensaad, K. and Vousden, K. H. (2007). p53: new roles in metabolism. *Trends in Cell Biology* 17, 286-291.

Bereczki, O., Ujfaludi, Z., Pardi, N., Nagy, Z., Tora, L., Boros, I. M. and Balint, E. (2008). TATA binding protein associated factor 3 (TAF3) interacts with p53 and inhibits its function. *BMC Molecular Biology* 9.

Bergmann, A., Yang, A. Y.-P. and Srivastava, M. (2003). Regulators of IAP function: Coming to grips with the Grim reaper *Current opinion in cell biology* 15, 717-724.

Blume, R. R. (1984). Euoniticellus intermedius (Coleoptera: Scarabaeidae): Description of Adults and Immatures and Biology of Adults *Environmental Entomology* 13, 1064-1068.

Bodai, L., Pardi, N., Ujfaludi, Z., Bereczki, O., Komonyi, O., Balint, E. and Boros, I. M. (2007). Daxx-like protein of Drosophila interacts with Dmp53 and affects longevity and Ark mRNA level. *J Biol Chem.* 282, 36386-93.

Boman, H. G. (1991). Antibacterial peptides: Key components needed in immunity. *Cell* 65, 205-207.

Bourdon, J.-C. (2007). p53 Family Isoforms. *Curr Pharm Biotechnol* 8, 332-336.
Bourdon, J.-C., Fernandes, K., Murray-Zmijewski, F., Liu, G., Diot, A., Xirodimas, D. P., Saville, M. K. and Lane, D. P. (2005). p53 isoforms can regulate p53 transcriptional activity. *Genes & Dev.* 19, 2122-2137

Brodsky, M. H., Nordstrom, W., Tsang, G., Kwan, E., Rubin, G. M. and Abrams, J. M. (2000). Drosophila p53 Binds a Damage Response Element at the reaper Locus. *Cell* 101, 103-113.

Brodsky, M. H., Weinert, B. T., Tsang, G., Rong, Y. S., McGinnis, N. M., Golic, K. G., Rio, D. C. and Rubin, G. M. (2004). Drosophila melanogaster MNK/Chk2 and p53 Regulate Multiple DNA Repair and Apoptotic Pathways following DNA Damage. *Mol. Cell. Biol* 24, 1219-1231.

Brooks, C. L. and Gu, W. (2006). p53 Ubiquitination: Mdm2 and Beyond. *Molecular Cell* 21, 307-315

Brown, A. E., France, R. M. and Grossman, S. H. (2004). Purification and Characterization of Arginine Kinase From the American Cockroach (Periplaneta americana). *Archives of Insect Biochemistry and Physiology* 56, 51-60.

Bruck, D. J. and Lewis, L. C. (2001). Adult Diabrotica spp. (Coleoptera: Chrysomelidae) infection at emergence with Iindigenous Beauveria bassiana (Deuteromycotina: Hyphomycetes). *J Invertebr Pathol* 77, 288-9.

Bruning, M., Lummer, M., Bentele, C., Smolenaars, M. M. W., Rodenburg, K. W. and Ragg, H. (2007). The Spn4 gene from Drosophila melanogaster is a multipurpose defence tool directed against proteases from three different peptidase families. *Biochem. J.* 401, 325-331.

Bulet, P., Cociancich, S., Dimarq, J., Lambert, J., Reichart, J. M., Hoffmann, D., Hetru, C. and Hoffmann, J. A. (1991). Isolation from a coleopteran insect of a novel inducible antibacterial peptide and of new members of the insect defensin family. *J. Biol. Chem.* 266, 24520-24525.

Bulet, P., Hetru, C., Dimarcq, J. L. and Hoffmann, D. (1999). Antimicrobial peptides in insects; structure and function. *Dev Comp Immunol* 23, 329-44.

Bulet, P., Stöcklin, R. and Menin, L. (2004). Anti-microbial peptides: from invertebrates to vertebrates. *Immunol Rev* 198, 169-84.

Buszczak, M. and Segraves, W. A. (2000). Insect metamorphosis: Out with the old, in with the new. *Current Biology* 10, R830-R833.

Cambefort, Y. (1991a). Biogeography and Evolution: In Dung Beetle Ecology. New Jersey: Princeton University Press.

Cambefort, Y. (1991b). From saprophagy to coprophagy. In Dung beetle Ecology. New Jersey: Princeton University Press.

Cambefort, Y. and Hanski, I. (1991). Dung beetle popultion biology. In Dung beetle ecology. New Jersey: Princeton University Press.

Cañadillas, J. M. P., Tidow, H., Freund, S. M. V., Rutherford, T. J., Ang, H. C. and Fersht, A. R. (2006). Solution structure of p53 core domain: Structural basis for its instability. *Proc Natl Acad Sci U S A* 103, 2109-2114.

Cashio, P., Lee, T. V. and Bergmann, A. (2005). Genetic control of programmed cell death in Drosophila melanogaster. *Seminars in Cell & Developmental Biology* 16, 225-235.

Castegna, A., Palmeiri, L., Spera, I., Porcelli, V., Palmieri, F., Fabis-Pedrini, M. J., Kean, R. B., Barkhouse, D. A., Curtis, M. T. and Hooper, D. C. (2011). Oxidative Stress and Reduced Glutamine Synthetase Activity in the Absence of Inflammation in the Cortex of Mice with Experimental Allergic Encephalomyelitis *Neuroscience* 185, 97-105.

Cerella, C., D'Alessio, M., Cristofanon, S., Nicola, M. D., Radogna, F., Dicato, M., Diederich, M. and Ghibellib, L. (2009). Subapoptogenic Oxidative Stress Strongly Increases the Activity of the Glycolytic Key Enzyme Glyceraldehyde 3-Phosphate Dehydrogenase:

Possible Involvement in an Adaptative Response to the Stress. *Ann. N.Y. Acad. Sci.* 1171, 583-590.

Chen, G., Gharib, T. G., Huang, C. C., Taylor, J. M., Misek, D. E., Kardia, S. L., Giordano, T. J., Lannettoni, M. D., Orringer, M. B. and Hanash, S. M. (2002). Discordant protein and mRNA expression in lung adenocarcinomas. *Mol Cell Proteomics* 1, 304-313.

Cheung, E. C. and Vousden, K. H. (2010). The role of p53 in glucose metabolism. *Curr Opin Cell Biol* 22, 186-191.

Chibi, M., Meyer, M., Skepu, A. G., Rees, D. J., Moolman-Smook, J. C. and Pugh, D. J. (2008). RBBP6 interacts with multifunctional protein YB-1 through its RING finger domain, leading to ubiquitination and proteosomal degradation of YB-1. *J Mol Biol* 384, 908-16.

Chung, M.-K., ·, C.-Y. K. and Kim, J.-C. (2007). Reproductive toxicity evaluation of a new camptothecin anticancer agent, CKD-602, in pregnant/lactating female rats and their offspring. *Cancer Chemother Pharmacol* 59, 383-395.

Chung, M.-K., Han, S.-S. and Kim, J. C. (2006). Evaluation of the toxic potentials of a new camptothecin anticancer agent CKD-602 on fertility and early embryonic development in rats. *Regulatory Toxicology and Pharmacology* 45, 273-281.

Coea, H. and Michalaka, M. (2010). ERp57, a multifunctional endoplasmic reticulum resident oxidoreductase. *The International Journal of Biochemistry & Cell Biology* In press.

Cooper, D. M., Granville, D. J. and Lowenberger, C. (2009). The insect caspases. *Apoptosis* 14, 247-56.

Corona, M., Velarde, R. A., Remolina, S., Moran-Lauter, A., Wang, Y., Hughes, K. A. and Robinson, G. E. (2007). Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. *Proc Natl Acad Sci U S A* 104, 7128-7133.

Davis, M. M., Primrose, D. A. and Hodgetts, R. B. (2008). A member of the p38 mitogen-activated protein kinase family is responsible for transcriptional induction of Dopa decarboxylase in the epidermis of Drosophila melanogaster during the innate immune response. *Mol Cell Biol* 28, 4883-95.

Deb, S. P. (2003). Cell Cycle Regulatory Functions of the Human Oncoprotein MDM2. *Molecular Cancer Research* 1, 1009-1016.

DeGregori, J. and Johnson, D. G. (2006). Distinct and Overlapping Roles for E2F Family Members in Transcription, Proliferation and Apoptosis. *Current Molecular Medicine* 6, 739-748.

Desai, S. D., Liu, L. F., Vazquez-Abad, D. and D'Arpa, P. (1999). Ubiquitin-dependent Destruction of Topoisomerase I Is Stimulated by the Antitumor Drug Camptothecin. *J. Biol. Chem.* 272, 24159-24164.

Destoumieux, D., Bulet, P., Loew, D., Dorsselaer, A. V., Rodriguez, J. and Bachere, E. (1997). Penaeidins, a new family of antimicrobial peptides isolated from the shrimp *Penaeus vannamei* (Decapoda). *J. Biol. Chem.* 272, 28398-28406.

Dimopoulos, G., Richman, A., Müller, H. M. and Kafatos, F. C. (1997). Molecular immune responses of the mosquito Anopheles gambiae to bacteria and malaria parasites. *Proc Natl Acad Sci U S A.* 94, 11508-13.

Doube, B. M. (1991). Dung Beetles of Southern Africa. In Dung Beetle Ecology. New Jersey: Princeton University press.

Du, W. and Dyson, N. (1999). The role of RBF in the introduction of G1 regulation during Drosophila embryogenesis. *The EMBO Journal* 18, 916-925.

Dushay, M. S., Asling, B. and ., D. H. (1996). Origins of immunity: Relish, a compound Rel-like gene in the antibacterial defense of Drosophila. *Proc Natl Acad Sci U S A* 93, 10343-7.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* 32, 1792-1797.

Edwards, K., Davis, T., Marcey, D., Kurihara, J. and Yamamoto, D. (2001). Comparative analysis of the Band 4.1/ezrin-related protein tyrosine phosphatase Pez from two Drosophila species: implications for structure and function. *Gene* 275, 195-205.

Efron, B. (1982). The Jackknife, the Bootstrap and Other Resampling Plans. In *CBMS-NSF Regional Conference Series in Applied Mathematics, Monograph 38*. Philadelphia.: SIAM,.

Ehret-Sabatier, L., Loew, D., Goyffon, M., Fehlbaum, P., Hoffmann, J. A. and Bulet, P. (1996). Characterization of novel cysteine rich antimicrobial peptides from scorpion blood. *J. Biol. Chem.* 271, 29537-29544.

Engstro¨m, Y., Loseva, O. and Theopold, U. (2004). Proteomics of the Drosophila immune response. *TRENDS in Biotechnology* 22.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. . *Evolution* 39, 783-791.

Fernández-Ayala, D. G., Brea-Calvo, G., López-Lluch, G. and Navas, P. (2005). Coenzyme Q distribution in HL-60 human cells depends on the endomembrane system. *Biochim Biophys Acta.* 1713, 129-137.

Ferrandon, D., Jung, A. C., Criqui, M. C., Lemaitre, B., Reichhart, J. M. and Hoffmann, J. A. (1998). A Drosomycin-GFP reporter transgene reveals a local immune response in *Drosophila* that is not dependant on the Toll pathway. *EMBO journal* 17, 1217-1227.

Flatt, T., Tu, M.-P. and Tatar, M. (2005). Hormonal pleiotropy and the juvenile hormone regulation of Drosophila development and life history. *BioEssays* 27, 999-1010.

Frenzel, J., Richter, J. and Eschrich, K. (2005). Pyruvate Protects Glucose-Deprived Muller Cells From Nitric Oxide-Induced Oxidative Stress by Radical Scavenging. *GLIA* 52, 276-288.

Frolov, M. V., Huen, D. S., Stevaux, O., Dimova, D., Balczarek-Strang, K., Elsdon, M. and Dyson, N. J. (2001). Functional antagonism between E2F family members. *Genes Dev* 15, 2146-2160.

Gakh, O., Cavadini, P. and Isaya, G. (2002). Mitochondrial processing peptidases. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1592, 63-77.

Gamcsik, M. P., Kasibhatla, M. S., Adams, D. J., Flowers, J. L., Colvin, O. M., Manikumar, G., Wani, M., Wall, M. E., Kohlhagen, G. and Pommier, Y. (2001). Dual Role of Glutathione in Modulating Camptothecin Activity:Depletion Potentiates Activity, but Conjugation Enhances the Stability of the Topoisomerase I-DNA Cleavage Complex. *Molecular Cancer Therapeutics* 1, 11-20.

Garner, E. and Raj, K. (2008). Protective mechanisms of p53-p21-pRb proteins against DNA damage-induced cell death. *Cell Cycle* 7, 277-282.

Gemkow, M. J., Dichter, J. and Arndt-Jovin, D. J. (2001). Developmental Regulation of DNA-Topoisomerases during Drosophila Embryogenesis. *Experimental Cell Research* 262, 114-121.

Gibbs, R. S. (2008). The genome of the model beetle and pest Tribolium castaneum. *Nature* 452, 949-955.

Gnerer, J. P., Kreber, R. A. and Ganetzky, B. (2006). wasted away, a Drosophila mutation in triosephosphate isomerase, causes paralysis, neurodegeneration, and early death. *Proc Natl Acad Sci U S A.* 103, 14987-14993.

Goergel, P., Meister, M., Kappler, C., Lemaitre, B., Riechhart, J. M. and Hoffmann, J. A. (1993). Insect immunity: The diptericin promoter contains multiple functional regulatory sequences homologous to mammalian acute-phase response elements. *Biochem. and Biophys. Res. Comm.* 197, 508-511.

Gradishar, W. J. and Schilsky, R. L. (1988). Effects of cancer treatment on the reproductive system. *Critical Reviews in Oncology/Hematology* 8, 153-171.

Greenbaum, D., Colangelo, C., Williams, K. and Gerstein, M. (2003a). Comparing protein abundance and mRNA expression levels on a genomic scale. *Genome Biology* 4, 1-8.

Greenbaum, D., Colangelo, C., Williams, K. and Gerstein, M. (2003b). Comparing protein abundance and mRNA expression levels on a genomic scale. *Genome Biology* 4.

Greenberg, S. and Grinstein, S. (2002). Phagocytosis and innate immunity. *Curr. Opin. Immunol.* 14, 136-145.

Gross, I., Goergel, P., Kappler, C., Reichart, J. M. and Hoffmann, J. A. (1996). *DrosophilaI* immunity: a comparitive analysis of the Rel proteins dorsal and Dif in the induction of the genes encoding diptericin and cecropin. *Nucleic acid research* 24, 1238-1245.

Gruntenko, N. E. and Rauschenbach, I. Y. (2008). Interplay of JH, 20E and biogenic amines under normal and stress conditions and its effect on reproduction. *Journal of Insect Physiology*,, 902- 908.

Grusch, M., Polgar, D., Gfatter, S., Leuhuber, K., Huettenbrenner, S., Leisser, C., Fuhrmann, G., Kassie, F., Steinkellner, H., Smid, K. et al. (2002). Maintenance of

ATP favours apoptosis over necrosis triggered by benzamide riboside. *Cell Death and Differentiation* 9, 169-178.

Guedesa, S. d. M., Vitorinoa, R., Dominguesa, R. r., Tomerb, K., Correiaa, A. J. F., Amadoa, F. and Dominguesa, P. (2005). Proteomics of immune-challenged Drosophila melanogaster larvae hemolymph. *Biochemical and Biophysical Research Communications* 328, 106-115.

Habig, W. H., Pabst, M. J. and Jakoby, W. B. (1974). Glutathione S-Transferases: The first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* 249, 7130-7139.

Haine, E. R., Moret, Y., Siva-Jothy, M. T. and Rolff, J. (2008). Antimicrobial Defense and Persistent Infection in Insects. *Science* 322, 1257-1258.

Han, Z. S. and Ip, Y. T. (1999). Interaction and specificity of Rel-related proteins in regulating *Drosophila* immunity Gene expression. *J. Biol. Chem.* 274, 21355-21361.

Hanski, I. and Cambefort, Y. (1991). Spatial Processes In Dung Beetle Ecology. New Jersey: Princeton University Press.

Haraa, T., Ishiib, T., Fujishirob, M., Masudac, M., Takaaki Itod, Nakajimae, J., Inouea, T. and Matsuseb, T. (2004). Glutathione S-transferase P1 has protective effects on cell viability against camptothecin. *Cancer Letters* 203, 199-207.

Hardie, D. G. (2004). The AMP-activated protein kinase pathway - new players upstream and downstream. *Journal of Cell Science* 117, 5479-5487.

Harper, J. W. (2004). Neddylating the Guardian: Mdm2 Catalyzed Conjugation of Nedd8 to p53. *Cell* 118, 2-4.

Ho, J. and Benchimol, S. (2003). Transcriptional repression mediated by the p53 tumor suppressor. *Cell Death and Differentiation.* 18, 404-408.

Hoffmann, J. A. and Hetru, C. (1992). Insect defensins: Inducible antibacterial peptides. *Immunology today* 13, 411-415.

Hoffmann, J. A. and Reichhart, J. M. (2002). Drosophila innate immunity: an evolutionary perspective. *Nat Immunol* 3, 121-6.

Holden, J. A., Wall, M. E., Wani, M. C. and Manikumar, G. (1999). Human DNA Topoisomerase I: Quantitative Analysis of the effects of Camptothecin Analogs and the Benzophenanthridine Alkaloids Nitidine and 6-Ethoxydihydronitidine on DNA Topoisomerase I induced DNA strand Breakage. *Archives of Biochemistry and Biophysics* 370, 66-76.

Horvath, C. M. (2000). STAT proteins and transcriptional responses to extracellular signals. *Trends in Biochemical Sciences* 20, 496-502.

Howell, S. and Shalet, S. (1998). Gonadal damage from chemotherapy and radiotherapy. *Endocrinol Metab Clin North Am* 4, 927-943.

Hull, R. and Ntwasa, M. M. (2010). Glycolytic flux occurs in Drosophila melanogaster recovering from camptothecin treatment. *Anticancer Drugs* 21, 945-957.

Hwang, J.-S., Lee, J., Kim, Y.-J., Bang, H.-S., Yun, E.-Y., Kim, S.-R., Suh, H.-J., Kang, B.-R., Nam, S.-H., Jeon, J.-P. et al. (2009). Isolation and Characterization of a Defensin-Like Peptide (Coprisin) from the Dung Beetle, Copris tripartitus. *International Journal of Peptides* Article ID 136284, 5.

Igaki, T., Kanda, H., Yamamoto-Goto, Y., Kanuka, H., Kuranaga, E., Aigaki, T. and Miura, M. (2002). Eiger, a TNF superfamily ligand that triggers the Drosophila JNK pathway. *The EMBO Journal* 21,, 3009-3018.

Imamura, M., Wada, S., Koizumi, N., Kadotani, T., Yaoi, K., Sato, R. and Iwahana, H. (1999). Acaloleptins A: inducible antibacterial peptides from larvae of the beetle, *Acalolepta luxuriosa*. *Arch Insect Biochem Physiol* 40, 88-98.

Ip, Y. T., Reach, M., Engstrom, Y., Kadalay, L., Cal, H., Gonzalez-Crespo, S., Tatel, K. and Levine, M. (1993). *Dif*, a dorsal related gene that mediates an immune response in *Drosophila*. *Cell* 75, 753-763.

Ishibashi, J., Saido-Sakanaka, H., Yang, J., Sagisaka, A. and ., M. Y. (1999). Purification, cDNA cloning and modification of a defensin from the coconut rhinoceros beetle, Oryctes rhinoceros. *Eur J Biochem* 266, 616-23.

Ivanov, G. S., Ivanova, T., Kurash, J., Ivanov, A., Chuikov, S., Gizatullin, F., Herrera-Medina, E. M., Rauscher, F., Reinberg, D. and Barlev, N. A. (2007). Methylation-Acetylation Interplay Activates p53 in Response to DNA Damage. *Mol Cell Biol.* 27, 6756-6769.

Jagelska, E. B., Brazda, V., Pecinka, P. and Miroslav, F. (2008). DNA topology influences p53 sequence specific DNA binding through structural transitions within target sites. *biochem J* 412, 57-63.

Jaklevic, B., Uyetake, L., Wichmann, A., Bilak, A., English, C. and Su, T. T. (2008). Modulation of ionizing radiation-induced apoptosis by bantam microRNA in Drosophila. *Dev Biol* 320, 122-130.

Jaklevica, B. R. and Su, T. T. (2004). Relative Contribution of DNA Repair, Cell Cycle Checkpoints, and Cell Death to Survival after DNA Damage in Drosophila Larvae. *Curr Biol* 14, 23-32.

Jang, I.-H., Chosa, N., Kim, S.-H., Nam, H.-J., Lemaitre, B., Ochiai, M., Kambris, Z., Brun, S., Hashimoto, C., Ashida, M. et al. (2006). A Spätzle-Processing Enzyme Required for Toll Signaling Activation in Drosophila Innate Immunity. *Developmental Cell* 10, 45-55.

Jassim, O. W., Fink, J. L. and Cagan, R. L. (2003). Dmp53 protects the Drosophila retina during a developmentally regulated DNA damage response. *EMBO J* 22, 5622-5632.

Jin, S., Martinek, S., Joo, W. S., Wortman, J. R., Mirkovic, N., Sali, A., Yandell, M. D., Pavletich, N. P., Young, M. W. and Levine, A. J. (2000). Identification and characterization of a p53 homologue in Drosophila melanogaster. *Proc Natl Acad Sci U S A.* 97, 7301-7306.

Joerger, A. C. and Fersht, A. R. (2008). Structural biology of the tumor suppressor p53. *Annu Rev Biochem* 77, 557-82.

Jones, C., Reifegerste, R. and Moses, K. (2006). Characterization of Drosophila mini-me, a Gene Required for Cell Proliferation and Survival. *Genetics.* 173, 793-808.

Kambris, Z., Brun, S., Jang, I.-H., Nam, H.-J., Romeo, Y., Takahashi, K., Lee, W.-J., Ryu Ueda and Lemaitre, B. (2006). Drosophila Immunity: A Large-Scale In Vivo RNAi Screen Identifies Five Serine Proteases Required for Toll Activation. *Current Biology* 16, 808-813.

Kanada, H., Igaki, T., Kanuka, H., Yagi, T. and Miura, M. (2002). Wengen, a Member of the Drosophila Tumor Necrosis Factor Receptor Superfamily, Is Required for Eiger Signaling. *J. Biol. Chem.* 277, 28372-28375.

Kang, D., Liu, G., Gunne, H. and Steiner, H. (1996). PCR differential Display of Immune gene expression in *Trichoplusia ni*. *Insect Biochem. Molec. Biol.* 26, 177-184.

Kang, D., Lui, G., Lundstrom, A. and Steiner, H. (1998). A peptidoglycan recognition protein in innate immunity conserved from insects to humans. *Proc. Natl. Acad. Sci. USA* 95, 10078-10082.

Kang, Y.-H., Chung, S.-J., Kang, I.-J., Park, J. H. Y. and Bünger, R. (2001). Intramitochondrial pyruvate attenuates hydrogen peroxide-induced apoptosis in bovine pulmonary artery endothelium. *Molecular and Cellular Biochemistry* 216, 37-46.

Kenjiro Uedaa, A. S., Morikazu Imamurab, Nami Miuraa, Shogo Atsumia, Hiroko Tabunokia, Ayako Watanabea, Madoka Kitamia and Ryoichi Sato. (2005). Purification and cDNA cloning of Luxuriosin, a novel antibacterial peptide with Kunitz domain from the Longicorn Beetle, Acalolepta luxuriosa. *Biochimica et Biophysica Acta (BBA) - General Subjects* 1722, 36-42.

Khanyile, L. M., Hull, R. and Ntwasa, M. (2008). Dung beetle database: comparison with other invertebrate transcriptomes. *Bioinformation.* 3, 159-161.

Khush, R. S., Cornwell, W. D., Uram, J. N. and Lemaitre, B. (2002). A ubiquitin-proteasome pathway represses the Drosophila immune deficiency signaling cascade. *Curr Biol* 12, 1728-37.

Kim, C. H., Shin, Y. P., Noh, M. Y., Jo, Y. H., Han, Y. S., Seong, Y. S. and Lee, I. H. (2010). An Insect Multiligand Recognition Protein Functions as an Opsonin for the Phagocytosis of Microorganisms. *JBC* In Press.

Kim, M., Lee, J. H., Lee, S. Y., Kim, E. and Chung, J. (2006). Caspar, a suppressor of antibacterial immunity in Drosophila. *Proc Natl Acad Sci U S A* 103, 16358-16363.

Kim, T. and Kim, Y. J. (2005). Overview of Innate Immunity in *Drosophil*. *Journal of Biochemistry and Molecular Biology* 38, 121-127.

Kim, Y. S., Ryu, J. H., Han, S. J., Choi, K. H., Nam, K. B., Jang, I. H., Lemaitre, B., Brey, P. T. and Lee, W. J. (2000). Gram negative bacteria-binding Protein, a pattern recognition receptor for lipopolysaccharide and B-1,3-Glucan that mediates the signaling for thw induction of innate immune genes in *Drosophila melanogaster* cells. *J. Biol. Chem* 275, 32721-32727.

Kleino, A., Myllymäki, H., Kallio, J., Vanha-aho, L.-M., Oksanen, K., Ulvila, J., Hultmark, D., Valanne, S. and Rämet, M. (2008). Pirk is a negative regulator of the Drosophila Imd pathway. *J Immunol* 180, 5413-22.

Kohanski, M. A., Dwyer, D. J. and Collins, J. J. (2010). How antibiotics kill bacteria: from targets to networks. *Nat Rev Microbiol.* 8, 423-435.

Konig, C. and Schmidt-Hempel, P. (1995). Foraging activity and immunocompetence in workers of the bumble bee, *Bombus terestris*. *Proc. R. Soc. Lond. B.* 260, 225-227.

Kornbluth, S. and White, K. (2005). Apoptosis in Drosophila: neither fish nor fowl (nor man, nor worm). *Journal of Cell Science* 118, 1779-1787.

Korsloot, A., Gestel, C. A. M. v. and Straalen, N. M. v. (2004). Environmental stress and Cellular Response in Arthropods. Boca Raton: CRC Press.

Koumenis, C., Alarcon, R., Hammond, E., Sutphin, P., Hoffman, W., Murphy, M., Derr, J., Taya, Y., Lowe, S. W., Kastan, M. et al. (2001). Regulation of p53 by Hypoxia: Dissociation of Transcriptional Repression and Apoptosis from p53-Dependent Transactivation. *Mol Cell Biol* 21, 1297-1310.

Lane, D. P. and Benchimol, S. (1990). p53: oncogene or anti-oncogene? *Genes Dev* 4, 1-8.

Larade, K. and Storey, K. B. (2004). Accumulation and translation of ferritin heavy chain transcripts following anoxia exposure in a marine invertebrate. *The Journal of Experimental Biology* 207, 1353-1360.

Lauth, X., Nesin, A., Briand, J. P., Roussel, J. P. and Hetru, C. (1998). Isolation, characterisation and chemical synthesis of a new insect defensin from *Chironomus plusmosus* (Diptera). *Insect Bioc. Mol. Biol.* 28, 1059-1066.

Lee, J. Y., Boman, A., Sun, C. X., Andersson, M., Jörnvall, H., Mutt, V. and Boman, H. (1989). Antibacterial peptides from pig intestine: isolation of a mammalian cecropin. *Proc. Natl. Acad. Sci. USA* 86, 9159-62.

Lee, K. S., Park, H. J., Bae, J. S., Goo, T. W., Kim, I., Sohn, H. D. and Jin, B. R. (2001). Molecular cloning and expression of a cDNA encoding the luciferase from the firefly, Pyrocoelia rufa. *J Biotechnol* 92, 9-19.

Lee, M. P., Brown, S. D., Chen, A. and Hsieh, T. (1993). DNA topoisomerase I is essential in Drosophila melanogaster. *Proc Natl Acad Sci U S A* 90, 6656-6660.

Lee, S. Y., Moon, H. J., Kurata, S., Kurama, T., Natori, S. and Lee, B. L. (1994). Purification and molecular cloning of cDNA for an inducible antibacterial protein of larvae of a coleopteran insect, *Holotrichia diomphalia. J Biochem (Tokyo)* 115, 82-6.

Lee, Y.-J., Kang, I.-J., Bu¨nger, R. and Kanga, Y.-H. (2003). Mechanisms of pyruvate inhibition of oxidant-induced apoptosis in human endothelial cells. *Microvascular Research* 66, 91-101.

Lehane, M. J., Wu, D. and Lehane, S. M. (1997). Midgut-specific immune molecules are produced by the blood-sucking insect *Stomoxys calcitrans*. *Proc. Natl. Acad. Sci. USA* 94, 11502-11507.

Levashina, E. A., Langley, E., Green, C., Grubb, D., Ashburner, M., Hoffmann, J. A. and Reichhart, J. M. (1999). Constitutive Activation of Toll-Mediated antifungal defense in serpin deficient *Drosophila*. *Science* 285, 1917-1919.

Levison, M. E. and Levison, J. H. (2009). Pharmacokinetics and pharmacodynamics of antibacterial agents. *Infect Dis Clin North Am.* 23, 791-815.

Levy, F., Bulet, P. and Ehret-Sabatier, L. (2004). Proteomic Analysis of the Systemic Immune Response of Drosophila. *Molecular & Cellular Proteomics* 3, 156-166.

Li, J., Choo, Y. M., Lee, K. S., Je, Y. H., Woo, S. D., Kim, I., Sohn, H. D. and Jin, B. R. (2005). A serine protease gene from the firefly, Pyrocoelia rufa: gene structure,expression, and enzyme activity. *Biotechnology Letters* 27, 1051-1057.

Li, L., Deng, B., Xing, G., Teng, Y., Tian, C., Cheng, X., Yin, X., Yang, J., Gao, X., Zhu, Y. et al. (2007). PACT is a negative regulator of p53 and essential for cell growth and embryonic development. *Proc Natl Acad Sci U S A* 104, 7951-7956.

Li, Q.-J., Pazdera, T. M. and Minden, J. S. (1999). Drosophila embryonic pattern repair: how embryos respond to cyclin E-induced ectopic division. *Development* 126, 228-2307.

Lichtinghagen, R., Musholt, P. B., Lein, M., Romer, A., Rudolph, B., Kristiansen, G., Hauptmann, S., Schnorr, D., Loening, S. A. and Jung, K. (2002). Different mRNA and protein expression of matrix metalloproteinases 2 and 9 and tissue inhibitor of metalloproteinases 1 in benign and malignant prostate tissue. *Eur Urol* 42, 398-406.

Lorence, A. and Nessler, C. L. (2004). Camptothecin, over four decades of suprising findings. *Phytochemistry* 65, 2735-2749.

Lu, W.-J. and Abrams, J. M. (2006). Lessons form p53 in non-mammalian models. *Cell Death and Differentiation* 23, 909-912.

Ma, W., Sung, H. J., Park, J. Y., Matoba, S. and Hwang, P. M. (2007). A pivotal role for p53: balancing aerobic respiration and glycolysis. *Journal of Bioenergetics and Biomembranes* 39, 243-246.

Mandal, S., Guptan, P., Owusu-Ansah, E. and Banerjee, U. (2005). Mitochondrial Regulation of Cell Cycle Progression during Development as Revealed by the tenured Mutation in Drosophila. *Developmental Cell* 9, 843-854.

Mandel, M. and Higa, A. (1970). Calcium-dependent bacteriophage DNA infection. *J. Mol. Biol.* 53, 159-162.

Marchand, C., Antony, S., Kohn, K., Cushman, M., Loanoviciu, A., Staker, B., Burgin, A., Stewart, L. and Pommier, Y. (2006). A novel norindenoisoquinoline structure reveals a common interfacial inhibitor paradigm for ternary trapping of the topoisomerase 1-DNA covalent complex. *Mol. Cancer Ther.* 5, 287-295.

Mari, M., Bai, J. and Cederbaum, A. I. (2002). Toxicity by pyruvate in Hep G2 cells depleted of Glutathione: Role of Mitochondria *Free Radical Biology & Medicine* 32, 73-83.

Marina, T.-B. (1990). Common Dung Beetles in Pastures of South-eastern Austrailia. Sydney: CSIRO

Marmaras, V. J. and Lampropoulou, M. (2009). Regulators and signalling in insect haemocyte immunity. *Cellular Signalling* 21, 186-195.

Masova, A., Sanda, M., Jiracek, J. and Selicharova, I. (2010). Changes in the proteomes of the hemocytes and fat bodies of the flesh fly Sarcophaga bullata larvae after infection by Escherichia coli. *Proteome Science* 8, 1-11.

Mather, A., Rakghotho, M. and Ntwasa, M. (2005). SNAMA, a novel protein with a DWNN domain and a RING finger-like motif:A possible role in apoptosis. . *Biochim Biophys Acta.* 1727, 169-176.

Mather, A. S. (2005). The Identification and characterisation of a novel apoptotic gene, *SNAMA*, in *Drosophila melanogaster*. In *School of Molecular and Cell Biology*, vol. Doctor of Philosophy (ed. Johannesburg: University of the Witwatersrand.

Matoba, S., Kang, J.-G., Patino, W. D., Wragg, A., Boehm, M., Gavrilova, O., Hurley, P. J., Bunz, F. and Hwang, P. M. (2006). p53 Regulates Mitochondrial Respiration. *Science* 312., 1650 - 1653.

Maxton-Kuchenmeister, J., Handel, K., Schmidt-Ott, U., Roth, S. and Jackle, H. (1999). *Toll* homolog expression in the beetle *Tribolium* suggests a different mode of dorsoventral patterning than in *Drosophila*. *Mechanisms of Development* 83, 107-114.

Means, T. K., Golenbock, D. T. and Fenton, M. J. (2000). The biology of Toll-like receptors. *Cytokine and growth factor reviews* 11, 219-232.

Medzhitov, R. and Janeway, C. A. (1997). Innate immunity: the virtues of a nonclonal system of recognition. *Cell* 91, 295-298.

Meek, D. W. and Anderson, C. W. (2009). Posttranslational Modification of p53: Cooperative Integrators of Function. *Cold Spring Harbor Perspectives in Biology* 1, 1-16.

Meirow, D. (2000). Reproduction post-chemotherapy in young cancer patients. *Molecular and Cellular Endocrinology* 169, 123-131.

Miotto, B., Sagnier, T., Berenger, H., Bohmann, D., Pradel, J. and Graba, Y. (2006). Chameau HAT and DRpd3 HDAC function as antagonistic cofactors of JNK/AP-1-dependent transcription during Drosophila metamorphosis. *Genes & Dev* 20, 101-112.

Miyanoshita, A., Hara, S., Sugiyama, M., Asaeka, A., Taniai, K., Yukuhire, F. and Yamakawa, M. (1996). Isolation and Characterization of a New Member of the Insect Defensin Family from a Beetle, Allomyrina dichotoma. *Biochem. Biophys. Res. Comm.*. 224, 526-531.

Moon, H. J., Lee, S. Y., Kurata, S., Natori, S. and Lee, B. L. (1994). Purification and Molecular Cloning of cDNA for an inducible antibacterial protein from larvae of the coleopteran, Tenibrio molitor. . *J Biochem* . 116 53-58.

Moon, N. S., Stefano, L. D., Morris, E. J., Patel, R., White, K. and Dyson, N. J. (2008). E2F and p53 induce apoptosis independently during Drosophila development but intersect in the context of DNA damage. *PLoS Genet* 4.

Moreno, E., Yan, M. and Basler, K. (2002). Evolution of TNF Signaling Mechanisms:JNK-Dependent Apoptosis Triggered by Eiger, the Drosophila Homolog of the TNF Superfamily. *Current Biology* 12, 1263-1268.

Moret, Y. and Schmid-Hempel, P. (2000). Survival for immunity: The price of imune system activation for bumblebee workers. *Science* 290, 1166-1168.

Mowlds, P., Barron, A. and Kavanagh, K. (2008). Physical stress primes the immune response of Galleria mellonella larvae to infection by Candida albicans. *Microbes and Infection* 10, 628-634.

Mukae, N., Yokoyama, H., Yokokura, T., Sakoyama, Y. and Nagata, S. (2002). Activation of the innate immunity in Drosophila by endogenous chromosomal DNA that escaped apoptotic degradation. *Genes Dev* 16, 2662-2671.

Mulinari, S., Häcker, U. and Castillejo-López, C. (2006). Expression and regulation of Spätzle-processing enzyme in Drosophila. *FEBS Lett.* . 580, 5406-10.

Muluk, N. B., Kisa, Ü., Kacmaz, M., Alpaslan and Koc, C. (2005). Efficacy of topotecan treatment on antioxidant enzymes and TBA-RS levels in submandibular glands of rabbits:An experimental study. *Otolaryngol Head Neck Surg* 132, 136-40.

Ollmann, M., Young, L. M., Como, C. J. D., Karim, F., Belvin, M., Robertson, S., Whittaker, K., Demsky, M., Fisher, W. W., Buchman, A. et al. (2000). Drosophila p53 Is a Structural and Functional Homolog of the Tumor Suppressor p53. *Cell* 101, 91-101.

Ondrousková, E., Soucek, K., Horváth, V. and Smarda, J. (2008). Alternative pathways of programmed cell death are activated in cells with defective caspase-dependent apoptosis. *Leuk Res* 32, 599-609.

Oren, M. (1999). Regulation of the p53 Tumor suppressor protein. *The journal of biological chemistry* 274, 36031-36034.

Orntoft, T. F., Thykjaer, T., Waldman, F. M., Wolf, H. and Celis, J. E. (2002). Genome-wide study of gene copy numbers, transcripts, and protein levels in pairs of non-invasive and invasive human transitional cell carcinomas. *Mol Cell Proteomics* 1, 37-45.

Otvos, L. (2000). Antibacterial peptides isolated from insects. *J Pept Sci.* 6, 497-511.

Otvos, L. (2002). The short proline-rich antibacterial peptide family. *Cell Mol Life Sci* 59, 1138-50.

Ou, H. D., Löhr, F., Vogel, V., Mäntele, W. and Dötsch, V. (2007). Structural evolution of C-terminal domains in the p53 family. *EMBO J* 26, 3463-3473.

Owusu-Ansah, E., Yavari, A., Mandal, S. and Banerjee, U. (2008). Distinct mitochondrial retrograde signals control the G1-S cell cycle checkpoint. *Nat Genet* 40, 356- 361.

Pal, S. and Wu, L. P. (2009). Pattern recognition receptors in the fly: Lessons we can learn from the Drosophila melanogaster immune system. *Fly* 3, 121-129.

Parenta, N., Winstallb, E., Beauchemina, M., Claudie Paqueta, Poirierb, G. G. and Bertranda, R. (2009). Proteomic analysis of enriched lysosomes at early phase of camptothecin-induced apoptosis in human U-937 cells. *Journal of Proteomics* 72, 960-973.

Pelte, N., Robertson, A. S., Zou, Z., Belorgey, D., Dafforn, T. R., Jiang, H., Lomas, D., Reichhart, J.-M. and Gubbf, D. (2006). Immune challenge induces N-terminal cleavage of the Drosophila serpin Necrotic. *Insect Biochem Mol Biol* 36, 37-46.

Perkins, D. N., Pappin, D. J. C., Creasy, D. M. and Cottrell, J. S. (1999). Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 20.

Peters, M., Deluca, C., Hirao, A., Stabolic, V., Potter, J., Zhou, L., Hiepa, J., Snow, B., Arya, A., Wong, J. et al. (2002). Chk2 regulates irradiation-induced, p53 mediated apoptosis in Drosophila. *Proc Natl Acad Sci* 99, 11305-11310.

Petersen, U. M., Bjorklund, G., Ip, Y. T. and Engstrom, Y. (1995). The *dorsal*-related immunity factor, Dif, is a sequence specific trans-activator of *Drosophila* cecropin gene exprerssion. *EMBO Journal* 14, 3146-3158.

Peterson, C., Carney, G. E., Taylor, B. J. and White, K. (2002). Reaper is required for neuroblast apoptosis during Drosophila development *Development* 129, 1467-1476.

Polager, S. and Ginsberg, D. (2008). E2F - at the crossroads of life and death. *Trends Cell Biol* 11, 528-35.

Pretorius, A. (1999). The isolation of Novel components of the Antigen Presentation and Processing Pathway via MHC class I molecules using a Retroviral Promoter Trap in Chinese Hamster Ovary (CHO) cells In *Faculty of Science*, vol. Master of Science (ed. Cape Town: University of The Western Cape.

Price, D. M., Jin, Z., Rabinovitch, S. and Campbell, S. D. (2002). Ectopic expression of the Drosophila Cdk1 inhibitory kinases, Wee1 and Myt1, interferes with the second mitotic wave and disrupts pattern formation during eye development. *Genetics* 161, 721-731.

Pugh, D. J. R., Eiso, A. B., Faro, A., Lutya, P. T., Hoffmann, E. and Rees, D. J. G. (2006). DWNN, a novel ubiquitin-like domain, implicates RBBP6 in mRNA processing and ubiquitin-like pathways. *BMC Struct Biol* 6.

Pujol, N., Link, E. M., Liu, L. X., Kurz, C. L. o., Alloing, G. v., Tan, M.-W., Ray, K. P., Solari, R., Johnson, C. D. and Ewbank, J. J. (2001). A reverse genetic analysis of components of the Toll signaling pathway in Caenorhabditis elegans. *Current Biology* 11, 809-821.

Qi, D., Larsson, J. and Mannervik, M. (2004). Drosophila Ada2b is required for viability and normal histone H3 acetylation. *Molec. Cell. Biol.* 24, 8080-8089.

Ragimov, N., Krauskopf, A., Navot, N., Rotter, V., Oren, M. and Aloni, Y. (1993). Wild-type but not mutant p53 can repress transcription initiation in vitro by interfering with the binding of basal transcription factors to the TATA motif. *Oncogene* 8, 1183-1193.

Rakgotho, P. M. (2007). Identification of proteins that interact with DWNN domain of SNAMA a member of a novel protein superfamily. In *School of Molecular and Cell Biology*, vol. Master of Science (ed. Johannesburg: University of the Witwatersrand.

Rao, X.-J. and Yu, X.-Q. (2010). Lipoteichoic acid and lipopolysaccharide can activate antimicrobial peptide expression in the tobacco hornworm Manduca sexta. *Developmental and Comparative Immunology* 34, 1119-1128.

Rebollar, E., Valadez-Graham, V., Vazquez, M., Reynaud, E. and Zurita, M. (2006). Role of the p53 homologue from Drosophila melanogaster in the maintenance of histone H3 acetylation and response to UV-light irradiation. *FEBS Letters* 580, 642-648.

Rees, J. A., Moniatte, M. and Bulet, P. (1997). Novel Antibacterial peptides isolated from a European Bumblebee, *Bombus pascuorum* (Hymenoptera, Apoidea). *Insect Biochem. Molec. Biol.* 27, 413-422.

Remedios, C. G. d., Berry, D. A., Carter, L. K., Coumans, J. V., Heinke, M. Y., Kiessling, P. C., Seeto, R. K., Thorvaldson, T., Trahair, T., Yeoh, T. et al. (1996). Different electrophoretic techniques produce conflicting data in the analysis of myocardial samples from dilated cardiomyopathy patients: protein levels do not necessarily reflect mRNA levels. *Electrophoresis* 17, 235-8.

Renuci, M., Strambi, C., Strambi, A., Augier, R. and Charpin, P. (1990). Ovaries and Regulation of Juvenile Hormone Titer in Achefa domesticus L. (Orthoptera). *General and Comparative Endocrinology* 78, 137-149.

Richardson, H. and ., S. K. (2002). Death to Flies: Drosophila as a model system to study cell death. *Journal of immunological methods* 265 21-38.

Richardson, H. and Kumar, S. (2002). Death to flies: Drosophila as a model system to study programmed cell death. *Journal of Immunological Methods* 265, 21-38.

Richer, M. J., Keays, C. A., Waterhouse, J., Minhas, J., Hashimoto, C. and Jean, F. (2004). The Spn4 gene of Drosophila encodes a potent furin-directed secretory pathway serpin. *Proc. Natl. Acad. Sci. USA* 101.

Roh, K.-B., Kim, C.-H., Lee, H., Kwon, H.-M., Park, J.-W., Ryu, J.-H., Kurokawa, K., Ha, N.-C., Lee, W.-J., Lemaitre, B. et al. (2009). Proteolytic Cascade for the Activation of the Insect Toll Pathway Induced by the Fungal Cell Wall Component. *J Biol Chem* 284, 19474-19481.

Rolff, J. and Siva-Jothy, M. T. (2002). Copulation corrupts immunity: A mechanism for a cost of mating in insects. *Proc Natl Acad Sci U S A.* 99, 9916-8.

Römer, L., Klein, C., Dehner, A., Kessler, H. and Buchner, J. (2006). p53--a natural cancer killer: structural insights and therapeutic concepts. *Angew Chem Int Ed Engl* 45, 6440-6460.

Rossa, J., Jianga, H., Kanostb, M. R. and Wang, Y. (2003). Serine proteases and their homologs in the Drosophila melanogaster genome: an initial analysis of sequence conservation and phylogenetic
relationships. *Gene* 304, 117-131.

Rougon, D. and Rougon, C. (1991). Dung Beetles of the Sahel Region. In Dung Beetle Ecology. New Jersey: Princeton University Press.

Russo, J., Dupas, S., Frey, F., Carton, Y. and Brehelin, M. (1996). Insect immunity: early events in the encapsulation process of parasitoid (Leptopilina boulardi) eggs in resistant and susceptible strains of Drosophila. *Parasitology* 112, 135-142.

Sadd, B. M. and Siva-Jothy, M. T. (2006). Self-harm caused by an insect's innate immunity. *Proc. R. Soc. B* 273, 2571-2574.

Sagisaka, A., Tanaka, H., Furukawa, S. and Yamakawa, M. (2004). Characterization of a homologue of the Rel/KF-KB transcription factor from a beetle, Allomyrina dichotoma. . *Biochemica et Biophys acta* 1678, 85-93.

Saito, A., Ueda, K., Imamura, M., Atsumi, S., Tabunoki, H., Miura, N., Watanabe, A., Kitami, M. and Sato, R. (2005). Purification and cDNA cloning of a cecropin from the longicorn beetle, Acalolepta luxuriosa. *Comp Biochem Physiol B Biochem Mol Biol* 142, 317-23.

Sakai, Y., Saijo, M., Coelho, K., Kishino, T., Niikawa, N. and Taya, Y. (1995). cDNA Sequence and Chromosomal Localisation of a Novel Human Protein, RBQ-1 (RBBP6), That Binds to the Retinoblastoma Gene Product. *Genomics* 30, 98-101.

Sax, J. K. and El-Deiry, W. S. (2003). p53 downstream targets and chemosensitivity. *Cell Death and Differentiation* 10, 413-417.

Schmidt, F., Rieger, J., Wischhusen, J. r., Naumann, U. and Weller, M. (2001). Glioma cell sensitivity to topotecan: the role of p53 and topotecan-induced DNA damage. *European Journal of Pharmacology* 412, 21-25.

Scholtz, C. H. and Holm, E. (1996). Insects of Southern Africa. Durban: Butterworths.

Schroder, J. M. (1999). Epithelial antimicrobial peptides: innate local host response elements. *Cell. Mol. Life. Sci.* 56, 32-46.

Schumacher, B., Hofmann, K., Boulton, S. and Gartner, A. (2001). The C. elegans homolog of the p53 tumor suppressor is required for DNA damage-induced apoptosis. *Current Biology* 11, 1722-1727.

Scott, R. E., Giannakouros, T., Gao, S. and Peidis, P. (2003). Functional potential of P2P-R: A role in the cell cycle and cell differentiation related to its interactions with proteins that bind to matrix associated regions of DNA? *Journal of Cellular Biochemistry* 90, 6-12.

Seameco, T. (2004). Investigation into the role of DWNN in cell death. In *Department of Biotechnology*, vol. Masters in Science (ed. Cape Town: University of the Western Cape.

Sekelsky, J. J., Burtis, K. C. and Hawley, R. S. (1998). Damage Control: The Pleiotropy of DNA Repair Genes in Drosophila melanogaster. *Genetics* 148, 1587-1598.

Seo, Y. R., Fishel, M. L., Amundson, S., Kelley, M. R. and Smith, M. L. (2002). Implication of p53 in base excision DNA repair: in vivo evidence. *Oncogene* 21, 731-737.

Serbus, L. R. and Sullivan, W. (2007). A Cellular Basis for Wolbachia Recruitment to the Host Germline. *PLoS Pathogens* 3, 1930-1937.

Shin, S. W., Kokoza, V., Lobkov, I. and Raikhel, A. S. (2003). Relish-mediated immune deficiency in the transgenic mosquito Aedes aegypti. . *Proc. Nat. Acad. Sci. USA* 100, 2616-2621.

Shin, Y.-K., Hong, Y. S., Chang, H. J., Jung, K. H., Jeong, S.-Y. and Park, J.-G. (2009). Upregulation of glycolytic enzymes in proteins secreted from human colon cancer cells with 5-fluorouracil resistance. *Electrophoresis* 30, 2182-2192.

Sigrist, S., Jacobs, H., Stratmann, R. and Lehner, C. F. (1995). Exit from mitosis is regulated by Drosophila fizzy and the sequential destruction of cyclins A, B and B3. *EMBO J* 14, 4827-4838.

Silva, J. B. D., Albuquerque, C. M. R. D., Araujo, E. C. D., Peixoto, C. A. and Hurd, H. (2000). Immune defense Mechanisms of *Culex quinquefasciatus* against *Candida albicans* infection. *Journal of invertebrate Pathology* 76, 257-262.

Simon, A. F., Shih, C., Mack, A. and Benzer, S. (2003). Steroid Control of Longevity in Drosophila melanogaster. *Science* 299,, 1407-1410.

Simons, A., Melamed-Bessudo, C., Wolkowicz, R., Sperling, J., Sperling, R., Eisenbach, L. and Rotter, V. (1997). PACT: cloning and characterization of a cellular p53 binding protein that interacts with Rb. *Oncogene.* 2, 145-55.

Skepu, A. (2005). Identification and characterisation of a novel gene, DWNN, isolated from promoter-trapped Chinese hamster ovary cells. In *Faculty of Science*, vol. Doctor of Philosophy (ed. Cape Town: University of the Western Cape.

Smith, M. L., Ford, J. M., Hollander, C., Bortnick, R. A., Amundson, S. A., Seo, Y. R., Deng, C.-X., Hanawalt, P. C. and Fornace, A. J. (2000). p53-Mediated DNA Repair Responses to UV Radiation: Studies of Mouse Cells Lacking p53, p21, and/or gadd45 Genes. *Mol Cell Biol* 20, 3705-3714.

Sogame, N., Kim, M. and Abrams, J. M. (2003). Drosophila p53 preserves genomic stability by regulating cell death. *Proc Natl Acad Sci U S A* 100, 4696-4701.

Soller, M., Bownes, M. and KublI, E. (1999). Control of Oocyte Maturation in Sexually Mature Drosophila Females. *Developmental Biology* 208, 337-351.

Song, J. and Tanouye, M. A. (2008). From bench to drug: Human seizure modeling using Drosophila. *Progress in Neurobiology* 84 182-191.

Song, Y.-H., Mirey, G., Betson, M., Haber, D. A. and Settleman, J. (2004). The Drosophila ATM Ortholog, dATM, Mediates the Response to Ionizing Radiation and to Spontaneous DNA Damage during Development. *Current Biology* 14, 1354-1359.

Song, Y. H. (2005). Drosophila melanogaster: a model for the study of DNA damage checkpoint response. *Mol Cells.* 19, 167-79.

Stallings, D. M., Hepburn, D. D. D., Hannah, M., Vincent, J. B. and O'Donnell, J. (2006). Nutritional supplement chromium picolinate generates chromosomal aberrations and impedes progeny development in Drosophila melanogaster. *Mutation Research* 610, 101-113.

Steiner, H., Hultmark, D., Engstrom, A., Bennich, H. and Boman, H. G. (1981). Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature* 292, 246-248.

Stronach, B. (2005). Dissecting JNK signaling, one KKKinase at a time. *Developmental Dynamics* 232, 575-584.

Sun, A., Bagella, L., Tutton, S., Romano, G. and Giordano, A. (2007). From G0 to S phase: a view of the roles played by the retinoblastoma (Rb) family members in the Rb-E2F pathway. *J Cell Biochem* 102, 1400-1404.

Sutcliffe, J. E. and Brehm, A. (2004). Of flies and men; p53, a tumour suppressor. *FEBS Lett* 567, 86-91.

Sutcliffe, J. E., Korenjak, M. and Brehm, A. (2003). Tumour suppressors-a fly's perspective. *European Journal of Cancer* 39, 1355-1362.

Tabata, M., Tabata, R., Grabowski, D. R., Bukowski, R. M., Mahrukh K. Ganapathi and Ganapathi, R. (2001). Roles of NF-kB and 26 S Proteasome in Apoptotic Cell Death Induced by Topoisomerase I and II Poisons in Human Nonsmall Cell Lung Carcinoma. *J. Biol. Chem* 276, 8029–8036.

Takeba, Y., Sekine, S., Kumai, T., Matsumoto, N., Nakay, S., Tsuzuki, Y., Yanagida, Y., Nakano, H., Asakura, T., Ohtsubo, T. et al. (2007). Irinotecan-Induced Apoptosis Is Inhibited by Increased P-Glycoprotein Expression and Decreased p53 in Human Hepatocellular Carcinoma Cells. *Biol. Pharm. Bull.* 30, 1400-1406.

Takeda, K., Noguchi, T., Naguro, I. and Ichijo, H. (2008). Apoptosis signal-regulating kinase 1 in stress and immune response. *Annu Rev Pharmacol Toxicol* 48, 199-225.

Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986). Staurosporine, a potent inhibitor of phospholipid/Ca++dependent protein kinase. *Biochemical and Biophysical Research Communications* 135, 397-402.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution* In Press.

Tanaka, H., Sagisaka, A., Fujita, K., Kaneko, Y., Imanishi, S. and Yamakawa, M. (2009). Lipopolysaccharide elicits expression of immune-related genes in the silkworm, Bombyx mori. *Insect Molecular Biology* 18, 71-75.

Tanaka, T., Utsunomiya, T., Utsunomiya, H. and Umesaki, N. (2008). Irinotecan HCl, an anticancer topoisomerase I inhibitor, frequently induces ovarian failure in premenopausal and perimenopausal women. *Oncology Reports* 19: , 1123-1133.

Thangaraju, M., Gopal, E., Martin, P. M., Ananth, S., Smith, S. B., Prasad, P. D., Sterneck, E. and Ganapathy, V. (2006). SLC5A8 triggers tumor cell apoptosis through pyruvate-dependent inhibition of histone deacetylases. *Cancer Res.* 66.

Toledo, F., Lee, C. J., Krummel, K. A., Rodewald, L.-W., Liu, C.-W. and Wahl, G. M. (2007). Mouse Mutants Reveal that Putative Protein Interaction Sites in the p53 Proline-Rich Domain Are Dispensable for Tumor Suppression *Moleular and Cellular Biology* 27, 1425-1432.

Tomicic, M. T., Christmann, M. and Kaina, B. (2005). Topotecan-triggered degradation of topoisomerase I is p53-dependent and impacts cell survival. *Cancer Res* 65, 8920-8926.

Tomie, T., Ishibashi, J., Furukawa, S., Kobayashi, S., Sawahata, R., Asaoka, A., Tagawa, M. and Yamakawa, M. (2003). Scarabaecin, a novel cysteine-containing antifungal peptide from the rhinoceros beetle, *Oryctes rhinoceros. Biochem Biophys Res Commun.* 307, 261-6.

Tower, J. (2006). Sex-specific regulation of aging and apoptosis. *Mechanisms of Ageing and Development* 127, 705-718.

Trenczek, T. (1997). Biological mediators of insect immunity. *Annu. Rev. Entomol* 42, 611-643.

Tweedie, S., Ashburner, M., Falls, K., Leyland, P., McQuilton, P., Marygold, S., Millburn, G., Osumi-Sutherland, D., Schroeder, A., R. Seal et al. (2009). FlyBase: enhancing Drosophila Gene Ontology annotations.
. *Nucleic Acids Research* 37, D555-D559.

Tyndale-Biscoe, M. (1978). Physiological age-grading in females of the dung beetle Euoniticellus intermedius (Reiche) (Coleoptera: Scarabaeidae). *Bulletin of Entomological Research* 6, 207-217.

Utsunomayi, T., Tanaka, T., Utsunokiya, H. and Umesaki, N. (2008). A novel molecular mechanism for anticancer drug-induced ovarian failure: Irinotecan HCl, an anticancer topoisomerase I inhibitor, induces specific FasL expression in granulosa

cells of large ovarian follicles to enhance follicular apoptosis. *International Journal of Oncology* 32, 991-1000.

Valanne, S., Kleino, A., Myllymäki, H., Vuoristo, J. and Rämet, M. (2007). Iap2 is required for a sustained response in the Drosophila Imd pathway. *Dev Comp Immunol* 31, 991-1001.

Varela, P. F., Llera, A. S., Mariuzza, R. A. and Tormo, J. (2002). Crystal Structure of Imaginal Disc Growth Factor-2. *J. Biol. Chem.* 277, 13229–13236.

Vasselon, T. and Detmers, P. A. (2002). Toll Receptors: a central element in Innate Immune Responses. *Infection and Immunity* 70, 1033-1041.

Vermeulen, C. J. and Loeschcke, V. (2007). Longevity and the stress response in Drosophila. *Exp Gerontol* 42, 153-9.

Vierstraete, E., Verleyen, P., Baggerman, G., D'Hertog, W., Bergh, G. V. d., Arckens, L., Loof, A. D. and Schoofs, L. (2004). A proteomic approach for the analysis of instantly released wound and immune proteins in Drosophila melanogaster hemolymph. *PNAS* 101, 470-475.

Villa, P., Kauffmann, S. H. and Earnshaw, W. C. (1997). Caspases and caspase inhibitors. *TIBS* 22, 388-93.

Vilmos, P. and Kurucz, E. (1998). Insect immunity: evolutionary roots of the mammalian innate immune system.. *Immunology letters* 62, 59-66.

Wang, L. and Ligoxygakis, P. (2006). Pathogen recognition and signalling in the Drosophila innate immune response. *Immunobiology* 211, 251-261.

Wang, Z. and Wang, G. (2004). APD: the Antimicrobial Peptide Database. *Nucleic Acids Res.* 32, D590-2.

Warburg, O. (1956). On the origin of cancer cells. *Science* 123, 309-314.

Weber, A. N. R., Moncrieffe, M. C., Gangloff, M., Imler, J.-L. and Gay, N. J. (2005). Ligand-Receptor and Receptor-Receptor Interactions Act in Concert to Activate Signaling in the Drosophila Toll Pathway. *J. Biol. Chem.* 280, 22793-22799.

Wells, B. S., Yoshida, E. and Johnston, L. A. (2006). Compensatory Proliferation in Drosophila Imaginal Discs Requires Dronc-Dependent p53 Activity. *Current Biology* 16, 1606-1615.

Werner, T., Liu, G., Kang, D., Ekengren, S., Steiner, H. and Hultmark, D. (2000). A family of peptidoglycan recognition proteins in the fruit fly *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* 97, 13772-13777.

Whitten, M. M. A., Tew, I. F., Lee, B. L. and Ratcliffe, N. A. (2004). A Novel Role for an Insect Apolipoprotein (Apolipophorin III) in B-1,3-Glucan Pattern Recognition and Cellular Encapsulation Reactions. *J Immunol* 172, 2177-2185.

Wichmann, A., Jaklevic, B. and Su, T. T. (2006). Ionizing radiation induces caspase-dependent but Chk2- and p53-independent cell death in Drosophila melanogaster. *Proc Natl Acad Sci U S A* 103, 9952-9957.

Witte, M. M. and Scott, R. E. (1997). The proliferation potential protein-related (P2P-R) gene with domains encoding heterogeneous nuclear ribonucleoprotein association and Rb1 binding shows repressed expression during terminal differentiation. *Proc. Natl. Acad.Sci. USA* 94, 1212-1217.

Wu, Q.-Y., Li, F., Zhu, W.-J. and Wang, X.-Y. (2007). Cloning, expression, purification, and characterization of arginine kinase from Locusta migratoria manilensis. *Comparative Biochemistry and Physiology* 148 355-362.

Yamaauchi, H. (2001). Two novel insect defensins from larvae of the cupreous chafer *Anomala cuprea:* purification, amino acid sequences and antibacterial activity. *Insect Bioc. Mol. Biol.* 32, 75-84.

Yamada, Y., Davis, K. D. and Coffman, C. R. (2008). Programmed cell death of primordial germ cells in Drosophila is regulated by p53 and the Outsiders monocarboxylate transporter. *Development.* 135, 207-216.

Yoon, H. S., Lee, C. S., Lee, S. Y., Choi, C. S., Lee, I. H., Yeo, S. M. and Kim, H. R. (2003). Purification and cDNA cloning of inducible antibacterial peptides from Protaetia brevitarsis (Coleoptera). *Archives of Insect Biochemistry and Physiology* 52, 92 - 103.

Yoshida, H., Kinoshita, K. and Ashida, M. (1996). Purification of a peptidoglycan recognition protein from hemolymph of the silkworm, *Bombyx mori. Journal of biological Chemistry* 271, 13854-13860.

Yoshiyama, T., Namiki, T., Mita, K., Kataoka, H. and Niwa, R. (2006). Neverland is an evolutionally conserved Rieske-domain protein that is essential for ecdysone synthesis and insect growth. *Development* 133, 2565-2574.

Yu, Y., Wang, L.-S., Shen, S.-M., Xia, L., Zhang, L., Zhu, Y.-S. and Chen, G.-Q. (2007). Subcellular Proteome Analysis of Camptothecin Analogue NSC606985-Treated Acute Myeloid Leukemic Cells. *Journal of Proteome Research* 6, 3808-3818.

Zamaraeva, M. V., Sabirov, R. Z., Maeno, E., Ando-Akatsuka, Y., Bessonova, S. V. and Okada, Y. (2005). Cells die with increased cytosolic ATP during apoptosis: a bioluminescence study with intracellular luciferase. *Cell Death and Differentiation* 12, 1390-1397.

Zhang, Y., Zhang, J., Jiang, X., Wang, G., Luo, Z., Fan, Y., Wu, Z. and Pei, Y. (2010). Requirement of a Mitogen-Activated Protein Kinase for Appressorium Formation and Penetration of Insect Cuticle by the Entomopathogenic Fungus Beauveria bassiana. *Applied and Environmental Microbiology* 76, 2262-2270.

Zheng, J., Edelman, S. W., Tharmarajah, G., Walker, D. W., Pletcher, S. D. and Seroude, L. (2005). Differential patterns of apoptosis in response to aging in Drosophila. *Proc Natl Acad Sci U S A* 102, 12083-8.

Zhou, R., Silverman, N., Hong, M., Liao, D. S., Chung, Y., Chen, Z. J. and Maniatis, T. (2005). The role of ubiquitination in Drosophila innate immunity. *J Biol Chem* 280, 34048-55.

Zou, Z., Evans, J. D., Lu, Z., Zhao, P., MichaelWilliams, Sumathipala, N., Hetru, C., Hultmark, D. and Jiang, H. (2007). Comparative genomic analysis of the Tribolium immune system. *Genome Biology* 8, R177.

Zuco, V., Benedetti, V. and Zunino, F. (2009). ATM- and ATR-mediated response to DNA damage induced by a novel camptothecin, ST1968. *Cancer Letters*.

Research output

Conference proceedings attended

Rodney Hull, Ricardo Antunes, Tobias Ntuli, MphoRakgotho and Monde Ntwasa, Characterisation of the ubiquitin like protein SNAMA in *Drosophila melanogaster:*The third International Conference Ubiquitin, Ubiquitin-like Proteins & Cancer:February 9-11, 2006 The University of Texas M. D. Anderson Cancer Center, Houston, Texas

Rodney Hull, and Monde Ntwasa, Proteomic analysis of molecules involved in *E*uoniticellus *intermedius* immune response: 2006 MCBG Symposium: 5th October, 2006. University of the Witwatersrand Medical School

Ricardo Antunes, Rodney Hull, and Monde Ntwasa, Ubiquitin like protein SNAMA: Ubiquitin ligase activity and its role in DNA damage response pathways: 2006 MCBG Symposium: 5th October, 2006. University of the Witwatersrand Medical School

Monde Ntwasa, Chris Arnot, Rodney Hull and Tobias Ntuli, International Conference on Systems Biology.: October 19-24, 2005: Protein networks that are activated in response to DNA damage.: Boston. USA

Rodney Hull, Chris Arnot and Monde Ntwasa.Identification of proteins involved in the immune system of the beetle *Euoniticellus intermedius*: 16th-20th January 2005: *SASBMB XIXth Conference. Stellenbosch University*

Articles in peer reviewed journals

1. **Rodney Hull and Monde Ntwasa, (2010).** Glycolytic flux occurs in Drosophila melanogaster recovering from camptothecin treatment. *Anticancer Drugs*, **21** (10), 945-57.

2. **Lucky M Khanyile, Rodney Hull and Monde Ntwasa (2008**). Dung beetle database: comparison with other invertebrate transcriptomes, Bioinformation.**3** (4), 159–161.

3. Rodney Hull, Lucky Khanyile, Marcus Byrne and Monde Ntwasa (2011). Lifestyle and host defence mechanisms of the dung beetle *Euoniticellus intermedius* (Coleoptera: Scarabaeidae) .*Journal of Insect Science* **(submitted).**

Collaborations :

In 2010 I visited the laboratory of our collaborators in Strasbourg University to learn advanced Mass Spectrometry

In 2011 I visited another collaborator's laboratory at Tohoku University, Japan to learn RNA interference techniques.