Universidade de Aveiro
2004

Departamento de Biologia

Sofia de Morais Correia Pereira Guedes

Resposta imunológica da *Drosophila melanogaster*: uma abordagem utilizando metodologia proteómica

Drosophila melanogaster immune response: a proteomic approach

Universidade de Aveiro
2003

Departamento de Biologia

Sofia de Morais Correia Pereira Guedes

Resposta imunológica da *Drosophila melanogaster*: uma abordagem utilizando metodologia proteómica

Drosophila melanogaster immune response : a proteomic approach

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia Molecular, realizada sob a orientação científica do Doutor Pedro Miguel Dimas Neves Domingues, Professor Auxiliar do Departamento de Química da Universidade de Aveiro, e do Doutor Francisco Manuel Lemos Amado, Professor Auxiliar do Departamento de Química da Universidade de Aveiro

O júri

presidente

Doutor Edgar Figueiredo da Cruz e Silva
professor associado da Universidade de Aveiro

Doutora Ana Maria de Jesus Bispo Varela Coelho
professora associada do Instituto de Tecnologia Química e Biológica da Universidade Nova de Lisboa

Doutor Francisco Manuel Lemos Amado
professor auxiliar da Universidade de Aveiro

Doutor Pedro Miguel Dimas Neves Domingues
professor auxiliar da Universidade de Aveiro

Acknowledgments

My advisers, Prof. Pedro Domingues and Prof. Francisco Amado, for scientific guidance and for constant support during this journey.

Prof. Kenneth Tomer (Triangle Area Mass Spectrometry, NIEHS, USA) for the excellent collaboration in the protein identification.

Rui for the spot identifications and for always being there for me, with never-ending patience, support and care.

Prof. Rosário Domingues for the friendship and for the smile and optimism at all times.

My lab colleagues, Rita, Henrique, Judite, Helena S., Sofia, Ana, for the help and friendly support through tough times.

Cristina Barros for always attending me, nice and friendly.

My friends who care for me.

My family and, most of all, my mother. Without their love, comprehension and support none of this would have been achieved.

resumo

Os Insectos, tal como *Drosophila melanogaster*, são capazes de reconhecer eficientemente microorganismos invasores e montar uma rápida e potente resposta imunológica quer face a infecções bacterianas quer face a infecções fúngicas. Durante a última década, a mosca da fruta emergiu como um modelo animal promissor para o estudo da imunidade inata, em parte devido ao profundo conhecimento da sua genética. Os inúmeros estudos realizados neste tema, a resposta imunológica da *Drosophila*, indicam claramente que quando activado o sistema de defesa do organismo um elevado número de genes é induzido/reprimido, em adição aos já bem estudados genes dos peptídeos antimicrobianos. No entanto, a sua contribuição no combate à infecção ainda não foi aprofundada. Através da utilização dos avanços mais recentes na análise do proteoma foi construído, por electroforese bidimensional, um mapa das proteínas da hemolinfa de larvas de *Drosophila*. 105 pontos foram retirados e submetidos a identificação por espectrometria de massa e um total de 99 identificações positivas foram obtidas utilizando uma combinação de espectros de MALDI-TOF/TOF MS e MS/MS. Na lista de proteínas identificadas estão incluídas enzimas metabólicas, proteínas estruturais, componentes da maquinaria translacional, proteínas de choque térmico e proteínas envolvidas em mecanismos de defesa imunológica e antioxidante. O estudo da sua função celular, assim como o seu enquadramento nas vias metabólicas correspondentes, contribuirá para uma melhor compreensão dos mecanismos moleculares da resposta imunológica de *Drosophila*.

abstract

Insects, including *Drosophila melanogaster*, are able to efficiently recognize invading intruders and to mount a potent and rapid innate immune reponse to both bacterial and fungal infections. In the last decade, the fruit fly has emerged as a promising invertebrate model to investigate innate immunity, in part because of it's well characterized genetics. The information provided by the inumerous reports on *Drosophila*'s immune response indicates that a high number of genes are both up- and down-regulated, in addition to the well-known antimicrobial peptide genes, upon immune challenge. Nevertheless, their contribution in fighting off infection has not been seriously addressed. With the application of recent advances in proteomics, a 2-DE protein map of *Drosophila* larvae hemolymph was constructed. A total of 105 protein spots were excised and submitted to identification by mass spectrometry, using a combination of MALDI-TOF/TOF MS and MS/MS spectra and resulting in 99 positive identifications. The list of identified protein spots includes metabolic enzymes, structural proteins, translational apparatus components, heat shock proteins and proteins involved in defence mechanisms, such as antioxidant and immunological reactions. The study of their cellular function, as well as enchaining the overall biochemical information, will contribute to a better understanding of the underlying molecular mechanisms of *Drosophila*'s immune response.

Index

Abbreviattions

2-DE	Two dimensional electrophoresis
2D-PAGE	Two dimensional polyacrylmide gel electrophoresis
APS	Ammonium persulphate
ATP	Adenosin triphosphate
BSA	Bovine serum albumin
CD36	Cell surface antigen 36
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate
CRQ	Croquemort
Da	Daltons
DIF	Dorsal-related immunity factor
DmFADD	*Drosophila melanogaster* FAS-associated death domain protein
DNA	Desoxyribonucleic acid
DREDD	Death related ced-3/NEDD2-like protein
DTAK 1	*Drosophila* transforming growth factor β activated kinase
DTT	Dithiothreitol
EDTA	Ethylenodiaminetetraacetic acid
EMP	Epithelial membrane protein
EMS	Ethyl methane sulfonate
$FADH_2$	flavin adenine dinucleotide (reduced form)
FK506BP	FK506 binding protein

GNBP	Gram-negative bacteria-binding protein
GSH	Glutathione (reduced form)
GSSG	Glutathione disulfide (oxidized form)
Hsp	Heat shock protein
IEF	Isoelectric focusing
IkB	Nuclear factor I-kappa B
IKK	I-Kappa kinase
IKKβ	Nuclear factor I-kappa B β-kinase
IKKγ/NEMO	I-Kappa B kinase gamma modulator
IL-1R	Interleukin receptor 1
Imd	Immune deficiency
IPG	Immobilized pH gradient
KDa	KiloDaltons
LPP	Lipopolysaccharide
MALDI	Matrix-assisted laser desorption ionization
MAPKKK	Mitogen-activated protein kinase kinase kinase
mRNA	Ribonucleic acid messenger
MS	Mass spectrometry
Mw	Molecular weight
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NDP	Nucleoside diphosphate
NF-kB	Nuclear factor kappa-B
OD	Optical density

PAMP	Pathogen associated molecular patterns
PGR	Peptidoglycan recognition
PGRP	Peptidoglycan recognition protein
pI	Isoelectric point
PO	Phenol oxidase
PPIase	Peptidyl-prolyl isomerase
PPO	Prophenol oxidase
PRR	Pattern recognition receptor
RIP	Receptor interacting protein
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
Rpm	rounds per minute
RT	Recovery time
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoreis
SR	Scavenger receptor
TEMED	N,N,N',N'-tetramethylenediamine
TNF-α	Tumor necrosis factor-α
TOF	Time-of-flight
TRAP1	Tumor necrosis factor receptor associated protein 1
Tris-HCl	Tris (hydroxymethyl)-aminoethane chloride
UV	ultraviolet

1. Introduction

Insects are probably the first successful scholars of a combinatorial chemistry (Otvos, 2000). At present, they are found in most of the biological niches, nearly all terrestrial and freshwater habitats.

The class Insecta contains far more species than any other class of animals or the entire plant kingdom. More than one million of insect species have been described and it is estimated that as many as 30 million species may exist. They demonstrate a remarkable evolutionary success as these creatures are constantly exposed to potentially pathogenic microorganisms and eukaryotic parasites. Their great diversity, as we know it today, was achieved not by high reproduction rates but rather by low extinction rates when compared to other animal groups (Labandeira and Sepkoski, 1993). The resistance of insects to infectious agents has certainly contributed to their extreme proliferation and diversity (Bulet *et al.*, 1999), and it reveals as being very important and essential not only for their own survival but also for the health of the plant, animal and human populations with which they closely interact (Dimopoulos, 2003). Several of the major human diseases are spread by insects and are rapidly expanding as a result on the development of insecticide resistance in vectors and drug resistance in parasites.

Historically, the study of insect immunity started with much of the very early work on the general question immunology in the 1920's, a period of considerable scientific activity. By the early twenties, it was understood that septic injury induced the appearance in the cell-free

hemolymph of large-spectrum potent antimicrobial activity (Glaser, 1918; Metalnikow, 1920; Paillot, 1920; all reviewed in Hetru *et al.*, 2003). In the late 1950's, when it was discovered that certain immunity could be induced in some insects, a second rush of work on insect immunity started. The most recent period of research started in 1980 (with the first isolation and purification of an induced antibacterial factor), when these activities were attributed to inducible cationic antimicrobial peptides as the cecropins and attacins revealed by Steiner *et al.*'s (1981) work. An increasing number of antimicrobial peptides were then identified from insects and, later, from mammalian species. It is now thought that all metazoans rely on various types of these bio-molecules as part of their host defence (Ganz *et al.*, 1999; reviewed in Hetru *et al.*, 2003). The discovery of antimicrobial peptides represented the turning point in the appreciation and research on the innate immunity in detriment of the adaptive immunity.

Unlike vertebrates, which possess acquired immunity with "immunological memory" based on antigen-specific selection of antibodies and receptors, the innate immune response of insects relies on the recognition of common microbial strucutures (such as peptidoglycans or lipopolisaccharides) to mount a generic and systemic response, which is considered to be invariant against all types of infections and unaltered to sub-sequent challenges (Carton and Nappi, 2001). Nevertheless, our knowledge today indicates us that the innate immune system of insects comprises a variety of components and

mechanisms that can discriminate between different microorganisms and mount specific responses to control pathogenic infections.

Much of the attention that the innate immunity subject has received in the past few years can be largely attributed to the model organism *Drosophila*. In fact, an impressive body of knowledge on insect´s innate immunity has been generated from studies in *Drosophila* (reviewed in Hoffmann, 2003). It becomes pertinent to ask ourselves why *Drosophila* is considered a useful animal model for studying immunity.

1.1. *Drosophila* as an animal study model

O'Kane (O'Kane, 2003) reviews the use of *Drosophila* and *C. elegans* as powerful tools for studying diseases, as these organisms possess many pathways that are well conserved between them and humans.

Although having no adaptive immune response and being like most invertebrates highly resistant to microbial infections, *Drosophila* is particularly well suited to the study of innate immunity (Hoffmann and Reichhart, 2002). The power of molecular genetics (together with the fully sequenced genome) and biochemistry makes *Drosophila* the probably best available model to date to investigate innate immunity. Despite this "genomic appreciation", this organism represents an

undemanding laboratory pet and can easily be handled and bred in large numbers. In addition, little space is needed and the feeding medium is inexpensive and rapidly prepared (Mota, 2003). Furthermore, it is now becoming clear that humans, worms and flies share many genes and cellular mechanisms in common and that flies, for instance, can suffer from diseases in a way similar to those of humans. Studies focusing on innate immunity, in model insects as *Drosophila*, can be used to gain insights into cellular mechanisms of combating an infectious disease. Because molecular mechanisms controlling these specific biological processes are conserved between *Drosophila* and mammals, extrapolation can be done in order to better understand these subjects. This is also true for the understanding of normal cellular functions, for instance, subjects like ageing (Mandavilli *et al.,* 2002, Orr *et al.,* 2003), cancer (Richardson *et al.,* 2002; Claveria *et al.,* 2003) and other disorders of physiological control (Hendricks, 2003; Lasko, 2002).

1.2. *Drosophila*'s innate immune response

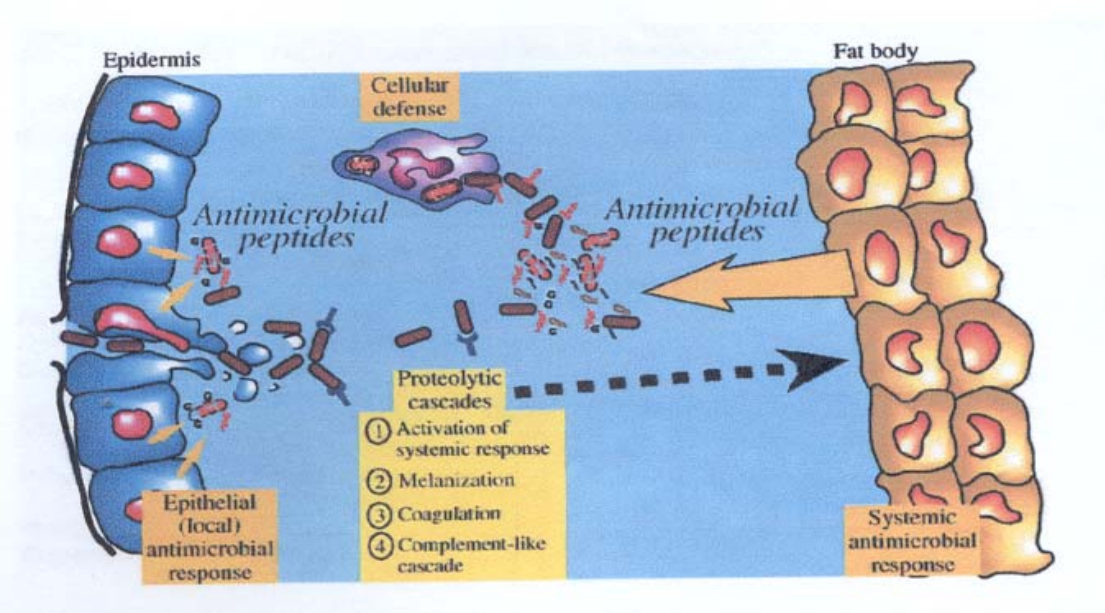
Insects are exposed to a variety of infectious microbes in their habitats throughout their life cycle (Dimopoulos, 2003), co-existing with microorganisms in numerous ways. For instance, insect larvae often develop in decaying organic matter, and insect adults often serve as vectors for microbes, including the ones that cause plant and animal

disease. It is not surprising, then, that insects have sensitive mechanisms for identifying pathogens and an array of strategies for defending themselves against microbial attack (Khush and Lemaitre, 2000).

In order to cope with the risk of infection, due to this frequent and diverse exposure, *Drosophila* like other insects have then developed several structural barriers and a multifaceted innate immune system comprising a variety of synergistic mechanisms. This multifaceted process of *Drosophila* immune response (reviewed in Hetru *et al.*, 2003) involves humoral and cellular reactions that culminate in the destruction of invading organisms by lytic peptides.

The first line of defence against microbes is represented by the structural barriers (Hoffmann and Reichhart, 2002; Dimopoulos, 2003). These include the hardened outer exoskeleton, the peritrophic matrix of the midgut and the epidermis (cells of the digestive and genital tracts) of the tracheae and of the Malpighian tubules which produce antimicrobial peptides that inhibit microbial growth. In addition, *Drosophila* maintains a low pH and accumulates digestive enzymes and antibacterial lysozymes in it´s midgut. The exoskeleton protects the insect organs and the hemolymph from direct exposure and upon fissure it is rapidly sealed through coagulation and melanization reactions (Söderhäll and Cerenius, 1998; Theopold *et al.*, 2002; reviewed in Dimopoulos, 2003), with the production of cytotoxic molecules at the site of wounding.

Pathogens that successfully enter the general body cavity (called the hemocoele) will encounter the host innate immune system that



comprises both cellular and humoral defence mechanisms (figure 1-1).

Figure 1-1: The antimicrobial host defence of *Drosophila* - bacteria are illustrated as brown rods; recognition protein factors as purple pincers; and putative opsonizing proteins as red T-shapes (adapted from Hoffmann and Reichhart, 2002).

The cellular defences consist essentially of phagocytosis by macrophage-like cells, called the plasmatocytes. Nevertheless, other cell types present in the hemolymph are important in controlling infection. Whereas these cells are capable of engulfing microbes, larger pathogens are encapsulated by a specialized flattened blood cell called the lamellocyte (De Gregorio *et al.*, 2001; Hoffmann and Reichhart, 2002). Furthermore, the crystal cells release granules containing components required for clotting and melanization reactions at the wounding site (Wu *et al.*, 2001).

The hallmark of the humoral reactions is the systemic antimicrobial response. It corresponds to the challenge-induced synthesis and release of antimicrobial peptides by the fat body (an organ equivalent to the mammalian liver), in result of signalling pathways activation. These peptides are directly secreted into the hemolymph (analogous to the circulatory system of mammals, as *Drosophila* lacks a true one), where their combined concentrations can reach 300 μM in infected flies (Hoffmann and Reichhart, 2002). Figure 1-2 summarizes our today understanding of these petides.

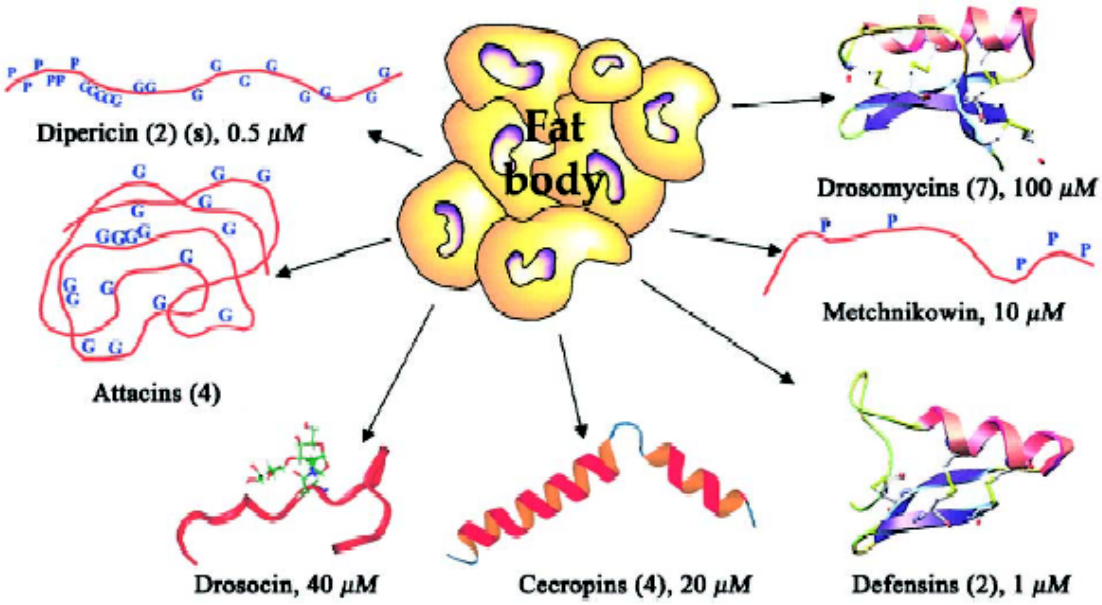


Figure 1-2: The *Drosophila*'s fat body produces 7 distinct antimicrobial peptides (or peptide families). Drosomycins, metchnikowin, defensins, cecropins and drosocin have been biochemically isolated from immune-challenged flies and their genes have been cloned. Concentrations refer to values observed in hemolymph 24 hours after immune challenge. The numbers in parentheses

represent the number of paralogues for each type of gene in the *Drosophila melanogaster* genome (adapted from Hetru *et al.*, 2003).

These antimicrobial peptides have mostly a large spectrum of activity, although they have preferential targets. In regard to their activity spectra, it is well known that drosomycin and metchnikowin are essentially antifungal molecules; defensin (and also metchnikowin) is active against gram-positive bacteria; cecropins, drosocin, attacins and diptericins are active against gram-negative bacteria and some fungal strains (Hoffmann *et al.*, 1999; Bulet *et al.*, 1999; Otvos, 2000; Meister *et al.*, 2000; Imler and Hoffmann, 2000). None of these peptides is cytotoxic for the host at the concentrations found in the hemolymph. Their mode of action is not fully understood and probably is not unique. They are essentially membrane active and induce perturbations on the prokaryotic or fungal cell membrane or interference with membrane assembly or bacterial proteins, leading consequently to efflux of solutes and often to rapid death of the pathogen.

The humoral reactions also involve several proteolytic cascades. One example is shown on figure 1-3, the melanization cascade.

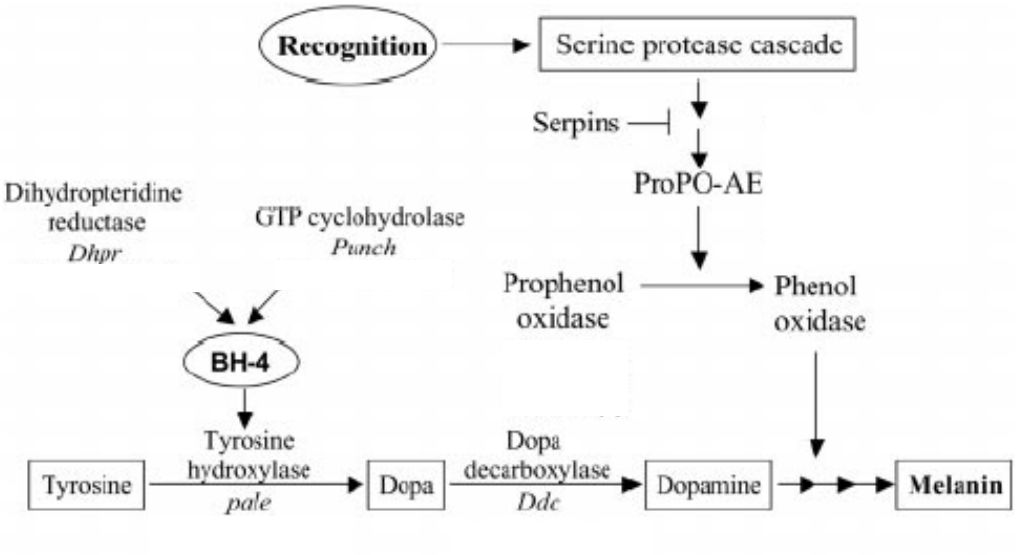


Figure 1-3: Schematic representation of the *Drosophila* melanization cascade (adapted from De Gregorio *et al.*, 2001).

Of paramount importance among the humoral reactions is precisely the melanization cascade, which leads to localized generation of quinones and toxic oxygen intermediates and culminates in the production of melanin at wound sites or around engulfed microorganisms. As other insects, for instance *Anopheles gambiae*, *Drosophila* has the equivalent of a complement-like cascade that may contribute to microorganism opsonization (De Gregorio *et al.*, 2001), as a cellular reaction defence. Finally, hemolymph zymogen cascades play a crucial role in activating the synthesis of antimicrobial peptides in the fat body; however, wether and how hemolymph coagulation participates in the host defence remains to be clearly established (Hoffmann and Reichhart, 2002).

These cellular and humoral defence reactions interact is a question being investigated. Elrod-Erickson et al. (2000) reported some intriguing results, where mutants with defects in the humoral immune response were further immunocompromised when phagocytosis was blocked and thus the cellular immune response impaired. These observations indicated that cellular and humoral immune reactions can, and probably do, cooperate or act in concert in order to fight bacterial infections in the fly *Drosophila*.

1.2.1. Recognition

Pathogen recognition by the innate immune system is believed to rely on interactions between conserved molecular structures present on the surface of pathogens and host proteins called pattern recognition receptors (PRRs). These PRRs are capable of specifically binding to the conserved molecular structures of pathogens, briefly PAMPs (pathogen associated molecular patterns) (Medzhitov and Janeway Jr., 2002). PRRs can mediate microbial killing directly, through phagocytosis, or indirectly by triggering serine protease cascades that in turn can activate defence reactions, such as melanotic encapsulation or initiate intracellular immune signalling pathways which regulate the transcription of antimicrobial peptides and other effector genes (Hoffmann *et al.*, 1996; Hoffmann and Reichhart, 2002) (figure 1-4).

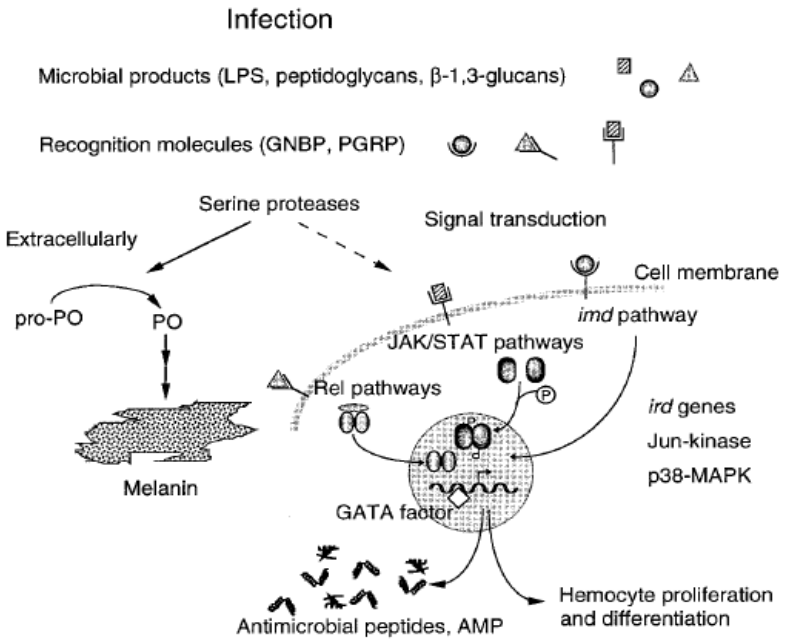


Figure 1-4: Model of the immune response activation in insects (adapted from Engstrom, 1999).

In turn, a number of microbial patterns (PAMPs) found at the surface of pathogens have been identified that elicit immune responses, both in invertebrates and vertebrates. They are diverse and characteristic of various classes of microorganisms, such as the lipopolysaccharides (LPSs) of gram-negative bacteria, the peptidoglycan

of gram-positive bacteria, the β-1,3-glucan of yeasts and the phosphoglycan of parasites (Franc and White, 2000).

This recognition question, even in case of *Drosophila*, has not been fully addressed during investigations in the last few years. But important advances have been made since 1999, when the study of the necrotic mutation clearly showed that Toll, a transmembrane receptor (Toll pathway receptor, see section1.2.2.2.), did not directly sense microbial infection (Levashina *et al.*, 1999). It was shown that Toll is activated (as during embryogenesis) by a cleaved form of cysteine knot cytokine-like polypeptide, Spaetzle. At this time, recognition of infectious non-self was known to occur upstream in the pathway, but the quest for the actual pattern recognition receptor of fungal and gram-positive bacterial infection remained open. The breakthrough came with the generation by Michel and co-workers (Michel *et al.*, 2001) of a mutant fly line, called "semmelweis", that failed to activate Toll in response to gram-positive bacterial challenge and had consequently a dramatically lowered resistance to this type of infection. The gene mutated by random ethyl methane sulfonate (EMS) mutagenesis, in these experiments, turned out to be PGRP-SA, a member of the large evolutionary conserved family of PGRPs (peptidoglycan recognition proteins). Peptidoglycan recognition proteins have been isolated from both insects and vertebrates; they share a highly conserved PGR-region (of about 160 residues) and comprise both secreted transmembrane and cytoplasmatic forms (Werner *et al.*, 2000). To date, as many as 13 members of the PGRP gene family have been identified in the *Drosophila*

genome, which also has representatives in humans, mice and cattle (Kang *et al.*, 1998; Liu *et al.*, 2001).

In *Drosophila*, PGRPs do not appear to play redundant roles. For the semmelweis mutation referred as affecting PGRP-SA, only the Toll-activation by gram-positive bacteria is blocked, while the Toll-activation by fungal infection remains wild type in these mutants. Later, researchers on 3 independent studies reported the discovery of the role of another PGRP family member that specifically activated the Imd pathway (see section 1.2.2.2., Imd pathway), upon stimulation with gram-negative bacteria infection (Choe *et al.*, 2002; Gottar *et al.*, 2002; Ramet *et al.*, 2002). The candidate gene in this case encodes a putative transmembrane receptor, PGRP-LC, and potentially can form several splice isoforms, although it is not yet clear how PGRP-LC can bring out the immune response reactions activation.

Relatively to the insetc's PGRP genes in general, they can produce several isoforms through alternative splicing with potential different ligand binding specificities. Interestingly, some of these isoforms are differentially regulated upon immune challenge suggesting, thus, splice selection by immune signals (Christophides *et al.*, 2002). The fact that the binding specificities of the PGRP family are not restricted to peptidoglycan and that, together, they are most likely providing a broad recognition repertoire for a variety of microorganisms makes them a key peace in the insect's immune surveillance system (Dimopoulos, 2003).

Another PRR family that has been implicated in intracellular immune signalling pathways activation in *Drosophila* is the gram-

negative bacteria binding protein family (GNBP). GNBPs contain a region with high similarity to β-1,3-glucan binding domains of bacterial glucanases and were initially isolated as a gram-negative bacteria-binding protein from *Bombyx mori*, in a report by Lee *et al.* (1996) (reviewed in Dimopoulos, 2003). Published works report the molecular characterization and expression of GNBP encoding genes, in both *Bombyx mori* and in the mosquito *Anopheles gambiae*, as well as their strongly up-regulation upon bacterial infection (Lee *et al.*, 1996; Dimopoulos *et al.*, 1997; all reviewed in Dimopoulos, 2003). In *Drosophila*, at least three genes encode for proteins that share similarities with GNBPs family. Kim *et al.* (2000) also report that one of the *Drosophila* GNBPs exist in both soluble and membrane bound forms and that it is capable of activating immune response mechanisms upon LPS challenge in *Drosophila melanogaster* cells.

Along with this, the *Drosophila* genome reveals the presence of a large number of putative genes encoding homologues of proteins that might function as recognition factors in other organisms. In an effort to discover PRRs in *Drosophila*, Abrams *et al.* (1992) showed that both Schneider cells and embryonic hemocytes exhibited a similar ability to recognize and take up modified low density lipoproteins, a property known as scavenger activity. Using this binding affinity approach and an expression cloning strategy, a scavenger receptor (SR) – DSR-CI – was characterized in *Drosophila*, few years later by Pearson *et al.* (1995). Other SRs were also identified at that time: epithelial membrane protein (EMP) and croquemort (CRQ), both homologues of the mammalian CD36

receptor (Hart *et al.*, 1993; Franc *et al.*, 1996). Both CRQ and DSR-CI are expressed in hemocytes and macrophages: CRQ is required for the phagocytosis and uptake of apoptotic cells but not bacteria (Franc *et al.*, 1999), while DSR-CI, like many other mammalian scavenger receptors, has a high affinity binding to a broad array of polyanionic ligand motifs including microbial β-glucan, suggesting it's involvement in microbial recognition (Krieger, 1997). The *Drosophila* genome appears to encode, at least, three additional EMP-like proteins, four additional CRQ-like proteins and two more DSR-CI-like proteins. It is likely, thus, that some of these proteins will function in microbial recognition (Khush and Lemaitre, 2000).

1.2.2. Transduction of immune response signals

1.2.2.1. Serine Protease Cascades

Pattern recognition receptors that have bound to PAMPs, in recognizing the infection, are believed to activate proteolytic cascades involving serine proteases and serpins (serine proteases inhibitors) (Dimopoulos, 2003). These cascades will transduce and amplify the immune response signal by activating intracellular signalling pathways that control antimicrobial gene expression, or effector systems such as melanization reactions.

Most of the serine proteases implicated in the invertebrate innate immune response, studied so far, possess a mosaic structure consisting of a carboxyl-terminal serine protease domain and a variety of other domains in their amino-terminal region. In this region, the clip-domain is the most frequently found amino-terminal domain (Dimopoulos, 2003; Gorman *et al.*, 2000) and consists of a cysteine knot. Clip-domains are thought to be implicated in interactions with other proteins and have a similar structure to big defensin (Jiang and Kanost, 2000; Gorman and Paskewitz, 2001). The *Drosophila* genome contains about 200 serine proteases, of which 35 carry clip-domains (Christophides *et al.*, 2002), and many others that resemble proteins with fibrinogen- and complement-like domains (Khush and Lemaitre, 2000).

In mammals, cytokines are key signalling molecules of their immune system. They are responsible for an effective immune response by rapidly transmitting information from the infection site to immune-responsive tissues. However, these molecules are poorly conserved between vertebrates species (Khush and Lemaitre, 2000) and, not surprisingly, no homologues of the vertebrate cytokines have been found in *Drosophila* genes. One possibility is that, if they exist in *Drosophila*, they are structurally divergent. Following this view point, the *Drosophila* genome does contain a high number of putative protease encoding genes as stated above. Some of these proteases will eventually regulate coagulation and melanization cascades as defence mechanisms, while others take part in proteolytic cascades that control the immune-responsive signalling pathways. For instance, a clip-

domain serine protease – phersephone – and a serpin – Spn43Ac – have been reported to be linked in activating the Toll pathway in *Drosophila* (Levashina *et al.*, 1999; Ligoxygakis *et al.*, 2002).

Moreover, studies using other animal models report the involvement of both serine proteases and serpins in the control of melanization reactions in moths (Jiang and Kanost, 1997; Satoh *et al.*, 1999; Park *et al.*, 2000) and in the mosquito *Anopheles gambiae* (AgSP14D1, AgSp14D2, AgSP14A) with sequence characteristics of phenoloxidase-activating enzymes (Dimopoulos *et al.*, 2001).

For all the immune-related clip-domain serine proteases, studies indicate, so far, that they are found constitutively expressed in the insect hemolymph, where rapidly activate defence responses.

1.2.2.2. Intracellular signalling pathways

The intracellular signalling pathways will thus function in the transduction of the signal for immune defence, from the serine proteases cascades to the transcriptional machinery of immune gene mRNAs production.

To date, studies on insect immune response regulation have largely focused on antimicrobial gene expression in *Drosophila*. Consequently, some of the signalling pathways that control *Drosophila* antimicrobial gene expression are well characterized, being the Toll and the Imd pathways the majors immune signalling pathways identified and

studied. *Drosophila* flies that carry mutations in both pathways do not express any antimicrobial peptides and are extremely susceptible to fungal, gram-negative and gram-positive bacterial infection (Lemaitre *et al.*, 1996).

In the mid 1990s, it was known that *Drosophila* produced seven distinct antimicrobial peptides with activity directed against fungi, gram-positive and gram-negative bacteria (table 1-1).

The promoters of the genes encoding these peptides contain sequence motifs related to mammalian NF-kB response elements, as

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

revealed by their cloning in the early 1990s (Engström *et al.*, 1993; Kappler *et al.*, 1993). Establishment of transgenic fly lines with mutated elements indicated that they are mandatory for immune inducibility of these genes (Hoffmann and Reichhart, 1997; Hoffmann *et al.*, 1999; Engström, 1999).

Table 1-1: Inducible antimicrobial peptides in *Drosophila* (adapted from Hoffmann and Reichhart, 2002).

At the time that data were obtained, regarding the role of kB-like response elements in *Drosophila*, a single NF-kB family member was known in the fly, Dorsal (a Rel protein involved in dorsoventral patterning in the early embryo) (Reichhart *et al.*, 1993). Since then, two additional family members have been discovered in *Drosophila*, DIF (dorsal-related immunity factor) (Ip *et al.*, 1993) and Relish (Dushay *et al.*, 1996). Moreover, independent studies had already pointed to significant similarities between the activation of the NF-kB-related transactivator Dorsal, by the Toll pathway, during dorsoventral patterning in the early *Drosophila* embryo and the cytokine-induced activation of NF-kB in immune-responsive mammalian cells (Belvin and Anderson, 1996). These similarities encouraged genetic analysis screens of the immune detection of antimicrobial peptides in Toll pathway mutants of *Drosophila* (Hetru *et al.*, 2003). So, Hoffmann *et al.* (1996) showed that induction of the antimicrobial peptide Drosomycin and, more generally, resistance to fungal infections did require a wild-type Toll receptor and several other members of the embryonic Toll signalling pathway (Lemaitre *et al.*, 1996). But, in contrast, induction of antibacterial peptides, such as Diptericin, was found to be Toll-independent needing a wild-type copy of a yet unknown gene then referred to as *immune deficiency* (imd) (Lemaitre *et al.*, 1995). In other words, mutants of the Toll pathway are highly susceptible to fungal infections, but behave as wild type when challenged with gram-negative bacteria, whereas imd mutants show high susceptibility to gram-negative infection but resist fungal infection, as if they were wild type.

These observations led to the conclusion that, at least, 2 distinct signalling pathways controlled the resistance to microbial attack and the induction of the various antimicrobial peptides.

Figures 1-5 and 1-6 show a schematic representation of both signalling pathways, as well as their components.

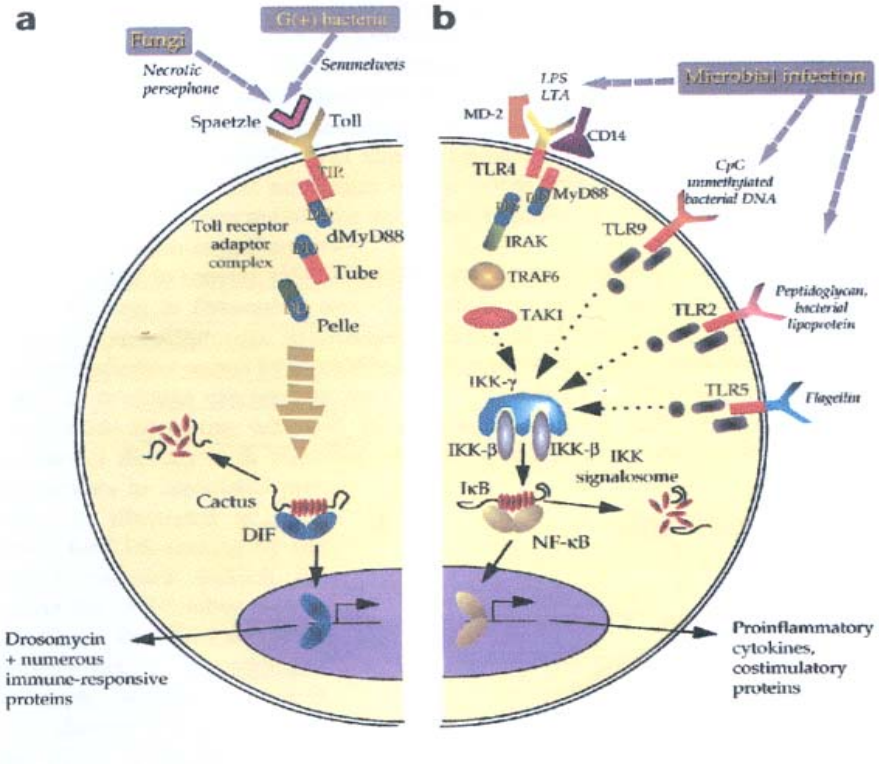


Figure 1-5: Toll pathway in *Drosophila* and mammals. (a) Present view of Toll-dependent induction of immune genes upon fungal and gram-positive bacterial infections in *Drosophila*. This scheme is valid for systemic response by the fat body cells. (b) TLR signalling of microbial infection by the mammalian innate immunity (adapted from Hoffmann and Reichhart, 2002).

The Toll pathway

Toll is a transmembrane receptor (Hashimoto *et al.*, 1988). Its extracellular domain contains leucine-rich repeats and its intracytoplasmic region shows significant sequence similarity with the corresponding region of the interleukin receptor 1 (IL-1R) and is referred to as the Toll-IL-1R (TIR) domain (reviewed in Imler and Hoffmann, 2001).

Toll is activated via a cleaved form of the polypeptide Spaetzle, which is structurally similar to mammalian nerve growth factor (Mizuguchi *et al.*, 1998), in result of a proteolytic cascade. A receptor-adaptor complex is formed on the intracytoplasmic side of Toll, which comprises 3 death domain proteins: DmMyD88 (which possess, as Toll, a TIR homology domain), Tube and the kinase Pelle. In turn, this complex signals to the ankyrin domain protein Cactus, which is phosporylated by a yet unknown kinase, and dissociation between Cactus and the NF-kB/Rel protein DIF occurs (figure 1-5). DIF is the predominant transactivator in the antifungal and anti-gram-positive bacterial defence in *Drosophila* adults (Manfruelli *et al.*, 1999; Meng *et al.*, 1999). In larvae, Dorsal can substitute for DIF (Manfruelli *et al.*, 1999). So, after Cactus degradation, DIF translocates into the nucleus and directs the transcription of the drosomycin and metcnhikowin genes plus that of some 350 additional genes induced by natural fungal infection, many with unknown function (Irving *et al.*, 2001; De Gregorio

et al., 2001), as revelead by genome-wide analysis of *Drosophila* immune response. It is assumed that the products of this large number of genes act in concert to fight off the infection, as resistance to it is by no means explained solely by the antimicrobial peptides induction (Hoffmann and Reichhart, 2002).

The Imd pathway

The Imd pathway governs mainly the defence reactions against gram-negative bacteria and controls resistance to these microorganisms. The transmembrane receptor of this pathway has not been firmly identified yet (figure 1-6). It is also not known whether this putative receptor is activated by direct interaction with microbial patterns or by the end-product of a proteolytic cascade (as for Toll). As for the Toll pathway, the immune induction of antibacterial peptides genes relies on a Rel family member of inducible transactivators, Relish. But, in contrast, this Rel protein is not inhibited by Cactus, it carries its own inhibitory sequences in form of several COOH-terminal located ankyrin repeat domains (Dushay *et al.*, 1996).

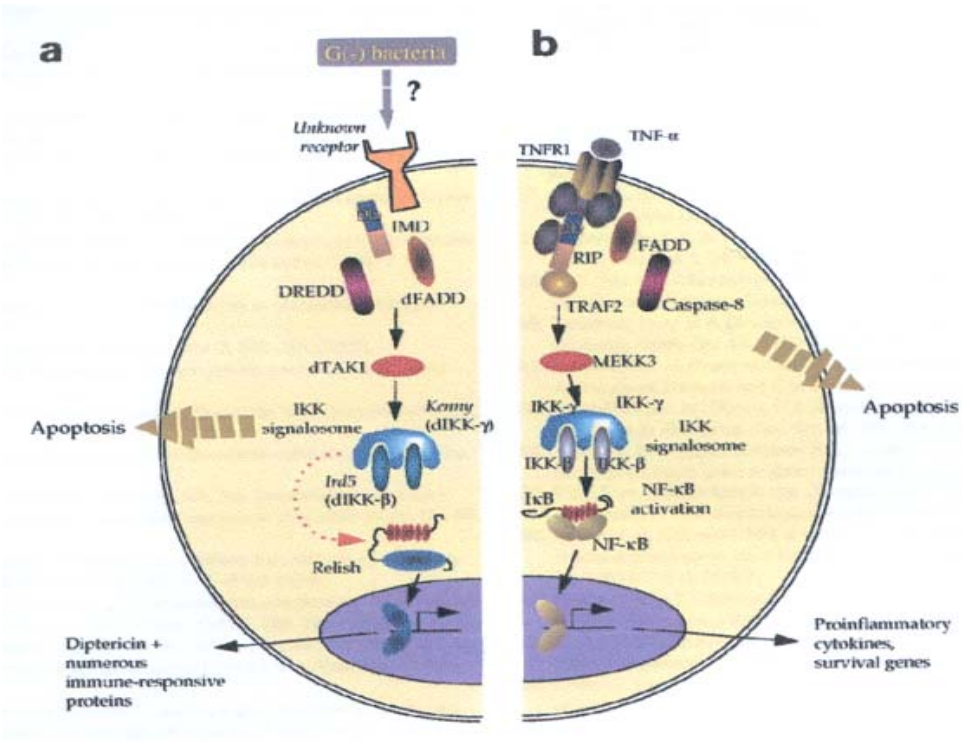


Figure 1-6: The Imd pathway in *Drosophila* and the TNF-α receptor pathway in mammals. (a) Present view of Imd pathway regulation of immune genes induction upon gram-negative bacterial infections in *Drosophila*. This pathway can also promote apoptosis. (b) Outlines of the mammalian TNF-α receptor signalling pathway (adapted from Hoffmann and Reichhart, 2002).

Recently, the imd gene was identified as a protein containing a death domain with significant sequence similarity to mammalian tumor necrosis factor-α (TNF-α) receptor-interacting protein (RIP) (Georgel *et al.*, 2001). The Imd protein probably interacts with DmFADD (Leulier *et al.*, 2002; Naitza *et al.*, 2002) and the caspase-8 homologue DREDD (Leulier *et al.*, 2000; Elrod-Erickson *et al.*, 2000). It has been

demonstrated that loss of function mutations in the genes encoding both DmFADD and DREDD silence the Imd pathway (Leulier *et al.*, 2002; Naitza *et al.*, 2002; Leulier *et al.*, 2000; Elrod-Erickson *et al.*, 2000). The mitogen-activated protein kinase kinase kinase – MAPKKK – dTAK1 acts downstream of Imd/DmFADD and activates an IkB kinase – IKK – signalosome equivalent (Vidal et al., 2001), consisting of *Drosophila* homologues of mammalian IKKβ and IKKγ/NEMO (NF-kB essential modifier) (Silverman et al., 2000; Lu et al., 2001; Rutschmann et al., 2000). Posteriorly, the protein Relish is cleaved, although by a still unknown caspase, and the Rel homology domain translocates into the nucleus while the ankyrin repeat domain remains in the cytoplasm (Stoven et al., 2000). Just as DIF, in the Toll pathway, cleaved Relish activates the transcription of the genes encoding antibacterial peptides, such as diptericin, and also many others (up-regulation of more than 220 genes by gram-negative bacterial infection)(Irving *et al.*, 2001).

Nonetheless, microorganisms can carry numerous structural patterns on their cell walls and, when introduced by septic injury, a given pathogen can concomitantly activate both described pathways. It has been reported that a cross-talk exists between the two pathways (Govind, 1999; Han and Ip, 1999). This cross-talk occurs via the transcription factors thanks to the heterodimerization between the various Rel proteins (observed in *Drosophila* cell lines) and it would variably affect antimicrobial peptide genes expression (Han and Ip, 1999).

1.2.3. Killing mechanisms

Antimicrobial peptides

In *Drosophila*, the antimicrobial peptides are primarily produced in the fat body and then rapidly secreted into the hemolymph. Within few hours after infection, these peptides can reach, all together, micromolar concentrations creating a very hostile environment to intruders (Hultmark, 2003). Moreover, in addition to this systemic response, antimicrobial peptides are produced locally in epithelial tissues (like gut, salivary glands, genital and digestive tracts) as well, except for their different regulation from that of the fat body cells (Hoffmann *et al.*, 1999).

Including those peptides referred in section 1.2, as many as 24 immune inducible peptides, with antimicrobial activity and regulatory dependence on Toll and Imd pathways, have been identified in *Drosophila melanogaster* through a powerful mass spectrometry approach (Uttenweiller-Joseph *et al.*, 1998). They share common features such as low molecular weight (below 5 kDa), a positive net charge at physiological pH and, for most of them, a structure consisting of amphiphilic α-helices or hairpin-like β-sheets or even mixed structures (Bulet *et al.*, 1999). Although their activity is, in most cases,

specific for different classes of pathogens, in general, the killing mechanism is believed to rely on bacterial membrane disintegration or interference with membrane assembly or bacterial proteins (Otvos, 2000).

In the future, advantages of these biomolecules such as rapidly killing target microorganisms, broad activity spectra and ineffectivity against mammalian cells, should lead to their use as candidates to overcome the alarming problem of acquired bacterial drug-resistance and the emergence of opportunistic pathogens in immunosupressed hosts (Bulet *et al.*, 1999).

Phagocytosis and melanotic encapsulation

Cellular defences, as already mentioned, refer to hemocyte-mediated immune responses such as phagocytosis, nodulation and encapsulation (Schimdt *et al.*, 2001; Carton and Nappi, 2001). In *Drosophila*, they involve primarily the following hemocyte cell types: plasmatocytes and lamellocytes. In contrast, at larvae stages plasmatocytes remain the most abundant hemocyte type in circulation, although other two cell types are present, the lamellocytes and the crystal cells (reviewed in Lavine and Strand, 2002).

Phagocytosis refers to the engulfment of intruders by an individual cell and subsequent degradation. It is mediated by PRRs (section 1.2.1.) that bind to the intruder and triggers intracellular cascades leading to its internalisation through an actin-dependent

mechanism (Aderem and Underhill, 1999). Besides taking up biotic targets like bacteria, yeast and apoptotic bodies, plasmatocytes (main class phagocytically active) are also able to engulf abiotic targets like synthetic beads, an ability very handy for studying the underlying molecular mechanisms of phagocytosis (de Silva et al., 2000).

When the intruder corresponds to a multicellular pathogen too large to be phagocytosed, the most common response is encapsulation (Johnson *et al.*, 2003). This results in the production of lamellocytes, because of a more specialized cellular reaction due to host infection by these larger parasites, as a result of hemocyte proliferation and differentiation into this other class (Hultmark, 2003). This class of large flattened cells are thus implicated in the formation of a cellular capsule around the parasite, through successive cell layers deposition. Encapsulation is often accompanied by the deposition of melanin and protein-phenol complexes in a reaction termed melanotic encapsulation. It is thought that crystal cells, characterized by the presence of crystalline inclusions in the cytoplasm and by carrying phenol oxidase (PO), are involved in melanin deposition around foreign encapsulated pathogens and wounds (Kurucz *et al.*, 2003). Melanotic encapsulation requires the activation of PO, which is an oxidoreductase that catalyses phenols conversion into quinones (providing quinonoid products for melanization reactions), present as an inactive precursor termed prophenol oxidase (PPO) (Ligoxygakis *et al.*, 2002). Activated PO is highly "sticky" (Nappi and Sugumaran, 1993) and this property aids

the deposition of PO on the parasite surface where melanization reaction occurs (Sugumaran, 2002).

1.3. Work's aim

The fruit fly *Drosophila melanogaster* has been one of the most intensively studied organism in the past few years, serving as a model system for the investigation of many developmental and cellular processes common to higher eukaryotes, including humans (Adams *et al.*, 2000).

It is also the model animal chosen for the present study. Due to the ease of genetic and molecular analysis, combined with a complete genomic sequence and novel techniques such as microarray analysis screens, makes this organism an essential tool for deciphering innate immunity. This kind of investigation has generated a huge amount of information and few issues remain to be clearly understood in *Drosophila* multifaceted immune response (Hoffmann *et al.*, 2002; De Gregorio *et al.*, 2001; Imler *et al.*, 2000; all reviewed in Hoffmann, 2003). Even though several hundred genes are up-regulated upon immune challenge, in addition to the antimicrobial peptide genes, their contribution in fighting off infection has not yet been acutely addressed.

The general aim of this study is to identify proteins that can be correlated with *Drosophila* immune defence reactions. For that reason, a

2DE-MS approach was developed in order to attempt identification of protein spots that vary in result of the immune challenge. The results will serve to increase the current knowledge on *Drosophila* innate immune response from a biochemical viewpoint.

2. Material and Methods

2.1. Animals

Drosophila melanogaster Berlin K were kept in 600mL bottles and fed with agar medium prepared from 100g yeast, 100g sucrose, 12g agar and 5mL propionic acid per litre of water. The growth conditions, including a controlled temperature of 21°C, were established according to Mota (Mota, 2003).

2.2. Infection procedure

Sets of 40 *Drosophila melanogaster* third instar larvae were immunised through miming a natural infection procedure by feeding on a conditioned medium. This conditioned medium was prepared in a similar way from that of normal growth conditions, plus the addition of a microorganisms membranes suspension (Biopental OM, OM Portuguesa). Each mililiter of this suspension contained bacterial lisates from 400 milion of all the following bacteria, *Diplococcus pneumoniae*, *Neisseria catarrhalis* and *Staphylococcus aureus*, and bacterial lisates from 200 milion of all the following bacteria, *Klebsiella pneumoniae*, *Haemophilus influenza* and *Streptococcus pyogenes*. A 1:10 proportion of microorganisms membranes suspension:feeding medium was used in every experimental procedure. Larvae were placed one by one in this

medium and maintained in theses conditions for a period of 24 hours. Subsequently, the initial set of immunised larvae was divided into two according to the recovery times (RTs) of 6 and 24 hours, respectively. For both recovery times, larvae were replaced into normal growth conditions, similar to the control (C) set.

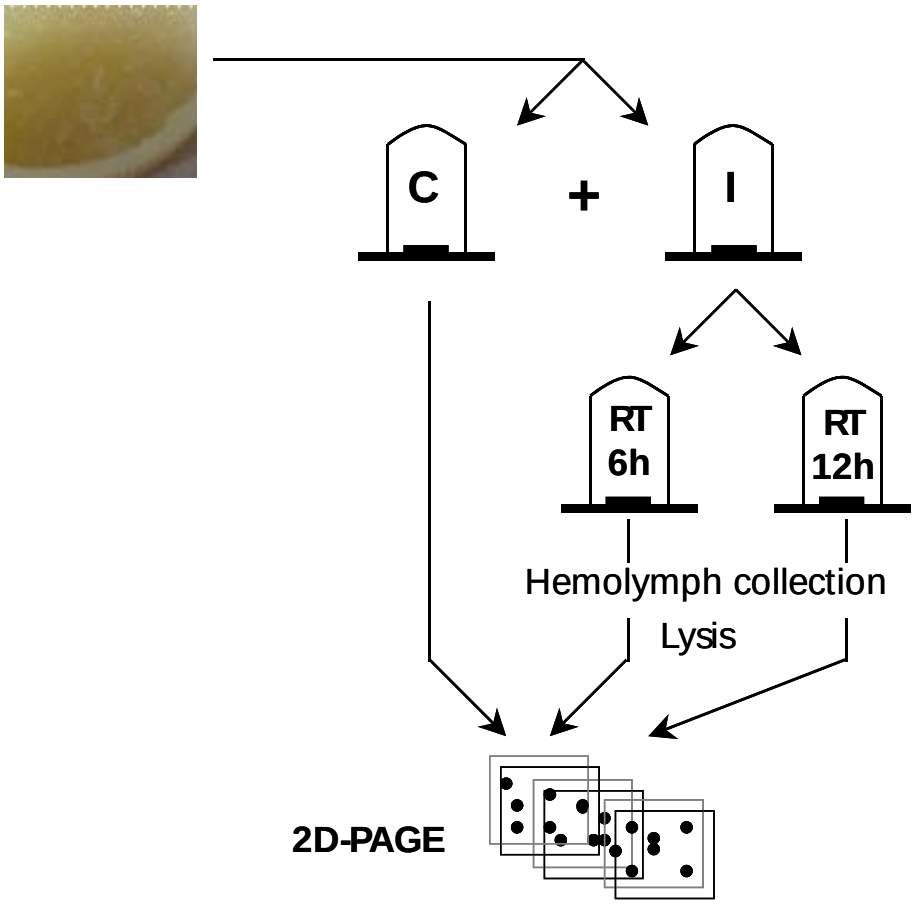


Figure 2-1: Schematic representation of the *Drosophila* larvae immunisation. C, Control larvae set; I, Immunised larvae set; RT, recovery time.

2.3. *Drosophila* larvae hemolymph extraction

The hemolymph of 40 *Drosophila melanogaster* third instar larvae was collected according to Braun et al. (1998). The animals were previously anaesthetized with ether embedded cotton for about three minutes and then pricked and gently squeezed to collect hemolymph, expelled as a droplet upon pressure. Larvae hemolymph was suspended

in 400μL of a lysis solution (8M urea, 2M thiourea, 1% CHAPS, 13mM DTT, 4μL of a protease inhibitor cocktail (Sigma, P2714)), followed by a centrifugation step of 15 minutes at 14000*g* at 4°C.

2.4. Protein quantification

2.4.1. Lowry assay

The *Lowry* (Lowry *et al.*, 1954) method comprises a combination of *biuret* complexes with the *Folin-Ciocalteau* reagent giving a blue coloured product with absorption maximum at 750nm. The formation of this *molybdenum* or *tungsten blue* is due to the reduction of heteropolyphosphor acids of molybdenum and tungsten (*Folin-Ciocalteau* reagent) to blue coloured hydroxides of molybdenum and tungsten (*molybdenum* or *tungsten blue*) with varying stoichiometry. This can be attributed to the presence of reducing amino acids such as tyrosin, tryptophan and cysteine as well as the formation of the *biuret* complex and subsequent reduction of the *Folin-Ciocalteau* reagent. This method has a sensitivity of 2-100μg/mL.

Just for the quantification step, samples were dialysed against water using a membrane with a cut-off of 3000Da (Pierce). A blank consisting only of lysis solution was used in an attempt to control dialysis. Briefly, 10μL of dialysate was incubated with 3,5mL of reagent solution (a solution of 0,5% Copper(II) sulphate-pentahydrate and 1% *tri*-Sodium citrate di-hydrate plus a solution of 2% Sodium carbonate anhydrous and 0,4% Sodium hydroxide) for 10 minutes at room temperature. A dilution of 1:1 of the Folin Cicolteau reagent was add

and samples incubated for 30 minutes, followed by absorbance reading at 750nm. The sample protein concentration was estimated by interpolation with BSA standards of 2, 5, 10, 12, 16, and 30μg curve.

2.5. SDS-PAGE

Polyacrylamide gel results from the polymerisation of acrylamide monomer into long chains and the crosslinking of these by bifunctional compounds such as *N,N'*- methylene bisacrylamide reacting with free functional groups at chain termini. The gel pore dimensions are dependent of acrylamide/bisacrylamide (solution) used in polymerisation. SDS-PAGE has been extremely useful as an analytical tool for the separation and quantification of protein species from complex mixtures (O´Farrell, 1975). Protein complex mixtures are separated under denaturing conditions by heating at 100ºC in the presence of excess of SDS, a thiol reagent to cleave disulphide bonds and a tracking dye (bromophenol blue). SDS binds to the protein in a constant ratio and the net charge acquired is stronger compared with the intrinsic charges. In this way, the protein-SDS complex run only based on weight when an electric field is applied.

SDS-PAGE is composed by two different types of gel: a stacking gel (4% acrylamide/bisacrylamide) and a resolving gel (12,5% acrylamide/ bisacrylamide). The stacking gel is useful to concentrate the

entire sample on the top of the resolving gel avoiding, this way, spreading effects.

The resolving gel, 1mm thick, was cast in a Hoefer 600 apparatus. After resolving gel polymerisation occurred, a stacking gel was cast on top of it. The sample was boiled for 5 minutes in a water bath in SDS loading buffer. The samples were subsequently loaded as well as molecular weights standards (Low molecular weight range, Amersham Pharmacia). The electrophoresis chamber was filled with 1xSDS running buffer. The electrophoresis was performed at 200V, 100mA, 25W until tracking dye reached the bottom of the separating gel. The gel was stained or transferred onto a nitrocellulose membrane.

2.6. Two dimensional gel electrophoresis (2D-PAGE)

Improvements in separation and characterisation of proteins were addressed by the combination of isoelectric focusing (IEF), in the first dimension, with SDS-PAGE in the second dimension (O´Farrel, 1975). In the first dimension the proteins are brought on a strip that contains an immobilised pH gradient. By applying an electric field over the strip, the proteins will migrate until they reach the pH area on the strip where they will be neutral. In the second dimension, proteins are separated on their size/mass. This conjugation allows the separation of thousands of different cellular proteins.

However, this technique has limitations since the impossibilty to resolve proteins over 200000Da and to resolve proteins extremly acid or basic. Recently, novel IPGs have become available for separating acid or basic proteins with the application of pH ranges of 8-11, 9-12 or 1-3 (Gorg *et al.*, 2000). Changes on the composition of the IPG strips to *N,N*-dimethylacrylamide or *N*-acryloylminoethoxyethanol improve the separation of the referred proteins.

The isoelectric focusing was performed in a horizontal IPGphor (Amersham Pharmacia). The hemolymph protein extract was solubilized in rehydration buffer (8M urea, 2M thiourea, 12mM DTT, 0,5% immobilines pH 3-10, 1% CHAPS) and each sample was then pipeted into a strip holder, IPG strips of 13cm with a linear pH range of 3-10. A cover oil was applied to protect the strip during the focusing time against dehydratation. The strip holders were then placed in the IPGphor and the electrophoresis was started by rehydration step (12h at 50mA/strip). The focusing program was 1h at 500V, 1h at 1000V and 2h at 8000V.

After the first dimension, the strips were removed and were equilibrated with an equilibration buffer (see appendix).

For the second dimension, gels were cast in Hoefer 600 apparatus as described previously for SDS-PAGE. The polyacrylamide stacking gel was replaced by agarose gel. The running conditions performed were the same as described previoulsy.

2.7. Staining procedures

2.7.1. Coomassie blue staining

Gels were stained in coomassie solution for about 1 hour and destained by incubation in destaining solution until the background was clear. Gel image was acquired using the GS-710 calibrated imaging densitometer (Bio-Rad) and analyzed with the Quantitaty One 4.2.1 software. This procedure was only used in SDS-PAGE.

2.7.2. Colloidal coomassie blue staining

This procedure was used in 2D-PAGE for mass spectrometry. The colloidal coomassie blue staining detection limit is 10 times more sensitive than normal coomassie blue, it has a very low background and it doesn't penetrate the gel matrix. The ideal conditions to perform mass spectrometry analysis.

The gel was incubated in fixing solution for 30min, stained overnight and destained in several washes of 25% methanol until the spots became visible.

2.7.3. Silver Staining

In this procedure, the gel is impregnated with soluble silver ions and developed by treatment with an aldheyde (formaldeyde or gluteraldheyde), which reduces silver ions to form an insoluble brown precipitate. The presence of proteins promotes this reduction.

The silver staining procedure was performed according to Yan *et al.* (2000). Briefly, gels were incubated in fixing solution for 30min and then changed to sensibilization solution for 30min. Two washes of 10min each with water were done. Following the washes, the gels were impregnated in silver solution for 20min in the dark. To remove the excess of silver ions, an additional wash was realized. The visualization of spots was possible by the placement of gel in a developing solution. The reaction was stopped with EDTA (powder). Finally, gels were washed with distilled water.

2.8. Two dimensional gels image analysis

Each gel image was acquired using the GS-710 calibrated imaging densitometer (Bio-Rad) and analyzed with the PdQuest v7.1 software (Bio-Rad). The software allowed background subtraction, automatic spot detection, as well as automatic gel matching, and subsequential comparative analysis of normalized spot optical densities (ODs).

Although automatically, spot detection in each gel was verified by visual inspection in order to obtain an image pattern as similar as

possible with the original gel. After detection, comparative analysis was performed by matching the gels with a control gel as reference (called "master"), which also required visual inspection because the software relies basically in spot position and checking false matchings turns out to be absolute essential. Before exporting results to Microsoft Excel program in order to analyse quantitative changes between control and immunised *Drosophila* larvae gel samples, spots intensities were normalized using the normalization formula in PD-Quest software: ("raw" spot volume) x (scaling/normalizing factor). The "raw" spot volume is the unnormalized volume or intensity of each spot and the scaling factor provides a meaningful value, for example, ppm (x 10^6) or percent (x 100). The normalization factor is calculated for each gel and, because of inconsistent staining times produce gel-to-gel variations (for instance, background and spots number), the total volume or intensity of all valid spots in each gel was the factor used for normalizing each spot volume.

2.9. Protein identification

Mass spectrometry is now firmly entrenched as the first choice methodology for protein identification and characterisation. It is continuing to evolve rapidly and diversify into an array of technologies, with each variant adapted to specific applications. Recently, the

development of a new MS/MS generation apparatus with a MALDI source allowed proteins to be directly identified from a common sample, using either the peptide mass fingerprinting and/or high quality tandem MS/MS data (Larsen *et al.*, 2001, Griffin *et al.*, 2001, Doneanu *et al.*, 2001).

For mass spectrometry analysis, the 2D-PAGE gels were dyed with colloidal coomassie blue; the sopts were excised manually and transferred to an Investigator ProGest automated digester rack (Genomic Solutions, Ann Arbor, MI, USA). A total remotion of the dye it is necessary before the addtion of trypsin. The spots were then washed twice with 25mM Ammonium Bicarbonte in 50% Acetonitrile, and dried with nitrogen flow.

The dried spots were incubated with sequence grade modified porcine trypsin (Promega, V1115) at 37ºC during 8 hours. The tryptic digests thus obtained were lyophilised and ressuspended in 10μL of 50% acetonitrile in 0.1% formic acid solution. Samples aliquots (0.350μL) were spotted onto the MALDI sample target plate using a SymBiot XVI Sample Workstation (Applied Biosystems, Foster City, CA, USA) previously spotted with a matrix (0.350μL) of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% formic acid.

Peptide mass spectra were obtained on a MALDI-TOF-TOF mass spectrometer (4700 Proteomics Analyzer, Applied Biosystems, Foster City, CA, USA) in the positive ion reflector mode. Spectra were obtained in a mass range between 800 and 4000Da with ca. 1500 laser shots. Trypsin autolysis peaks were used as an internal calibration of the mass

spectra, allowing a routine mass accuracy better than 25ppm. A spectra interpretation method was created for each spot set, in order to select the two non-matricial peaks of higher intensity and to exclude the matricial and trypsin autolysis peaks for subsequent MS/MS data acquisition. Atmosferic air was used for MS/MS spectra acquisition.

Spectra were processed and analysed by the Global Protein Server Workstation (Applied Biosystems, Foster City, CA, USA), which uses internal Mascot (Matrix Science Ltd, U.K.) software for searching the peptide mass fingerprints and MS/MS data. Searches were performed against the NCBI non-redundant protein database. Further confirmation of protein identifications was obtained by duplicating the protein identifications using Protein Prospector (www.prospector.ucsf.edu, from the University of California at San Francisco) and/or Prowl (www.prowl.rockfeller.com, Rockefeller University at New York Universities) software.

3. *Drosophila* larvae protein map:

Results & Discussion

A huge amount of information on gene repertoires of various organisms, including *Drosophila melanogaster*, has been supplied by a number of genome sequencing projects in the past few years. However, in order to obtain less "static" information it is necessary to investigate the dynamics and functions of genes and their products, as well as the relationship between them (Kaji *et al.,* 2000). Although global gene expression analysis achieved by a microarray DNA chip is an extremely powerful technological tool, biological system functions are essentially protein controlled not only through location and quantity, but also through several post-translational modifications (such as phosphorylation, glycosylation, proteolytic cleavage, etc.). Therefore, the understanding of the mechanisms underlying biological events is not completely accomplished without a proteomic analysis (Kaji *et al.,* 2000). Proteomics refers to the global analysis of complex protein mixtures, which involves the ability to rapidly analyse and identify the measurable protein profile in cells, tissues, body fluids or even small organisms. A classic proteomics approach involves the association of the high resolution two dimensional gel electrophoresis (2-DE) displaying a large number of separated proteins from a given sample with mass spectrometry. Recently, proteomics has been expanded to cover more broadly studies on global changes in protein expression in tissues or body fluids in different states of development or environment influence (Yergey *et al.,* 2002).

To date, there have been only a few reports on *Drosophila* and 2-DE. Ericsson *et al.* (1997) presented the first on-line 2-DE protein

database of adult *Drosophila melanogaster* (http://tyr.cmb.ki.sc), even though the *url* is no longer available. In this work, 2-DE protein patterns of male and female head, thorax and abdomen were presented and compared with computer-assisted image analysis (PDQUEST software, v5.0). The proteins spots reported were identified based on the comparison of the 2-DE patterns of other *Drosophila* organs, and by using the Compute pI/Mw tool on the Expasy server and miming sequences with amino acid sequences in the SWISS-PROT database. The aim of that work was the 2-DE comparative analysis of protein patterns of male and female head, thorax, and abdomen to obtain information on differentially expressed proteins between different genders and organs. Those identified spots were mainly muscle proteins (such as myosins and actins) (Mogami *et al.,* 1982, Becker *et al.,* 1992, Saide *et al.,* 1989, Hiromi *et al.,* 1985, Karlik *et al.,* 1986, Basi *et al.,* 1984, Ayme-Southgate *et al.*, 1989, Falkenthal *et al.,* 1984), except for a set of six identified as yolk proteins by Western blotting (Ericsson *et al.*, 1997).

Recently, Vierstraete *et al.* (2003) reported the identification of *Drosophila* larvae hemolymph proteins using a MALDI-TOF/MS approach. This first attempt in constructing a 2-DE database of *Drosophila* larvae hemolymph resulted in the identification of 32 protein spots. The positive identifications included a set of storage and transport proteins, enzymes (glycolytic, proteolytic and hydrolytic), two serine protease inhibitors, growth factors and structural proteins, demonstrating for the first time (except for ferritins and hexamerins) their presence in the larvae hemolymph.

3.1. Drosophila melanogaster larvae hemolymph proteins 2-DE pattern

Larvae hemolymph proteins were separated using two dimensional gel electrophoresis and identified by MALDI-TOF/TOF mass spectrometry. The 2-DE pattern obtained for larvae hemolymph proteins is shown in figure 3-1. Proteins observed ranged from molecular mass of 10,000-120,000Da and pI of pH 3-10.

During preliminary experiments the 2-DE gels were silver stained, in order to obtain as much information as possible, and about 315 spots were detected in all samples. But, to ensure enough protein amount for identification by mass spectrometry, 700μg of total protein was loaded in the 2-DE gel and with coomassie colloidal staining a total of 289 spots were observed, as shown in figure 3-2. The numbers in the image near the spots indicate that they were submitted to mass spectrometry identification, as well as how they are indexed in the identification list (table 3-1).

With this attempt the 2-DE pattern of both staining procedures turned out to be very similar, as confirmed by the 2-DE analysis using PD-Quest software (v7.1). Although the applied protein was excessively high, precipitation or underfocusing effects weren't observed.

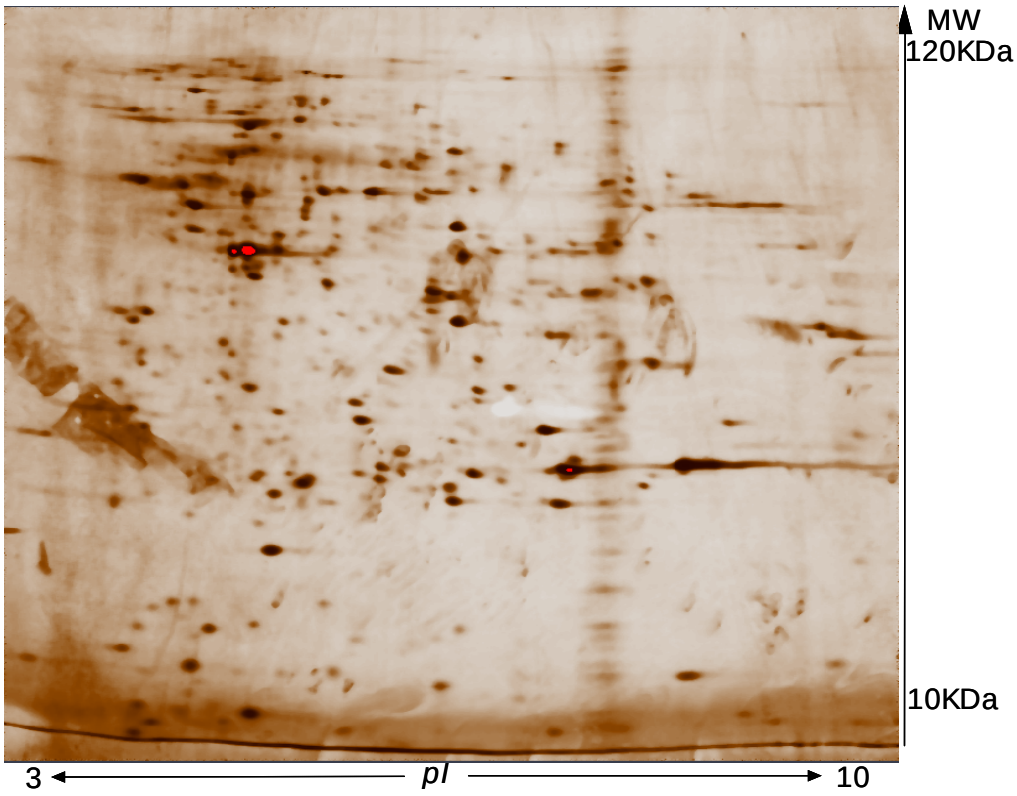


Figure 3-1: Protein map of *Drosophila* larvae hemolymph silver stained.

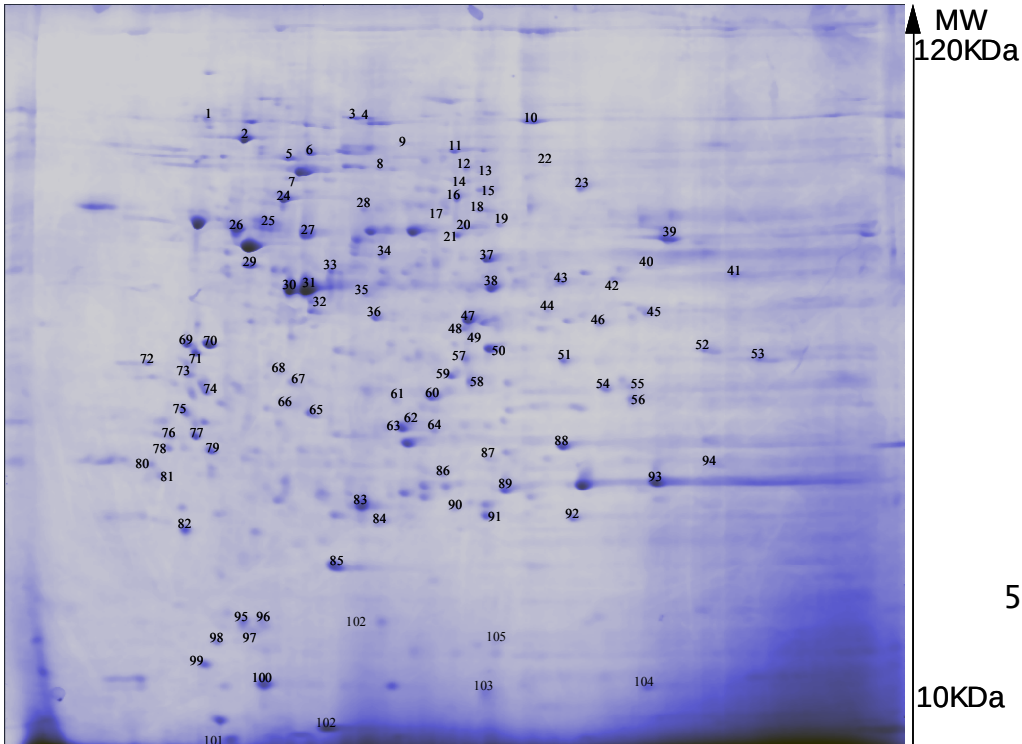


Figure 3-2: Protein map of *Drosophila* larvae hemolymph coomassie colloidal staining.

3.2. Identification of *Drosophila melanogaster* larvae hemolymph proteins

As indicated in figure 3-2, a total of 105 spots including some of the most intense (35 spots) and the remaining less intense spots were excised and digested. Ninety-nine of the total excised spots were identified (corresponding to 89%) as summarised in table 3-1.

Table 3-1: List of identified hemolymph proteins in *Drosophila* third instar larvae.

Spot ID.	Protein name	Cellular function	Seq. cov. (%)	NCBI entry	SWISS-PROT	pI	Mr (kDa)
1	GM08240p or Aminopeptidase	Proteolysis	22	16768538	Q95S62	4,9	73,49
2	Heat shock protein 83 (Hsp 82)	Molecular chaperone	40	123661	P02828	4,9	81,81
3, 4	Paramyosin	Structural component of myofibrils filaments from muscles	19	10959	P35415	5,5	102,16
5	Heat shock 70 kD protein cognate precursor (GRP 78)	Molecular chaperone (stress response)	43	808951	P02825	5,2	72,19
6, 9	Larval serum protein gamma chain precursor (hexamerin 1 gamma)	Larval storage protein for biosynthesis	20	13124784	P11997	5,3	93,34
7	CG9468 gene product or Lysosomal α-mannosidase	Complex carbohydrate metabolism	15	22945989	Q9VLH9	5,2	112,88
8	CG2118 gene product	Unknown	22			5,8	75,00
10	Elongation factor 2 (EF2)	Elongation factor for protein biosynthesis	32	24585711	P13060	6,2	94,41
11	Larval serum protein 2 precursor (hexamerin 2)	Larval storage protein for biosynthesis	24	2495185	Q24388	6	85,42

Spot ID.	Protein name	Cellular function	Seq. cov. (%)	NCBI entry	SWISS-PROT	pI	Mr (kDa)
12	Phosphoenolpyruvate carboxykinase mitochondrial precursor	Carbohydrate metabolism (gluconeogenesis, krebs cycle)	18	68039	P20007	6,3	71,08
13	Hsp 90 related protein TRAP1 (tumor necrosis factor receptor-associated protein 1)	ATPase	38	6466460	Q9V9D1	6,9	77,37
14	Succinate dehydrogenase flavoprotein subunit mitochondrial precursor	Krebs cycle, oxidative phosphorylation	36	13124721	Q94523	6,7	72,29
15	CG10724-PB gene product	Unknown				6,3	66,11
16	Malic enzyme	Carbohydrate metabolism (pyruvate metabolism)	31	7248646	Q9NIW2	6,1	63,91
17	CG4830 gene product or Luciferase	Unknown	27	7299573	Q9VGC5	5,9	59,20
18	Past1 gene product or Receptor mediated endocitosis protein 1 isoform a	Unknown	38	24646379	Q8T8W3	6	55,38
19	CG9629 gene product or Serine proteinase	Immune pathways activation	40	23093143	-	8,7	64,11

20	CG7430 gene product or Dihydrolipoamide dehydrogenase precursor	Carbohydrate metabolism	24	24666147	Q9VVL7	6,4	53,05
21	Gr gene product or Glutathione reductase (Thioredoxin reductase)	Stress response (detoxification)	51	1857433	P91938	5,9	53,19
22	CG8193 gene product or Prophenol oxidase	Immune response	30	13774118	Q9BLD9	6,5	79,24
23	CG8036 gene product or Transketolase	Carbohydrate metabolism (pentose phosphate pathway)	33	16768650	Q95S19	6,7	67,99

Spot ID.	Protein name	Cellular function	Seq. cov. (%)	NCBI entry	SWISS-PROT	pI	Mr (kDa)
24	Heat shock protein 60 kD mitochondrial precursor (Hsp 60)	Molecular chaperone (stress response)	30	12644042	O02649	5,4	60,77
25	Tubulin 1 α-chain	Major microtubules constituent	24	85172	P06603	5	49,87
26, 27	BetaTub56D gene product or Tubulin 1 β-chain	Major microtubules constituent	57	24655741	Q8IME1	4,8	50,11
28	SD02216p or Chaperonin 2 β-subunit	Molecular chaperone	49	16198301	Q9W392	5,6	58,02
29	CG17109 gene product or Aminoacylase 1	Proteolysis, urea cycle	34	7300957	Q9VCR0	4,9	44,94

30	Actin 57B	Cell motility	48	1703117	P53501	5,2	41,80
31, 32	Actin	Cell motility	29	13124689	P18091	5,3	41,79
33	eIF-4a gene product or Eukaryotic translational initiation factor 4A	Elongation initiation regulation	24	24582075	Q02748	5,7	44,32
34	CG1970 gene product or NADH dehydrogenase Fe-S protein 2	Oxidative phosphorylation, ubiquinone biosynthesis	27	22759353	Q9V4E0	6,7	51,06
35	CG9498 gene product	unknown	40			5,6	48,73
36	CG3902 gene product or Acyl-Coenzyme A dehydrogenase	Fatty acid oxidation, oxidative phosphorylation	45	7293851	Q9VVU1	6,3	45,33
37	Enolase	Carbohydrate metabolism	65	119351	P15007	6,1	46,53
38	CG7176 gene product or Isocitrate dehydrogenase1	Carbohydrate metabolism (krebs cycle)	31	7295109	Q9VSI6	6,3	46,63
39	ATP synthase α-chain mitochondrial precursor (Bellwether protein)	Oxidative phosphorylation	41	5921205	P35381	9,1	59,38
40	CG4094 gene product or Fumarase precursor	Carbohydrate metabolism (krebs cycle)	11	22831834	Q9W3X6	6,4	44,08

41	CG3861 gene product or Citrate synthase mitochondrial precursor	Carbohydrate metabolism (krebs cycle)	45	7290712	Q9W401	8,9	51,54

Spot ID.	Protein name	Cellular function	Seq. cov. (%)	NCBI entry	SWISS-PROT	pI	Mr (kDa)
43	Translational elongation factor 1 gamma-chain	Elongation factor for protein biosynthesis	49	6716514	Q9NJH0	6,2	48,95
44	CG6543 gene product or Enoyl-CoA hydratase (mitochondrial)	Fatty acid metabolism	44	7303266	Q9V6U5	8,9	31,56
45, 46	Fructose-biphosphate aldolase	Carbohydrate metabolism	33	543797	P07764	6,9	39,02
47	Arginine kinase	Aminoacid metabolism (phosphotransferase)	54	1346366	P48610	5,7	39,59
48	CG12233 gene product or Isocitrate dehydrogenase α	Carbohydrate metabolism (krebs cycle)	38	22832574	Q8IQW9	6,9	40,82
49	CG17427 gene product	unknown	32			9,9	18,75
50	CG6084 gene product or Aldehyde reductase	Carbohydrate metabolism, complex lipide metabolism	32	23093630	Q9VTK9	6,9	34,15
51	CG5362 gene product or Malate dehydrogenase	Carbohydrate metabolism (krebs cycle, pyruvate metabolism)	32	22946147	Q9VKX2	6,9	33,73

52	Glyceraldehyde 3-phosphate dehydrogenase 1 (GAPDH I)	Carbohydrate metabolism	44	120640	P07486	8,2	35,29
53	Glyceraldehyde 3-phosphate dehydrogenase 2 (GAPDH II)	Carbohydrate metabolism	46	14286123	P07487	8,5	35,34
54	CG9914 gene product or Hydroxyacyl-CoA dehydrogenase	Fatty acid oxidation	17	7293203	Q9VXI1	6,2	32,60
56	Guanine nucleotide-binding protein β-subunit like protein	Transmembrane signalling transduction and/or modulating	33	14286121	O18640	7,1	35,59
57, 58	Glycerol-3-phosphate dehydrogenase 2 (NAD^+)	Complex lipid metabolism	45	84980	P13706	6,2	39,30

Spot ID.	Protein name	Cellular function	Seq. cov. (%)	NCBI entry	SWISS-PROT	pI	Mr (kDa)
61	CG10997 gene product or Chloride intracellular channel 5	Voltage-gated channel protein	42	7292935	Q9VY78	5,9	30,15
62	CG8560 gene product or Carboxypeptidase B	Proteolysis	10	28380588	Q961J8	5,4	39,26
64	CAH1 protein or Carbonic anhydrase	Carbonate dehydratase activity	50	7287779	Q9V396	5,9	29,93

66	CG17633 gene product or Carboxypeptidase A	Proteolysis	15	7297556	Q9VL86	5,2	47,63
67	CG1532 gene product	unknown	50			5,2	31,62
68	CG11911 gene product or Chymotrypsin-like serine protease precursor	Immune response (proteolysis)	13	7296216	Q9VPN8	5,4	30,12
69	Tm1 gene product or Tropomyosin1	Filament actin binding in calcium-dependent regulation of muscle contraction	57	24647089	Q9VF97	4,7	32,72
70	Laminin receptor		71	157810	P38979	4,8	28,39
71	Tropomyosin 2	Filament actin binding in calcium-dependent regulation of muscle contraction	41	136072	P09491	4,7	32,78
72	Alpha NAC (Nascent polypeptide associated complex protein α-chain)	Transcriptional coactivator	34	2407247	O16813	4,6	23,00
73, 74	Tropomyosin 1 non-muscle isoform (Cytoskeletal tropomyosin)	Not clear	72	136075	P06754	4,8	29,34

75	14-3-3 Protein epsilon	Signal transduction pathways, cell cycle	59	3023178	P92177	4,7	29,55

Spot ID.	Protein name	Cellular function	Seq. cov. (%)	NCBI entry	SWISS-PROT	pI	Mr (kDa)
76	CG4912 gene product or Translational elongation factor 1 δ-chain	Elongation factor for protein biosynthesis	48	7297627	Q9VL18	4,6	25,83
77	14-3-3-like protein (Leonard protein)	Signal transduction pathways, cell cycle	41	112683	P29310	4,8	28,21
78	CG9673 gene product or Serine protease K15/F2R3	Immune response (proteolysis)	29	7293268	Q9VXC7	4,5	27,85
79	CG17870-PA	Unknown	47			4,8	28,27
80	BcDNA.GM07659 or FK506-binding protein	Peptidyl-prolyl cis-trans isomerase (PPIase activity)	60	24657035	Q8MLW1	4,7	23,95
82	Translationally controlled tumor protein homolog (TCTP)	Not clear	69	9979174	Q9VGS2	4,7	19,63
83	Thioredoxin peroxidase 1	Stress response (detoxification)	47	7230426	Q9V3P0	5,5	21,72
84	Thioredoxin peroxidase 3	Peroxidase activity	35	11935114	Q9VEJ0	7,0	26,36

85	CG18594 gene product or Phosphatidylethanolamine binding protein	Not clear	63	7300863	Q9VD01	5,7	19,59
86	Transient receptor potential locus C protein (GIP-like protein)		35	549123	P36951	6,1	29,07
87	Phosphoglycerate mutase	Carbohydrate metabolism	40	1079115	Q9GU73	6,6	28,70
88	Voltage dependent anion selective channel (VDAC)	Porin protein	65	6174942	Q94920	6,4	30,53
90	CG18030 gene product or Serine protease 1	Immune pathways activation	17	23172694	Q9VA66	7,7	25,21
91	Glutathione s-transferase (GST class-theta)	Stress response (detoxification)	39	121694	P20432	8,3	22,55
92	Glutathione transferase D1	Stress response (detoxification)	49	66614	Q963F1	6,9	23,72
93	Alcohol dehydrogenase	Carbohydrate metabolism, lipid metabolism	85	113424	P00334	6,9	27,72

Spot ID.	Protein name	Cellular function	Seq. cov. (%)	NCBI entry	SWISS-PROT	pI	Mr (kDa)
94	Enoyl-CoA hydratase mitochondrial	Fatty acid metabolism	37	7293203	Q9VXI1	8,8	31,56
95	Eukaryotic translational initiation factor 5A	Elongation initiation regulation	27	25090601	Q9GU68	5,0	17,59

96	eIF-5a gene product or Eukaryotic translational initiation factor 5A	Elongation initiation regulation	22	7291729	Q9GU68	4,9	17,58
97	CG4944-PB	Unknown	59			5,2	14,40
98	CG6891 gene product or Cyclic AMP-regulated protein like protein		53	7293507	Q9VWQ7	4,8	18,55
99	CG2968 gene product or ATP synthase mitochondrial (F1 complex δ-subunit)	Oxidative phosphorylation	68	7291126	Q9W2X6	5,7	16,72
100	CG14558 gene product	Unknown	47			5,1	16,22
101	Thioredoxin 2	Stress response (detoxification)	45	27734590	Q9V429	4,7	11,73
102	CG31305-PI or Fatty acid binding protein (lipocalin domain)		70	23171010	Q9VGM2	5,6	14,54
103	Nucleoside diphosphate kinase (NDP kinase)	Nucleoside triphosphates synthesis, signal transduction	32	127980	P08879	7,8	17,16
104	Peptidyl-prolyl cis-trans isomerase (cyclophilin)	PPIase activity	38	118099	P25007	8,4	17,89
105	Cofilin/Actin depolymerizing factor homolog (Twinstar protein)	Actin binding activity	38	1168731	P45594	6,7	17,14

42,	
55,	
59,	
60,	Unknown
63,	
65,	
89	

A large number of spots excised in this work are considered faint. This was done intentionally considering that frequently the most intense spots represent structural proteins, as can be observed for spots 25 and 27 that represent tubulin subunits or for spots 30 and 31 that represent actin. The identified faint spots represent, almost exclusively, non-structural proteins such as metabolic enzymes (spot 37 identified as Enolase or spot 57 identified as Glycerol-3-phosphate dehydrogenase).

The information on their functional characteristics was obtained from the SWISS-PROT and KEGG (http://www.genome.ad.jp/kegg/metabolism) pages.

Thirty-five percent of the identified spots represent proteins that have not been isolated or characterized before on *Drosophila* and so their cellular role was predicted based on specific homology domains shared with protein identifications from other species.

The most recent work of Vierstraete *et al.* (2003) reported a total of 32 spots identified using MALDI-TOF/MS approach. Five of these spots, which include actin, enolase, arginine kinase, fatty acid binding protein (lipocalin), larval serum protein gamma chain (precursor), were

also identified in this in spots numbered 30-32, 37, 47, 102 and 6, respectively. The remaining spots identified by Vierstraete *et al.* (2003) belong to protein families such as ferritins, serpins (serine proteases inhibitors), glycolytic enzymes, senescence marker proteins and lectins. Although none of these identifications were accomplished, taking into account the number of unexcised spots, it is possible that they are present in the 2-DE map obtained in this study. Albeit these previously results on *Drosophila* and 2-DE, 94 new proteins were identified.

3.3. Classification of *Drosophila melanogaster* larvae hemolymph identified proteins

About 50% of all identified protein spots with known or predicted function (searched on Pubmed, KEGG, and SWISS-PROT) in cellular processes belong to the functional class of metabolic enzymes. Within this class, the identification of a number of enzymes involved in carbohydrate metabolism (including glycolysis, gluconeogenesis, pentose phosphate pathway, krebs cycle), lipid metabolism (fatty acid oxidation) and protein metabolism including translational apparatus components, which account for the so called “house-keeping” pathways supporting cellular activity, was achieved.

Not only insects hemolymph (as is the case of other extracellular fluids) constitute a support for biological functions like oxygen or

metabolic transport or water and nutrients reserve, but also a source and transport of components of the humoral immune response, acting thereby as an important barrier to infection. The responses of *Drosophila* to an immunological challenge are now almost completely established (Hoffmann *et al.*, 2002, De Gregorio *et al.*, 2001, Hetru *et al.*, 2003, Meister *et al.*, 2000), and important components including recognition factors, signaling molecules, and effector molecules are likely to be continuously present in the hemolymph. In this context, proteins with defence properties were identified and represent 11% of total proteins listed in table 3-1. Among them serine proteases correspond to spots numbered 19, 68, 78 and 90. These proteins have been implicated in many physiological processes in which specific proteins are activated by proteolytic cleavage. Recent studies (Gorman *et al.,* 2001, Robertson *et al,* 2003) report serine proteases as playing a central role in insect immune response in regulating important processes like hemolymph coagulation, melanization of pathogen surfaces and antimicrobial peptide synthesis (as exposed in the introductory section).

In addition to the serine proteases, another important enzyme involved in melanization as a defence mechanism (Ligoxygkis *et al.,* 2002, Johnson *et al.*, 2003) was identified, which is prophenol oxidase (spot 22). When in it's active form (through proteolytic cleavage activation), the enzyme phenol oxidase is one of the enzymes responsible for melanin biosynthesis and also helps localized melanization onto pathogens by adsorbing to their surfaces

(Suguraman, 2002). The deposition of melanin prevents pathogen multiplication and thus its growth.

The insect immune responses which protect them from parasites, in conjunction with other molecular mechanisms such as adaptation to oxidative stress and aging of aerobic living organisms, are biological process where re-establishment of cellular redox homeostasis is crucial. Reported investigations (Kanzok *et al.,* 2001, Bauer *et al.,* 2002) document the absence of glutathione reductase in *Drosophila melanogaster*, being the thioredoxin system responsible for maintaining sufficiently high levels of reduced glutathione. Herein spot proteins were positively identified as important components that participate in this protective system: thioredoxin 2 (spot 101), thioredoxin peroxidase 1 (spot 83), thioredoxin reductase (spot 21) and glutathione transferase (spot 91). As documented, the high levels of cytosolic reduced glutathione are preserved by the thioredoxin system through thioredoxin reductases that are able to perform a dithiol-disulfide reaction with glutathione disulfide (GSSG) producing glutathione (GSH). The thioredoxin peroxidases have regulatory functions in redox state-associated signaling and act as electron acceptors from thioredoxin reductases for reducing hydrogen peroxides (as well as other organic peroxides) (Bauer *et al.,* 2002).

Also recently implicated in the modulation of immunological stress response are the heat shock proteins (Hsp). These proteins are also known as stress proteins that possess a cytoprotective function allowing cells to survive in stressful conditions that would be lethal in

their absence. Under normal conditions, they play an important role in intracellular "house-keeping" due to their molecular chaperone activity (Parcellier *et al.,* 2003). Within this class, Hsp's like the identified Hsp 82 (spot 2), Hsp 70 (spot 5) and Hsp 60 (spot 24) act as molecular chaperones by taking part in functions such as transporting proteins into cellular compartments, folding of newly synthesised proteins in the cytosol, endoplasmic reticulum and mitochondria, degradation of unstable or unfolded proteins and prevention of their aggregation, thus contributing to cell maintenance. Along with these Hsp's, a related protein to Hsp 90 (protein TRAP1, spot 13) was identified and, in a recent work (Felts *et al.,* 2000), was reported to be an ATP-binding protein and to exhibit ATPase activity. Due to this attributes it is still placed among the Hsp90 family proteins, but there is no evidence of interactions with co-chaperones.

Spot numbered 80 corresponding to a FK506-binding protein and spot numbered 104 as a cyclophilin are also proteins with chaperone properties. These proteins with PPIase activity belong to the Peptidyl-prolyl cis-trans isomerase family and are implicated in protein folding as well as other cellular processes like protein trafficking and chaperone activity.

In regard to the unknown or unclassified proteins, into cellular processes, an extensive search was done although no satisfactorily clear information was obtained.

:

4. Proteomic Analysis of *Drosophila melanogaster* immune response:

Results & Discussion

Life on Earth has radiated to fill virtually every conceivable habitat and niche. In each of these, organisms much interact with their environment and deal with some degree of variations in its parameters (Storey, 1999). Examples include temperature, pressure, oxygen and water availability, both ions and metals levels variations, numerous potentially toxic compounds and pathogen or parasite attack, as well. To some extent, variation in these parameters is accommodated with ease but, beyond certain limits stress is imposed. This kind of situation stimulates the organism to undergo metabolic adjustments that will allow the counteraction of the stress related negative effects. Studies on the interactions between organisms, their organs and/or cells, and the changes occurring in the environmental parameters cover an enormous part of Modern Biology and Clinical studies within Medicine. This is easily understandable as life is a continuous interplay between environmental stimulus and cellular responses.

Some areas of metabolic response to stress have been particularly explored, including adaptive responses of cellular energy metabolism, enzyme regulation and protein or membrane structures to intense exercise, ischemic stress, oxidative stress and aging. This comparative biochemistry approach has made, over the last 40 years, major advances in understanding how metabolism can be adapted and/or protected in order to allow life to persist through changing environments or at extreme situations.

Herein, an attempt is made to enhance the existing knowledge on the *Drosophila* innate immune response by comparative biochemistry,

using an immunological challenge as the environmental stimulus provoking stress. The effects of an immune challenge are investigated, through the immunisation procedure referred in section 2.2, on the protein expression levels of *Drosophila* third instar larvae by a mass spectrometric proteome analysis. The intent is to characterize protein expression differences between control and immunised larvae sets that are linked solely to the *Drosophila* immune response.

4.1. Drosophila melanogaster 2-DE protein pattern analysis in result of the immune challenge

In order to investigate the immune challenge effects in protein expression, *Drosophila* 2-DE protein patterns of control and immunised larvae (both recovery times) were obtained according to the methodology described in sections 2.6 and 2.7, using two replicate gels per sample. An example of a 2-DE protein pattern is shown in both figures 3-1 and 3-2 for reversible silver stained and colloidal stained 2-DE maps, respectively. Their comparison was made via the PD-Quest v7.1 software analysis, as exposed in section 2.8.

Initially, and prior to mass spectrometry protein identification, the reversible silver staining procedure was the procedure used for the 2-DE methodology, due to its higher sensitivity as well as reasonable trustworthy in semi quantifying protein maps. So, relatively to silver stained 2-DE protein patterns, the software allowed the detection of about 336 spots in the control gels, 315 spots in the 6 hours recovery gels and 320 in the 24 hours recovery gels.

This software analysis also revealed that, during the recovery period, both qualitative and quantitative changes occurred (for gel images see appendix II). Briefly, qualitative changes refer to presence and/or absence of spots when comparing 2-DE protein patterns of control and immunised larvae with 6 and 24 hours of recovery, whereas quantitative changes refer to optical density differences in spots present both in 2-DE protein patterns of control and immunised larvae, indicating their induction or repression. In fact, concerning the qualitative changes, after 6 hours of recovery, 17 spots were absent from the control gels. None of these spots were identified, as most of them are not visible in the colloidal coomassie stained gels essentially because of their faintness. Interestingly, 12 of the 14 spots that appear in the 24 hours recovery gels, by comparison with the 6 hours recovery time, are in common with the group of the 17 spots referred previously. Again, none of these spots were identified in the previous chapter. Finally, after 24 hours recovery, 26 spots are still absent from the control gels.

Concerning the quantitative changes that occurred during the recovery period, and taking into account the information displayed in table 3-1 regarding the identified hemolymph proteins, the following results were obtained:

- After 6 hours of recovery: 36 spots were induced and a total of 16 were identified as protein spots involved in functional classes such as fatty acid oxidation, respiration, protein metabolism, cellular defence mechanisms, as well as structural proteins. Along with this, 20 spots are found to correspond to repressed proteins and 7 of these spots were identified (table 4-1).

Table 4-1: Quantitative changes that occurred during the 6 hours recovery time.

Cellular function	Protein name	
	Induction	Repression
Stress response	Hsp 82 Thioredoxin 2 chaperonin 2 β-subunit	
Immune response	Serine proteinase	
Protein metabolism	Translational elongation factor 2 Aminoacylase Translational elongation factor 1 γ-chain	Larval serum protein 2

Carbohydrate metabolism	Transketolase	Malic enzyme Aldehyde reductase Glyceraldehyde 3-phosphate dehydrogenase
Fatty acid oxidation	Enoyl-CoA hydratase Hydroxyacyl-CoA dehydrogenase	
Cellular respiration	NADH dehydrogenase	
Structural proteins	Paramyosin Tubulin 1 α-chain Tubulin 1 β-chain Actin 87E	Actin 57B Actin Tropomyosin 1
Total number of spots	**36** (16 identified spots)	**20** (7 identified spots)
Total number of spots altered during the RT	**56**	

♦ Concerning the recovery period between the 6 hours and the 24 hours recovery time, differences in optical densities were observed for a total of 93 spots. Indeed, 27 spots were induced and positive identifications were accomplished for 8 spots, whereas a total of 66 spots were repressed and from those 29 spots were positively identified, including proteins implicated in various cellular functions as carbohydrate, protein and lipid metabolisms, cellular defence mechanisms and signal transduction as well as structural proteins. It is worthy to emphasize that, during this recovery period, one can find

repressed spots that were induced after 6 hours recovery as well as the opposite.

Table 4-2: Quantitative changes that occurred during the recovery period between the 6 hours and the 24 hours recovery time..

Cellular function	Protein name	
	Induction	**Repression**
Stress response	Hsp 82	Hsp 90 related protein TRAP1 thioredoxin reductase chaperonin 2 β-subunit thioredoxin peroxidase 1 thioredoxin peroxidase 3 thioredoxin 2
Immune response		serine proteinase chymotrypsin-like serine protease serine protease PPIase
Protein metabolism	larval serum protein 1 γ-chain larval serum protein 1 α-chain larval serum protein 2	arginine kinase translational elongation factor 1 γ-chain carboxypeptidase A carboxypeptidase B carbonic anhydrase

Carbohydrate metabolism	citrate synthase fructose-biphosphate aldolase	phosphoenolpyruvate carboxykinase fumarase isocitrate dehydrogenase malate dehydrogenase glycerol-3-phosphate dehydrogenase 2
Cellular function	**Protein name**	
	Induction	**Repression**
Cellular respiration		succinate dehydrogenase flavoprotein subunit
Structural proteins		Paramyosin tubulin 1 β-chain chloride intracellular channel 5 tropomyosin 1 non-muscle
Others	NDP kinase	14-3-3 protein epsilon 14-3-3-like protein GIP-like protein Twinstar protein
Total number of spots	**27** (8 identified spots)	**66** (29 identified spots)
Total number of spots altered during the RT	**93**	

- Finally, after 24 hours of recovery, there are still spots that do not return to control levels. A total of 31 spots are still induced and 8 of those were positively identified as protein spots involved mainly in protein biosynthesis, fatty acid oxidation and signal transduction. Nevertheless, once again the majority of OD differences is represented by the repressed spots that account for 50 spots..

Table 4-3: Quantitative changes that occurred during the overall 24 hours recovery period.

Cellular function	Protein name	
	Induction	Repression
Stress response		Hsp 90 related protein TRAP1 thioredoxin reductase glutathione transferase
Immune response		serine proteinase chymotrypsin-like serine protease serine protease
Protein metabolism	larval serum protein 1 γ-chain larval serum protein 1 α-chain	carboxypeptidase B
Cellular function	**Protein name**	
	Induction	**Repression**
Carbohydrate metabolism	citrate synthase fructose-biphosphate aldolase	phosphoenolpyruvate carboxykinase malic enzyme

		dihydrolipoamide dehydrogenase enolase isocitrate dehydrogenase aldehyde reductase glycerol-3-phosphate dehydrogenase 2 phosphoglycerate mutase
Fatty acid oxidation	Enoyl-CoA hydratase	
Cellular respiration		succinate dehydrogenase flavoprotein subunit
Structural proteins	tubulin 1 α-chain	chloride intracellular channel 5 tropomyosin 1
Others	NDP kinase	receptor mediated endocytosis 14-3-3 protein epsilon 14-3-3-like protein GIP-like protein
Total number of spots	**8** (from a total of 31 spots)	**24** (from a total of 50 spots)
Total number of spots altered during the recovery time	**81**	

4.1.1. 2-DE protein pattern comparative analysis *versus* staining procedure

In an ideal 2-DE proteomics analysis approach study, all replicate gel images of any sample would contain the same spots with the same individual spot volumes (or intensities) and each protein spot would migrate to the same position every time. However, as already known, due to the nature of 2-DE proteomic methodologies there will always be "process variability" factors in any dataset collected in this way. Some of these factors, among others, will arise during sample preparation, gel running, and staining, or from gel analysis procedure. According to Smales *et al.* (2003), when using silver staining technology, the reproducibility of generated data is dependent on individual protein spots intensities (which in turn is correlated with the coefficient of variation) and is protein specific. So, for expression analysis studies, comparison with confidence of individual spots intensities, which correspond to proteins of interest between gels and samples, is feasible provided the coefficients of variation for those individual spots are known. This is especially important when the spot corresponds to a low abundant protein, as it is for low spot volumes that match the highest coefficients of variation (Smales *et al.*, 2003). This can be overcome by considering alternative staining procedures such as SYPRO Ruby and Coomassie, which present a higher linearity

measure of relative protein abundance in a gel map than does silver staining.

Taking into account the exposed, the coomassie alternative was chosen for this proteomic analysis approach study of *Drosophila* immune response, although compromising sensitivity was a disadvantage. Then, as for protein mass spectrometry identification in the previous chapter, the alternative staining procedure was the colloidal coomassie staining, for it is 10 times more sensitive than normal coomassie blue. From this point forward, every comparison in terms of quantitative changes between control and immunised *Drosophila* larvae protein patterns was performed using colloidal protein patterns for each gel and sample. Plus, this staining procedure allowed spot intensities normalization by taking into consideration the spot volume from a marker band (for its precise protein composition provides the possibility to find out the protein quantity present in each marker band) equally chosen in each gel, besides the spots normalization already referred in section 2.8.

4.2. Drosophila melanogaster identified hemolymph proteins differentially expressed in result of the immune challenge

In order to investigate the immune challenge effects in protein expression, the comparison in terms of quantitative changes between control and immunised *Drosophila* was performed for the hemolymph proteins identified in the previous chapter, using *Drosophila* 2-DE colloidal coomassie protein patterns and via three replicate gels per sample. Their comparison was made via the PD-Quest v7.1 software analysis, as exposed in section 2.8, and the variability factors for both recovery times are shown in table 4-4. The 24 hours recovery values were calculated via the 6 hours recovery values in an attempt to verify what was occurring during the recovery period.

With the purpose of attributing spot volume (intensity) differences between samples as significant changes in protein expression, i. e. not related to gel-to-gel variations, statistical analysis via the Student's *t* test (P=0,05) was performed taking into consideration the three replicates per sample (Smales *et al.*, 2003). The spots that presented a confidence interval larger than 10% between the replicates were not taken for the variability factors estimations between samples, as they were not considered to be reliable.

Table 4-4: Table of the quantitative changes in the identified *Drosophila* hemolymph proteins after immune challenge for both recovery times. ▲means up-regulation whereas ▼means down-regulation; **nv** means no significant variability and occurs whenever the variability factor is less than 10% between samples.

Metabolism	Identified proteins	Recovery time (hours)	
		6h	24h
Carbohydrate metabolism	Fructose-biphosphate aldolase	20%▲	nv
	Transketolase	21%▲	nv
	Glyceraldehyde 3-phosphate dehydrogenase I	nv	37%▼

Metabolism	Identified proteins	Recovery time (hours)	
		6h	24h
Carbohydrate metabolism	Glyceraldehyde 3-phosphate dehydrogenase II	13%▲	67%▼
	Phosphoglycerate mutase	14%▲	24%▼
	Enolase	nv	14%▼
	Phosphoenolpyruvate carboxykinase	31%▼	11%▲
	Citrate synthase	22%▲	nv
	Isocitrate dehydrogenase 1	nv	20%▼
	Isocitrate dehydrogenase 3 (NAD^+) subunit	36%▲	61%▼
	Succinate dehydrogenase (ubiquinone flavoprotein)	15%▼	76%▼
	Fumarase	nv	10%▲
	Malate dehydrogenase (malic enzyme)	16%▲	78%▼
	Cytosolic malate dehydrogenase	52%▲	23%▼
	Alcohol dehydrogenase	13%▼	27%▲
	Aldehyde reductase	10%▼	19%▼
	Dihydrolipoamide dehydrogenase	47%▲	44%▼
Energy metabolism Oxidative phosphorylation	NADH dehydrogenase Fe-S protein	29%▼	12%▼
	Succinate dehydrogenase (ubiquinone flavoprotein)	15%▼	76%▼
ATP synthesis	ATP synthase mitochondrial F1 complex δ-subunit	35%▲	10%▼
	ATP synthase α-chain	58%▲	37%▼
Lipid metabolism Fatty acid β-oxidation	Enoyl-CoA hydratase mitochondrial	70%▲	38%▼
	Acyl-CoA dehydrogenase	42%▲	48%▼

Metabolism	Identified proteins	Recovery time (hours)	
		6h	24h
Protein metabolism			
	Aminoacylase	47%▲	51%▼
	Arginine kinase	nv	32%▼
Proteolysis and peptidolysis	Carboxypeptidase A	60%▲	31%▼
	Carboxypeptidase B	27%)▲	36%▼
Biosynthesis	Larval serum protein 1 γ-chain	22%▼	23%▲
	Larval serum protein 1 α-chain	17%▼	23%▲
	Larval serum protein 2	41%▼	117%▲
	Translational elongation factor 2	31%▼	39%▲
	Translational elongation factor 1γ	27%▼	21%▼
	Eukaryotic translational initiation factor 4A	nv	27%▼
	Eukaryotic initiation factor 5A	25%▼	nv
	Alpha NAC	55%▼	10%▼

Stress response	Identified proteins	Recovery time	
		6h	24h
Heat shock response (and chaperone properties)	Heat shock protein 83	27%▲	25%▼
	Heat shock protein 70	19%▲	25%▲
	Heat shock protein 60	nv	16%▼

	Heat shock protein 90 related protein TRAP1	10%▲	62%▲
	Chaperonin 2 β-subunit	18%▲	55%▼
Immune response	Prophenol oxidase	28%▼	29%▲
	Chymotrypsin -like serine protease	42%▲	39%▼
	Serine proteinase	37%▲	48%▼
	Serine protease 1	28%▲	44%▼
	PPIase	45%▲	nv

Stress response	**Identified proteins**	**Recovery time**	
		6h	**24h**
Detoxification	Thioredoxin	43%▲	30%▼
	Thioredoxin reductase	40%▲	41%▼
	Thioredoxin peroxidase 1	30%▲	65%▼
	Thioredoxin peroxidase 3	37%▲	50%▼
	Glutathione transferase	42%▲	27%▼

Cellular function	**Identified proteins**	**Recovery time**	
		6h	**24h**
Structural	Paramyosin	39%▲	32%▼
	Paramyosin, long form	23%▲	46%▼
	Tubulin 1 β-chain	44%▲	32%▼
	Actin	16%▼	nv
	Actin 57B	28%▲	nv
	Actin 87E	50%▲	18%▼
	Tropomyosin 1	27%▼	nv

	Tropomyosin 2	24%▲	39%▲
Voltage-gated channel	Chloride intracellular channel protein	45%▲	57%▼
Porin protein	VDAC	36%▲	30%▼
Cell communication (signal transduction)	14-3-3 protein epsilon	nv	25%▼
	14-3-3 like protein (Leonard protein)	49%▲	29%▼
Unclassified	Nucleoside diphosphate kinase (NDP kinase)	50%▼	nv
	Cyclic AMP-regulated protein like-protein	14%▼	Nv
	Receptor mediated endocytosis protein 1, isoform a	117%▲	61%▼
	Guanine nucleotide-binding protein β-subunit like protein	13%▲	18%▼
	Fatty acid binding protein	nv	31%▼
	Twinstar protein (cofilin/actin depolymerizing factor homolog)	18%▲	72%▼

By observing and interpreting the information contained in table 4-4, it is possible to notice, because of the immune challenge and during the recovery period, the occurrence of a significant reprogramming of protein synthesis and/or expression pattern in *Drosophila* larvae. Cellular processes such as carbohydrate, lipid, and

protein metabolisms, in addition to antioxidant and immunological defence devices, are shown to be affected.

4.2.1. *Drosophila melanogaster* hemolymph proteins differentially expressed after 6 hours of recovery

4.2.1.1 Proteins induced by the immune challenge

The up-regulated proteins, sorted into their functional classes, represent enzymes implicated in the carbohydrate metabolism, which comprises pathways such as glycolysis, pentose phosphate, and krebs cycle, enzymes implicated in the fatty-acid β-oxidation pathway, and enzymes implicated in protein degradation (proteolysis), in addition to stress response related proteins.

Nearly all the identified glycolytic enzymes present an increase in their relative abundance after 6 hours of recovery. The enzymes involved in this pathway are fructose-biphosphate aldolase, glyceraldehyde 3-phosphate dehydrogenases I and II, phosphoglycerate mutase, enolase, and dihydrolipoamide dehydrogenase. Enzymes for instance, isocitrate dehydrogenase, fumarase, malate dehydrogenase, directly implicated in the Krebs cycle, also show a similar behaviour.

Although there is no clear information in the literature concerning analogous studies, these results suggest that even though a reorganization of metabolism network is needed, at cellular level, the organism is using these pathways for one of their major purposes: ATP production. The increase in the ATP synthesis, shown by the up-regulation of the subunit enzymes ATP synthase F1 complex δ-subunit and ATP synthase α-chain, and the decline of phosphoenolpyruvate carboxykinase gluconeogenesis enzyme also propose that ATP production is more obligatory to cells than does the use of these pathways for generating buildings blocks to fatty acid or aminoacid biosynthesis, for instance. At the cellular level, ATP depletion results in proteotoxic stress that can lead to dysfunction, destabilization, and aggregation of many cellular proteins including enzymes, ion pumps, and constituents of cytoskeletal and contractile structures (Kabakov *et al.*, 2002). Reports focusing on the main effects of heat shock reveal that intracellular ATP abundance declining is one of the primary events (Findly *et al.*, 1983, Lambowitz *et al.*, 1983). The assumption that identical consequences would occur, results that these pathways involved in cellular respiration are induced in result of the cells demands in ATP synthesis and energy production. Thus, cells seem to readjust their metabolic activities according to their energetic requirements and, if necessary, at the cost of their biosynthetic capabilities.

Along with this, an enzyme involved in the pentose phosphate pathway, transketolase, was also identified and stimulated after 6 hours of recovery. This pathway is known to be the major source of NADPH, as

a result of glucose oxidation, a very important element in reductive biosynthesis (for instance, fatty acid and nucleotide biosynthesis and detoxification reactions). Still concerning NADPH production, all identified enzymes implicated in fatty acid β-oxidation – enoyl-CoA hydratase, acyl-CoA dehydrogenase, and hydroxyacyl-CoA dehydrogenase – showed to be induced upon immune challenge. In fatty acid β-oxidation pathway, fatty acids are firstly activated and then transported into mitochondria for degradation where they are broken down into acetyl-CoA, resulting as well as the pentose phosphate pathway in NADPH production (and $FADH_2$). These results can suggest that either this raise in NADPH cell requirements is caused by an increase in energy demand by ATP production through cellular respiration, or is caused by an increase in the antioxidant defence reactions for cellular redox homeostasis maintenance.

In fact, all the identified proteins involved in adaptive response mechanisms like immune response or detoxification, as well as the heat shock proteins were also induced after 6 hours of recovery.

The higher stimulation indexes observed in table 4-4 correspond to the proteins involved in both immune response and detoxification defence mechanisms.

Concerning the immune response, proteins such as serine proteases, one PPIase (immunophilin) and prophenoloxidase were identified. As exposed in section 3.3, serine proteases have been reported as important molecules playing a central role in insect immune response by regulating important processes like hemolymph

coagulation, melanization of pathogen surfaces and antimicrobial peptide synthesis, via proteolytic cleavage activation of specific proteins. So, their increase after 6 hours recovery is likely related with *Drosophila* larvae immune response reactions.

In this context, the PPIase up-regulation is justified in a similar manner. PPIases, as already referred, belong to the peptidyl-prolyl cis-trans isomerases family and are responsible for catalysing the isomerisation of peptide bonds N-terminal to proline residues in polypeptide chains (Shaw, 2002), having roles in the folding of newly synthesised proteins. Based on drug specificity, PPIases have been divided into three distinct classes - cyclosporine A binding cyclophilins; FK506-binding proteins (FKBPs); and parvulin-like PPIases- and are also structurally divergent. Even though, cyclophilins and FKBPs are collectively referred to as immunophilins (Schreiber, 1991, cited in Luan, 1998). Among the identifications presented in table 3-1, there are two positive identifications for a KFBP (spot numbered 80) and a cyclophilin (spot numbered 104); however, in the 2-DE colloidal protein patterns used for the proteomics analysis of *Drosophila* immune response, the correspondent spot for FKBP was absent. During the past several years, a growing number of immunophilins has been characterized from sources from bacteria to yeast and higher plants (Schreiber, 1991, Fruman *et al.*, 1994, reviewed in Luan, 1998). Their high level of conservation and ubiquitous distribution among divergent organisms and in almost all subcellular compartments indicates that these proteins participate in important cellular processes. Since then, they have been

implicated in facilitating protein folding *in vivo* (due to its rotamase activity) (Shaw, 2002), protein trafficking (Stammes *et al.*, 1991), and can function as chaperones, a possibility supported by reports that demonstrate the heat-shock responsive expression of cyclophilin mRNAs in yeast (Sykes *et al.*, 1993) and in a higher plant (Luan *et al.*, 1994a). Recently, they have been also associated to oxidative stress response (Santos *et al.*, 2000, cited in Cavarec *et al.*, 2002), probably because of its roles in the referred cellular processes. Therefore, PPIase up-regulation suggests it is an immune-associated protein because of its capabilities in protein folding and in oxidative stress, both consequences of the immune defence reactions.

Also directly implicated in insects innate immune response is the enzyme phenol oxidase. In this study, the inactive form of this enzyme, prophenol oxidase, was identified and among the other immune-related proteins is the only to be repressed upon immune challenge (and after 6 hours recovery). In the introductory section, one of the killing mechanisms described as associated with the insect immune response was the encapsulation (cellular defence reaction). Encapsulation is often accompanied by the deposition of melanin and protein-phenol complexes around the intruder, in a reaction termed melanotic encapsulation, which requires the proteolytic activation of phenol oxidase present as an inactive precursor termed prophenol oxidase. Hence, the results propose that the prophenol oxidase decline probably is coupled with the enzyme active form rising, thus contributing to the melanization reactions as a cellular defence mechanism.

Besides the directly immune-related proteins referred, proteins with antioxidant properties also show to be induced upon immunological stress. After 6 hours of recovery, proteins like thioredoxin, thioredoxin reductase, thioredoxin peroxidases 1 and 3, and glutathione transferase, presented stimulation indexes comparable to those of immune-related proteins. Nowadays, it is well known that oxidative stress is concurrent with immune response, including the insects' innate immune response. Cellular defences, such as phagocytosis, melanotic encapsulation, results in cytotoxic reactive oxygen intermediates (ROI) production, as well as reactive nitrogen intermediates (RNI) and associated enzymes. The signalling pathways involved in the activation and consequent generation of ROI (for instance, O_2^- or H_2O_2) are not completely understood, in part because of the "cross-talk" that exists between the signal transduction messengers. It is hard to known which event is the first to occur: it is known that immune system activation, through the pattern recognition receptors (PRRs), triggers specific signal transduction pathways (see section 1.2.2) and one of the consequent events is for certain the orchestrated ROI production brought to help elimination of pathogens; on the other hand, upon ligand binding of phagocytic cells, the generated ROI initiate the activation "program" that coordinates the production of proteins with protective functions. Nevertheless, these reactive oxygen species can damage various components of living cells, such as unsaturated lipids (giving rise to organic peroxides), proteins, or nucleic acids (Rabilloud *et al.*, 2002). To counter these deleterious events, cells use several

protective systems that either repair the various types of damage (for instance, DNA repair enzymes), or destroy the reactive oxygen species which implies the action of enzymatic systems like the peroxidases. Peroxidases, like the thioredoxin peroxidase 1, destroy peroxides by reducing them to the corresponding alcohol (or water) with the simultaneous oxidation of a specific co-substrate (thioredoxin 2). This oxidized co-substrate is then reduced back using NADPH as the reducing agent, being the overall reaction the peroxides destruction to the corresponding alcohols (and/or water) and consumption of NADPH.

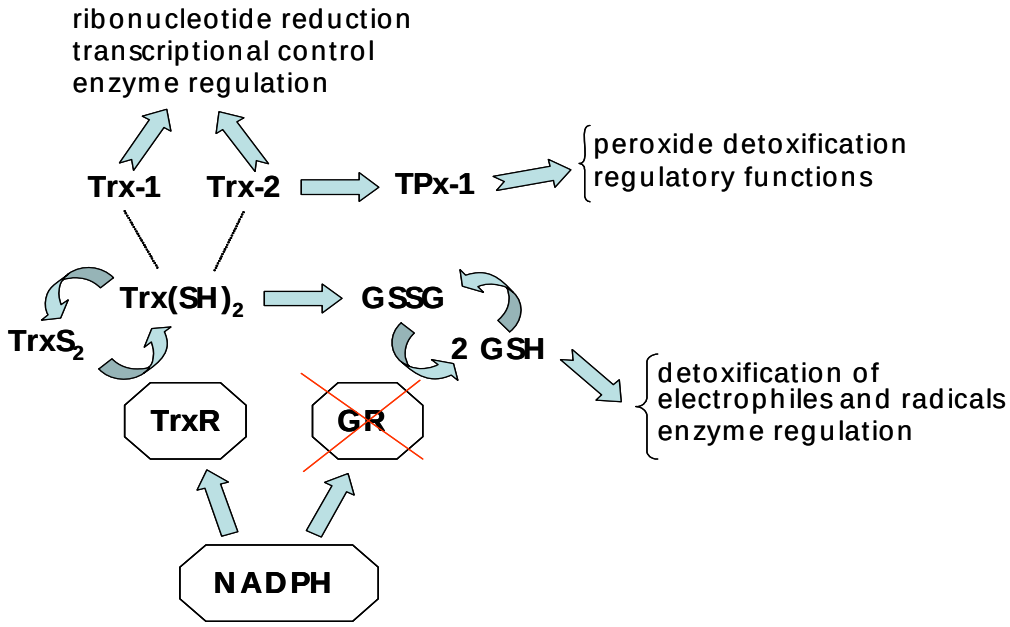


Figure 4-1: Thioredoxin system components interaction in *Drosophila melanogaster*. NADPH is the reducing co-substrate in this system, used by

thioredoxin reductase for reducing both thioredoxins and glutathione in stress detoxification reactions, envolving thioredoxin peroxidase as well. It is also shown that only thioredoxin 2 serve as substrate for thioredoxin peroxidase 1. Trx, thioredoxin; TPx, thioredoxin peroxidase; $Trx(SH)_2$, reduced thioredoxin; $TrxS_2$, disulfide (oxidized) form; TrxR, thioredoxin reductase; GR, glutathione reductase; GSSG, glutathione disulfide; GSH, reduced glutathione (adapted from Bauer *et al.*, 2002).

In *Drosophila*, the system responsible for re-establishment of cellular redox homeostasis is the thioredoxin system, as already described, and its components interact in a similar way of that presented in figure 4-1. Furthermore, the results about this antioxidant system suggest that, rather than contributing to ATP production, the consumption of NADPH is mainly directed to the protective role of antioxidant defences.

Finally, the heat shock proteins analysed in this study are also induced upon immune challenge, as expected, although with a stimulation index comparatively lower than the stimulation index of the immune- and detoxification-related proteins discussed above. Nonetheless, their inducibility was expected as these proteins are up-regulated in response, not only to heat shock, but also to other stresses in nearly all organisms, where they promote stress tolerance mainly by functioning as molecular chaperones. Although Hsps are best known for their inducibility by heat, it has been reported that the presence of non-native proteins within cells is sufficient to induce their expression (Krebs and Feder, 1997). Therefore, their inducibility can be attributed to a variety of stresses, including immunological stress responses, as a

direct reaction to cells and/or tissues damages, whether directly caused or whether caused by disorders in cell maintenance due to ROS production, lipids oxidation or proteolytic activity. The results indicated in table 4-4 reveal that, among the identified Hsps, a relative low increase was associated with the cytoplasmatics Hsp 82 and Hsp 70, after 6 hours of recovery. Although it is to be considered that the stress imposed in this study was not severe, for the immunisation procedure did not include pricking larvae, in a report by Krebs and Feder (1997) Hsp 70 expression in third-instar *Drosophila* larvae at severe heat shoch treatment (38,5 °C) was initially very low, increasing many hours afterwards. A similar behaviour is suggested by these results associated with Hsps, mainly for Hsp 70 which at the 24 hours of recovery is still being up-regulated. When concerning the mitochondrial Hsps, Hsp 60 and Hsp 90 related protein, results suggest that the application of the immunological challenge did not severely affected them or the normal functioning of mitochondria, as already implied by the up-regulated fatty acid β-oxidation pathway.

Besides functioning as molecular chaperones, Hsps are implicated in regulation protein turnover (Parsell and Linquidist, 1993), by facilitating recognition and degradation of unstable or unfolded proteins via the ubiquitin system proteolytic degradation. Polyubiquitinated proteins are subsequently degraded by a large ATP-dependent complex, the proteasome. Although none of the identifications presented in this study succeeded in positive identifications of components implicated in this pathway, it is well established that almost any cellular stress

response is associated with protein accumulation and subsequent degradation. However, three proteins –carboxypeptidase A and B, and aminoacylase (urea cycle) – with proteolytic activity were identified and up-regulated after 6 hours of recovery. In addition, for both colloidal coomassie and silver staining 2-DE protein patterns, it is possible to observe a decline in the total spots number after immune challenge. This too might indicate an increase in proteolytic activity due to the stress imposed, although probably coupled with repression of protein biosynthesis.

4.2.1.2 Proteins repressed by the immune challenge

The proteins repressed due to immune challenge were mainly proteins involved in biosynthetic processes, such as protein biosynthesis (translational apparatus components), complex lipid metabolism (glycerol metabolism) and precursor's biosynthesis (for generating aminoacids, nucleotides, lipids, purines and pirimidines, and porfyrins, etc.).

Concerning protein biosynthesis, all the identified proteins, such as larval serum proteins (storage proteins for biosynthesis), translational initiation and elongation factors, are down-regulated after 6 hours of recovery indicating the occurrence of a global slowdown in protein translation. Like this biosynthetic pathway, the enzymes glycerol-3-phosphate dehydrogenase, aldehyde reductase, and alcohol dehydrogenase, that stand for the glycerol metabolism, also show a

decline in their expression after the immune challenge. These results are in agreement with the observed carbohydrate metabolism results, indicating that those pathways are directed to the production of ATP and NADPH and not to the consumption of energy as it is the case of glycerol metabolism.

Taken these results all together, the stress response is stimulating the important switch of the biosynthetic normal cellular activity towards cellular protective functions by inducing Hsps, immune-related proteins and *Drosophila* antioxidant system components.

4.2.2. *Drosophila melanogaster* hemolymph proteins differentially expressed after 24 hours of recovery

After 24 hours of recovery, nearly all hemolymph proteins analysed in the previous point (section 4.2.1) are still being down-regulated, when compared with the correspondent values of the 6 hours recovery time. Basically, the enzymes involved in the carbohydrate, energy, and lipid metabolisms, as well as the proteins involved in the overall stress response, including elements of the immune and antioxidant defence reactions, Hsps, and proteins with proteolytic activity, were still down-regulated (indicating a little recuperation from the immunological stress at cellular level).

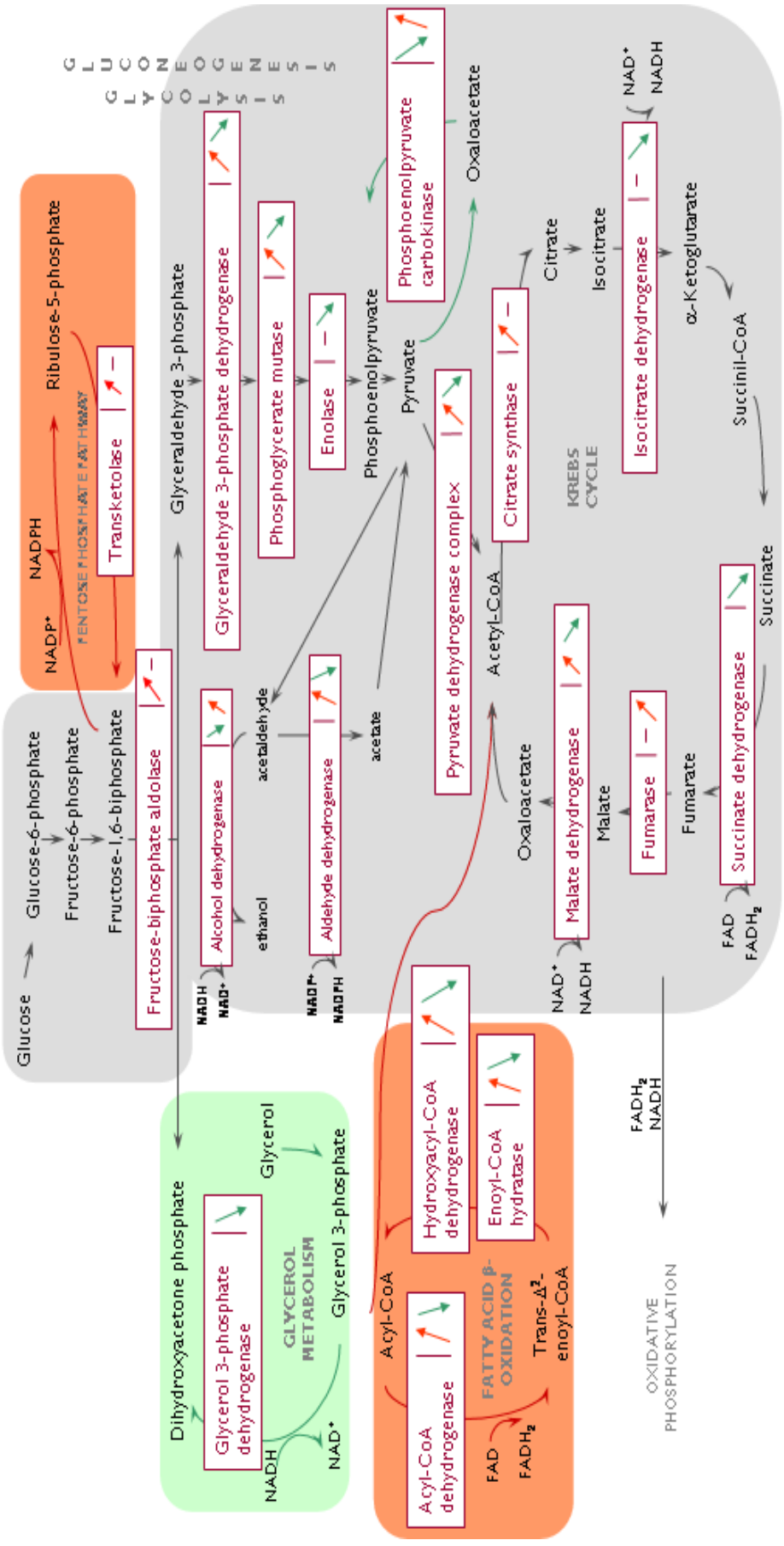
Yet, as expected, part of the identified proteins implicated in protein biosynthesis were induced after 24 hours of recovery, most likely indicating that, at this point, a little recuperation in anabolism was starting to occur at the cellular level.

Furthermore, at this time, there were still cellular processes that did not returned to their control values. By comparison with the control sets, only the metabolic proteins implicated in the ATP synthesis, protein biosynthesis, and fatty acid β-oxidation appeared to regain their normal functioning. Another exception is the enzyme prophenol oxidase, involved in cellular defences, which also returned to its control values. Unlike the later and regarding the stress-related proteins, Hsps such as Hsp 70, Hsp 90 related protein, and PPIase, at this point they still have stimulation indexes above the control values. This seem to be in agreement with already referred increase in the protein biosynthesis, for these proteins with molecular chaperone properties will surely be involved in this cellular process.

As a whole, these results illustrate that, after reorganization of the metabolism and stress adaptive responses functioning, i. e., up-regulation of stress-related proteins and concomitant protein degradation with global biosynthesis slowdown, at this time resources are being redirecting to normal development. With the diminished energy demand after stress recuperation, at cellular level, the regain of normal development can be interpreted with the emergence of anabolism in detriment of catabolism due to metabolic adaptation, as pointed up by the results.

In figure 4-2 an overall picture of the results concerning the metabolic re-adjustments, upon the immune stress imposed in this study, is presented for both the recovery times.

Figure 4-2: Metabolic pathways altered with the immune challenge for both recovery times. Boxes show both identified proteins and their variability at 6 and 24 hours of recovery, respectively; means induction, whereas corresponds to repression; indicates that no significative variability occurred at that recovery time.


GLUCONEOGENESIS
GLYCOLYSIS
PENTOSE PHOSPHATE PATHWAY
Glucose
Glucose-6-phosphate
Fructose-6-phosphate
Fructose-1,6-biphosphate
Fructose-biphosphate aldolase
Transketolase
Ribulose-5-phosphate
NADP+
NADPH
Glyceraldehyde 3-phosphate
Glyceraldehyde 3-phosphate dehydrogenase
Phosphoglycerate mutase
Enolase
Phosphoenolpyruvate
Phosphoenolpyruvate carbokinase
Oxaloacetate
Pyruvate
Alcohol dehydrogenase
acetaldehyde
ethanol
NADH
NAD+
Aldehyde dehydrogenase
acetate
NAD+
NADH
Pyruvate dehydrogenase complex
Acetyl-CoA
Citrate synthase
Citrate
KREBS CYCLE
Isocitrate
Isocitrate dehydrogenase
NAD+
NADH
α-Ketoglutarate
Succinil-CoA
Succinate
Succinate dehydrogenase
FAD
FADH2
Fumarate
Fumarase
Malate
Malate dehydrogenase
NAD+
NADH
Oxaloacetate
Dihydroxyacetone phosphate
Glycerol
Glycerol 3-phosphate dehydrogenase
NADH
NAD+
GLYCEROL METABOLISM
Glycerol 3-phosphate
Acyl-CoA
Hydroxyacyl-CoA dehydrogenase
Enoyl-CoA hydratase
FATTY ACID β-OXIDATION
Trans-Δ2-enoyl-CoA
Acyl-CoA dehydrogenase
FAD
FADH2
FADH2
NADH
OXIDATIVE PHOSPHORYLATION

5. Conclusions

Although a vast amount of essential information has been supplied by the completion of the *Drosophila melanogaster* genome sequence, the understanding of mechanisms underlying biological events is not completely accomplished without performing a proteomic analysis investigation. This kind of investigation, where a protein profile analysis can be used for studying global changes in protein expression in tissues or body fluids in different developmental or environmental conditions, turns out to be essential as it is entrenched that any biological system functions are mainly protein controlled.

Hence, in this study, a proteomics analysis approach is reported on the investigation of hemolymph proteins of *Drosophila melanogaster* larvae. By using 2-DE with extended pH range (3-10) and high sensitivity protein identification through MALDI-TOF/TOF, the identification of 99 protein spots out of a total of 105 excised spots was achieved, which corresponds to 89% of positive protein identifications. The major percentage of these identifications were metabolic enzymes implicated in biochemical pathways such as glycolysis and gluconeogenesis, krebs cycle, fatty acid oxidation and protein biosynthesis apparatus components. Additionally, important components of the *Drosophila* immune response machinery, as well as Hsps and antioxidant defence components of the thioredoxin system, were also located in the 2-DE map. As a whole, this work presented for the first time the identification and location of 94 proteins in the hemolymph of *Drosophila* larvae.

The effects of an immune challenge in the overall modification of *Drosophila* 2-DE protein patterns were investigated by proteome analysis, taking into consideration the already accessible information on the *Drosophila* hemolymph proteins. The intent was to investigate protein expression differences between control and immunised larvae sets which could be related solely to the *Drosophila* immune response, which corresponded to the main goal of this work. The results suggested that the response to the immune challenge stimulated the switch of the biosynthetic normal cellular activity (with the repression of, mainly, protein and lipid metabolisms), towards cellular protective functions by inducing Hsps and other proteins with chaperone properties, immune-related proteins and *Drosophila* antioxidant system components (and their co-factors, NADPH and $FADH_2$), which account for 11% of the total of 99 identified proteins. Both their presence and up-regulation demonstrated their importance and contribution in maintaining cellular redox homeostasis, in addition to immune signalling and fighting off infection.

In conclusion, the results achieved in this work increase the present knowledge of the *Drosophila* immune response, supported essentially on mRNA-based approaches showing genes transcriptionally modified after infection. This sort of approach measures messengers abundances in the total gene expression profile analysis and not the actual proteins, the real mediators in cellular processes. Additionally, mRNA-based approaches can not be used for analysing fluids like hemolymph, an important support and barrier for insect survival. With

this kind of proteome analysis tool, similar investigations in studying expression levels, modifications and interactions of immune-related proteins can be carried out using other species than *Drosophila* (for which genome information is also available), leading to a further comprehensive understanding of innate immunity.

6. Bibliography

Abrams, J. M., Lux, A., Steller, H. and Krieger, M. (1992). Macrophages in *Drosophila* embryos and L2 cells exhibit scavenger receptor-mediated endocytosis. Proc. Natl. Acad. Sci. USA 89, 10375-10379.

Adams M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., *et al.* (2000). The genome sequence of *Drosophila melanogaster*. Science 287, 2185-2195.

Aderem, A. and Underhill, D. M. (1999). Mechanisms of phagocytosis in macrophages. Annu. Rev. Immunol. 17, 593-623.

Ayme-Southgate, A., Lasko, P., French, C. and Pardue, M. L. (1989). Characterization of the gene for mp20: a *Drosophila* muscle protein that is not found in asynchronous oscillatory flight muscle. J. Cell Biol. 108, 521-531.

Basi, G. S., Bordman, M. and Storti, R. V. (1984). Alternative splicing of a *Drosophila* tropomyosin gene generates muscle tropomyosin isoforms with different carboxyl-terminal ends. Mol. Cell. Biol. 4, 2826-2836.

Bauer, H., Kanzok, S. M. and Schirmer, R. H. (2002). Thioredoxin-2 but not thioredoxin-1 is a substrate of thioredoxin peroxidase-1 from *Drosophila melanogaster*. J. Biol. Chem., 277, 17457-17463.

Becker, K. D., O'Donnell, P. T., Heitz, J. M., Vito, M. and Bernstein, S. I. (1992). Analysis of *Drosophila* paramyosin: identification of a novel isoform which is restricted to a subset of adult muscles. J. Cell Biol. 116, 669-681.

Belvin, M. P. and Anderson, K. V. (1996). A conserved signalling pathway: the *Drosophila* Toll-Dorsal pathway. Annu. Rev. Dev. Biol. 12, 393-416.

Braun, A., Hoffmann, J. A. and Meister, M. (1998). Analysis of the *Drosophila* host defence in *domino* mutant larvae, which are devoid of hemocytes. Proc. Natl. Acad. Sci. USA 95, 14337-14342.

Bulet, P., Hetru, C., Dimarcq, J. and Hoffmann, D. (1999). Antimicrobial peptides in insects: structure and function. Dev. Comp. Immunol. 23, 329-344.

Carton, Y. and Nappi, A. J. (2001). Immunogenetics aspects of the cellular immune reponses of *Drosophila* against parasitoids. Immunogenetics 52, 157-164.

Cavarec, L., Kamphausen, T., Dubourg, B., Callebaut, I., Lemeunier, F., Métivier, D., *et al.* (2002). Identification and characterization of Moca-cyp. J. Biol. Chem. 277, 41171-41182.

Choe, K. M., Werner, T., Stoven, S., Hultmark, D. and Anderson, K. V. (2002). Requirement for a peptidoglycan recognition protein (PGRP) in Relish activation and antibacterial immune responses in *Drosophila*. Science 296, 359-362.

Christophides, G., Zdobnov, E. M., Barillas-Mury, C., Birney, E., Blandin, S., Blass, C., *et al.* (2002). Immunity-related genes and gene families in *Anopheles gambiae*. Science 298, 159-165.

De Gregorio, E., Spellman, P. T., Rubin, G. M. and Lemaitre, B. (2001). Genome-wide analysis of the *Drosophila* immune response by using oligonucleotide microarrays. Proc. Natl. Acad. Sci. USA 98, 12590-12595.

De Silva, J. B., de Albuquerque, C. M. R., de Araujo, E. C., Peixoto, C. A. and Hurd, H. H. (2000). Immune defence mechanisms of *Culex quinquefasciatus* (Diptera: *Culicidae*) against *Candida albicans* infection. J. Invert. Pathol. 76, 257-262.

Dimopoulos, G. (2003). Insect immunity and its implication in mosquito-malaria interactions. Cellular Microbiol. 5, 3-14.

Dimopoulos, G., Müller, H. M., Levashina, E. A. and Kafatos, F. C. (2001). Innate immune defence against malaria infection in the mosquito. Curr. Opin. Immunol. 13, 79-88.

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. USA 94, 11508-11513.

Doneanu, C. E., Griffin, D. A., Barofsky, E. L. and Barofsky, D. F. (2001). An exponential dilution gradient system for nanoscale liquid chromatography in combination with MALDI or nano-ESI mass spectrometry for proteolytic digests. J. Am. Soc. Mass Spectrom. 12, 1205-1213.

Dushay, M. S., Asling, B. and Hultmark, D. (1996). Origins of immunity: Relish, a compound Rel-like gene in the antibacterial defence of *Drosophila*. Proc. Natl. Acad. Sci. USA 93, 10343-10347.

Elrod-Erickson, M., Mishra, S. and Schneider, D. (2000). Interactions between the cellular and humoral immune responses in *Drosophila*. Curr. Biol. 10, 781-784.

Engström, Y. (1999). Induction and regulation of antimicrobial peptides in *Drosophila*. Dev. Comp. Immunol. 23, 345-358.

Engström, Y., Kadalayil, L., Sun, S. C., Samakovlis, C., Hultmark, D. and Faye, I. (1993). KappaB-like motifs regulate the induction of immune genes in *Drosophila*. J. Mol. Biol. 232, 327-333.

Ericsson, C., Petho, Z. and Mehlin, H. (1997). An on-line two-dimensional polyacrilamide gel electrophoresis protein database of adult *Drosophila melanogaster*. Electrophoresis 18, 484-490.

Falkenthal, S., Parker, V. P., Mattox, W. M. and Davidson, N. (1984). *Drosophila melanogaster* has only one myosin alkali light-chain gene which encodes a protein with considerable aminoacid sequence homology to chicken myosin alkali light chains. Mol. Cell. Biol. 4, 956-965.

Felts, S. J., Owen, B. A. L., Nguyen, P., Trepel, J., Donner, D. B. and Toft, D. O. (2000). The hsp-90-related protein TRAP1 is a mitochondrial protein with distinct functional properties. J. Biol. Chem. 275, 3305-3312.

Findly, R. C., Gillies, R. J. and Shulman, R. G. (1983). In vivo phosphorus-31 nuclear magnetic resonance reveals lowered ATP during heat shock of Tetrahymena. Science 219, 1223-1225.

Franc, N. C., Dimarcq, J. L., Lagueux, M., Hoffmann, J. A. and Ezekowitz, R. A. (1996). Croquemort, a novel *Drosophila* hemocyte/macrophage receptor that recognizes apoptotic cells. Immunity 4, 431-443.

Franc, N. C., Heitzler, P., Ezekowitz, R. A. B. and White, K. (1999). Requirement for croquemort in phagocytosis of apoptotic cells in *Drosophila*. Science 284, 1991-1994.

Franc, N. C. and White, K. (2000). Innate recognition systems in insect immunity and development: new approaches in *Drosophila*. Microbes and Infection 2, 243-250.

Fruman, D. A., Burakoff, S. J. and Bierer, B. E. (1994). Immunophilins in protein folding and immunosuppression. FASEB J. 8, 391-400.

Ganz, T. and Lehrer, R. I. (1999). Antibiotic peptides from higher eukaryotes: biology and applications. Mol. Med. Today 5, 292-297.

Georgel, P., Naitza, S., Kappler, C., *et al.* (2001). *Drosophila* immune deficiency (IMD) is a death domain protein that activates antibacterial defence and can promote apoptosis. Dev. Cell 1, 503-514.

Girardin, S. E., Sansonetti, P. J. and Philphot, D. J. (2002). Intracellular *vs* extracellular recognition of pathogens – common concepts in mammals and flies. Trends Miicrobiol. 10, 193-199.

Glaser, R. W. (1918). On the existence of immunity principles in insects. Psyche 25, 38-46.

Gorg, A., Obermain, C., Boguth, G., Harder, A., Scheibe, B, Wildgruber, R, *et al.* (2000). The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 21, 1037-1053.

Gorman, M. J., Andreeva, O. V. and Paskewitz, S. M. (2000). Molecular characterization of five serine protease genes cloned from *Anopheles gambiae* hemolymph. Insect Biochem. Mol. Biol. 30, 35-46.

Gorman, M. J. and Paskewitz, S. M. (2001). Serine proteaes as mediators of mosquito immune responses. Insect Biochem. Mol. Biol. 31, 257-262.

Gottar, M., Gobert, V., Michel, T., Belvin, M., Duyk, G., Hoffmann, J. A., *et al.* (2002). The *Drosophila* immune response against gram-negative bacteria is mediated by a peptidoglycan recognition protein. Nature 416, 640-644.

Govind, S. (1999). Control of development and immunity by Rel transcriptional factors in *Drosophila*. Oncogene 18, 6875-6887.

Griffin, T. J., Gygi, S. P., Rist, B., Aebersold, R., Loboda, A., Jilkine, A., *et al.* (2001). Quantitative proteomic analysis using a MALDI quadrupole time-of-flight mass spectrometer. Anal. Chem. 73, 978-986.

Han, Z. and Ip, Y. T. (1999). Interaction and specificity of Rel-related proteins in regulating *Drosophila* immunity gene expression. J. Biol. Chem. 274, 21335-21361.

Hart, K. and Wilcox, M. (1993). A *Drosophila* gene encoding an epithelial membrane protein with homology to CD36/LIMPII. J. Mol. Biol. 234, 249-253.

Hashimoto, C., Hudson, K. L. and Anderson, K. V. (1988). The Toll gene of *Drosophila*, required for dorsoventral embryonic polarity, appears to encode a transmembrane protein. Cell 52, 269-279.

Hetru, C., Troxler, L. and Hoffmann, J. A. (2003). *Drosophila melanogaster* antimicrobial defence. The J. Inf. Dis. 187, S327-334.

Hiromi, Y. and Hotta, Y. (1985). Control elements of the *Drosophila* segmentation gene fushi tarazzu. EMBO J. 4, 1681-1687.

Hoffmann, J. A. (2003). The immune response of *Drosophila*. Nature 426, 33-38.

Hoffmann, J. A., Kafatos, F. C., Janeway Jr., C. A. and Ezekowitz, R. A. B. (1999). Phylogenetic perspectives in innate immunity. Science 284, 1313-1318.

Hoffmann, J. A. and Reichhart, J. M. (2002). *Drosophila* innate immunity: an evolutionary perspective. Nat. Immunol. 3, 121-126.

Hoffmann, J. A. and Reichhart, J. M. (1997). *Drosophila* immunity. Trends Cell Biol. 7, 309-316.

Hoffmann, J. A., Reichhart, J. M. and Hetru, C. (1996). Innate immunity in higher insects. Curr. Opin. Immunol. 8, 8-13.

Hultmark, D. (2003). *Drosophila* immunity: paths and patterns. Curr. Opin. Immunol. 15, 12-19.

Imler, J. and Hoffmann, J. A. (2001). Toll receptors in innate immunity. Trends Cell Biol. 11, 304-311.

Imler, J. and Hoffmann, J. A. (2000). Signalling mechanisms in the antimicrobial host defence of *Drosophila*. Curr. Opin. Microbiol. 3, 16-22.

Ip, Y. T., Reach, M., Engstrom, Y., Kadalayil, L., Cai, H., Gonzalez-Crespo, S., *et al.* (1993). Dif, a dorsal-related gene that mediates an immune response in *Drosophila*. Cell 75, 753-763.

Irving, P., Troxler, L., Heuer, T. S., Belvin, M., Kopczynski, C., Reichhart, J. M., *et al.* (2001). A genome-wide analysis of immune responses in *Drosophila*. Proc. Natl. Acad. Sci. USA 98, 15119-15124.

Jiang, H. and Kanost, M. R. (2000). The clip-domain family of serine proteinases in arthropods. Insect Biochem. Mol. Biol. 30, 95-105.

Jiang, H. and Kanost, M. R. (1997). Characterization and functional analysis of 12 naturally occurring reactive site variants of serpin-1 from *Manduca sexta*. J. Biol. Chem. 272, 1082-1087.

Johnson, J. K., Rocheleau, J. F., Hillyer, J. F., Chen, C. C., Li, J. and Christensen, B. M. (2003). A potential role for phenylalanine hydroxylase in mosquito immune responses. Insect Biochem. Mol. Biol. 33, 345-354.

Kabakov, A. E., Budagova, K. R., Latchman, D. S. and Kampinga, H. H. (2002). Stressful preconditioning and Hsp 70 overexpression attenuate proteotoxicity of cellular ATP depletion. Am. J. Physiol. Cell Physiol. 283, C521-C534.

Kaji, H., Tsuji, T., Mawuenyega, K. G., Wakamiya, A., Taoka, M. and Isobe, T. (2000). Profiling of *Caenorhabditis elegans* proteins using two-dimensional gel electrophoresis and matrix assisted laser desorption/ionization-time of flight mass spectrometry. Electrophoresis 21, 1755-1765.

Kang, D., Liu, G., Lundstroem, A., Gelius, E. and Steiner, H. (1998). A peptidoglycan recognition protein in innate immunity conserved from insects to humans. Proc. Natl. Acad. Sci. USA 95, 10078-10082.

Kanzok, S. M., Fechner, A., Bauer, H., Ulschmid, J. K., Müller, H., Botella-Munoz, J., *et al.* (2001). Substitution of the thioredoxin system for the glutathione reductase in *Drosophila melanogaster*. Science 291, 643-646.

Kappler, C. Meister, M., Lagueux, M., Gattef, E., Hoffmann, J. A. and Reichhart, J. M. (1993). Insect immunity. Two 17 bp repeats nesting a kappaB-related sequence confer inducibility to the diptericin gene and bind a polypeptide in bacteria-challenged *Drosophila*. EMBO J. 12, 1561-1568.

Karlik, C. C. and Fyrberg, E. A. (1986). Two *Drosophila melnogaster* tropomyosin genes: structural and functional aspects. Mol. Cell. Biol. 6, 1965-1973.

Khush, R. S. and Lemaitre, B. (2000). Genes that fight infection. Trends Genet. 16(10), 442-449.

Khush, R. S., Leulier, F. and Lemaitre, B. (2001). *Drosophila* immunity: two paths to NF-kB. Trends Immunol. 22(5), 260-264.

Kim, Y. S., Ryu, J. H., Han, S. J., Choi, K. H., Nam, K. B., Jang, I. H., *et al.* (2000). Gram-negative bacteria-binding protein, a pattern recognition receptor for lipopolysaccharide and beta-1,3-glucan that mediates the signalling for the induction of innate immune genes in *Drosophila melanogaster* cells. J. Biol. Chem. 275, 32721-32727.
Kimbrell, D. A. and Beutler, B. (2001). The evolution and genetics of innate immunity. Nat. Rev. Genet. 2, 256-267.

Krebs, R. A. and Feder, M. E. (1997). Tissue-specific variation in Hsp70 expression and thermal damage in *Drosophila* melanogaster larvae. J. Exp. Biol. 200, 2007-2015.

Krieger, M. (1997). The other side of scavenger receptors: pattern recognition for host defence. Curr. Opin. Lipid. 8, 275-280.

Kurucz, E., Zettervall, C., Sinka, R., Vilmos, P., Pivarcsi, A., Ekengren, S., *et al.* (2003). Hemese, a hemocyte-specific transmembrane protein, affects the cellular immune response in *Drosophila*. Proc. Natl. Acad. Sci. USA 100, 2622-2627.

Labandeira, C. C. and Sepkoski Jr., J. J. (1993). Insect diversity in the fossil record. Science 261, 310-315.

Lambowitz, A. M., Kobayashi, G. S., Painter, A. and Medoff, G. (1983). Possible relationship of morphogenesis in pathogenic fungus, Histoplasma capsulatum, to heat shock response. Nature 303, 806-808.

Larsen, M. R., Larsen, P. M., Fey, S. J. and Roepstorff, P. (2001). Characterization of differently processed forms of enolase 2 from Saccharomyces cerevisiae by two-dimensional gel electrophoresis and mass spectrometry. Electrophoresis 22, 566-575.

Lavine, M. D. and Strand, M. R. (2002). Insect hemocytes and their role in immunity. Insect Biochem Mol. Biol. 32, 1295-1309.

Lee, W. J., Lee, J. D., Kravchenko, V. V., Ulevitch, R. J. and Brey, P. T. (1996). Purification and molecular cloning of an inducible gram-negative bacteria- binding protein from the silkworm, *Bombyx mori*. Proc. Natl. Acad. Sci. USA 93, 7888-7893.

Lemaitre, B., Kromer-Metzger, E., Michaut, L., Nicolas, E., Meister, M., Georgel, P., *et al.* (1995). A recessive mutation, immune deficiency (imd), defines two distinct control pathways in the *Drosophila* host defence. Proc. Natl. Acad. Sci. USA 92, 9465-9469.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M. and Hoffmann, J. A. (1996). The dorsoventral regulatory gene cassette Spaetzle/Toll/Cactus controls the potent antifungal response in *Drosophila* adults. Cell 86, 973-983.

Leulier, F., Rodriguez, A., Khush, R. S., Abrams, J. M. and Lemaitre, B. (2000). The *Drosophila* caspase DREDD is required to resist gram-negative bacterial infection. EMBO Rep. 1, 353-358.

Leulier, F., Vidal, S., Saigo, K., Ueda, R. and Lemaitre, B. (2002). Inducible expression of double-stranded RNA reveals a role for DFADD in the regulation of the antibacterial response in *Drosophila* adults. Curr. Biol. 12, 996-1000.

Levashina, E. A., Langley, E., Green, C., Gubb, D., Ashburner, M., Hoffmann, J. A., *et al.* (1999). Constitutive activation of Toll-mediated antifungal defence in serpin-deficient *Drosophila*. Science 285, 1917-1919.

Ligoxygakis, P., Pelte, N., Ji, C., Leclerc, V., Duvic, B., Belvin, M., *et al.* (2002). A serpin mutant links Toll activation to melanization in the host defence of *Drosophila*. EMBO J. 21, 6330–6337.

Liu, C., Xu, Z., Gupta, D. and Dziarski, R. (2001). Peptidoglycan recognition proteins: a novel family of four human innate pattern recognition molecules. J. Biol. Chem 276, 34686–34694.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the Folinphenol reagent. J. Biol. Chem. 193, 265–267.

Lu, Y., Wu, L. P. and Anderson, K. V. (2001). The antibacterial arm of the *Drosophila* innate immune response requires an IkB kinase. Genes Dev. 15, 104–110.

Luan, S. (1998). Immunophilins in animals and higher plants. Bot. Bull. Acad. Sin. 39, 217–223.

Luan, S., Albers, M. W. and Schreiber, S. L. (1994a). Light-regulated, tissue specific immunophilins from a higher plant. Proc. Natl. Acad. Sci. USA 91, 984–988.

Manfruelli, P., Reichhart, J. M., Stewart, R., Hoffmann, J. A. and Lemaitre, B. (1999). A mosaic analysis in *Drosophila* fat body cells of the control of antimicrobial peptide genes by the Rel proteins Dorsal and DIF. EMBO J. 18, 3380–3391.

Medzhitov, R. and Janeway Jr., C. A. (2002). Decoding the patterns of self and nonself by the innate immune system. Science 296, 298–300.

Meister, M., Hetru, C. and Hoffmann, J. A. (2000). The antimicrobial host defence of *Drosophila*. Curr. Top. Microbiol. Immunol. 248, 17–36.

Meng, X., Khanuja, B. S. and Ip, Y. T. (1999). Toll receptor-mediated *Drosophila* immune response requires DIF, an NF-kB factor. Genes Dev. 13, 792-797.

Metalnikow, S. (1920). Immunité naturelle ou acquise des chenilles de *Galleria mellonella*. CR Acad. Sci. Paris 83, 5958-5966.

Michel, T., Reichhart, J. M., Hoffmann, J. A. and Royet, J. (2001). *Drosophila* Toll is activated by gram-positive bacteria through a circulating peptidoglycan recognition protein. Nature 414, 756-759.

Mizuguchi, K., Parker, J. S., Blundell, T. L. and Gay, N. J. (1998). Getting knotted: a model for the structure activation of Spaetzle. Trends Biochem Sci 23, 239-242.

Mogami, K., Fujita, S. C. and Hotta, Y. (1982). Identification of *Drosophila* indirect flight muscle myofibrillar proteins by means of two-dimensional electrophoresis. J. Biochem. 91, 643-650.

Mota, M. P. (2003). Influência genética e estocástica no fenómeno de envelhecimento. University of Trás-os-Montes & Alto Douro, Portugal, pp. 52-53.

Naitza, S., Rosse, C., Kappler, C., Georgel, P., Belvin, M., Gubb, D., *et al.* (2002). The *Drosophila* immune defence against gram-negative infection requires the death protein DFADD. Immunity 17, 575-581.

Nappi, A. J. and Sugumaran, M. (1993). Some biochemical aspects of eumelanin formation in insect immunity. In: Pathak JPN. Insect Immunity. New Delhi, Oxford & IBH Publishing Co., 131-148.

O'Farrel, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250, 4007-4021.

O'Kane, C. J. (2003). Modelling human diseases in *Drosophila* and *Caenorhabditis*. Sem. Cell and Dev. Biol. 14, 3-10.

Otvos Jr., L. (2000). Antibacterial peptides isolated from insetcs. J. Peptide Sci. 6, 497-511.

Paillot, A. (1920). L'immunité acquise chez les insects. CR Acad. Sci. Paris 83, 278-280.

Parcellier, A., Gurbuxani, S., Schmitt, E., Solary, E. and Garrido, C. (2003). Heat shock proteins as cellular chaperones that modulate mitochondrial cell death pathways. Biochem. Biophys. Res. Commun. 304, 505-512.

Park, D. S., Shin, S. W., Hong, S. D. and Park, H. Y. (2000). Immunological detection of serpin in the fall webworm, *Hyphantria cunea* and its inhibitory activity on the prophenoloxidase system. Mol. Cell 10, 186-192.

Parsell, D. A. and Lindquist, S. (1993). The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. Annu. Rev. Genet. 27, 437-496.

Pearson, A., Lux, A. and Krieger, M. (1995). Expression cloning of dSR-CI, a class C macrophage specific scavenger receptor from *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA 92, 4056-4060.

Rabilloud, T., Heller, M., Gasnier, F., Luche, S., Rey, C., Aebersold, R., *et al.* (2002). Proteomics analysis of cellular response to oxidative stress. J. Biol. Chem. 31, 277, 19396-19401.

Ramet, M., Manfruelli, P., Pearson, A., Mathey-Prevot, B. and Ezekowitz, R. A. (2002). Functional genomic analysis of phagocytosis and identification of a *Drosophila* receptor for E. coli. Nature 416, 644-648.

Reichhart, J. M., Georgel, P., Meister, M., Lemaitre, B., Kappler, C. and Hoffmann, J. A. (1993). Expression and nuclear translocation of the rel/NF-kB-related morphogen dorsal during the immune response of *Drosophila*. CR Acad. Sci. (Paris) 316, 1207-1217.

Robertson, A. S., Belorgey, D., Lilley, K. S., Lomas, D. A., Gubb, D. and Dafforn, T. R. (2003). Characterization of the necrotic protein that regulates the Toll-mediated immune response in *Drosophila*. J. Biol. Chem. 278, 6175-6180.

Rutschmann, S., Jung, A. C., Zhou, R., Silverman, N., Hoffmann, J. A. and Fernandon D. (2000). Role of *Drosophila* IKK gamma in a Toll-independent antibacterial immune response. Nat. Immunol. 1, 342-347.

Saide, J. D., Chin-Bow, S., Hogan-Sheldon, J., Busquests-Turner, L., Vigoreaux, J. O., Valgeirsdottir, K. and Pardue, M. L. (1989). Characterization of components of Z-bands in the fibrillar flight muscle of *Drosophila melanogaster*. J. Cell Biol. 109, 2157-2167.

Santos, A. N., Korber, S., Kullertz, G., Fischer, G. and Fischer, B. (2000). Oxygen stress increases prolyl cis/trans isomerase activity and expression of cyclophilin 18 in rabbit blastocysts. Biol. Reprod. 62, 1-7.

Satoh, D., Horii, A., Ochiai, M. and Ashida, M. (1999). Prophenoloxidase-activating enzime of the silkworm, *Bombyx mori*. Purification, characterization and cDNA cloning. J. Biol. Chem. 274, 7441-7453.

Schmidt, O., Theopold, U. and Strand, M. R. (2001). Innate immunity and evasion by insect parasitoids. Bioessays 23, 344-351.

Schreiber, S. L. (1991). Chemistry and biology of immunophilins and their immunosuppressive ligands. Science 251, 283-287.

Shaw, P. E. (2002). Peptidyl-prolyl isomerases: a new twist to transcription. EMBO Rep. 3, 521-526.

Silverman, N., Zhou, R., Stoven, S., Pandey, N., Hultmark, D. and Maniatis, T. (2000). A *Drosophila* IkappaB kinase complex required for Relish cleavage and antibacterial immunity. Genes Dev. 14, 2461-2471.

Smales, C. M., Birch, J. R., Racher, A. J., Marshall, C. T. and James, D. C. (2003). Evaluation of individual protein errors in silver-stained two-dimensional gels. Biochem. Biophys. Res. Commun. 306, 1050-1055.

Söderhäll, K. and Cerenius, L. (1998). Role of the prophenoloxidase-activating system in invertebrate immunity. Curr. Opin. Immunol. 10, 23-28.

Stamnes, M. A., Shieh, B. -H., Chuman, L., Harris, G. L. and Zuker, C. S. (1991). The cyclophilin homolog NinaA is a tissue-specific integral membrane protein required for the proper synthesis of a subset of *Drosophila rhodopsins*. Cell 65, 219-227.

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.

Storey, K. B. (1999). Environmental Stress and Gene Regulation. BIOS Scientific Publishers Ltd., Oxford. UK.

Stoven, S., Ando, I., Kadalayil, L., Engstrom, Y. and Hultmark, D. (2000). Activation of the *Drosophila* NF-kB factor Relish by rapid endoproteolytic cleavage. EMBO Rep. 1, 347-352.

Sugumaran, M. (2002). Comparative biochemistry of eumelanogenesis and the protective roles of phenoloxidase and melanin in insects. Pigment Cell Res. 15, 2-9.

Sykes, K., Gething, M. -J. and Sambrook, J. (1993). Proline isomerases function during heat shock. Proc. Natl. Acad. Sci. USA 90, 5853-5857.

Theopold, U., Li, D., Fabbri, M., Scherfer, C. and Schimdt, O. (2002). The coagulation of insect hemolymph. Cell Mol. Life Sci. 59, 363-372.

Uttenweiller-Joseph, S., Moniatte, M., Lagueux, M., Dorsselaer, A. V., Hoffmann, J. A. and Bulet, P. (1998). Differential display of peptides induced during the immune response of *Drosophila*: a matrix-assisted

laser desorption ionization time-of-flight mass spectrometry study. Proc. Natl. Acad. Sci. USA 95, 11342-11347.

Vidal, S., Khush, R. S., Leulier, F., Tzou, P., Nakamura, M. and Lemaitre, B. (2001). Mutations in the *Drosophila* dTAK1 gene reveal a conserved function for MAPKKKs in the control of Rel/NF-kB-dependent innate immune responses. Genes Dev. 15, 1900-1912.

Vierstraete, E., Cerstiaens, A., Baggerman, G., Van den Bergh, G., De Loof, A. and Schoofs, L. (2003). Proteomics in *Drosophila melanogaster*: first 2D database of larval hemolymph proteins. Biochem. Biophys. Res. Commun. 304, 831-838.

Werner, T., Liu, G., Kang, D., Ekengren, S., Steiner, H. and Hultmark, D. (2000). A family of peptidolgycan recognition proteins in the fruit fly *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA 97, 13772-13777.

Wu, L. P., Choe, K., Lu, Y. and Anderson, K. V. (2001). *Drosophila* immunity: genes on the third chromosome required for the response to bacterial infection. Genetics 159, 189-199.

Yan, J. X., Wait, R., Berkelman, T., Harry, R. A., Westbrook, J. A., Wheeler, C. H. and Dunn, M. J. (2000). A modified silver staining protocol for visualization of proteins compatible with matrix-asssisted laser desorption/ionization and electrospray ionization mass spectrometry. Electrophoresis 21, 3666-3672.

Yergei, A. L., Coorssen, J. R., Backlund, P. S., Blank, P. S., Humphrey, G. A. and Zimmerberg, J. (2002). De novo sequencing of peptides using MALDI-TOF/TOF. J. Am. Soc. Mass Spectrom. 13, 784-791.

Appendix

Appendix I

Equipments and reagents

Equipments

Sigma 2K15 centrifuge (12148 rotor), spectrometer (Shimaduzu), pH meter (Denver Instrument), Water bath (Grant).

Electrophoresis unit (SE 600-Hoefer), isoelectric focusing system (IPGphor), strip holders of 13 cm from Amersham-Pharmacia.

Reagents

Urea (17-1319-01, Amersham-Pharmacia), Thiourea (Riedel), CHAPS (17-1314-01, Amersham-Pharmacia), DTT (17-1318-01, Amersham-Pharmacia), Acrylamide (Fluka), Bisacrylamide (Fluka), Tris (17-1321-01, Amersham-Pharmacia), SDS (17-1313-01, Amersham-Pharmacia), Glycerol (17-1325-01, Amersham-Pharmacia), Ammonium persulphate (17-1311-01, Amersham-Pharmacia), TEMED (17-1312-01, Amersham-Pharmacia), Glycine (17-1323-01, Amersham-Pharmacia), IPG cover fluid (17-1335-01, Amersham-Pharmacia), Molecular weight standards (17-0446-01, Amersham-Pharmacia),

Bromophenol blue (Panreac), 2-mercaptoethanol (Fluka), Cupper sulphate (Riedel), Silver nitrate (Sigma-Aldrich), Formaldehyde (Panreac), Sodium carbonate (Fluka), Sodium hydroxyde (Panreac), Sodium citrate (Panreac), EDTA (Panreac), Methanol (Panreac), Coomassie brilliant Blue R250 (Fluka), Coomassie brilliant blue G-250 (Fluka), Agarose (Fluka), Strips pH 3-10, 13 cm (17-6001-15, Amersham-Pharmacia), Ampholites pH 3-10 (17-6000-87, Amersham-Pharmacia), Sodium thiosulphate (Panreac), Acetic acid (Panreac), Sodium acetate (Panreac).

Buffers and solutions

Quantification method (Lowry assay)

Solution A

0.5% $CuSO_4.5H_2O$

1% $Na_3C_6H_5O_7(2H_2O)$

Solution B

2% Na_2CO_3

0.4% NaOH

Solution C

1mL Solution A

50 mL Solution B

Solution D

1 (H_2O): 1 (Folin Cicolteau's reagent)

SDS-PAGE

SDS running buffer

25 mM Tris

192 mM Glycine

0.1% SDS

pH should be about 8.3, but adjustment is not necessary.

SDS-PAGE Stacking gel (4%)

4% Acrylamide/bisacrylamide (30:0.8)

125mM Tris (pH 6.8)

0.1% SDS

0.1% APS

0.01% TEMED

SDS-PAGE Resolving gel (12.5%)

12.5% Acrylamide/bisacrylamide (30:0.8)

375mM Tris (pH 8.9)

0.1% SDS

0.1% APS

0.01% TEMED

SDS Sample loading buffer

250mM Tris (pH 6.8)

4% SDS

40% Glycerol

2% 2-mercaptoethanol

0.01% Bromophenol blue

2D-PAGE

Lysis buffer

8M Urea

2M Thiourea

13mM DTT

1% CHAPS

Rehydratation buffer

8M Urea

2M Thiourea

12.5mM DTT

2% CHAPS

1% Ampholites pH 3-10

0.01% bromophenol blue

Equilibration buffer

50mM Tris (pH 8.8)

6M Urea

30% Glycerol

2% SDS

Staining Solutions for the different procedures

Coomassie Blue Staining

Coomassie staining solution

0.1% Coomassie Brilliant Blue R250

25% Methanol

10% Acetic Acid

Destaining solution

25% Methanol

10% Acetic acid

Colloidal Coomassie staining solution

0.32% Coomassie Brilliant blue G250

20% Methanol

Silver Staining

Fixing solution

40% Methanol

10% Acetic acid

Sensibilization solution

30% Methanol

0.2% Sodium thiosulphate

6.8% Sodium acetate

Silver solution

2.5% Silver nitrate

Developing solution

2.5% Sodium carbonate

0.04% Formalin

Stop solution

1.46% EDTA

Appendix II

Comparative analysis of the 2-DE silver stained protein patterns

a) Global qualitative analysis of the 2-DE protein patterns

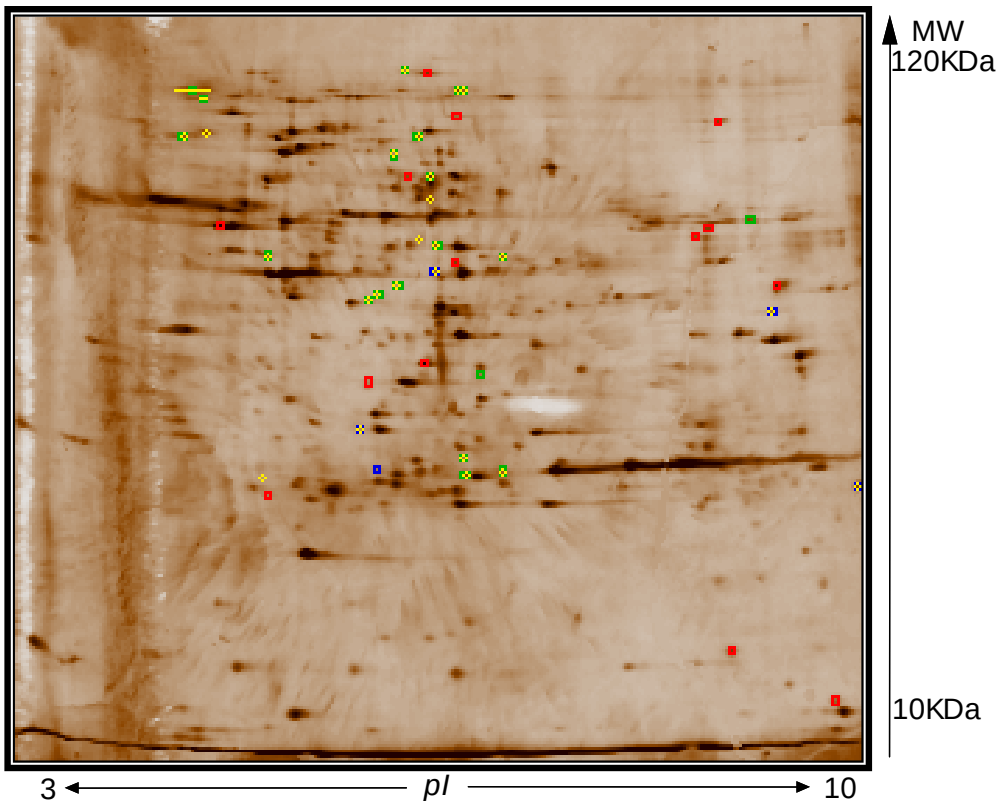


Figure 1: Comparative analysis of 2-DE protein patterns of control and immunised *Drosophila* larvae. refers to spots only present in control gels comparatively to the 6 hours recovery gels; refers to spots only present in the 6 hours recovery gels rather than in the 24 hours recovery gels; refers to spots only present in the 24 hours revovery

gels relatively to the 6 hours recovery gels; refers to spots only present in control gels rather than in the 24 hours recovery gels.

b) Quantitative analysis between the 2-DE protein patterns of control and immunised *Drosophila* larvae

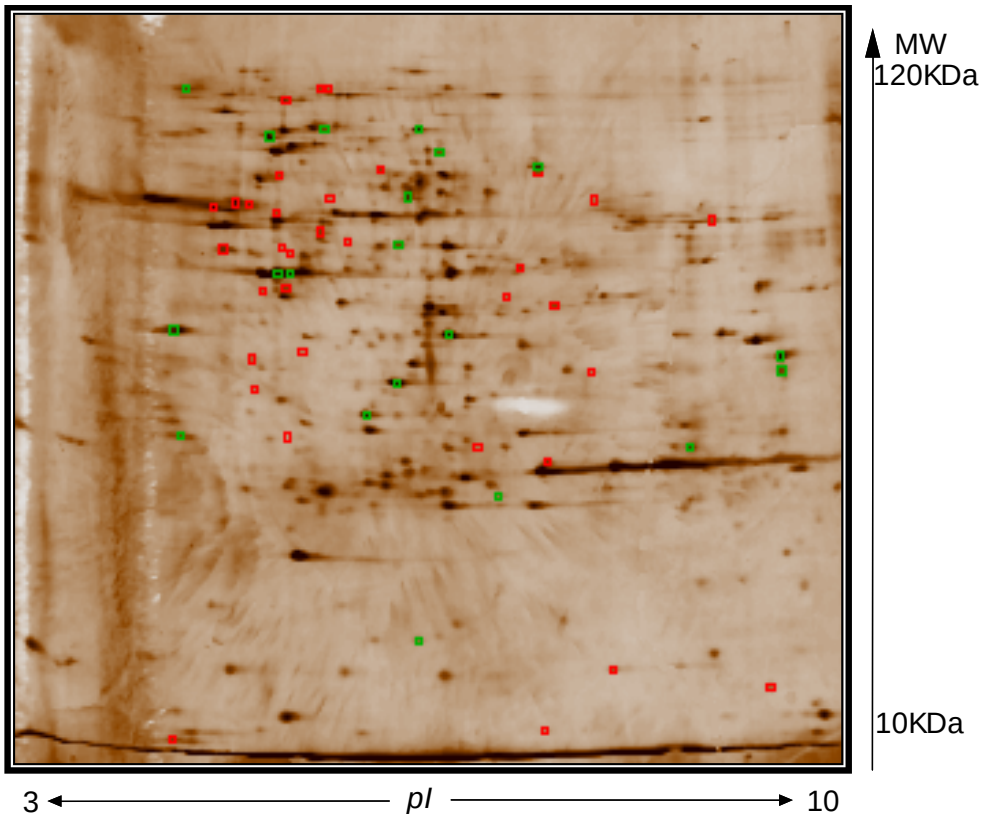


Figure 2: Comparative analysis of 2-DE protein patterns of *Drosophila* larvae between control and 6 hours recovery conditions. refers to spots that increase at least 2 fold after 6 hours of recovery; refers to spots that decrease at least by a factor of 0,5 after 6 hours of recovery.

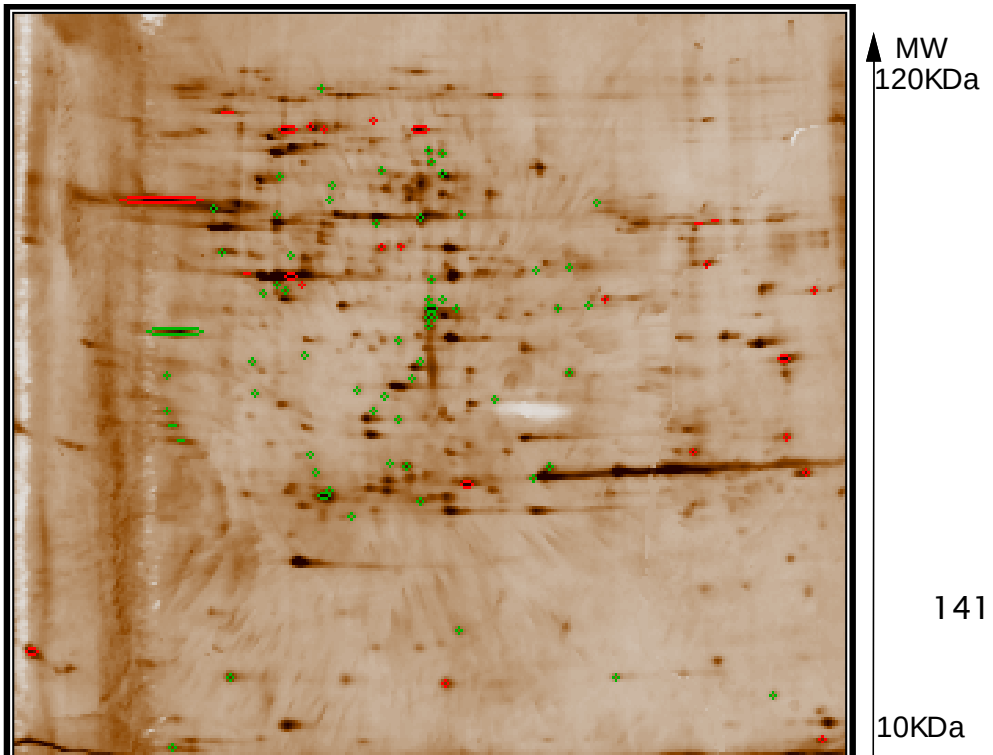


Figure 3: Comparative analysis of 2-DE protein patterns of *Drosophila* larvae between the two recovery times. refers to spots that increase at least 2 fold during the recovery time; refers to spots that decrease at least by a factor of 0,5 during the recovery time.

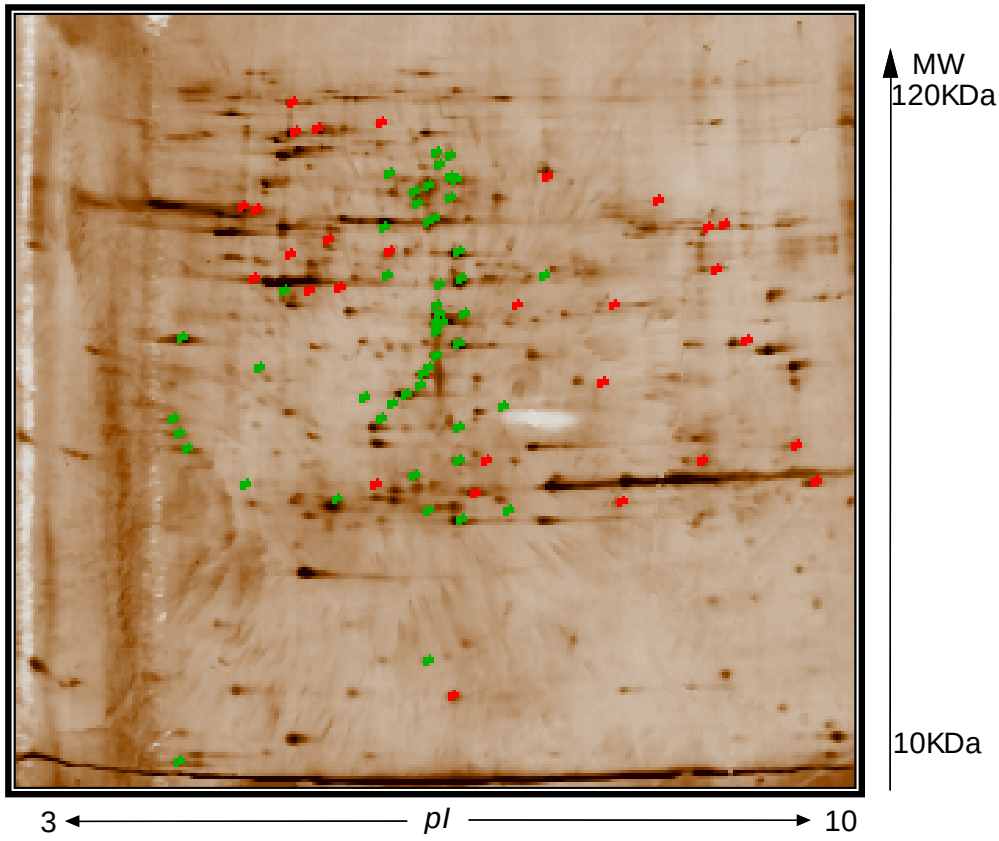


Figure 4: Comparative analysis of 2-DE protein patterns of *Drosophila* larvae after 24 hours of recovery. refers to spots that increase at least 2 fold after the entire recuperation period; refers to spots that decrease at least by a factor of 0,5 after the entire recuperation period.