Immune Reactions Involved in Parasitoid-Host Interactions

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A Thesis Submitted for the Degree of
Doctor of Philosophy
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February 2002

To my Grandmother, Yu-Lan Sun

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Abstract

Endoparasitoid wasps that deposit their eggs and subsequently develop inside the hemocoel of another insect use maternal secretions to interfere with the immune defence of the host. Virus-like particles (VLPs) that protect the parasitoid against its host immune defence constitute one layer of maternal protection. In addition to VLPs, mucinous components represent another layer of protection. These maternal protein secretions interfere with the host defence system and other host functions and so help provide the physiological environment required for the survival and growth of the parasitoid wasps inside the hemocoel of the host.

In this study, maternal protein secretions involved in parasitoid-host interactions were explored and insect coagulation reactions were investigated at a molecular level. The functions of maternal protein secretions of the endoparasitoid wasp *Venturia canescens* Gravenhorst (Hymenoptera: Ichneumonidae) were investigated regarding their role in providing protection against the host's immune system. Analysis of the deduced VLP1-protein sequence revealed three domains: an amino-terminal region, which is highly hydrophobic, a carboxyl-terminal domain with significant similarity to phospholipid hydroperoxide glutathione peroxidase (PHGPx) and a tandem repeat, linking the two domains (Heller *et al.,* 1996). Two variants of VLP1 gene exist because of an interval deletion in the tandem repeat domain in the laboratory asexual stocks. Furthermore, sexual and asexual *V. canescens* strains were obtained from the same location in southern France and isolated into lines homozygous for the VLP1 alleles. The genetic basis for the observed phenotypic differences was investigated by comparing the two asexual lines with the corresponding homozygous VLP1 genotypes in sexual strains. It was found that same pattern of morphological and functional differences observed between the laboratory VLP1 strains (Beck *et al.,* 1999; Beck *et al.,* 2001) also occurred in the two asexual VLP1 lines and in the two homozygous sexual VLP1 strains from the field. These data indicated that the VLP1 gene is genetically closely linked to the putative gene. In addition, some additional phenotypic differences were observed between the sexual and asexual lines from the field suggesting that another gene(s) is also involved in the phenotypes. To further explore the functions of VLP1, an enzymatic analysis of the VLP1 PHGPx domain

(VcPHGPxd) was performed by comparing the expression and tissue-specificity of VLP1 with a related PHGPx in *Drosophila*. The results indicated that the VcPHGPxd is not enzymatically active but may still be able to interact with modified phospholipids on the membrane, thereby masking or removing potentially damaging lipids from the surface of the eggs of parasitoid.

In addition to the VLPs, mucinous protein secretions were examined on the egg surface. A *Venturia* homologue of hemomucin, a surface mucin recently described in *Drosophila*, was identified in *Venturia* ovaries. This hemomucin-like protein is part of the mucinous layer on the egg and larval surface of the parasitoid. *Venturia* hemomucin-like protein can attract host hemolymph proteins, in particular lipophorin, to form a complex that is similar to the *Drosophila* hemomucin-lipophorin complex which has been shown to be a part of the *Drosophila* coagulation reactions (Theopold and Schmidt, 1997). Therefore a localised coagulation reactions, involving egg surface hemomucin and host lipophorin may protect the eggs against further host cellular attacks.

To better understand coagulation reactions and to identify other components involved in insect hemolymph coagulation reactions, a novel approach to isolate coagulation factors was employed using *Galleria mellonella* as a model system. An *in vitro* coagulation reaction with cell-free hemolymph and rabbit red blood cells was performed and the resulting complex was used to produce a specific antiserum. The antiserum reacted with a subset of hemolymph proteins as well as with granulocytes but not with other hemocyte types. Screening expression libraries with the antiserum identified a limited number of positive clones, which coded for both some previously characterised components of the immune activation cascade and some new candidates for clotting factors. The first category included lipophorin, prophenoloxidase (proPO) and mucin-like proteins, which are members of both the coagulation system and the prophenoloxidase cascade. Novel candidates for the insect clotting factors included a glutathione S-transferase (GST) and a distant member of the alpha-crystallin/small heat shock protein family. Members of the latter family are known to have tendency to aggregate and are known targets for transglutaminase, a key enzyme of clotting reactions in both vertebrates and crustaceans.

Statement

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Dongmei Li

February 2002

Acknowledgements

I would like to express my sincere gratitude to my supervisors Professor Otto Schmidt and Dr. Ulrich Theopold for their guidance, support and encouragement throughout the course of this study.

I wish to thank all my colleagues and my friends in the Insect Molecular Biology Laboratory, Dr Sassan Asgari and Dr Annette Reineke for their help, suggestion and discussion. I would like to acknowledge Ms Nicki Featherstone for her help in rearing caterpillars and Mr Harry Roberts for his help in statistics analysis and valuable suggestions. I'd like to thank all the people in the lab for providing a friendly and active atmosphere: Dr Markus Beck, Dr Mahmood Amad, Mr Richard Glatz, Mr Marco Fabbri, Mr Sarjan Mohammad, Mr Guangmei Zhang and Mr Mehdi Sohani.

My special thanks go to Dr Ian Riely for his critically reading and valuable comments.

I would also like to acknowledge Dr Leo Beukeboom and Ms Maria Vicki Schneider, University of Leiden, The Netherlands, for providing the parasitoid wasp strains from southern France.

My deep thanks go to my husband, Zengqi Zhao for his endless love, support and understanding. Special thanks to him for his contributions in parasitoid wasp analysis and RT-PCR works. Thanks to my son, Yu Zhao for providing more happiness to my life. Specific thanks to him for his help in spelling checks.

Special thanks to our best secretaries Mrs Anke Johnsen and Mrs Sue Eaton for their consistent help and friendship, Ms Heather Fraser, Mr Terry Feckner and Dr Gary Taylor and all the others in the Department of Applied and Molecular Ecology who helped me in one way or another.

I am also grateful to my extended family in China, my grandmother, my parents, my parents-in-law, my sisters and brothers for their understanding and support. Special thanks go to my younger sister, Yumei Li for great support and help.

Finally I would like to express my sincere gratitude to Adelaide University for rewarding me with a Adelaide Postgraduate Scholarship and Overseas Postgraduate Research Scholarship. Without these scholarships this thesis would have not been possible.

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Abbreviations

Ab	Antibody
Ac	Acetate
APS	Ammonium persulfate
p-APMSF	4-Amidinophenylmethanesulfonyl fluoride
BB4	*E. coli* strain
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
β-Me	β-Mercaptoethanol
bp	Base pair(s)
BPB	Bromophenol blue
BSA	Bovine serum albumin
cDNA	Complimentary deoxyribonucleic acid
conc.	Concentration
DAB	3,3'-Diaminobenzidine
dCTP	Deoxycytosine triphosphate
DDW	Double distilled water
DMF	Dimethylformamide
DmPHGPx	*Drosophila* PHGPx gene
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DL-DOPA	DL-3,4-Dihydroxyphenylalanine
EDTA	Ethylenediaminetetraacetic acid
FITC	Fluorescein isothiocyanate
g	Gram(s)
g	Gravity
GalNac	N-acetyl-D-galactosamine
H.p. lectin	*Helix promatia* lectin
HPLC	High performance liquid chromatography
IgG	Immunoglobuline G
IPTG	Isopropyl-b-D-thiogalactopyranoside

kb	Kilobase pair(s)
kDa	KiloDalton(s)
LB	Luria-Bertani
M	Molar
mA	Miliampere(s)
mCi	Milicurie
μg	Microgram(s)
μl	Microlitre(s)
min	Minute(s)
μM	Micromolar
mM	Milimolar
MOPS	3-(N-Morpholino)propanesulfonic acid
mRNA	Messanger RNA
MW	Molecular weight
NADPH	β-Nicotinamide Adenine Dinucleotide Phosphate (reduced form)
NBT	Nitro blue tetrazolium chloride
ng	Nanogram(s)
nm	Nanometre(s)
NP-40	Nonylphenoxy polyethoxy ethanol
ORF	Open reading frame
p35	The 35 kDa VLP protein
p40	The 40 kDa VLP protein
p52	The 52 kDa VLP protein
p60	The 60 kDa VLP protein
p80	The 80 kDa VLP protein
PAGE	Polyacrylamide gel electrophoresis
PFA	Paraformaldehyde
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PHGPx	Phospholipid-hydroperoxide glutathione peroxidase
PTU	Phenylthiourea

PDVs	Polydnaviruses
RAPD	Random amplified polymorphic DNA
RbCl	Rubidium chloride
RM	Repeat Minus
RNA	Ribonucleic acid
RNase	Ribonuclease
RP	Repeat Plus
r.p.m.	Rounds per minute
RT	Room temperature
SDS	Sodium dodecyl sulphate
sec	Second(s)
TAE	Tris-acetate/EDTA buffer
TBE	Tris-borate/EDTA buffer
TBST	Tris-buffered saline + Tween-20
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-Tetrametylethylenediamine
TFA	Trifluoroacetic acid
TR	Tandem repeat
Tris	Tris-hydroxymethyl-aminomethane
Tween 20	Polyoxyethylenesorbitan monolaurate
U	Unit(s)
UV	Ultraviolet light
V	Volt(s)
v	Volume(s)
VcPHGPxd	*Venturia* VLP1 PHGPx domain
VLPs	Virus-like particles
VLP1	The formerly 40 kDa VLP protein (p40)
VLPs	Virus-like particles
v/v	Volume per volume
w/v	Weight per volume
X-Gal	5-Bromo-4-chloro-3-indolyl-b-D-galactopyranoside

Chapter 1: Literature Review and Aims of the Study

1 Insect immunity and its evasion by parasitoids

1.1 Introduction

Insects are continuously exposed to potential pathogens and parasites, but only a few become infected. Insects used several strategies to survive the infections. To prevent infection, insect integument and gut epithiel are considered as the first physical barrier. However, if this physical barrier is breached, insects will respond actively and eventually kill pathogens.

Insects have a complex and efficient defence system to respond to microbial and parasitic infections. This system can be defined as insect innate immunity. In general, insect immune systems consist of three closely associated reactions (Hoffmann *et al.*, 1996). 1) Cellular reactions are mainly controlled by hemocytes and composed of phagocytosis, nodule formation, encapsulation (Ratcliffe and Rowley, 1979); 2) Humoral reactions include the induction of the immune proteins by fat body and hemocytes acting in the hemolymph to fight infections; 3) Wound healing and the induction of proteolytic reactions leading to activation of prophenoloxidase and hemolymph coagulation. Insect hemolymph coagulation was the focus of this investigation and a review of the relevant literature follows.

Interestingly, although the insect immune system is highly effective in handling microbial infection, some insect parasitoids are able to overcome their host immune reactions. Obviously the parasitoids must have evolved protective mechanisms to allow them to develop inside their hosts. Endoparasitoids lay their eggs inside host eggs or early instar larvae to avoid the host immune reactions which are more pronounced in later larval stages (Salt, 1968). Others oviposit into certain tissues of their host to finish their embryonic development without encountering host hemolymph (Salt, 1968). Furthermore, many insect endoparasitoids inject biochemical

compounds into their host during oviposition inducing changes of host physiology and development (Strand and Dover, 1991). Some actively suppress the host immune system using polydnaviruses (Asgari *et al.*, 1997); some passively evade the host by coating the egg or larva surface with virus-like particle (VLPs) (Schmidt and Schuchmann-Feddersen, 1989). In this part, research on VLPs will be discussed in detail, with emphasis on current literature on VLPs of *Venturia canescens*.

1.2 The insect immune system

Vertebrate immunity, especially mammalian immunity includes innate and acquired or adaptive reactions. Adaptive responses rely on highly antigen-specific receptors produced through clonal selection whereas the innate immune system is less specific and has to rely on the recognition of broad classes of foreign antigens (Strand and Pech, 1995a).

Insect immune responses are only innate, or non-clonal. In general, when a foreign invader enters the hemocoel of an insect, a number of hemolymph proteins are induced and interact with hemocytes to eliminate the invader. The insect innate immunity will be discussed in the following chapters. As mentioned before, cellular and humoral defence reactions are two main aspects of insect immunity. Cellular defence reactions are conducted by the insect cells, the hemocytes while the humoral defence reactions are mainly involved the cell-free hemolymph proteins. In addition, insect hemolymph coagulation and prophenoloxidase (proPO) cascades are dependent on both the cellular and humoral components of the insects. It is widely accepted that insect hemolymph coagulation reactions involve the interaction between cellular and humoral pro-coagulant activity (Bohn, 1986). The proPO activating system seems to be a humoral reaction, but it has its cellular basis, like proPO, itself, and other substrates involved in the cascade, are produced in hemocytes and released into hemolymph.

1.2.1 Cellular reactions

The cellular immune reactions involve mainly the insect blood cells or hemocytes and consist of adhesive reactions of hemocytes against microbes or parasites. Insect cellular defence reactions have been studied for about 100 years. Changes in morphology, behaviour and types of cell composition during an infection have been widely researched with microscopy, lectin and monoclonal antibody markers (McKenzie and Preston, 1992; Strand, 1994; Theopold *et al.,* 1995; Theopold *et al.,* 1996). Hemocytes phagocytose bacteria, trap microbes in nodules and encapsulate large parasitoid eggs (Prevost *et al.,* 1990). Hemocytes are also involved in other immune responses such as the phenoloxidase cascade and hemolymph coagulation (Gregoire, 1974; Brehelin, 1979).

Hemocytes from different insect species vary in their morphological and functional characteristics. Among these various hemocytes, the granulocytes and plasmatocytes are thought to be the most important hemocytes involved in insect cellular defence reactions (Ratcliffe, 1993). According to the number and size of the foreign organisms in the insect hemocoel, three major cellular defence reactions can be classified: Phagocytosis, encapsulation and nodule formation.

1.2.1.1 Phagocytosis

Since phagocytosis is a ubiquitous process within the animal kingdom, occurring in numbers of all invertebrate phyla and vertebrates, it may be regarded as the primary cellular response against invading microorganisms. The main steps for phagocytosis are recognition, endocytosis, ingestion and killing (Götz and Boman, 1985; Gupta, 1991). These processes have been studied in different insect species. Generally, granulocytes and plasmatocytes are the major hemocytes involved in phagocytosis. The surface of the hemocytes contains binding sites that recognise cell wall components of foreign organisms. Thus, when the foreign organisms encounter the hemocytes, the surface components of their cell wall will be recognised by the

hemocytes. The bound organisms are then surrounded by pseudopods and ingested by coated vesicles. Finally the engulfed organisms are lysed by lysozyme and antibacterial peptides (Götz and Boman, 1985).

1.2.1.2 Encapsulation

Foreign organisms such as nematodes, insect parasitoids and copepods, too large to be phagocytosed, are often encapsulated. In contrast to phagocytosis, encapsulation is a multicellular process. Similar to phagocytosis, both the granulocytes and plasmatocytes are involved. However, in *D. melanogaster,* lacking granulocytes, plasmatocytes and their modification, lamellocytes are the cells forming the capsule around foreign objects (Russo *et al.,* 1996).

The major steps for encapsulation are the recognition of foreign organisms by granulocytes, the degranulation of the granulocytes on the surface of the foreign organisms, the attraction of plasmatocytes and the formation of a capsule (Rowley and Ratcliffe, 1981). However, Pech and Strand (1996) reported that granulocytes complete the capsule. Wiegand *et al.* (1996) also showed that plasmatocytes initiated cellular encapsulations of or aggregated around the large organisms *in vitro* culture.

1.2.1.3 Nodule formation

In some invertebrates that are unable to deal adequately with large numbers of small foreign particles, such as bacteria, using phagocytosis, nodule formation occurs (Ratcliffe, 1993). This defence response is similar to encapsulation and also includes hemocyte degranulation. Generally, nodules comprise a central core of entrapped foreign organisms, surrounded by multicellular hemolytic aggregates. This formation can be induced by surface components of foreign organisms such as lipopolysaccharides (LPS), zymosan, laminarin and some glycoproteins (Lackie, 1988). During nodule formation, the granulocytes degranulate after having recognised the foreign organisms. The degranulated and lysed granulocytes together with the

entrapped and phagocytosed foreign organisms form a coagulum, around this coagulum, further hemocytes start to attach and flatten, finally forming large multicellular hemolytic aggregates or nodules. The nodule may adhere to the foreign organisms and eventually the organisms will be encapsulated.

1.2.2 Humoral reactions

The humoral reactions are based on antibacterial peptides and additional proteins which are either normally present or are induced (Boman and Hultmark, 1987). Since the first insect anti-bacterial peptide, lysozyme was discovered in 1968 by Mohrig and Messner, more than 170 antimicrobial peptides or polypeptides have been identified in insects (Bulet *et al.*, 1999) and 25 antimicrobial peptide families have been characterised (Trenczek, 1998). These anti-microbial proteins can be classified into five specific and one miscellaneous group (Hoffmann, 1995; Trenczek, 1998) (Table 1).

The first insect lysozymes are all closely related to the lysozymes found in vertebrates, the other induced peptides or proteins are all novel factors that were first discovered in insects (Hultmark, 1993). In general, lysozymes are widely distributed enzymes that degrade peptidoglycan in the bacterial cell wall by hydrolysis (Stryer, 1988). Two types of lysozymes are distinguished in vertebrates: the goose-type and the chicken-type. The insect lysozymes are closely related to the chicken-type lysozymes (Faye and Hultmark 1993). Their main function seems to remove the bacterial murein sacculus that is left after the action of other antibacterial proteins (Boman and Hultmark, 1987).

Table 1 Anti-microbial peptides (Modified after Trenczek, 1998)

Group of anti-microbial peptides	Characteristics	Examples
Chicken-type-like lysozyme	First antibacterial protein against gram-positive bacteria closely related to vertebrate lysozyme.	Lysozymes
Ceropin-like peptides	Basic peptides, without cysteine, 2 -helices with short intermediate stretch, ca. 4 kDa against gram negative bacteria preferentially	Bactericidin Cecropin Sarcotoxin I
Proline-rich peptides	Proline up to 25%, without cysteine, group a) unsubstituted, group b) O-glycosylated, ca. 2-3 kDa, against gram-negative bacteria predominantly	Abaecin Apidaecin Drosocin Lebocin Metalnikowin Metchnikowin Pyrrhocoricin
Glycine-rich peptides	Rich in glycine, some are aimdated, some are O-glycosylated, ca. 9-3-kDa, against Gram-negative bacteria preferentially	AFP (antifungal peptide) Attacin Coleoptercin Diptericin Hemiptericin Holotricin 2, 3 Hymenoptaecin Sarcotoxin II Tenecin 3
Cysteine-rich peptides	1-4 disulfide-bridges, against fungi and bacteria	Drosomycin Insect defensin (sapecin) Thanatin
Miscellaneous	Individual characteristics, active against bacteria, fungi, lytic against erythrocytes	Andropin Ceratotoxin Gallysin-1 Royalisin

The cecropins were first discovered in the giant silk moth, *Hyalophora cecropia*, and were named accordingly (Hultmark *et al.,* 1980). Cecropins are a family of peptides with 35-39 amino acid residues and are generally amphipathic molecules that interact with lipid membranes. They increase the permeability of the cell membrane leading to cell lysis (Gazit *et al.,* 1994). Normally eukaryotic cells are resistant to cecropin concentrations which are able to kill and lyse most of the bacteria (Hultmark, 1993). Interestingly, it was reported that besides their immune function, cecropins are also involved in insect development (Tryselius *et al.,* 1992).

Proline-rich peptides are found in several insects and have different functions. For example, abaecin and apidaecin were found in the hemolymph of bacteria-challenged honeybees, abaecin is bactericidal while apidaecin is bacteriostatic (Hetru *et al.*, 1994). Drosocin, another proline-rich peptide, was identified from *Drosophila* and is active against gram-negative bacteria at very low concentration (Hultmark, 1993).

The glycine-rich peptides were identified in several insects, including attacin, initially discovered in a moth, sacrotoxins II and diptericons in flies and coleoptercin from a beetle (Hultmark, 1993). They can affect dividing cells of *E. coli* and some other gram-negative bacteria, causing them to grow in long chains (Hultmark, 1993).

Insect defensins are one group of the cyclic antibacterial peptides and were initially thought to be homologous to mammalian defensins. However, they show structural similarity to potassium-channel-blocking scorpion toxins like charybdotoxin (Bontems *et al.,* 1991). Generally insect defensins are 36-46 amino acid long and have the same cysteine pairing: Cys1-Cys4, Cys3-Cys5 and Cys3-Cys6 (Bulet *et al.,* 1999). Similar to cecropins, insect defensins were involved in development as well (Matsuyama and Natori, 1988).

Furthermore, the antibacterial peptides can be produced in several tissues such as hemocytes, epidermis, pericardial cells, midgut, nerve cells, ovary and testes. However, fat body is the major tissue to produce peptides and proteins (Hultmark,

1993). The fat body can respond to bacterial infection and synthesize antibacterial peptides or proteins. For example, *Drosophila* adults can produce more than 10 different molecules such as drosocin, drosomycin, defensin, diptericin and so on. Dragonfly, *Aeschna cyanea* only synthesize one single defensin (Bulet *et al.*, 1999).

1.2.3. Immune recognition proteins

In vertebrates, antibodies can recognise and bind to microbes or parasites through adaptive responses. In invertebrates, though lacking specific immunoglublins, also posse recognition molecules which can discriminate and bind pathogens. These recognition proteins react typically with highly conserved structures of the pathogen (Trenczek, 1998), which were named pathogen associated molecular patterns (PAMPs). The recognition molecules are called pattern recognition receptors (PRRs, Medzhitov, 1997). The pathogenic structures, PAMPs, are lipopolysaccharides (LPS), petidoglycans (PG) and ß-1, 3-glycans. Many binding proteins in arthropod are known (Duvic and Söderhäll, 1990; Cerenius *et al.*, 1994) with some in insects characterised to molecular level (Matha *et al.*, 1990 ; Xu *et al.*, 1995;). In addition, some other proteins that can bind foreign organisms and thus induce or adjust insect immune reactions (Trenczek, 1998). A number of the LPS- and glucan-binding proteins and other recognition proteins have been identified in different insects (Table 2). Although only some examples of insect recognition proteins have been described in this literature review, there is a wide range of them and these are listed in Table 2.

Table 2 Recognition proteins in insects (Modified after Trenczek, 1998)

Species	Description
	LPS binding proteins
Bombyx mori	11 kDa proteins, lipophorin, Xu *et al.*, 1995, Kato *et al.*, 1994
Ceratitis capitata	47 kDa protein, Charalambidis *et al.*, 1995
Galleria mellonella	24 kDa, 26 kDa, 17 kDa proteins, lipophorin, Klunner *et al.*, 1994; Dunphy and Halwani, 1997
Periplaneta americana	Pa-LBP, Pa-lectin, Jomori *et al.*, 1990; Jomori and Natori, 1991; Kawasaki *et al.*, 1993
Rhodinius prolixus	A lectin, Pereira *et al.*, 1981
Triatoma infestans	A hemagglutinin, Hypsa and Grubhoffer, 1995
	ß-1,3-lucan binding proteins
Bombyx mori	BGBP, Ochiai *et al.*, 1988
Galleria mellonella	A lectin, Matha *et al.*, 1990
	Proteins involved in the immune response
Pseudoplusia includens	Adhesion peptide, Clark *et al.*, 1997
Drosophila melanogaster	Calpain , Theopold *et al.*,1995 Croqquemort, Franc *et al.*, 1996 dSR-CI (Scavvenger receptor), Pearson *et al.*, 1995 Hemomucin, Theopold *et al.*, 1996, 1997 Peroxidasin , Nelson *et al.*, 1994; Tepass *et al.*, 1994
Manduca sexta	HAIP (Hemocyte Aggregation Inhibitor Protein), Kanost *et al.*, 1994 Hemolin, Ladendorf and Kanost, 1990 Scolexin, Kyriakides *et al.*, 1993, 1995
Bombyx mori	Hemocytin, Kotani *et al.*, 1995
Hyalophora ceropia	Hemolin, Sun *et al.*, 1990
Sarcophaga peregrina	200 kDa protein, Kobayashi *et al.*, 1995
Blaberus cranlifer	90 kDa protein , Rantamaki *et al.*, 1991
Ceratitis capitata	47 kDa protein, Marmaras *et al.*, 1992

Lectins (Jomori and Natori, 1992; Kawasaki *et al.,* 1993), hemolin (Sun *et al.,* 1990; Schmidt *et al.,* 1993) and hemomucin (Theopold *et al.,* 1996, Theopold and Schmidt, 1997) have been found to be involved in recognition of foreign organisms. In addition, Johansson and Söderhäll (1989) isolated a 76 kDa adhesion factor in crayfish, *Pacifastacus leniusculus*. The factor that can induce granulocyte degranulation and cell adhesion contains an R-G-D amino acid sequence. Interestingly, Rantamaki *et al.* (1991) identified a 90 kDa protein from the hemocytes of the insect, *Blaberus craniifer*. The 90 kDa factor can induce hemocyte degranulation, enhance adhesion to the substratum, but also cross-react with the 76 kDa cell adhesion factor. This indicates that very similar molecules may function in recognition of foreign organisms within invertebrates.

Hemolin, or P4, a 48 kDa protein found in the hemolymph of *Hyalophora cecropia* and *Manduca sexta* is a molecule with four C2-type Ig-domains belonging to the immunoglobulin superfamily (Sun *et al.,* 1990; Faye and Kanost, 1997). In normal larvae, hemolin is present at low levels. However, after bacterial infection, it increases up to 18 times (Andersson and Steiner, 1987). Hemolin binds to bacterial surfaces and forms a complex with two other proteins (Sun *et al.,* 1990). Furthermore, evidence also indicates that hemolin may bind to LPS (Daffre and Faye, 1997) and inhibit hemocyte aggregation as well (Ladendorff and Kanost, 1990; LanzMendoza *et al.,* 1996). Similarly, another protein called hemocyte aggregation inhibitor protein (HAIP), which is probably a dimeric lectin and also inhibit hemocyte aggregation (Kanost *et al.,* 1994). However, HAIP is not induced by bacterial infections (Kanost *et al.,* 1994). Insects also use other humoral recognition molecules, namely hemagglutinins (lectins). Lectins are proteins with multiple carbohydrate binding sites. Several lines of evidence demonstrated that some lectins have opsonic activity and can be considered as recognition molecules that elicit immune responses (Jomori and Natori, 1992; Kawasaki *et al.,* 1993). In *Periplaneta americana*, two lectins (Pa-LBP and Pa-lectin) can recognise LPS and participate in the clearing of bacteria from hemolymph (Jomori and Natori, 1992; Kawasaki *et al.,* 1993). The sequence of many insect lectins is related to C-type lectins from vertebrates (Jomori and Natori, 1992;

Kawasaki *et al.,* 1993). Interestingly, some C-type lectins in mammals are also involved in pattern recognition of microorganisms.

Hemomucin, an insect mucin named according to a typical mucin domain sequence (Theopold *et al.,* 1996) is another candidate for a recognition protein in insect defence. Hemomucin was isolated from a *Drosophila* cell line. Hemomucin can be labelled by lectins with specificity for N-Acetyl Galactosamine (GalNAc) such as *Helix pomatia* agglutinin (HPL) (Theopold *et al.,* 1996). In addition, hemomucin can be detected in insect microparticles, indicating that it may be involved in hemocyte adhesion and hemolymph clot formation (Theopold and Schmidt, 1997). In addition, a scavenger receptor (dSR-C1) with broad polyanionic binding specificity isolated from phagocytosing cells also has a mucin domain (Pearson *et al.,* 1995).

1.2.4 Hemolymph coagulation

1.2.4.1 Introduction

The innate immune system of vertebrates comprises a number of proteolytic activation cascades, such as the complement system and the coagulation cascade, which help to establish a first line of defence against foreign invaders (Cerenius and Söderhäll, 1995). In searching for similar systems in arthropods, two proteolytic cascades have been identified, the prophenoloxidase activating cascade (proPO) and the coagulation cascade in the horseshoe crab, *limulus* (Iwanaga *et al.,* 1998). Both cascades are activated by surface antigens of microbial invaders (such as LPS, peptidoglycan and ß-1, 3-D-glucan), is essential for survival (Iwanaga *et al.,* 1998; Muta and Iwanaga, 1996). Although the proPO cascade is mostly known to produce microbicial compounds, it is also involved in several other reactions of the invertebrate immune system including cellular reactions (Cerenius and Söderhäll, 1995). Accordingly, a cell-surface form of prophenoloxidase has been identified (Charalambidis *et al.,* 1996). Clotting factors and their mode of action in a number of non-insect species of arthropods have been identified and characterised at the molecular level (Muta and

Iwanaga, 1996; Iwanaga *et al.,* 1998). In the following, the non-insect clotting reactions will be discussed in detail. A summary of insect coagulation will also be described afterwards and an interaction of coagulation proteins in a parasitoid/host (*Venturia/Ephestia*) system will be mentioned as well.

1.2.4.2 Coagulation in non-insect arthropods

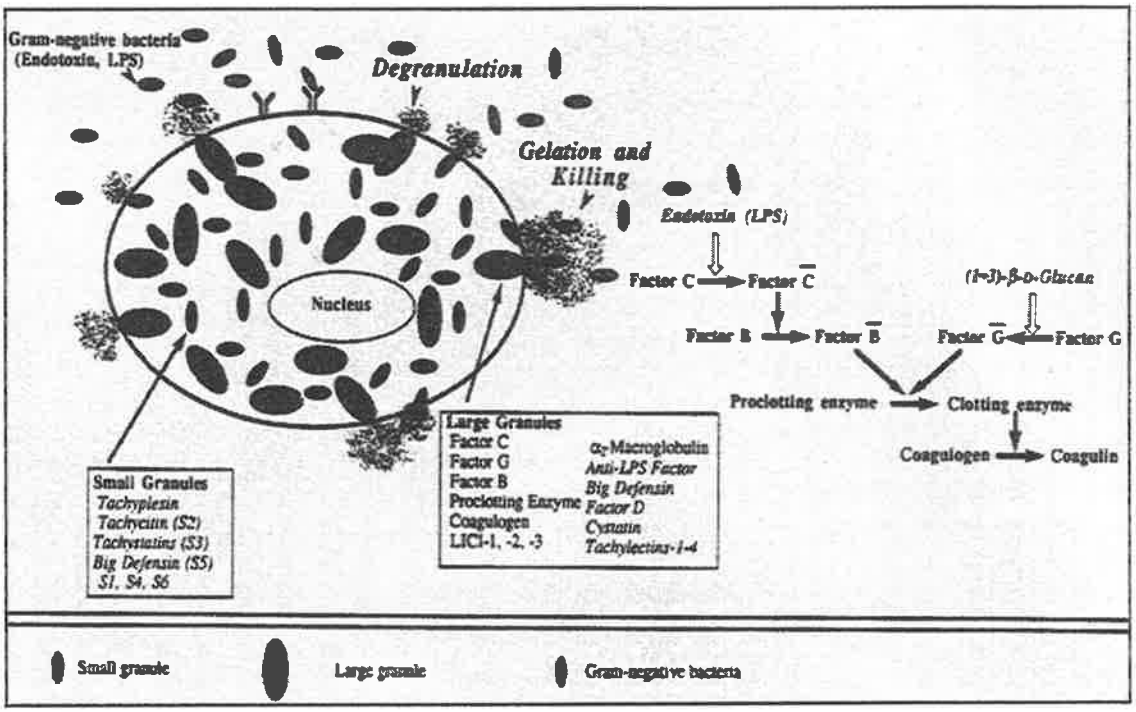
Most of the research on arthropod hemolymph coagulation has been performed in non-insect species, including crustaceans (Crayfish, Cerenius *et al.,* 1994) and two ancient chelicerates, the horseshoe crabs, *Limulus polyhemus* and *Tachypleus tridentatus*. In this part, some aspects that might have implications for the insect clotting system will be discussed in detail.

1.2.4.2.1 Clotting system in *Limulus*

Hemocytes in the hemolymph of the horseshoe crab, *Limulus,* are essential in its defence system. Granulocytes, the single type of hemocytes, contain large but diluted and small but dense granules (Toh, 1991). The granular cells are very sensitive to bacterial endotoxin, like LPS (Armstrong, 1991), leading to the LPS-activated clotting pathway. In addition, Kakinuma *et al.* (1981) reported that ß-1, 3-glucan does not cause hemocyte degranulation, but activates the hemolymph lysate protein, factor G; eventually, a ß-1, 3-glucan-induced clotting cascade is triggered. Accordingly, two kinds of clotting pathways in horseshoe crabs have been identified. Figure 1 (Iwanaga *et al.,* 1998) shows detailed processes of the *Limulus* coagulation cascade.

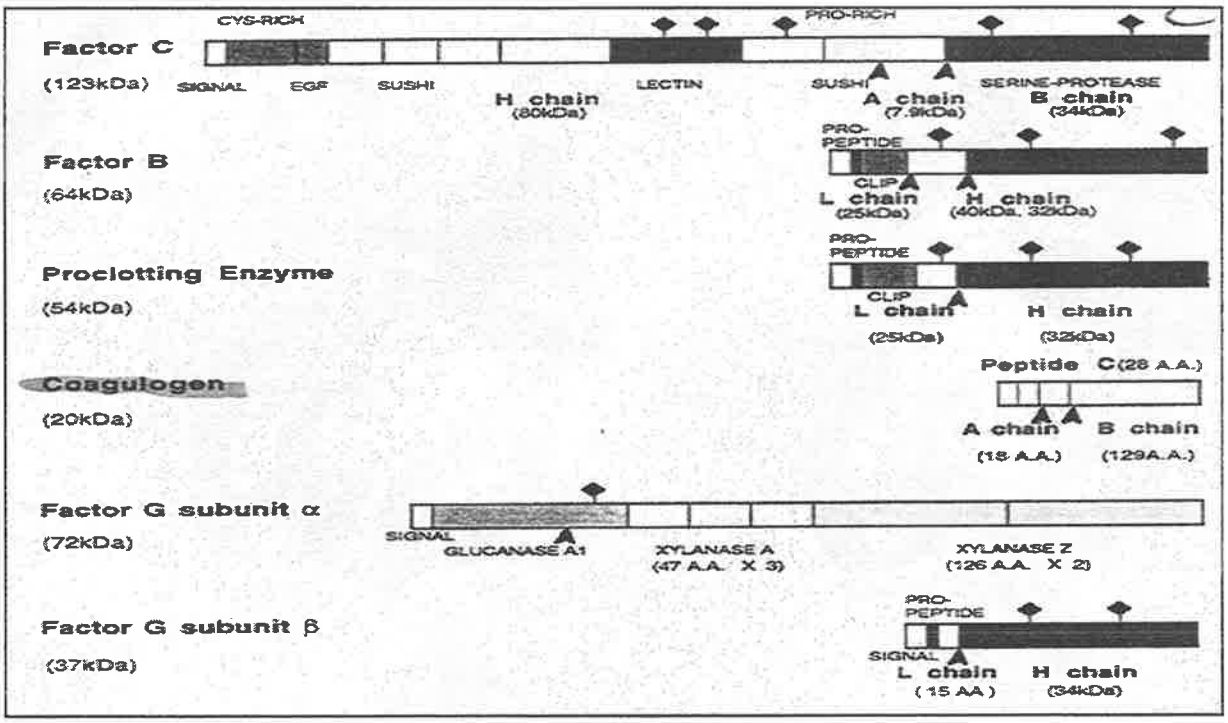
- LPS-activated clotting pathway

The LPS-activated clotting system comprises three proteases (factor C, B and proclotting enzyme, Fig. 1) which activate each other sequentially in a proteolytic cascade, leading finally to the precipitation of the clotting protein coagulogen into insoluble coagulin. Fig. 2 shows the protein domain of these clotting factors. A serine



After (Iwanaga *et al.*, 1998)

Fig. 1 Coagulation cascade in the horseshoe crab



After (Iwanaga *et al.*, 1998)

Fig. 2 Protein domains of clotting factors

protease domain is common in a number of factors, In addition, there are additional domains, including epidermal growth factor (EGF-like) domains, sushi domains, which are found in mammalian complement factors, C-type lectin domains and clip (or disulfide knotted) domains. The clip domain shows similarities to antibacterial peptides (big defensin). It was postulated that this and other peptides released during the activation of the cascade have antibacterial activity, adding to the efficacy of the clotting cascade. Antibacterial activity was recently shown for the clip domain of the proPO-activating enzyme from crayfish (Wang *et al.,* 2001). The sensitivity of horseshoe crab hemolymph towards LPS is in fact the basis for the so-called *Limulus* test, which is used in the clinical field.

- β-glucan clotting pathway

A second activation pathway is initiated by the fungal elicitor β-glucan and involves the heterodimeric factor G. The α-subunit of factor G contains several regions with similarities to bacterial enzymes involved in carbohydrate degradation, e.g. glucanases and xylanases (Fig. 2). Since some of the functionally essential amino acids of the active sites are missing in these domains, they are likely to retain substrate specificity of the enzymes but lack their activity. Although transglutaminase activity has been found in horseshoe crab hemocytes (Muta and Iwanaga, 1996), it does not seem to involve the crosslinking of gelation in horseshoe crabs as observed in other arthropods.

A mechanism to keep the coagulation reaction localised may involve a tight regulation by serine protease inhibitors (serpins). To date, three *Limulus* intracellular coagulation inhibitors (LICI-1, LICI-2 and LICI-3) have been identified and characterised (Miura *et al.,* 1994; Miura *et al.,* 1995; Lal Agarwala *et al.,* 1996). All these inhibitors are stored in L-granules and released into the hemolymph by rapid exocytosis. These serpins can be bound covalently to serine protease. Among them, LICI-1 inhibits factor C whereas LICI-2 and LICI-3 have inhibitory activities on factor C, factor G and the clotting enzyme. As a result of the function of these serpins, the clotting reaction is regulated.

1.2.4.2.2 Clotting system in crustaceans

In crustaceans, clotting has been shown to depend on the activity of the calcium-dependent enzyme transglutaminase. Transglutaminase is released by hemocytes or muscle cells upon wounding and cross-links clottable hemolymph proteins. Clotting proteins have been isolated from a number of crustacean species, including crayfish (Hall *et al.,* 1995b; Hall *et al.,* 1999), shrimp (Yeh *et al.,* 1999) and sand crayfish (Komatsu and Ando, 1998). They are often large (about 200 kDa) lipoproteins, with similarity to vitellogenins (Hall *et al.,* 1999).

1.2.4.2.3 Clotting system in vertebrates

Like in the horseshoe crab, the blood clotting system in vertebrates is dependent on the activity of a proteolytic cascade (including the protease thrombin) leading ultimately to the precipitation of fibrinogen into fibrin. It also involves a thrombin activating transglutaminase (blood coagulation factor XIIIa), which stabilizes fibrin aggregates through crosslinking. Although both horseshoe crabs and vertebrates employ proteolytic cascades to achieve hemostasis, there is little homology in the primary structure of the proteins involved in both reactions. However, some protein domains are shared between clotting factors from different animal groups. The best-studied example is the von Willebrand factor (vWBF), which plays a central role in blood clotting. Domains with similarity to vWBF can be found in a number of arthropod proteins that are involved in hemolymph clotting (Kotani *et al.,* 1995). One protein that does show good homology between arthropods and vertebrates is in fact transglutaminaes, which is involved in clotting reactions in both crayfish and vertebrates (Theopold *et al.,* 2001b).

1.2.4.3 Clotting reaction in insects

In contrast to other arthropods little is known about the clotting reaction of insect hemolymph, which is usually regarded as part of the hemostatic wound response. As in

Limulus, the insect clotting reaction can be modulated by microbial surface antigens, such as lipopolysaccharide (LPS), peptidoglycan and ß-1, 3-glucan (Hoffmann and Reichhart, 1997). Similar to the situation in vertebrates and non-insect arthropods, hemolymph coagulation in most insects involves cellular and humoral components, which are inactive on their own (Bohn, 1986). It has also been shown that the resulting coagulum of insect hemolymph coagulation acts as a trap for bacteria, which bind to fibrilla structures formed by hemocyte degranulation (Ratcliffe and Rowley, 1979).

During the last decades, research on insect hemolymph coagulation had focused on the morphology and aggregation of hemocytes (Geng and Dunn, 1988). The morphological characterisations of the cellular reactions were found in different insect species (Bohn, 1986), Furthermore, Gregoire (1974) described the formation of long, adhesive pseudopodia in hemocytes during coagulation in lepidoptera larvae and classified four types of coagulation patterns. Brehélin (1979) concluded that without hemocytes, the plasma did not clot. Recently, the formation of insect microparticles has been described as a kind of hemocyte coagulation reactions in *Drosophila* and *Galleria* (Theopold and Schmidt, 1997). At present, the molecular characterisation of the proteins involved in hemolymph clotting is lagging behind the detailed morphological characterisation of the cell-types involved.

Siakotos (1960) first reported lipoprotein as part of the coagulum in *Periplaneta americana.* Later Brehélin (1979) demonstrated that the plasma coagulogen in *Locusta migratoria* is a lipoglyco-protein complex. As research proceeded, lipophorin was confirmed as the clotting protein in a few insects such as *Lucusta migratoria;* the cockroach, *Leucophaea maderae* and *Drosophila melanogaster* (Barwig and Bohn, 1980; Gellissen, 1983; Barwig, 1985; Theopold and Schmidt, 1997). More recently, Duvic and Brehelin (1998) reported that a ß-1, 3 glucan-induced p260-P85 protein complex in locust plasma corresponds to locust lipophorin; it is involved in the clotting reaction and inhibits prophenoloxidase activities.

1.2.4.3.1 The plasma coagulogen

As mentioned above, the plasma coagulogen in a number of insect species has been identified physiologically as a hemolymph protein, lipophorin (Barwig and Bohn, 1980; Gellissen, 1983; Barwig, 1985; Theopold and Schmidt, 1997). Lipophorin is an abundant, multifunctional lipoprotein found in the cell-free hemolymph, the insect plasma (Kanost *et al.,* 1990) and is engaged in coagulation reactions (Bohn, 1986). Lipophorin is consists of three subunits, all of which have been implied in immunity. In addition, there is a report of a 22 kDa protein (hemofibrin) from *Manduca sexta,* which forms a fibrous clot that can be observed with electron microscopy (Geng and Dunn, 1988). In addition, a number of proteins from other insects have been reported in the induction of coagulations, including M13 (Minnick *et al.,* 1986) and scolexin (Finnerty *et al.,* 1999), both from *Manduca sexta* and hemocytin, a multi-domain protein from *Bombyx mori*, which has significant homology to von Willebrand factor (Kotani *et al.,* 1995).

When searching for clotting homologues from *Drosophila* genome database, however, only few arthropod proteins involved in hemolymph clotting can be considered true orthologues of vertebrate clotting factors. One of the genes that can be positively identified by performing sequence comparisons is the enzyme transglutaminase (or factor XIIIa in vertebrates), for which there is a good homology in the *Drosophila* genome (CG7356). No obvious homologues of crayfish clotting protein or the horseshoe crab coagulogen can be identified (Theopold *et al.,* 2001b). Although vitellogenin also exists in insects, its function in the clotting system may be different from crustaceans, since a vitellogenin-like protein has recently been implied in the melanisation reaction (Lee *et al.*, 2000a).

Although true homologues of proteins involved in clotting of other species are somewhat difficult to identify in the *Drosophila* genome, there are a number of annotated genes, which might prove to be important in hemolymph clotting or related processes (Theopold *et al.,* 2001b). There are a number of predicted gene products,

which contain domains known to exist in clotting factors from other species. These domains include the immunoglobulin fold, the EGF-like domain, domains with homology to von Willebrand factor, sushi domains, clip domains and C-type lectin domains (Theopold *et al.*, 1999; Adams *et al.*, 2000). In most cases, the functional importance of these domains still has to be confirmed. Furthermore, there are some predicted genes with unique combinations of domains, which seem worthy of further functional analysis. For example, a good homologue of *Bombyx* hemocytin exists in the *Drosophila* genome (CG7002), which will most likely turn out to be involved in immune reactions (Theopold *et al.*, 2001b).

1.2.4.3.2 The hemocyte coagulation

Granulocytes are the major hemocytes involved in insect hemolymph coagulation (Bohn 1986; Theopold and Schmidt 1997). Hemocyte coagulogen is formed in the first stage; then the activated factors from hemocytes cause plasma coagulation. Normally structural changes occur in hemocytes during the coagulation reactions. For example, in the blister beetle, *Epicauta cinerea,* the granulocytes extrude long, straight and thread-like structures and finally form a clot with other hemocytes (Gupta, 1969). Recent findings of Theopold and coworkers (Theopold and Schmidt, 1997; Theopold *et al.*, 2001b) clearly showed that there are a number of similarities, on a cellular basis, between the reaction of hemocytes in insects and platelets in vertebrates. Most prominent among the biochemical similarities is the formation of microparticles both during blood clotting and hemolymph coagulation (Theopold and Schmidt, 1997). The formation of platelet-derived microparticles is dependent on calcium (Pasquet *et al.*, 1996). The formation of these insect microparticles was also shown to depend on calcium and positive staining with annexin V, which suggested the presence of PS on the outer leaflet of the cell membrane (Theopold and Schmidt, 1997). Based on these observations, it is quite obvious that insect microparticles are part of the cellular coagulation reaction.

1.2.4.4 Coagulation and *Drosophila* dorsoventral pattern

Dorsoventral pattern formation leads to polarity in the embryo; the first visible sign is the blastoderm stage, which has a flat dorsal surface and a slightly convex ventral side (Govind and Steward, 1991). Development of dorsoventral pattern of the egg shell and embryo is established through the interaction of several maternal genes (Govind and Steward, 1991). Among these genes, *K10, capu* and *spir* only function in the germ line, whereas the *dorsal* groups and the *cactus* gene are very important for the embryo dorsoventral pattern (Anderson *et al.*, 1985; Anderson and Nusslein-Volhard, 1986). In the ventral gene group, the *torpedo* gene is the *Drosophila* homology of vertebrate epidermal growth factor receptor (EGF). In the *dorsal* gene groups, a few genes which are serine proteases such as *gastrulation defective, snake, easter* are involved in the formation of a ligand for the Toll receptor (Govind and Steward, 1991). Furthermore, Lemaitre *et al.* (1996) reported that some components of the dorsoventral signalling cascade also control the expression of the antifungal peptide drosomycin after injury. On the other hand, some clotting factors of *Limulus* showed similar protein domains to the *Dorsal* and *Ventral* genes. For example, the *Limulus* coagulogen, a 175 amino acid single chain polypeptide shows striking topological similarity to the neutrophin nerve growth factor (NGF) and belongs to a new member of the TGF $ß_2$ superfamily (Daopin, 1992). All the other *Limulus* clotting factors are typical glycoproteins and have a serine protease domain. In addition, factor C contains an EGF-like domain; factor B and the proclotting enzyme contain a clip-like domain, which share sequence similarity with *Drosophila* serine protease *snake* and *easter* genes.

Based on the above results, it can be concluded that the dorsoventral cascade is multifunctional and might have functions in both development and humoral immunity, including coagulation cascade. However, more evidence is needed to confirm that members of the dorsoventral cascade play a role in coagulation reactions and in cellular immunity.

1.2.5 The prophenoloxidase cascade

As mentioned previously, insect uses PRRs to distinguish PAMP from self-structures (Medzhitov and Janeway, 1997; Medzhitov and Janeway, 2000). These receptors recognise conserved molecular patterns shared by large groups of microorganisms. The recognition of the microbial elicitors by specialised receptors leads to activation of a range of immune functions (Hoffmann *et al.,* 1999; Medzhitov and Janeway, 2000) including the prophenoloxidase cascade. The proPO cascade is a proteolytic cascade and consists of a number of proteins, including several proteases, which ultimately activate prophenoloxidase. The activated phenoloxidase is involved in several reactions, including melanin production, production of reactive oxygen species (Medzhitov and Janeway, 2000) and sclerotisation as part of the formation of the cuticle, capsule formation and wound healing. The concept of pattern recognition has gained strong support through the identification of a number of proteins that recognise microbial elicitors and subsequently activate the proPO cascade (Jomori *et al.,* 1990; Duvic and Söderhäll, 1990; Jomori and Natori, 1991; Söderhäll *et al.,* 1994; Cerenius *et al.,* 1994; Yu *et al.,* 1999; Ma and Kanost, 2000; Lee *et al.,* 2000b; Yu and Kanost, 2000).

Normally proPO is present as an inactive form which is converted to an enzymatically active from during a serine protease cascade. The enzyme that activates proPO is the prophenoloxidase activating enzyme (PPAE). However, proPO can also be activated by non-microbial elicitors, such as denaturing agents, denatured proteins and certain phospholipids (Theopold *et al.,* 2001b).

Multiple phenoloxidase genes are present in several species, their functions may differ in their enzymatic activity and substrate specificity. For example, three genes have been identified in *Drosophila* and six in *Anopheles*. In addition, an unsolved issue is the mechanism by which prophenoloxidase is transported into hemolymph and to the cuticle (Asano and Ashida, 2001) in the absence of any signal peptide in the primary protein sequences (Aspen *et al.,* 1995; Fujimoto *et al.,* 1995; Hall *et al.,* 1995a;

Kawabata *et al.*, 1995). There have been recent reports on a mechanism, which proteins can be transferred across membrane independent of a signal peptide (Joliot *et al.*, 1997; Joliot *et al.*, 1998; Prochiantz, 2000). It remains to be determined if this mechanism also applies to phenoloxidase, or other proteins. Although the proPO-cascade has been discussed as an independent part of humoral immunity, it is likely that phenoloxidase cooperates with other branches of the immune system including the clotting system. By binding to the clot, it might enhance the crosslinking activity and/or add antibacterial activity to other immune mechanisms that are localised there (Ashida *et al.*, 1988) and aid in wound healing (Lai-Fook, 1966). Furthermore, some reports have suggested that the propheoloxidase and clotting systems are related to each other to some extent (Decker and Rimke, 1998; Nagai and Kawabata, 2000; Theopold *et al.*, 2001b).

1.2.6 Reactions in a parasitoid/host system

Endoparasitoid wasps that deposit their eggs and develop inside the caterpillar body cavity interfere with the host defence system (Beckage, 1997). Eggs and growing larvae from the wasp, *Venturia canescens* are effectively protected by a surface layer, leaving the host, *Ephestia kuehniella*, cellular defence intact (Schmidt and Schuchmann-Feddersen, 1989). One of the components of the layer is virus-like particles, which are produced by the calyx gland. Besides VLPs, mucinous components from ovarian glands have also been reported to protect the eggs from the host immune defence (Rotheram, 1973a; Osman and Fuhrer, 1979; Davies and Vinson, 1986).

1.2.6.1 A hemomucin homologue in *Venturia*

As mentioned above, an insect mucin (hemomucin) isolated from a *Drosophila* cell line can be labelled by lectins with binding specificity for N-Acetyl galactosamine (GalNAc), such as HPL (Theopold *et al.*, 1996). Using HPL, a protein with the same size of hemomucin was detected in *Venturia* hemocytes, ovaries and on the surface of newly emerged larvae (Kinuthia, 1996). This indicated that a hemomucin homologue

exists in *Venturia*. Furthermore, in the host, *Ephestia*, a smaller sized protein was found in the silk gland and hemocytes using HPL (Kinuthia, 1996). This indicated that a hemomucin-related protein exists in *Ephestia* hemocytes. However, the function inside the host and the role in the defence system is not known.

1.2.6.2 Formation of insect microparticles

During hemocyte coagulation in *Drosophila* and *Galleria*, vesicles, insect microparticles were formed (Theopold and Schmidt, 1997). Insect microparticles are formed through the formation of fragmentation of filopodia and can be stained with HPL. Similarly, *Venturia* microparticles seem to have the same appearance, and might function in coagulation and in protection of the parasitoid against its host (Kinuthia, 1996). Based on these research findings on the reactions in the parasitoid/host system, a preliminary hypothesis how the parasitoid may be protected against its host can be obtained, however the exact mechanism remains unknown.

1.3 Parasitoid defence system

Immune responses allow insects to effectively react against foreign organisms; however, parasitoids have developed strategies to circumvent the host immune system. Endoparasitic Hymenoptera complete larval development within the body cavity of host insects, which eventually leading to the death of the host. Oviposited eggs and developing larvae must evade or disable the host immune responses and different wasp species may have evolved different strategies to manipulate host immunity, growth and development. Some braconids and ichneumonids employ a mutualistic association with certain unusual viruses called polydnaviruses (PDVs), while others secret protein particles, known as virus-like particles. The role of VLPs and PDVs in parasitoid and host relationships has been a major focus of research in insect immunity. VLPs and PDVs are secreted by parasitoid calyx cells and co-injected simultaneously with eggs into the host hemocoel (Stoltz and Vinson, 1979a; Stoltz and Vinson, 1979b; Asgari *et al.*, 1996; Asgari *et al.*, 1997). Parasitoids successfully develop inside a host either by

suppressing the immune response or by passive immunoevasion, which results from a failure to be recognised as foreign organisms (Quicke, 1997).

1.3.1 Active suppression by polydnaviruses

Some of the most potent weapons parasitoids utilize in suppressing their host's defence system are polydnaviruses (Schmidt and Theopold, 1991; Beckage, 1995; Beckage, 1998). PDVs are a group of mutualistic viruses that can disrupt the host physiological system in some ichneumonid and braconid wasps (Lavine and Beckage, 1995; Strand and Pech, 1995b). PDVs comprise multiple circles or segments of double stranded DNA and have equally distinctive obligate mutualistic association with parasitoids. They have been described in ichneumonids and braconids and comprise two virus genera in this family, ichnovirus and bracovirus respectively, according to phylogenetic and morphological criteria (Webb and Cui, 1998). Ichnoviruses generally are posses fusiform or quasicylindrial nucleocapsids that are surrounded by two envelopes (Fleming, 1992). Bracoviruses have a uniform cylindrical nucleocapsid enclosed by a unit membrane envelope, but sometimes, several nucleocapsides are observed in one envelope (Stoltz *et al.*, 1984; Fleming, 1992). In spite of the morphological difference between ichnoviruses and bracoviruses, they have some important common features as well: multi-particle and segmented double stranded DNA, replication in the nucleus of calyx cells and integration of the viral genome into the wasp genome.

The lifecycle of PDVs is characterised by virus replication from proviral DNA in a parasitic wasp followed by pathogenic virus infection in the host caterpillar. PDV genes are expressed in wasp oviducts and in parasitised larvae. Individual viral gene may be expressed only in the wasp, only in the parasitised host, or in both (Theilmann and Summers, 1987). During the course of oviposition, female parasitoids simultaneously inject eggs and PDVs into the host body (Stoltz 1993). Virions were observed mainly associated with host basement membranes, fat body, tracheal epithelia (Stoltz and Vinson, 1979a) and hemocytes (Davies *et al.*, 1987; Prevost *et al.*,

1990). PDVs cause a number of physiological, behavioural and developmental alterations in parasitised hosts and play a role in suppression of the host immune response. Several reports suggest that the initial protection is provided by maternal components from the calyx fluid. The immediate suppression of the host immune response is then replaced by PDV-encoded products (Webb and Luckhart, 1994; Asgari *et al.,* 1996). In suitable hosts, PDVs enter various tissues with transcription of the viral genes in the apparent absence of replication over the period required for the wasp's progeny to complete the development (Strand and Pech, 1995a). Transcriptionally active PDVs induce several physiological alterations in hosts that are considered important for survival of the wasp's progeny. In addition, PDVs have immunosuppressive and juvenilizing effects on hosts (Whitfield, 1990; Fleming, 1992). In the presence of virus, host hemocytes are often unable to encapsulate parasitoids, whereas in the absence of virus, hemocytes rapidly encapsulate the parasitoid (Fleming, 1992). However, a study on the interaction of *Microplitis demolitor* and its host, *Spodoptera frugiperda,* demonstrated that *M. demolitor* are unable to develop although *S. frugiperda* polydnavirus (MdPDV) can disrupt the encapsulation response (Trudeau and Strand, 1998). This suggests that successful infection of a host by PDVs does not assure successful development of the parasitoid.

1.3.2 Immune evasion by virus-like particles

In addition to polydnaviruses, virus-like particles have been observed in parasitic wasps. Several observations indicate that VLPs are involved in the immunoprotective function against the host immune response (Salt, 1973; Feddersen *et al.,* 1986; Schmidt and Schuchmann-Feddersen, 1989).

The understanding of polydnaviruses and virus-like particles began with the early work by Salt who studied how the parasitoid wasp *Venturia canescens* avoided its host's encapsulation (Salt, 1964). He found that some secretions of the calyx surrounded the eggs and formed a protective layer that helped to avoid encapsulation by the host. Later Rotheram (1967, 1973) confirmed that the coatings are VLPs. Thereafter several

studies on this coating have been conducted. It turned out that viruses are widespread among the parasitoids. VLPs from *V. canescens* appear somehow unusual in both their structure and mode of action. In contrast to polydnaviruses, there is no detectable nucleic acid in VLPs (Bedwin, 1979). In addition, VLPs mediate protection only while adherent to the egg surface, but PDVs can operate remotely from the egg. Accordingly VLPs were placed apart from polydnaviruses until their relationship to PDVs is determined (Fleming, 1992). A number of studies were based on *Ephestia kuehniella* as a host. Therefore, the interaction of *Venturia* and its host, *Ephestia kuehniella,* especially the VLPs of the *Venturia* will be discussed further.

1.3.2.1 VLPs of *Venturia canescens*

Many ichneumonid parasitoids produce viruses or virus-like particles in their reproductive tissues, which are injected simultaneously into the host together with their eggs (Stoltz and Vinson, 1979b; Feddersen *et al.,* 1986; Fleming, 1992). These particles are involved in mediating change of host physiology like inactivation of the host defence system (Feddersen *et al.,* 1986), changes of hormone titre (Zitnan *et al.,* 1995) and other alterations of host homeostasis (Beckage, 1993).

VLPs have mainly been described in the ichneumonid wasp, *V. canescens*. Given these particles appear to lack detectible nucleic acids (Bedwin 1979; Feddersen *et al.,* 1986), they were named virus-like particles (Schmidt and Schuchmann-Feddersen, 1989). VLPs have some similar characteristics with DNA-containing PDVs such as morphology, assembly within the nucleus of the calyx cells and mode of secretion, but other typical viral properties, such as viral replication in parasitoids and infection of host tissue are absent or unknown (Schmidt and Schuchmann-Feddersen, 1989).

Virus-like particles of *Venturia* are assembled in the nuclei of the calyx cells. Particles can be detected in calyx gland cells from pupal stages (Rotheram, 1973a; Rotheram, 1973b). They are electron-dense particles within enlarged nuclei of calyx gland cells and are coated with an envelope (Schmidt and Schuchmann-Feddersen, 1989). They

are eventually secreted into the lumen of the calyx gland. As the eggs pass through the calyx gland, they are covered by the particles. Since they lack DNA, VLPs might not play an active role in suppressing the host immune response (Schmidt and Theopold, 1990; Schmidt *et al.,* 1990). Further experiments showed that removal of VLPs from the egg surface by using denaturing buffers or binding the eggs with anti-VLPs antibodies leads to encapsulation of the eggs (Feddersen *et al.,* 1986). However, when Sephadex beads were injected into previously parasitised larvae, they were encapsulated (Feddersen *et al.,* 1986). These results indicated that the host is still able to react to foreign invaders and leave the host immune system intact (Schmidt and Schuchmann-Feddersen, 1989). This provided further evidence that VLPs passively protect parasitoid wasp against its host.

At a molecular level, VLPs in *Venturia* comprised four major proteins with molecular weight around 60 (p60), 52 (p52), 40 (p40) and 35 (p35) kDa; as well as some minor proteins with 80 (p80) kDa (Schmidt and Schuchmann-Feddersen, 1989). Using anti-VLPs antibodies, a protein of 42 kDa can be detected in the larval fat body of its host *Ephestia.* p60, p52 and p80 show cross-reactivity with the 42 kDa protein. Thus VLP proteins are related to a host protein. Experiments also confirmed that p42 is conserved within the several tested lepidopteran species though the size of the proteins is slightly different (Schmidt and Schuchmann-Feddersen, 1989). Among these VLP proteins, p40 (named VLP1) was cloned, sequenced and characterised (Hellers *et al.,* 1996).

1.3.2.2 VLP1

When VLP proteins from *Venturia* wasp oviducts were analysed on Western blot using antibodies against VLPs, four major bands of 60, 52, 40 and 35 kDa were detected in equivalent amounts and one minor band of 80 kDa was less prominently. In order to characterise the VLPs, it is important to clone and sequence them. The clone of p40 was obtained by screening an expression library first and it was named VLP1. Further analysis of VLP1 sequence revealed three domains: an amino-terminal region which is highly hydrophobic, a carboxyl-terminal region with significant similarity to

phospholipid hydroperoxide glutathione peroxidase (PHGPx) and a tandem repeat in between (Hellers *et al.,* 1996). A deletion of the tandem repeat creates two allelic versions of the VLP1 gene (Hellers *et al.,* 1996). Two clonal strains were isolated from the laboratory parthenogenetic (asexual) population by using VLP1 gene as a diagnostic marker (Beck *et al.,* 1999). Studies on the two clonal lines have been carried out and several differences between the two strains were observed (Beck *et al.,* 1999 and below).

- Genetic differences

Since VLP1 has two allelic versions, VLP1.2 and VLP1.0, which differ by the presence or absence of the tandem repeat sequence, two clonal lines, RP (repeat plus) and RM (repeat minus) were established (Beck *et al.,* 1999). Analysis of the two laboratory populations showed that all individual wasps are homozygous for one of the two allelic VLP1 genes. Besides the VLP1 difference, additional differences between the two lines were detected. By using randomly amplified DNA polymorphism (RAPD) analysis, 9.5% polymorphic scorable bands were identified (Beck *et al.,* 1999). This indicates that the two wasp lines are stable and have been separated long enough to accumulate additional DNA polymorphisms. Further studies of the two lines showed that they have other differences as well (Beck *et al.,* 1999; Beck *et al.,* 2001).

- Phenotypic difference

Besides the genetic difference between RP and RM populations, phenotypic differences were observed (Beck *et al.,* 1999; Beck *et al.,* 2001). Since VLPs are produced in the calyx tissues of the ovaries, ovarian tissues from the two strains were compared, several differences were observed: 1) RP calyx glands are smaller and more compact than that in the RM strain; 2) RP calyx tissues shows regular cell shapes with relatively compact nuclei while RM calyx tissues displayed extended VLP-filled membrane systems with obvious fusions of membranes (Beck *et al.,* 1999; Beck *et al.,* 2001). Thus the membrane-like vesicles in the RM calyx tissues block the eggs

passage into oviduct. Subsequently two distinctive reproductive strategies were employed in the two VLP strains (Beck *et al.,* 1999; Beck *et al.,* 2001).

- Reproductive difference

When the two clonal lines were reared separately, more offspring were produced by RP than RM wasps. Surprisingly RM wasps produced higher number offspring when the two wasps were allowed to compete for the same hosts (Beck *et al.,* 1999; Beck *et al.,* 2001). RP wasps have relatively large egg reservoirs and lay eggs immediately, whereas RM wasps have fewer eggs and delay egg deposition. The two strains may have a selective advantage depending on two extreme conditions: the RP wasps might be more successful when host numbers are abundant and wasp numbers are low while the RM wasps might be more successful when host are down and superparasitism is frequent (Beck *et al.,* 1999; Beck *et al.,* 2001).

1.3.2.3 Functional analysis of VLP1

As mentioned above the carboxyl-terminal region of VLP1 sequence shows significant similarity to phospholipid hydroperoxide glutathione peroxidase (PHGPx). However, the amino acids which normally constitute the catalytic center are not conserved (Hellers *et al.,* 1996). Furthermore, enzymatic assay of VLP1 with hydrogen peroxide and butyl-hydroperoxide as substrates did not show any reductive activities (Hellers *et al.,* 1996). Therefore, it is still not clear whether VLP1 is enzymatically active.

Phospholipid hydroperoxide glutathione peroxidases (PHGPxs) are intracellular antioxidant enzymes that can directly reduce peroxidised phospholipids and cholesterol in membranes (Ursini *et al.,* 1985; Thomas *et al.,* 1990). Functional studies showed that PHGPx prevents lipid peroxidation and protect biomembranes against oxidative stress (Ursini *et al.,* 1982). Its abundant expression in spermatids and high reductive activity in postpubertal testis (Roveri *et al.,* 1992; Maiorino *et al.,* 1998;) indicates a role in male fertility. Surprisingly, recent report demonstrated that

mammalian PHGPx also functions as an enzymatically inactive structural protein of the mitochondrial capsule (Ursini *et al.,* 1999).

PHGPx has two transcripts coding for two proteins, one with molecular weight around 20 kDa and the other around 23 kDa (Pushparekha *et al.,* 1995), The 23 kDa PHGPx which contains a leader sequence of 27 amino acids is the precursor to the mitochondrial PHGPx. When this precursor is imported into the mitochondria, its leader sequence is cut to yield a mature 20 kDa PHGPx (Arai *et al.,* 1996). This indicates that the leader sequence is necessary for the import of the PHGPx into the mitochondria.

In mammals, PHGPx is a selenoenzyme in which selenium is inserted in the active site as selenocysteine (Maiorino *et al.,* 1990). Like other selenoproteins, the selenocysteine (Sec) in mammalian PHGPxs (referred as Sec-PHGPxs), is encoded by a UGA codon that normally functions as a stop codon (Ursini *et al.,* 1985; Thomas *et al.,* 1990). However, in plants, PHGPx sequences isolated so far show that a Cys-codon (UGU), encodes the presumed catalytic residue (Sugimoto *et al.,* 1997; Faltin *et al.,* 1998), producing a selenium-independent form of PHGPx (Cys-PHGPx). Selenium dependent-PHGPx has been isolated from several animals, such as human, pig and rat; and from several tissues, like brain, liver, heart, testis and placenta (Maiorino *et al.,* 1990; Godeas *et al.,* 1997). Selenium independent-PHGPxs have been identified in several plants, including spinach and citrus (Sugimoto *et al.,* 1997; Faltin *et al.,* 1998). These reports reveal that mammalian Sec-PHGPx antioxidant activities are much higher than that of the Cys-PHGPx (Faltin *et al.,* 1998). In addition, some Cys-PHGPxs display substrate specificity towards the same lipid hydroperoxides as for Sec-PHGPXs (Faltin *et al.,* 1998). In contrast, Cys-PHGPx in citrus could not catalyse the reduction of hydrogen peroxide (H_2O_2) whereas, Sec-PHGPxs display such reductive activity (Maiorino *et al.,* 1996).

Attempts to correlate enzymatic functions to the PHGPx domain (*Venturia canescens* PHGPx domain, VcPHGPxd) were not successful probably due to the lack of

structurally conserved amino acids, which normally constitute the catalytic centre of the PHGPx enzyme (Hellers *et al.*, 1996). In particular, a selenium-cysteine, coded by a terminator-codon in most mammalian PHGPx genes and perceived to be essential for the enzymatic function (Maiorino *et al.*, 1995), was replaced by the amino acid, Alanine, in the VcPHGPxd. Moreover, no trace of selenium has been found in VLPs (A. Reineke, pers. comm.). Nevertheless, the hydropathic pattern of the VcPHGPxd closely resembling those of other functionally intact PHGPx enzymes (Hellers *et al.*, 1996), indicates some functionally conserved PHGPx-properties other than enzymatic function.

Much more research on mammalian and plant PHGPx have been conducted, and less is known about insect PHGPxs. Therefore, functional studies of PHGPx homologues may able us to further understand the defence mechanism of VLP1.

2. Aims of the study

2.1 Genetic and functional analysis of virus-like particles

A deletion of the tandem repeat region of VLP1 created two allelic lines (Beck *et al.*, 1999). From studies on two asexual *Venturia* strains, it seems that allelic VLP1 contributes to in the phenotypic differences observed between the strains. Recently sexual *Venturia* populations have been obtained from southern France where both VLP1 alleles co-exist in the wild. Therefore, whether the sexual populations display the same phenotypes as the laboratory populations was investigated.

2.2 Protection mechanisms of parasitoid against its host

Virus-like particles have been suggested to provide a passive protection to parasitoid, but VLP1 has sequence similarity with phospholipid hydroperoxide glutathione peroxidase (PHGPx) domain. Therefore, functional studies of VLP PHGPx domain are

necessary in order to provide some insight into the protection mechanism of parasitoid wasps.

In addition to VLPs, some mucinous proteins have been found to have a possible protective function. A mucinous protein, hemomucin, was isolated from *Drosophila* and it is expressed on hemocytes, egg surface and microparticles, which provides larvae with protection (Theopold and Schmidt, 1997). Whether a similar protection also exists in *Venturia* is not known. In addition, hemomucin attracts hemolymph protein, lipophorin, which is involved in hemolymph coagulation (Theopold and Schmidt, 1997). Insect microparticles are also part of the hemolymph coagulation reactions (Theopold and Schmidt, 1997). *Venturia* hemocytes can form microparticles where hemomucin is found to be enriched as well (Kinuthia, 1996). All these results suggest that there may be some links between the coagulation reaction and the egg surface modification inside the host. Lipophorin functions in multiple ways, e.g. as mediator of hemocyte adhesion in locusts (Cho *et al.,* 1998), as a coagulogen in insect coagulation cascade (Bohn, 1986), as a growth promoter in *V. canescens* (Nakahara *et al.,* 1999). However the function of lipophorin and hemomucin in the defence reactions in *Venturia/ Ephestia* system remains to be determined.

2.3 Insect hemolymph coagulation

Insect hemolymph coagulation depends on the interaction of cellular components and soluble serum factors (Grégoire, 1970). It is widely accepted that lipophorin is one of the clotting factors which play an important role in insect clotting cascade. However, other hemolymph molecules engaged in coagulation and their functions in the coagulation cascade remain unknown.

Therefore, as part of a broader research project, seeking to understanding molecular and biological characteristics of insect innate immunity and its involvement in development, the specific objectives of this project were:

- Genetic analysis of two distinctive reproductive strategies in *Venturia canescens*.

- Overexpression and functional analysis of the phopsholipid hydroperoxide glutathione peroxidase (PHGPx) gene from *Drosophila melanogaster* and *Venturia canescens*.

- Investigation of the immune protection of parasitoid wasp, *Venturia canescens*, against its host, *Ephestia kuehniella*

- Identification and molecular characteristion of insect coagulation components using *Galleria mellonella* as a model system.

Chapter 2: Materials and Methods

2.1 Materials

All chemicals used were analytical reagent or molecular biology grade. Solutions and media were prepared with double-distilled water and autoclaved where appropriate. The compositions of the solutions are list in the appendix 1 and 2.

2.2 Methods

2.2.1 Insect culture

2.2.1.1 *Galleria mellonella*: *Galleria* were reared in the dark at 25°C, Larvae were fed on an artificial diet .

2.2.1.2 *Drosophila melanogaster*: Canton S flies were kept on cornmeal/yeast food at a photoperiod of 14 h light: 10 h dark at 25°C.

2.2.1.3 *Venturia canescens* and *Ephestia kuehniella: V. canescens* wasps were reared on final instar caterpillars of *E. kuehniella*. The insects were maintained in the laboratory at 25°C under a 14:10h (light:dark) photoperiod. *E. kuehniella* caterpillars were fed on bran:wheat germ:brewing yeast (10:2:1) and *V. canescens* adults on a 50% honey-water solution. To distinguish the larval stages of host and parasitoid, the term caterpillar is used for *E. kuehniella*, and larva for *V. canescens* larval stages.

2.2.2 Genetic analysis of parasitoid wasps

2.2.2.1 Establishment of clonal wasp lines

In hymenopteran species, two modes of reproduction are employed. One is the haplodiploidy, where non-fertilised eggs develop into haploid males (arrhenotokous

reproduction) and fertilised eggs develop into diploid females (sexual reproduction); the other is the thelytokous mode of reproduction, where diploid female, the only populations, are produced by asexual mechanisms. To distinguish the population that use different modes of reproduction, sexual population was used to refer the haplodiploidy and asexual population refer to the thelytokous reproduction in the following experiments.

V. canescens populations were collected from Mildura, SA and Mount Boron, southern France and named accordingly (Table 3). Asexual females from laboratory stocks were separated according to the VLP1 allelic differences, named RP(aL) and RM(aL) respectively. Asexual females from two locations near Mildura (Australia), corresponding to the laboratory RM(aL) and RP(aL) genotypes at the VLP1 locus, were used for phenotypic comparison with the laboratory strains. To distinguish the two lines from the laboratory strains, the terms RM(aM) and RP(aM) are used. Asexual and sexual individuals from one location (Mount Boron) in southern France were recovered (Malmaberg *et al.*, 2000) and used for a comparison of the phenotypes and reproductive success. The sexual genotypes were named RM(sB) and RP(sB) for homozygous females and RM/RP(sB) for heterozygous females, and the asexual genotypes named as RP(aB) and RM(aB). To obtain homozygous RM or RP offspring from mixed field population, non-mated female wasps were individually paired with a single male wasp and kept separately with host caterpillars. After egg deposition the genotype of the VLP1 gene was determined in both parents using PCR. Those crosses, where both parents were homozygous for the VLP1 gene, were kept isolated and continuously checked for the homozygous RM or RP status in subsequent generations, using random offspring checks.

Table 3 Names for different strains of *Venturia canescens*

Locations\Strains	RP		RM	
	Sexual	Asexual	Sexual	Asexual
Laboratory	_	RP(aL)	_	RM(aL)
Mildura, Australia	_	RP(aM)	_	RM(aM)
Mount Boron, France	RP(sB)	RP(aB)	RM(sB)	RM(aB)

RP, Having VLP1 tandem repeat region; RM, No VLP1 tandem repeat region.

2.2.2.2 Egg numbers of calyx and oviduct

The RM phenotype is not fully penetrant and becomes evident when females are kept for several days, allowing the passage of eggs into the oviduct (Beck *et al.*, 1999; Beck *et al.*, 2001). To examine the phenotype of the homozygous RM or RP populations, each female was kept for six days with honey-water without hosts. Egg distribution and egg numbers between calyx and oviduct were examined by dissecting ovaries and counting the number of eggs in the calyx and in the oviduct. To determine the phenotype of heterozygous females, offspring from a genetic cross between homozygous males and virgin females were kept for six days and egg numbers in the calyx and oviduct were examined as for homozygous females. More than 160 ovaries were recorded for each experiment and numbers analysed statistically using students' t tests.

2.2.2.3 Reproductive success

To assess the reproductive success of each wasp strain under conditions that favour superparasitism, wasps from one strain were exposed to hosts in the presence or absence of wasps from the other strain. Twenty-four hours before the start of an experiment, parasitoids and host patches were prepared. Twenty final instar *E. kuehniella* caterpillars were placed into a box together with the appropriate *Ephestia* diet. Freshly emerged wasps were collected from homozygous RP and RM cultures

and transferred to new culture vessels containing honey solution. On the day of the experiment, two apparently healthy wasps were placed together with 20 caterpillars into culture vessels (60 mm in diameter, 90 mm high) and allowed to parasitise the hosts for different time periods. To differentiate between short and long periods of oviposition, 24 h and 96 h, were chosen as two time periods in the competition experiments.

Three different experimental combinations were examined. In two conditions, the vessels contained either two homozygous RM-wasps or two homozygous RP-wasps. In another condition, the vessels contained one homozygous RP and one homozygous RM wasp to compare the competitive of the two wasps. The experiment included three replicates. The number and genetic identity of offspring were recorded and the average number of offspring per individual wasp was calculated. Although not enough evidence was obtained about the differences between male and female offspring successes, the sex ratio of the two homozygous populations was determined to exclude any bias based on male/female distributions in superparasitised hosts. In spite that the absolute number of offspring was different for the two homozygous populations, the male/female ratio was similar [RM(sB) sex ratio 0.591 and RP(sB) sex ratio 0.616], which excludes sex ratio differences as a possible predisposition for observed differences in the reproductive success. However, sex ratio determination in heterozygous populations is not reliable, since each cross depends on the fertilization rate, which can be quite variable with the possible consequence that the sex ratio of heterozygous offspring was different for each cross. No attempt was made to measure the reproductive success of heterozygous larvae in superparasitised caterpillars to avoid a possible bias of different sex ratios in each cross.

2.2.3 Extraction and analysis of DNA

2.2.3.1 Genomic DNA preparation

Genomic DNA was prepared using the protocol described in Heller *et al.* (1996). Detailed procedure sees Appendix 3.

2.2.3.2. Plasmid DNA preparation

For mini-preparations of plasmid DNA, the alkaline lysis method according to Sambrook *et al.* (1989) was employed with slight modifications. Before the phenol: chloroform extraction, an RNase digestion step was introduced. RNase (DNase free) was added to a final concentration of 20 μg/ml and the sample was incubated for 37°C for 20 min. Furthermore, the phenol:chloroform extraction was only conducted when higher quality preparations were needed.

When higher yields of pure plasmid DNA were needed, the BRESApure Plasmid Kit (Bresatec Ltd., Thebarton, SA, Australia) and Qiaprep Mini kits (QIAGEN Pty Ltd, Clifon Hill, Victoria, Australia) were used according to the supplier's protocols.

2.2.3.3 Polymerase chain reactions (PCR)

Genomic DNA from the abdomen of individual wasps was extracted. PCR were performed using two VLP1-specific primers, which were used to identify the tandem repeated sequence within the VLP1 protein following the method of Heller *et al.* (1996).

The primers used in identifing the tandem repeated sequence within the VLP1 protein were:

DINT: 5'-CTCAATATGTGGGGTGGTGG-3'

SAC: 5'-TGAAAAACTGAATAAGCGTTGG-3'
or 5"II: 5'-TCGCAGTGGCTTGTCAGAGT

F-54 5'-CTACCACAGTCCCGGACGAC-3'
R-54 5'-GTCGTCCGGGACTGTGGTAG-3'

2.2.3.4 DNA quantitation

Spectrophotometer: Larger amounts of DNA were measured and analysed spectrophotometrically as described by Sambrook *et al.* (1989).

Spot test: For small DNA amounts (< 250 ng/µl), a spot test was carried out. An appropriate serial dilution of the DNA sample was spotted (1 µl per spot) onto the surface of a 1% agarose slab gel containing ethidium bromide (0.5 µg/ml). Next to this serial dilution, 1 µl spots of a series of DNA concentration standards (e.g. 250, 125, 62.5, etc. µg/µl) were pipetted. The spots were allowed to dry, and then photographed under UV illumination. The amount of DNA was estimated by comparing the intensities of the photographed sample spots with the ones of the standards.

2.2.3.5 Isolation and purification of DNA from agarose gels

The Wizard™ PCR Preps DNA Purification kit (Promega) and Geneclean kit (BIO 101, California, USA) were used to isolate and purify DNA restriction fragments out of agarose gels according to the manufacturer's manuals.

2.2.3.6 DNA sequencing and oligonucleotide synthesis

DNA sequencing was performed by the Nucleic Acid and Protein Chemistry Unit of the Department of Plant Science, The University of Adelaide, Australia and Flinders University of South Australia.

Oligonucleotide synthesis was produced by the Nucleic Acid and Protein Chemistry Unit of the Department of Plant Science and Geneworks (Adelaide, South Australia).

2.2.3.7 Sequence analysis

Drosophila Fly BLAST and NCBI BLAST were used to search for sequence in the GenBank (Altschul *et al.*, 1990). The MegAlign program was used for the comparison of sequences.

2.2.4 Extraction and analysis of RNA

2.2.4.1 Extraction of total RNA

It was extremely important that all components were RNase free, so treatments were carried out before RNA extraction. All the solutions were made using diethylpyrocarbonate (DEPC)-treated water (except Tris containing solution). The glassware was washed thoroughly with DEPC-H_2O and autoclaved.

For RNA extraction, hot phenol method was used (See Appendix 4), in addition, RNeasy (QIAGEN) and QuickPrep (Amersham pharmacia biotech) total RNA extraction kit were used according to the manufacturer's instructions.

2.2.4.2. Extraction of mRNA

Poly A tract kit (Promega) was used for mRNA extraction according to the manufacturer's instructions.

2.2.4.3 Northern blots

2.2.4.3.1 RNA blots

The Northern blots were conducted with the methods described in the molecular biology manuals (Sambrook *et al.,* 1989). Detail procedures are listed in Appendix 5.

2.2.4.3.2 Hybridisation and autoradiography

Ready-to-go DNA labelling beads (Amrad Pharmacia Biotech, Boronia, Vic, Australia) were used according to the manufacturer's instructions. Subsequently, to remove the unincorporated radioactive from the DNA labelling reaction, ProbeQuant G-50 micro columns (Amrad Pharmacia Biotech, Boronia, Vic, Australia) were applied according to the supplier's instructions.

Prehybridisation and hybridisation were performed according to Sambrook *et al.* (1989) at stringency conditions.

2.2.4.4 RT-PCR

Total RNA was treated with RQ1 RNase-free DNase prior to RT-PCR according to the technical bulletin (Promega). RNA sample was afterwards purified with a standard Phenol: Chloroform extraction followed by an ethanol precipitation. For reverse transcription reactions, 2 μg RNA samples and 1 μl reverse primer (0.1 μg/μl) were heated to 95°C for 5 min and placed on ice immediately. Then the followings were added to the reaction: 3 μl 5x AMV buffer, 0.5 μl AMV reverse transcriptase (Promega, 10 U/μl), 0.3 μl RNasin (Promega, 30 U/μl), 0.5 μl dNTPs (15 mM) and DEPC-treated water to final volume of 15 μl. The reaction was incubated at 42°C for one hour followed by 5 min at 95°C to inactivate the enzymes. A PCR reaction was performed by adding the followings to the RT reaction: 1 μl reverse primer, 1 μl forward primer, 0.5 μl dNTPs, 3 μl 10x PCR buffer, 0.5 μl Taq DNA polymerase

(Promega, 5 U/µl) and water to the final volume of 50 µl. The reaction was subjected to 3 min at 95°C and 30 cycles of 30 seconds denaturation at 94°C, 45 seconds annealing at 56°C and 1 min extension at 72°C and a further extension of 5 min at 72°C. Amplified RT-PCR reactions were then analysed on 1% agarose gels.

Primers used in the expression analysis of the corresponded genes were:

α-crys/SHSP

Reverse primer: 5'-TACCGTCTTATAATTCTTCTA-3'
Forward primer: 5'-TACCAAATTAATAGTAACACG-3'

Ribosomal protein L7

Reverse primer : 5'-TGTCCGATGGCCCAAATA-3'
Forward primer: 5'-TGCAGCCTTCTTCAGACG-3'

ProPO

Reverse: 5'-CGTTCCCTTGGGTATCAG-3'
Forward: 5'-CATAGCATACTCGCATGA-3'

Mucin-like

Reserve: 5'-CATCAATATCCATTAGTGAA-3'
Forward: 5'-CCAGATGGTTCTACTACTAC-3'

Trypsin-like

Reserve: 5'-GAGATACACGAGCGTTGA-3'
Forward: 5'-CCATCTTGCGTCTCTCCT-3'

GST

Reserve: 5'-CCTCACTGTCTCGTACCATCTCTT-3'

Forward: 5'-CACACTGGTTGACGATGGATTC-3'

2.2.5 Preparation and transformation of competent cells

2.2.5.1 Preparation of competent cells

For routine transformations, competent *E. coli* cells, strain JM 109, were used. M15 was used for transformation of pQE30 expression vector. Competent cells were prepared as below.

1. Bacterial cells were plated out on LB-plates containing the appropriate antibiotic and incubated overnight at 37°C.
2. A single colony was picked and used to inoculate a starter culture of 1 ml LB medium (+ antibiotic). The culture was grown at 37°C with shaking until it reached mid log phase, and used to inoculate 100 ml of LB medium (+ antibiotic) in a 250 ml flask at 37°C with shaking.
3. The culture was allowed to grow until OD_{600} = 0.5, and then centrifuged at 2000 rpm for 5 min at 4°C. Afterwards, the supernatant was carefully discarded. From this step onwards the cells were always kept on ice.
4. The pellet was gently resuspended in cold (4°C) TFB1 buffer (30 ml/100 ml culture) and incubated on ice for 90 min. Then, the solution was centrifuged at 2000 rpm for 5 min at 4°C.
5. The supernatant was carefully discarded and the pellet gently dissolved in ice-cold TFB2 buffer (4 ml/100 ml culture).
6. Finally, 150 µl aliquots were prepared and snap frozen in liquid nitrogen. The competent cells were stored at -70°C.

2.2.5.2 Transformation of competent *E. Coli* Cells

1. Frozen (-70°C) competent *E. coli* cells were thawed for ca. 5 min on ice and gently mixed before use.

2. Per transformation 50 µl cells were added to the DNA (1-50 ng; volume < 10 µl) in a pre-chilled microfuge tube. The transformation mix was gently mixed several times and placed immediately on ice for 20 min.
3. The cells were heat shocked in a water bath at exactly 42°C for 90 sec without shaking and then immediately placed on ice for 2 min.
4. To the transformation mix 450 µl LB medium was added. The sample was incubated at 37°C for 60 min with shaking.
5. If the bacteria are supposed to be tested for a-complementation (white/blue screening), 200 µl of IPTG (20 mg/ml) and 20 µl of X-gal (50 mg/ml) were spread onto LB plates supplemented with the appropriate antibiotic (e.g. 100 µg/ml ampicillin). The solutions were allowed to dry for at least 30 min at 37°C.
6. Several volumes of the transformed *E. coli* cell (e.g. 10 µl, 100 µl and 400 µl, respectively) were plated out onto the pre-prepared antibiotic plates and incubated overnight at 37°C.
7. Afterwards, individual colony (white ones in case of a blue/white screening) was picked for doing PCR to confirm the insert. Alternatively individual colony was used to set up overnight culture for plasmid DNA preparation and restriction digestion to identify the insects.

2.2.6 Preparation and analysis of proteins

2.2.6.1 Isolation of hemolymph, hemocytes, fat body and gut

Final instar of *G. mellonella* larvae was surface sterilised with 70% ethanol and a pair of prolegs was cut off with micro-scissors, the larvae were gently squeezed and bled into a drop of insect ringer on a piece of Parafilm 'M' (American National Can, Chicago, IL, USA) on ice. To avoid melanogenesis, 10 larvae were bled into 200 ml of insect ringer and centrifuged at 760 ***g*** immediately to precipitate the hemocytes. The recovered supernatant is the cell-free hemolymph.

To isolate fat body and gut, the sterilised caterpillar was dissected in PBS with fine forceps under a stereo-microscope. Fat body and gut tissues were collected in PBS and kept at -80°C for further use. The fresh tissues can be used directly to extract RNA or run on polyacrylamide gel with addition of equal volume of 2x loading buffer.

2.2.6.2 SDS-PAGE and Western blots

SDS polyacrylamide-gel electrophoresis on a Mini-Protean II electrophoresis unit (Bio Rad) was essentially performed according to Laemmli (1970). The protein marker SeeBlue was used according to the supplier's instructions (Novex, San Diego, CA, USA). Proteins were blotted onto a nitrocellulose membrane (Amersham) as described previously (Theopold *et al.*, 1996). The transfer of the separated proteins onto nitrocellulose filter was carried out in a Mini Trans-Blot cell (Bio-Rad) applying 200 mA/70 V (or 220 mA/100 V) for about 1 h using transfer buffer containing 192 mM glycine, 25 mM Tris-base and 20% (v/v) methanol (Towbin *et al.*, 1979). After pre-incubation of the filters in first blocking solution (8% w/v non-fat milk powder and 0.02% sodium azide in PBS) for 1 h at RT with gentle agitation, the primary antiserum was added to a specific final concentration, and the incubation was continued for another 2 h or over night. Then, the filters were washed three times, 10 min each, with gentle agitation: twice in PBS and once in 150 mM NaCl, 50 mM Tris-HCl pH 7.5. If not stated otherwise, alkaline phosphatase-conjugated secondary anti-rabbit antibody (Pierce) diluted 1:10000 in second blocking solution (5% w/v non-fat milk powder, 150 mM NaCl, 50 mM Tris-HCl pH 7.5) was added to the blot. The blots were incubated for 2 h at room temperature. After the incubation in secondary antiserum, the filters were washed three times for 10 min each in 150 mM NaCl, 50 mM Tris-HCl pH 7.5 with moderate shaking. Finally, for the visualisation of the protein bands, the blots were stained with NBT and BCIP as described by Sambrook *et al.* (1989).

If blots were developed with *Helix pomata* lectin (HPL) conjugated with peroxidase, they were treated in the following way. After immunoblotting, the blots were washed three times in TBST for 15 min each with agitation. To the last wash, the HPL was

added to give a final dilution of 1:10000. The filters were then incubated for at least 2 h. Afterwards they were washed four times in TBST for 10 min each and eventually immersed into staining solution (a tip of a spatula DAB and 50 µl of H_2O_2 in 20 ml of 10 mM Tris-HCl pH 7.5). The staining reaction was stopped by rinsing the blots in DDW and the blots were dried and scanned.

First antibodies used in the Western blots were:

1. Anti-VLP1 or anti-VcPHGPxd antiserum (1: 3,000): against the VLP1 PHGPx domain and used as a marker to detect the allelic difference of the VLP1 gene.
2. Anti-DmPHGPx antiserum (1:10,000): against *Drosophila* PHGPx clone, CG12013.
3. Anti-clot antiserum (1: 10,000): against immune clot formed by rabbit blood cells and *Galleria* cell-free hemolymph proteins.
4. Anti-lipophorin antibody (1: 5,000): against *Lucia* lipophorin.
5. Anti-hemomucin antiserum (1: 3,000): against *Drosophila* hemomucin.
6. Anti-proPO antiserum (1: 3,000): against *Manduca sexta* prophenoloxidase.

2.2.6.3 Expression and purification of proteins in *E. coli*

For expression of recombinant proteins, the QIAexpress system (QIAGEN) was employed. The region coding for *Drosophila* PHGPx was amplified by PCR and subcloned in frame into type IV constructs (pQE-30-, -31, -32). Subsequently, the recombinant protein was purified through the 6xHis tag.

PCR were conducted using two primers (with SphI and Kpn I restriction sites) within the coding sequences of DmPHGPx gene. The resulting PCR fragment was digested with SphI and KpnI, subcloned into pQE30 expression vector. Afterwards, M15 cells were transformed and induced with 0.5 mM IPTG. The *E. coli* transformed with DmPHGPx was found to produce significant amounts of recombinant DmPHGPx protein. The induced fusion protein was mainly detected in the soluble fraction. Native

protein was purified with Ni-resin column and eluted with 100 mM-250 mM imidazole according to manufacturer's instructions.

Primers used for cloning DmPHGPx gene were:

DmPHGPx SphI forwards: 5'-TGGCATGCTCTGCTAACGGAGATTACA-3'
DmPHGPx KpnI reverse: 5'-CACGGTACCCTACAGCAGCTTTTCAA-3'

2.2.6.4 Purification and binding of hemomucin

2.2.6.4.1 Affinity purification of hemomucin on HPL column

Hemomucin was purified from mbn-2 cells as described using affinity purification on a HPL lectin column (Theopold *et al.*, 1996). Briefly, cells from 500 ml of a densely grown culture were spun down, washed in PBS, and brought up in a lysis buffer (0.1 M sodium phosphate buffer, pH 7.5, containing 0.5% Nonidet P-40, 6×10^{-2} trypsin inhibiting U/ml of aprotinin and 1% epsilon amino caproic acid), lysed for 5 min on ice, and centrifuged twice for 10 min in a table top centrifuge. The supernatant was transferred to a column (1 mg of the lectin bound to CNBr-activated Sepharose according to the instructions of the supplier) and bound to the column on a tilting table. The column was washed in 40 ml of the lysis buffer, followed by a wash with 200 ml of lysis buffer without protease inhibitors. The bound proteins were eluted with a solution of 200 mM GalNAc in the last washing buffer. The eluted protein was analysed on SDS-PAGE and stained with Coomassie blue to estimate the concentration and purity as well.

2.2.6.4.2 Binding of hemolymph proteins to hemomucin beads and to *Venturia* eggs

Purified hemomucin (30-40 µg) was coupled to cyanogen bromide activated Sepharose beads according to the instructions of the supplier. This yielded a final volume of

approximately 200 µl. From this preparation, 40 µl was used per binding assay. Hemolymph from final stage *Ephestia* larvae was collected into insect ringer (IR:100 mM Tricine-HCl, pH 6.8, 100 mM NaCl, 40 mM KCl, 15 mM $MgCl_2$, 4 mM $CaCl_2$, saturated with phenyl thiourea) and cells removed by centrifugation at 3,000 ***g*** in a table top centrifuge for 5 min. The cell free hemolymph was incubated for 10 min with hemomucin beads with occasional mixing. Thereafter, the beads were washed 6 times with IR, the last washing solution being replaced with 20 µl double concentrated gel loading buffer, incubated for 10 min at 65°C and analysed by polyacrylamide gel electrophoresis.

Eggs from the oviduct of 20 adult *Venturia* were collected in PBS and used for the binding assay as for the Sepharose beads.

2.2.6.5 Preparation of antiserum

For the preparation of a specific antiserum, proteins were purified accordingly or further purified on a preparative polyacrylamide gel and used for immunisation. The proteins were mixed with an equal volume of complete Freund's adjuvant for first injection and incomplete Freund's adjuvant for further boosters. Immunisations were performed according to Harlow and Lane (1988) and conducted by Animal Care Centre, Waite Campus, Adelaide University, Australia.

2.2.6.5.1 Anti-DmPHGPx antibodies

Native recombinant DmPHGPx protein was purified using Ni-column. Antisera against *Drosophila* PHGPx protein were obtained by injecting the purified *Drosophila* PHGPx protein into rabbits. Immunisation was followed standard procedures with one injection using complete Freund's adjuvant followed by three booster injections four weeks later with an incomplete Freund's adjuvant into rabbits. About 10 µg of the purified proteins were used for each injection. Finally the antiserum was recovered two weeks after the 4th injection.

2.2.6.5.2 Preparation of clot and antibodies

Rabbit blood was collected in 2% sodium citrate water solution to prevent clotting. Red blood cells were washed a few times with 2% sodium citrate solution and following by insect ringer, finally suspended with insect ringer, and kept at 4°C until used. Fifty individuals of the final instar of *G. mellonella* larvae were surface sterilised with 70% ethanol. A pair of prolegs were cut off with micro-scissors, the larvae gently squeezed and bled into a drop of insect ringer on a piece of Parafilm 'M' on ice. After all larvae were bled and aliquots of the cell-free hemolymph pooled, rabbit red blood cells were added and incubated at room temperature for 30 min with gentle shaking. Afterwards, the sample was washed six times with insect ringer to remove unbound hemolymph proteins. The clot was broken up in fragments, mixed well with adjuvant and used for immunisation. Immunisation was performed according to standard procedures with one injection using complete Freund's adjuvant followed by another two injections 2 weeks later with an incomplete Freund's adjuvant into the same rabbit.

2.2.6.6 Enzymatic assay

In order to test the proteins for enzymatic activity, a coupled assay of glutathione peroxidase with glutathione reductase was used as described by Flohe and Gunzler (1984) with slight modifications. Briefly, in a semi-micro cuvette, 500 µl of 0.1 M potassium phosphate buffer (pH 7.0), 0.1 mM EDTA; 100 µl protein samples; 100 µl glutathione reductase (final 0.24 U) in potassium phosphate buffer and 100 µl of 10 mM GSH solution in water were added and then the mixture was pre-incubated at 37°C for 10 minutes. Afterwards, 100 µl of 1.5 mM NADPH in 0.1% $NaHCO_3$ was added and the substrate-independent consumption of NADPH was monitored for about 3 min. The reaction was started by adding100 µl of pre-warmed (37°C) substrate solution and the decrease in absorption at 340 nm monitored for about 7 min. Three substrates for PHGPx were used in the experiment: 1.5 mM hydrogen peroxide, 12mM

t-butyl hydroperoxide and 12 mM cumene hydroperoxide. Hydrogen peroxide and t-butyl hydroperoxide were diluted in water while cumene hydroperoxide was diluted in ethanol. The following enzyme samples were used: glutathione peroxidase from bovine erythrocytes (as a positive control); 0.1 μg/μl purified recombinant fusionproteins of *D. melanogaster* PHGPx (DmPHGPx) and VLP1 PHGPx domain (VcPHGPxd).

2.2.7 Constructing and screening recombinant cDNA expression libraries

For hemocytes expression library, 600 larvae were bled and hemocytes were collected, frozen with liquid nitrogen and stored at -20°C. For the whole larva library, 500 mg *Galleria* larvae (2 larvae) were frozen in liquid nitrogen, homogenised into powder and used for RNA extraction immediately. Total RNA was isolated using RNAeasy kit (QIAGEN). From this preparation, mRNA was purified using the PolyAtract mRNA isolation system (Promega). About 5 μg mRNA was used to construct a cDNA expression library using the λZAP vector according to the manufacturer's instructions (Stratagene). The library yielded $2x10^8$ independent clones for the hemocyte/fat body specific library and $5x10^9$ clones for larval library.

The expression libraries were screened using anti-clot antiserum with 1:5000 dilution according to the method described previously (Theopold *et al.,* 1994). Positive clones were purified to homogeneity and subcloned by *in vivo* excision of pBluescript phagemids according to the manufacturer's manual (Stratagene).

2.2.8 Immunocytochemistry

2.2.8.1 Immunofluorescence labelling of hemocytes

Hemocytes were collected from final instar larvae in PBS, added on a glass slide and allowed to attach for 10 min or the indicated times. To induce microparticle formation calcium (1 mM $CaCl_2$) was added to PBS or was present in PBS during collection. In

hemolymph preparations the bleeding was performed directly into a drop of PBS containing calcium and allowed to react for 10 min. Incubation with the antiserum (1:100 if not stated otherwise) was performed for 1 h. Hemocytes were then washed four times in PBS. Afterwards FITC-conjugated secondary antibody (Goat anti-rabbit IgG; Sigma Chemical Co., St Louis, MI, USA) was added. The secondary antibody (1:100 with 0.05% Tween 20 in PBS) was applied for one 1 h and followed by four washes as above. The samples were inspected under fluorescent microscope. Control samples were prepared as described above except that the corresponding preserum and secondary antibody was applied only.

2.2.8.2 Immunofluorescence labelling of clots and ovaries

Clots were prepared as above (2.2.6.5.2) and ovaries were dissected in PBS. The clot or the ovaries were fixed in 4% formaldehyde (PFA) in PBS followed by four washes with PBS + 0.05% Tween 20. Incubation with the first antiserum (1:1000 in PBS + 0.5% Tween 20 for anti-clot antibodies and 1:100 for others) was performed for 8 h. The ovaries were then washed four times in PBS + 0.05% Tween 20. Afterwards FITC or TRITC-conjugated secondary antibody (Goat anti-rabbit IgG; Sigma Chemical Co., St Louis, MI, USA) was added. The secondary antibody (1:100 with 0.5% Tween 20 in PBS) was applied for 8 h and followed by four washes as above. The samples were inspected under a confocal microscope (Bio-Rad MRC-1000 UV confocal system employing a Nikon diaphot 300 inverted microscope in fluorescence mode with filters for excitation at 488/10 nm and for emission at 522/32 nm for FITC, and 568/10 nm and 605/35 for TRITC). Control samples were prepared as described above except that the corresponding preserum and second antibody was applied only.

For lectin staining, FITC or TRITC-conjugated lectins were applied to the ovary and the clot samples after the fixation.

Chapter 3: Results

3.1 Genetic analysis of two *Venturia canescens* strains that differ in the VLP1 gene

In the endoparasitoid wasp *V. canescens*, virus-like particles, produced in the calyx glands of the female ovaries, become attached to the egg surface where they protect the eggs against the host immune system (Feddersen *et al.*, 1986). Previous studies have shown that a host protein share antigenic similarities with VLPs, and a passive protection role based on molecular mimicry was suggested (Rotheram, 1967; Schmidt and Schuchmann-Feddersen, 1989; Schmidt and Theopold, 1990).

Molecular characterisation of VLP1 coding DNA revealed the existence of two allelic genes. The allelic genes differ in the presence or absence of a tandem repeat sequence of 54 bp (Beck *et al.*, 2001). Since both allelic genes retain an open reading frame in the VLP1 coding region, two distinct proteins are produced in the two strains, which can be used to distinguish individuals from the two strains. Accordingly two allelic strains were established from the laboratory asexual stocks. Further analysis of the two strains demonstrated a number of distinctive differences, including differences in ovarian morphology and egg deposition behavior (Beck *et al.*, 2001). The questions are whether the VLP1 difference is the cause of the phenotypic differences and whether other genes are involved in producing these differences. One approach is to examine the correspondence between the VLP1 genotype and the phenotypic differences for wasps from other locations. Of particular interest is the correspondence for a sexually reproducing strain. Since the genome is fixed in the asexual strains, the observed phenotypic differences between the two laboratory strains could be due to the chance fixation of unlinked genes. However, if this is the case then there should be no correlation between the VLP1 genotype and the phenotypic differences in a sexually reproducing population. Therefore, more strains were analysed in order to answer these questions.

To test the genetic contribution to the phenotypes, sexual and asexual wasps were obtained from the field in Mont Boron, southern France by Dr Leo Beukeboom and Ms Maria Vicki Schneider. Asexual wasps were also collected from the field in Mildura, South Australia. All the three field populations were found to have both allelic genotypes when tested with VLP1 DNA as a diagnostic marker. With these strains and the available diagnostic tools, it is possible to address the questions of whether the two genotypes in the asexual and sexual field populations differ phenotypically and whether the VLP1 gene is genetically linked to the phenotypic differences.

3.1.1 Establishment of allelic *Venturia canescens* strains

Two genetically different laboratory strains were established by Beck *et al.* (1999). Molecular analysis of the VLP1 gene in the sexual and asexual wasps from the field showed both the RP and RM alleles were present in all populations (Fig. 3). In the sexual population, individual male wasps were all homozygous or hemizygous for one of the two VLP1 genes while individual females were variously homozygous or heterozygous for VLP1 genes (Fig. 3, RP/RM). Interestingly, no heterozygous asexual wild types were found. That is, the individual asexual wasps from the field were all homozygous for one of the two VLP1 genes.

All these wasps, corresponding to the laboratory RP and RM genotypes in the VLP1 locus, were established in the laboratory. As described above, asexual females were collected from two locations near Mildura, South Australia. They were named RP(aM) and RM(aM), for asexual Mildura. However, no sexual strain was recovered from Australia. In contrast, both sexual and asexual individuals were recovered from the one location in southern France (Malmberg *et al.*, 2000). These strains were named RP(aB) and RM(aB) for *a*sexual Mont Boron, and RP(sB) and RM(sB) for *s*exual Mont Boron. Females heterozygous for the RM and RP genes were obtained as the F_1 offspring of a cross between one homozygous RM and one homozygous RP parent from the sexual Mont Boron lines and were named RM/RP(sB).

Following the establishment of stable lines of homozygous RP and RM wasps. All the sexual and asexual individuals from one location were used for phenotypic comparison of homozygous RM and RP phenotypes in order to investigate the relationship between the allelic VLP1 genes and the phenotypic differences, and the possible contribution of the VLP1 gene.

3.1.2 Calyx gland phenotype and egg load

The two laboratory strains differ in ovarian morphology, mainly in the calyx tissue, where the VLP1 gene is expressed (Beck *et al.,* 1999). RM wasps from the laboratory strains frequently show defective calyx tissues, which can be detected in dissected ovaries under the light microscope, in antibody-stained tissues and in frozen sections (Beck *et al.,* 1999; Beck *et al.,* 2001). The main features of the RM phenotype in calyx tissues are necrotic calyx cells, reduced expression of calyx proteins, conspicuous membranes surrounding VLPs inside the calyx lumen and the presence of multiple eggs blocking the passage of eggs from ovarioles into the oviduct (Beck *et al.,* 2001). To investigate whether the phenotypes of RM and RP field collected sexual and asexual strains are similar to those from laboratory strains, ovaries from the two homozygous populations were dissected and the phenotypes examined. The expression of VLP1 proteins in the ovaries was analysed using anti-VLP1 antibodies. These results revealed staining patterns that are similar to those from the laboratory strains. For the asexual strains, staining of RM-ovaries is reduced compared to RP-ovaries (Fig. 4A), and the VLP1-staining is confined to the calyx due to the presence of membranes surrounding the secreted VLPs (Fig. 4B). In contrast, RP-ovaries have more VLP1-stained calyx fluid, which is free to shift inside the calyx and frequently pushed into the ovarioles during dissection (Fig. 4A, arrows). Likewise, staining in the ovaries from homozygous sexual RP-females showed calyx fluid that was freely moving (Fig. 4C), whereas in the ovaries from sexual RM-females, staining of calyx fluid was restricted to calyx tissues (Fig. 4D). This indicates that the differences in VLP1 expression, observed in asexual laboratory strains, also exist in sexual field populations. Moreover, these differences correlate with the two VLP1 genotypes.

The presence of membranes inside the calyx lumen of RM-ovaries may inhibit eggs in their passage from ovarioles into the oviduct, reducing the number of eggs in the oviduct (Beck *et al.*, 2001). Moreover, the blockage of calyx tissue may slow down egg maturation in ovarioles, thereby reducing the total number of eggs. To test whether these phenotypic effects are also found in the two field-collected populations, newly emerged offspring from the RP(aB), RM(aB), RP(sB), RM(sB) and RM/RP(sB) lines were kept on honey-water without hosts for 6 days. Their ovaries were then dissected and the numbers of eggs inside the calyx tissues were determined. The data were analysed statistically using chi square test.

In the two laboratory strains, there are more eggs inside the calyx tissues while no or few eggs are found in the RP calyx tissues. Similarly in both field strains the RM-genotypes [Fig. 5, RM(aB), RM(sB)] had more eggs inside the calyx tissues compared to RP-genotypes [Fig. 5, RP(aB), RP(sB)]. This suggests that the phenotypic differences observed in the two lines of the asexual laboratory strains are also found in the two field-collected strains. To confirm the observed difference between the two genotypes, the two asexual lines from Australian field populations were isolated and calyx egg numbers compared with the field populations from southern France. The RP(aM) and RM(aM) calyx egg numbers are very similar to the RP and RM phenotypes, indicating that the phenotypic difference is based on the genotype rather than a local variation.

One of the predictions of the passage of eggs through calyx tissue being blocked is a variable number of eggs inside the oviduct. To examine a possible correlation of egg distribution across calyx and oviduct, the number of eggs inside the oviduct were counted. It was found that within strains the number of oviduct eggs in RP-genotypes [Fig. 6, RP(aB), RP(sB)] was higher than in RM-genotypes [Fig. 6, RM(aB), RM(sB)]. This could indicate that high egg numbers in the calyx correlate with low egg numbers in the oviduct. To test this assumption, the calyx egg numbers were compared to the corresponding oviduct egg numbers from all available ovaries. Indeed a negative

correlation (Fig. 7) was observed, with higher egg numbers in the calyx tissues correlated with lower egg numbers in the oviduct.

Surprisingly, when the mean numbers of eggs in the oviducts of the sexual and asexual strains were compared, the egg numbers in asexual RP-oviducts were significantly higher than those in sexual RP-oviducts. Likewise, egg numbers in asexual RM-oviducts were higher than those in sexual RM-oviducts (Fig. 6A, B). Furthermore, the calyx phenotype of the heterozygous RM/RP(sB) females was more similar to the RP calyx phenotype (Fig. 5), while the oviduct phenotype of the RM/RP(sB) wasps was more similar to the RM oviduct phenotype (Fig. 6). This could indicate that an unlinked gene interacts with the VLP1 gene and that allelic combinations of the two genes are fixed in the asexual strains to produce the observed phenotypes.

3.1.3 Reproductive success of sexual offspring

It has been suggested that the outcome of inter-larval competition in superparasitised caterpillars may be determined by the timing of egg deposition (Marris and Casperd, 1996). Previous observations of the asexual laboratory strain suggested that RM-females lay eggs over a longer period than RP-females. In the event of superparasitism it is thus more likely that RM eggs would be laid later, thus exploiting a possible window of opportunity inside superparasitised hosts, where younger larvae are able to kill older larvae. The relative reproductive success of the two strains is different when females from the two lines compete for the same hosts compared to a setting where females from each line are separated (Beck *et al.*, 1999; Beck *et al.*, 2001). To test whether the pattern of reproductive success is similar in females from the two homozygous sexual VLP1 allelic genotypes, competition experiments were conducted to examine the ratio of sexual RP and RM offspring under various egg deposition conditions.

When two mated females, one from each genotype, competed for the same hosts their relative success was different depending on the time available for egg deposition (Fig.

8). When females were allowed to deposit eggs for a limited period (24 h), the homozygous sexual RP females produced more offspring than sexual RM (Fig. 8A). When the two females were allowed to lay eggs for 96 h, sexual RM females were more successful (Fig. 8B). This is in agreement with similar findings for the asexual laboratory strains under inter-strain competition, where RP-females produce relative more offspring within 1 h of egg laying, compared to 3 h, where RM-females are more successful (Beck *et al.*, 2001). In contrast, when two sexual wasps from the same genotype were allowed to deposit eggs for 24 h or for 96 h, the relative number of sexual RP-offspring was equal or higher compared to the number of sexual RM-offspring (Fig. 8). However, the absolute number of offspring was significantly lower under the 96 h condition (Fig. 8). Since this was not correlated with more moths emerging from the superparasitised hosts, it implies parasitism failures in highly superparasitised caterpillars (see Chapter 4.1).

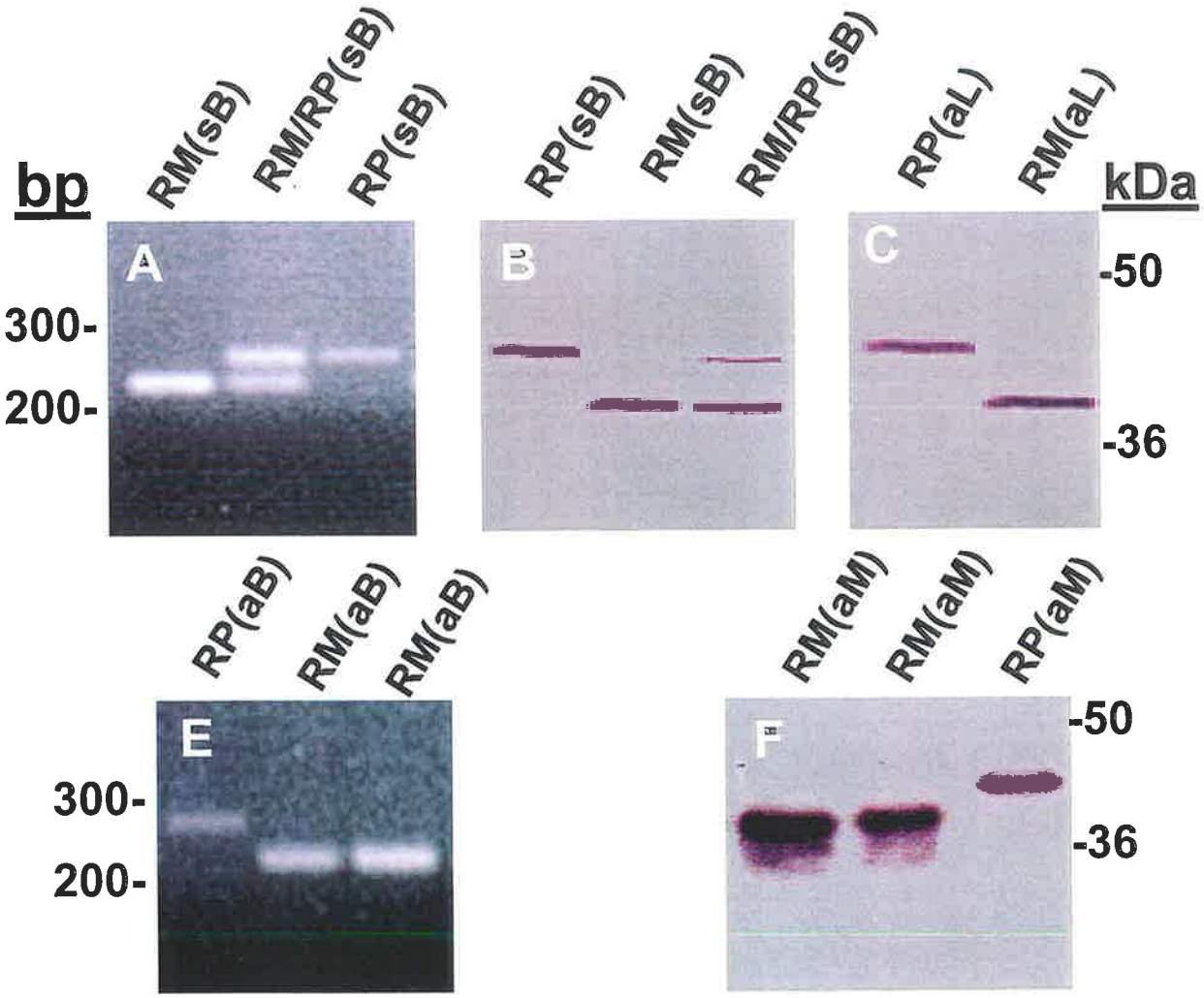


Fig. 3:

Molecular analysis of alleic genotypes at the VLP1 locus.

A) PCR analysis of genomic DNA from sexual strains originating in southern France, using primers flanking the site of the repeat sequence in the VLP1-coding region. Diagnostic amplified fragments from homozygous and heterozygous individuals are shown.

B) Western blot of protein extracts from sexual strains originating in southern France, using antibodies against recombinant VLP1 protein (Beck *et al.,* 1999). Diagnostic VLP1-protein bands from homozygous and heterozygous VLP1 alleles are shown.

C) Western blot of protein extracts from asexual wasps of the laboratory strain, using antibodies against recombinant VLP1 protein.

D) PCR analysis of asexual wasps from southern France.

E) Western blots of asexual wasps from South Australia.

Note the top labels are the names of the wasps from different locations and having different VLP1 alleles (See Chapter 2).

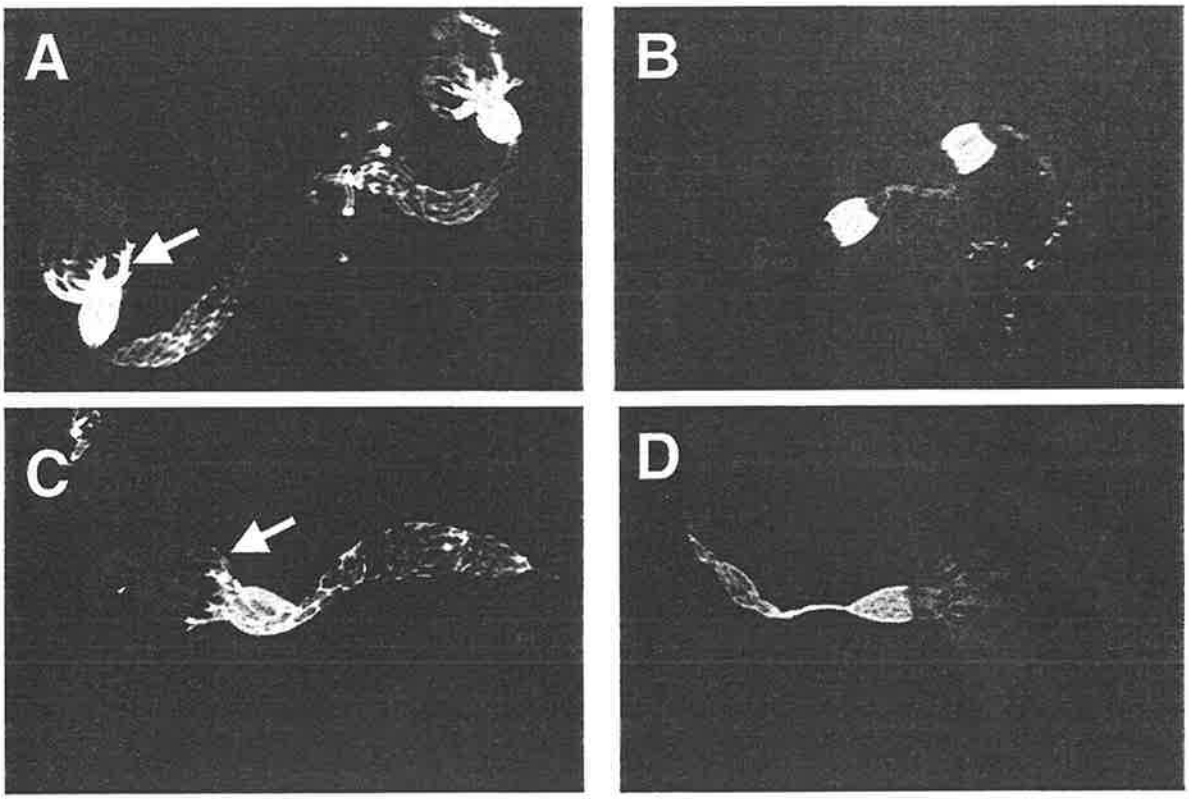


Fig. 4:

Antibody-stained ovaries from asexual and sexual strains. Whole mounts of ovaries from asexual laboratory strains and sexual field-collected strains incubated with anti-VLP antibodies, developed with FITC-conjugated secondary antibody and inspected using confocal microscopy. **A)** Ovaries from an RP(aL) female. **B)** Ovaries from an RM(aL) female. **C)** Ovaries from an RP(sB) female. **D)** Ovaries from an RM(sB) female. Note the staining of ovarioles in RP ovaries indicating free movement of VLPs in the calyx lumen (arrow).

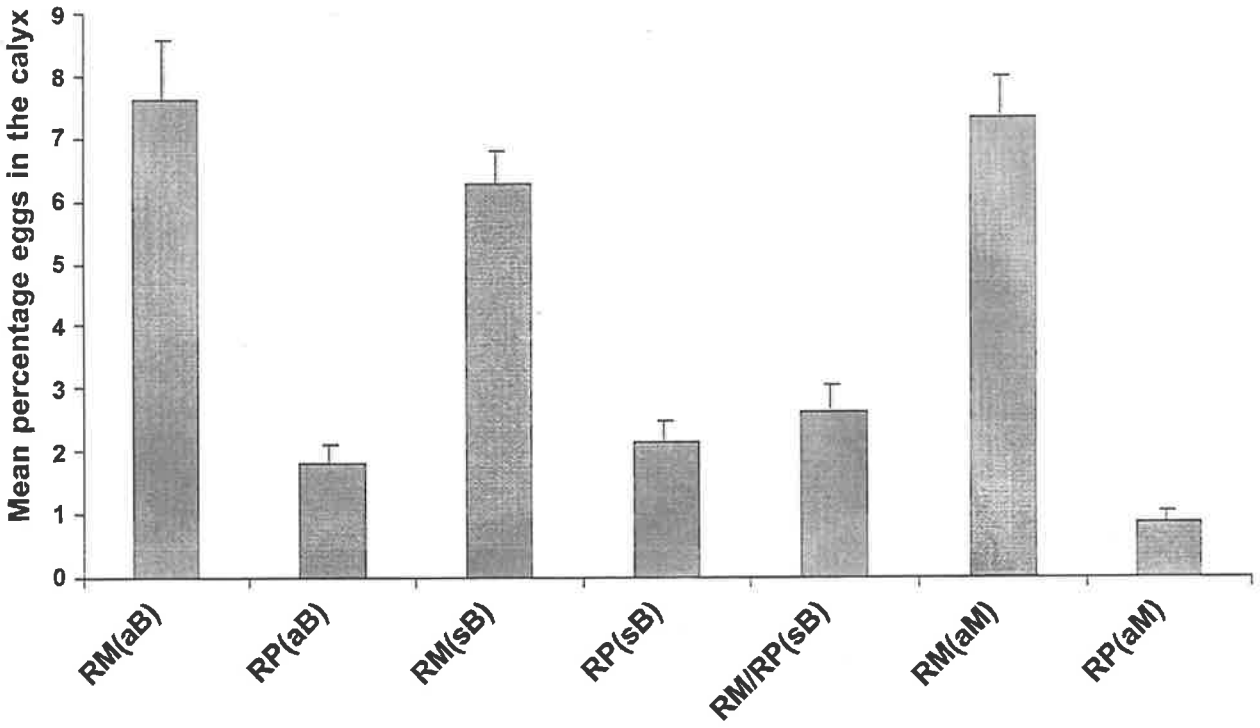


Fig.5:

Proportion of egg numbers in calyx tissue of field-collected sexual and asexual females.) Egg number found in calyx tissues as a percentage of the total egg load displayed on the Y-axis. Ovaries used in the experiments originate from: asexual females from Mont Boron (southern France) RP(aB) and RM(aB), from Mildura (South Australia) RP(aM) and RM(aM) and sexual homozygous and heterozygous females from Mont Boron RP(sB), RM(sB) and RP/RM(sB). Note that ovaries of heterozygous individuals resemble the RP phenotype. Error bars are indicated in the middle of each column.

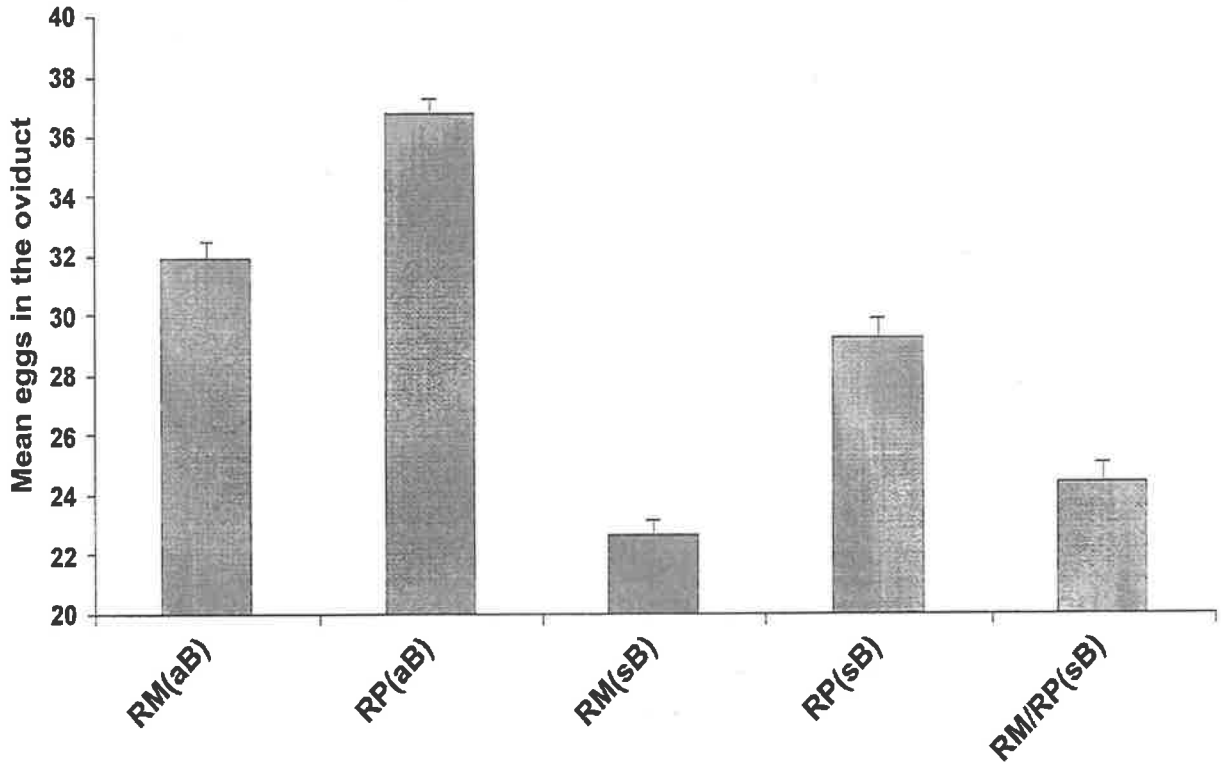


Fig. 6:

Proportion of egg in oviducts of sexual and asexual females from Mount Boron (southern France). Mean percentage of eggs found in oviducts are displayed on the Y-axis. Ovaries used in experiments were from asexual homozygous females from Mount Boron RP(aB) and RM(sB) and sexual homozygous and heterozygous females from Mount Boron RP(sB), RM(sB) and RP/RM(sB). Note that the sexual strains have less eggs compared to asexual strains, which indicating that additional genes may play a role. Error bars are indicated in the middle of each column.

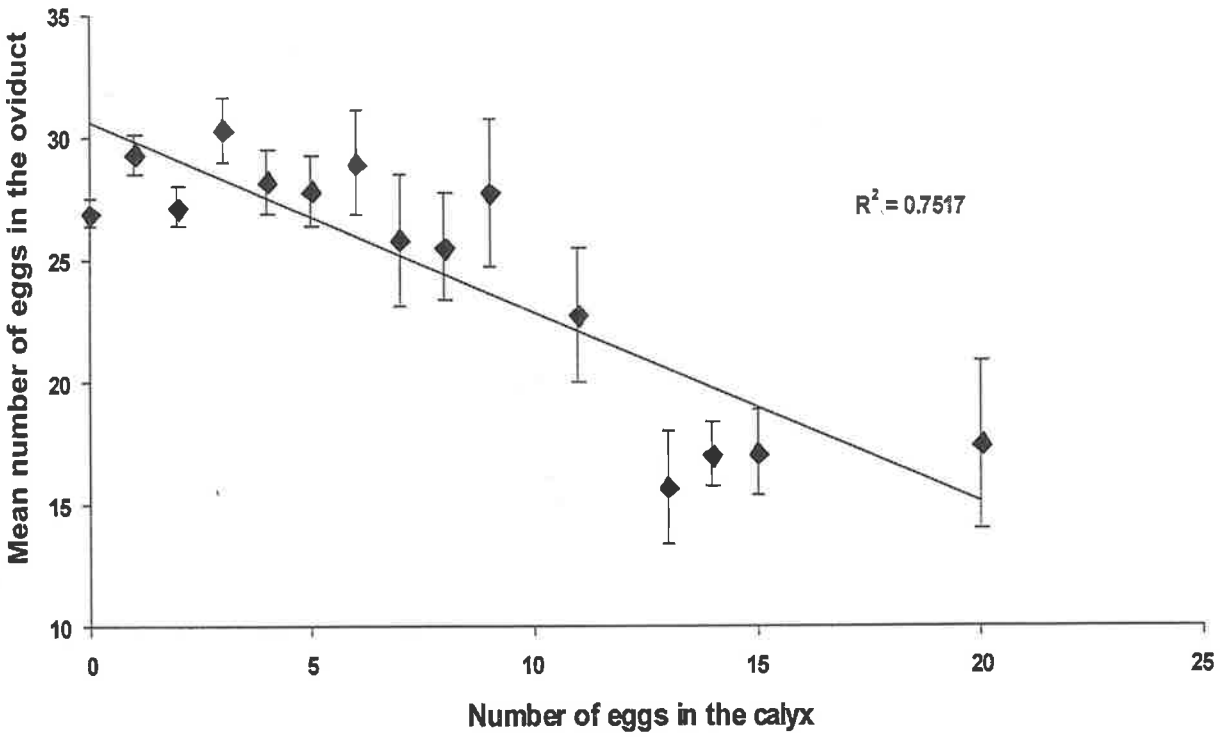


Fig. 7:

Regression between calyx morphology and egg load. Ovaries from the RM strains were grouped according to the number of eggs in calyx tissues. A negative linear relationship between egg numbers in calyx glands and in oviducts is demonstrated.

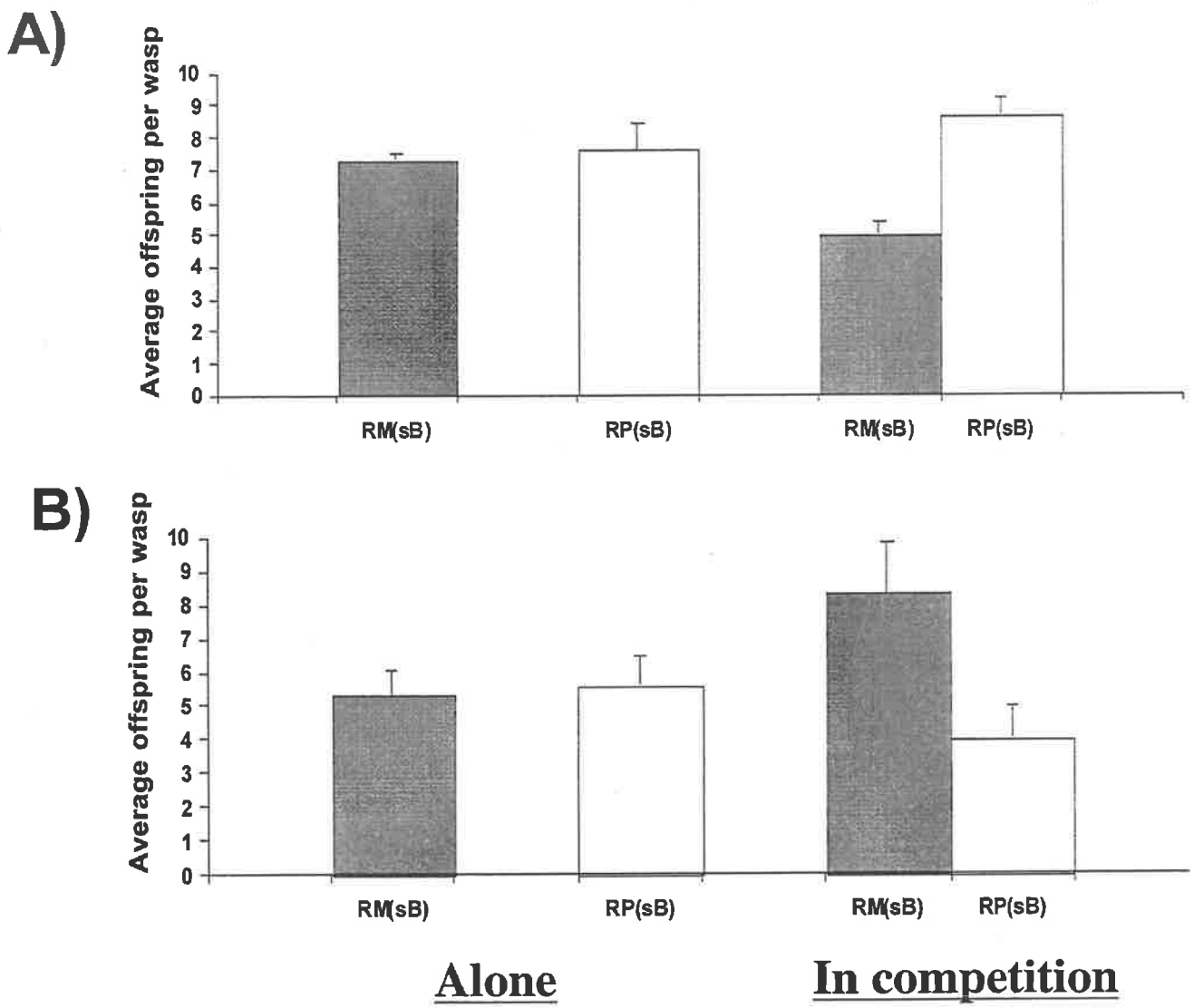


Fig. 8:

Reproductive success of asexual and sexual females under conditions where the two VLP1-genotypes compete inside host caterpillars. Two homozygous VLP1-females were allowed to parasitise for 24 hours **A)** or 96 hours **B).** Reproductive success was determined as a function of the average offspring per wasp. Error bars are indicated in the middle of each column.

In competition: Reproductive success of two wasps, one form each of the two homozygous VLP1-genotype, depositing eggs into the same hosts. Grey bars indicate average RM(sB) offspring per female and white bars are average RP(sB) offspring per female.

Alone: Reproductive success of two homozygous RP(sB) wasps depositing eggs into same hosts were compared with two homozygous RM(sB) wasps.

3.2 Egg surface protection of parasitoid wasp *Venturia canescens* against its host, *Ephestia kuehniella*

As mentioned above, one of the VLP proteins, VLP1, was sequenced (Hellers *et al.*, 1996) and its carboxyl terminal sequence show similarities with phospholipid hydroperoxide glutathione peroxidase (PHGPx). PHGPx is an anti-oxidant enzyme that can reduce phospholipid hydroperoxide on the membrane (Thomas *et al.*, 1990). The question is whether the VLP1 derived PHGPx domain has peroxidase activity. To answer this question, the enzymatic function of VLP1 was analysed.

3.2.1 Functional studies of virus-like particles

3.2.1.1 Sequence comparison and analysis of PHGPx genes

To identify genes that are related to the *Venturia* PHGPx domain (VcPHGPxd), the protein sequence was compared to other PHGPx protein sequences on the NCBI and BDGP servers. Similarities were detected with several vertebrate members of the PHGPx family, PHGPx-proteins from plants, nematode and a predicted PHGPx-gene from the *Drosophila* genome GenBank (Fig. 9). Most vertebrate members show a stop codon within the coding region, which in this case codes for a conserved selenocysteine at the active site (Maiorino *et al.*, 1995), while all plant, *Drosophila* and prokaryotic members display a canonical cys-codon in that position (Fig. 9, arrow). This indicates that incorporation of selenocysteine into the protein backbone is a conserved feature of most vertebrate genes (Kollmus *et al.*, 1996), whereas most of the plant and invertebrate members code for a cysteine at this position. Therefore, the apparent enzymatic activities for the corresponding gene products are probably based on the incorporation of selenium or other heavy metal ions into the protein backbone, by different mechanisms.

Since the predicted VcPHGPxd protein sequence has no cysteine at the active site, the question is whether VLP1 is enzymatically active. To investigate this, the VLP1 protein sequence was compared with *Drosophila* EST protein sequences. Amongst the *Drosophila* GPxs, the predicted protein product from CG12013 showed the highest similarity to VcPHGPxd (Fig. 10) and was therefore analysed further. In order to confirm the predicted sequence, the CG12013 fragment in the pOT_2 vector was fully sequenced. The complete DmPHGPx cDNA is 777 bp in length, with a 509 bp ORF encoding a 169 amino acid PHGPx-like protein. The cDNA contains a 65 bp 5'-UTR, a 207 bp 3'-UTR, an AATAAA polyadenylation signal and a poly A tail in the 3'-UTR (Fig. 11). Three conserved regions are present in the DmPHGPx amino acid sequences (Fig. 11, underlined) as well as the three catalytic residues (Fig. 11, embossed).

3.2.1.2 Expression and purification of PHGPx genes

To compare possible enzymatic activities between *Drosophila* PHGPx and VLP1, recombinant DmPHGPx protein was produced in a bacterial expression system. Using the pQE30 expression vector, pQEDmPHGPx plasmid was produced. Afterwards, M15 component cells were transformed with pQEDmPHGPx plasmid and protein was induced with 0.5mM IPTG. The pQEDmPHGPx-transformed cells were found to produce significant amounts of recombinant DmPHGPx protein (Fig. 12A, In). The resulting 24 kDa protein was mainly detected in the *E. coli* supernatant fraction (Fig. 12B, Sol). The recombinant native protein was purified with a Ni-resin column and eluted with 100 mM-250 mM imidazole (Fig. 12C). Imidazole (150 mM) gave a pure elution of DmPHGPx recombinant protein, thus these fractions were used for immunisation and enzymatic assays.

3.2.1.3 PHGPx expression in *Drosophila* and *Venturia*

PHGPx is a major protein in the testes of animals where it is involved in spermatogenesis as well as having a general antioxidant function (Nam *et al.*, 1998). Antibodies were produced by injecting the purified DmPHGPx protein into rabbits. In

order to investigate DmPHGPx expression in *D. melanogaster,* protein extracts from female and male flies were tested with antibodies against DmPHGPx. The results showed that higher amounts of the PHGPx protein were present in males than in females (Fig. 13A, B). To further confirm the gene expression, RNAs from females and males were extracted and Northern blot hybridisation was performed using DmPHGPx cDNA as a probe. *D. melanogaster* ribosomal protein RP49 cDNA fragment was used as an internal loading control. From the Northern blots with RP49 probe, it turned out that higher amount of RNA was loaded in female flies than in male flies (Fig. 13E). However, stronger signal was observed in the males with DmPHGPx cDNA probe than in females (Fig. 13D). Therefore, strong expression of PHGPx in males was concluded (Fig. 13C, D, E), which was consistent with the Western blot results. The results indicate that DmPHGPx might have a male specific function in insects similar to that in rats and humans (Imai *et al.,* 1995; Imai *et al.,* 2001). A more detailed analysis of PHGPx expression in the reproductive tract of *Drosophila* males revealed the protein was most highly expressed in the testes (Fig. 14A, B).

Since VLP1 has previously been shown expressed in the ovaries (Beck *et al.,* 1999), *Drosophila* ovaries were analysed for PHGPx expression. A signal was found in follicle cells and on the membranes of nurse cells (Fig. 14D). Lectin-binding to secreted O-glycosylated glycoproteins was used as an internal standard to compare the relative expression in reproductive tissues (Fig. 14C, E). In *Drosophila* male reproductive tissue, expression of PHGPx proteins was detected in the testicular duct (Fig. 14F, td), whereas no expression was observed in other tissues including the accessory gland (Fig. 14F, ag). Although VcPHGPxd is mainly expressed in the calyx tissues of *V. canescens* ovaries, some weak staining is also detected in ovarioles (Beck *et al.,* 1999). To test whether staining in ovarioles is due to cross-reactivity to other PHGPx proteins, protein extracts from *Venturia* ovaries were tested with the *Drosophila*-specific anti-PHGPx antiserum. In this assay the antiserum against DmPHGPx cross-reacted with VLP1 (Fig. 15, arrow) and with additional bands in *Venturia* ovaries (Fig. 15). This result indicated that antibodies against VcPHGPx reacted predominantly with VLP1, whereas antibodies against DmPHGPx cross-

reacted with VLP1 and related wasp PHGPx-proteins. This further confirmed the close similarities between VLP1 and DmPHGPx proteins.

3.2.1.4 Tissue distribution of DmPHGPx

To investigate the tissue distribution of DmPHGPx, protein extracts from different developmental stages and different tissues were obtained and Western blots were conducted with anti-DmPHGPx antiserum. It was found that DmPHGPx expression was ubiquitous. It was detected in larval, pupal and adult stages (Fig. 16A) and may be enriched in a number of tissues (Fig. 16B). Although DmPHGPx was expressed in all tissues tested, a tissue-specific accumulation was observed in imaginal discs (Fig. 16B, Ds), a result which needs further investigation.

3.2.1.5 Enzymatic activity

To examine DmPHGPx and VcPHGPxd for possible enzymatic activities, coupled assays were carried out with DmPHGPx and VcPHGPxd recombinant proteins. Three substrates for PHGPx, hydrogen peroxide, t-butyl hydroperoxide and cumene hydroperoxide were used in the assays. A positive control using peroxide from bovine erythrocytes (positive) and a negative control with buffer only (negative) were included in addition to the purified native proteins, DmPHGPx and VcPHGPxd. Relative peroxide activities were calculated using the positive enzyme activity as 100%. The results (Fig. 17) showed that the DmPHGPx had low activity with all three substrates while VcPHGPxd shows no peroxide activity. In addition, DmPHGPx displayed significantly higher activity towards cumene hydroperoxide than the other substrates examined. In order to rule out protein structure changes during the purification procedure, the soluble fractions of *E. coli* expressing DmPHGPx and VcPHGPxd genes were used for testing peroxidase activities, resulting in similar relative activities (not shown). In conclusion, the PHGPx homologue (CG12013) in *D. melanogaster* has peroxidase activity, while the VLP1 PHGPx-domain does not. Therefore a non-enzymatic function of PHGPx in VLPs was proposed and explored further.

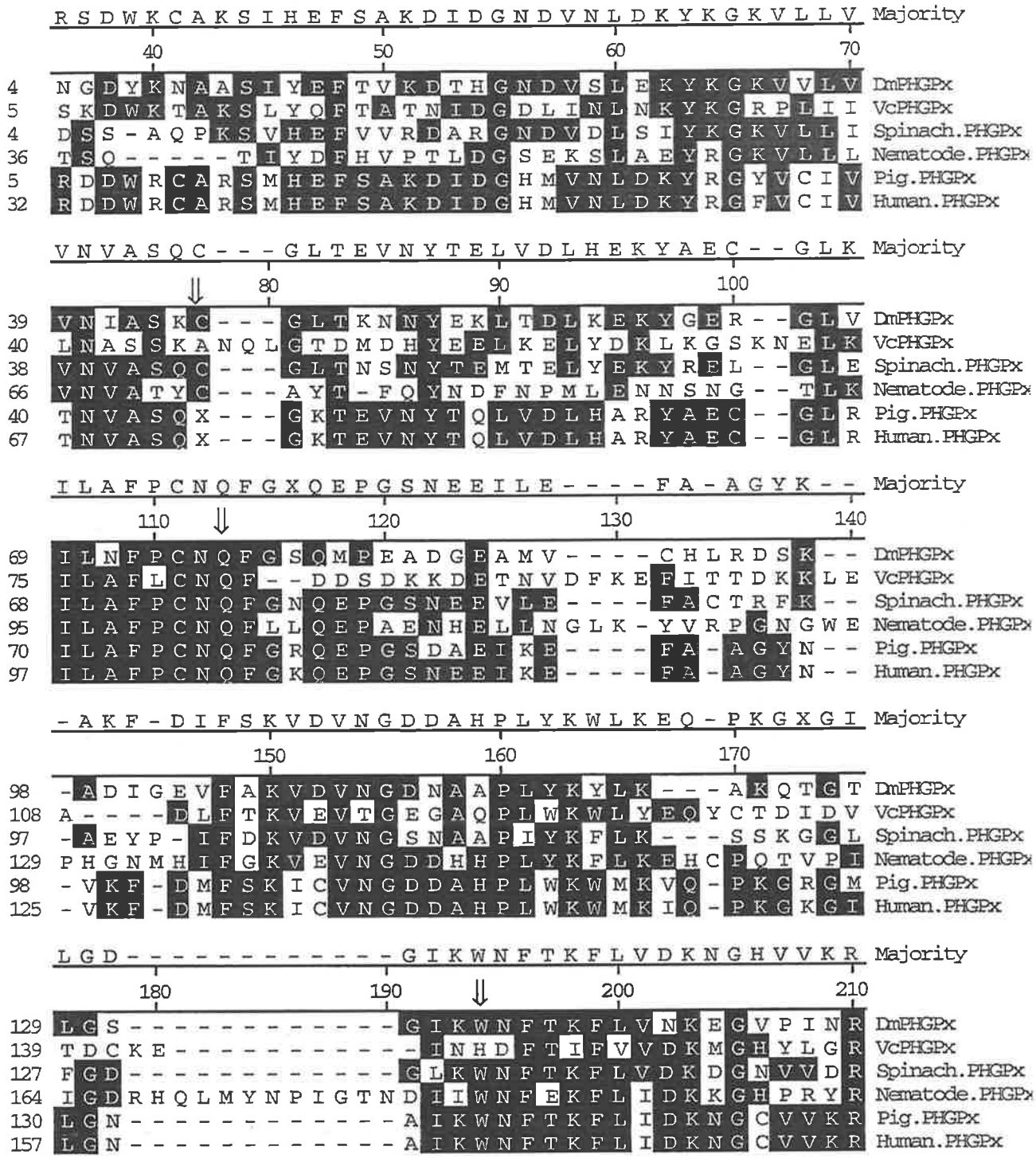


Fig. 9:

Amino acid sequence comparison of plant, insect and vertebrate PHGPx proteins. Catalytic residues are indicated by arrows. The genes were extracted from GenBank and their accession numbers are: VcPHGPx, *V. canescens* VLP1 PHGPx domain, AAK09374; DmPHGPx, *D. melanogaster* PHGPx annotated gene CG12013, AAF47761; Spinach.PHGPx, *Spinacia oleracea* PHGPx, JC5619; Nematode.PHGPx, *Dirofilaria immitis* PHGPx, P52033; Pig.PHGPx, *Sus scrofa* PHGPx, P36968; Human.PHGPx, *Homo sapiens* testis PHGPx, CAA50793.

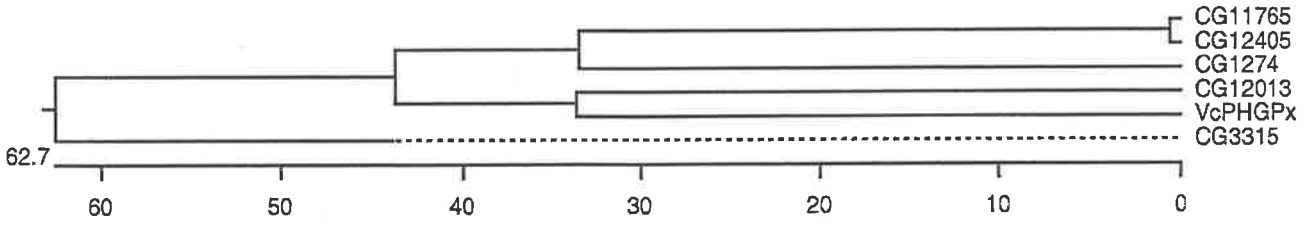


Fig. 10:

Phylogenetic comparison of VLP1 PHGPx domain with GPx homologues from *Drosophila melanogaster*. Phylogenetic comparison was constructed by using the ClustalW method with PAM250 residue weight table. Annotation numbers are used in the tree. The genes were obtained from GenBank and their accession numbers are as follows: VcPHGPx, AAK09374; CG12013, AAF47761; CG11765, AAF58779; CG12405, AAF58797; CG1274, AAF47704; CG3315, AAF46018.

```
                                                                    
   CGGCACGAGCGAAAGTGAGAAAGTGCCCAGTTCCATTAGTCATTGCTATTACG          53

            M  S  A  N  G  D  Y  K  N  A  A  S  I  Y  E           15
CCATAAATCGACATGTCTGCTAACGGAGATTACAAGAACGCCGCCTCGATCTACGAG       110

F  T  V  K  D  T  H  G  N  D  V  S  L  E  K  Y  K  G  K          34
TTCACCGTGAAGGATACCCATGGCAACGATGTTTCCCTGGAAAAGTACAAGGGCAAG       167

V  V  L  V  V  N  I  A  S  K  C  G  L  T  K  N  N  Y  E          52
GTGGTCCTGGTGGTGAACATCGCCTCCAAGTGCGGCCTGACCAAGAACAACTACGAG       224

K  L  T  D  L  K  E  K  Y  G  E  R  G  L  V  I  L  N  F          71
AAGCTGACGGATCTAAAGGAGAAGTACGGCGAGCGCGGCCTGGTGATCCTCAACTTC       281

P  C  N  Q  F  G  S  Q  M  P  E  A  D  G  E  A  M  V  C          90
CCGTGCAATCAGTTTGGGTCCCAGATGCCGGAGGCCGATGGAGAGGCCATGGTGTGC       338

H  L  R  D  S  K  A  D  I  G  E  V  F  A  K  V  D  V  N         109
CACCTGCGCGACTCCAAGGCTGACATCGGCGAGGTGTTCGCCAAGGTCGATGTGAAT       395

G  D  N  A  A  P  L  Y  K  Y  L  K  A  K  Q  T  G  T  L         128
GGAGACAATGCGGCGCCGCTGTACAAATACCTAAAGGCCAAGCAGACCGGCACCCTG       452

G  S  G  I  K  W  N  F  T  K  F  L  V  N  K  E  G  V  P         147
GGCAGCGGAATCAAGTGGAACTTCACCAAGTTTCTGGTGAACAAGGAGGGCGTGCCC       509

I  N  R  Y  A  P  T  T  D  P  M  D  I  A  K  D  I  E  K         166
ATCAACCGATATGCCCCGACCACCGATCCCATGGACATCGCCAAGGACATTGAAAAG       566

L  L  *
CTGCTGTAGATGTGCCCTAGACGTTAGCTGCTCTTTTGGACTGTGTTTTCGCGTAGC       623
TTTAGCTTGTAACCGTCTGGCCACAGATAGTCTTGTGGCCGGCCACATGCACCGCAT       680
GGTATATATATTGTCTACTCCTTACTGGTTTTGATTCCCACCCGCAAACATaataaa       737
CGCCATTCGTGGTTACCACACTaaaaaaaaaaaaaaaa                          777
```

Fig. 11:

cDNA and deduced amino acid sequences of DmPHGPx. Amino acid residues, shown in one-letter code, are aligned with the first nucleotide of each codon. The start codon is in bold and the stop codon is marked by an asterisk (*). The three conserved region are underlined and the catalytic residues are embossed.

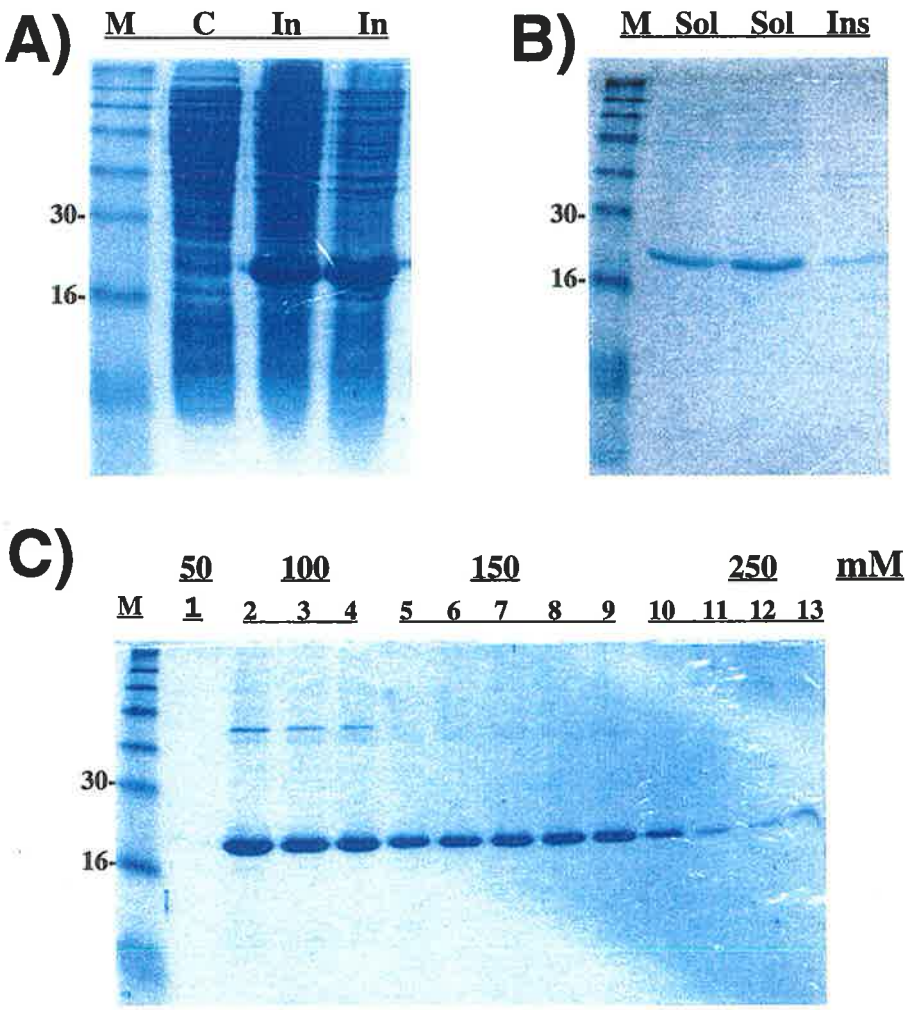


Fig. 12:

Expression of DmPHGPx recombinant protein

A) Induction and solubility of DmPHGPx protein. IPTG was added to the *E coli* cell cultures containing pQE30DmPHGPx plasmid. A protein about 24 kDa was induced (In) which was not present in the non-induced cells (C).

B) The induced cell pellet was sonicated, the protein was mainly existing in the soluble part (Sol) in comparison to the precipitates (Ins).

C) Purification of the fusionprotein DmPHGPx overexpressed in the construct pQE30. The fusion protein was purified under native conditions according to the manufacturer's instructions. Different concentrations of imidazole were applied to elute the DmPHGPx recombinant proteins. 10 µl aliquot form each 500 µ l imidazole elution fractions were separated by 15% SDS-PAGE and stained with Coomassie blue.

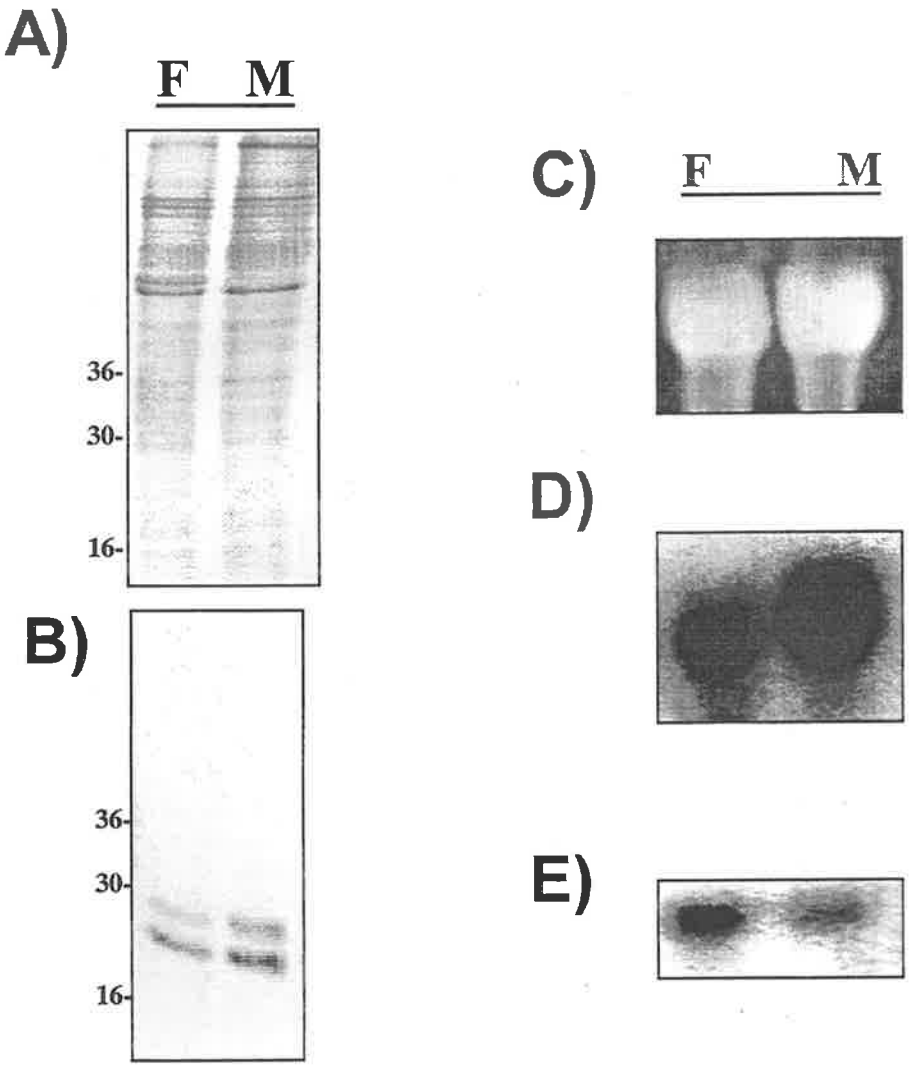


Fig. 13:

Expression of PHGPx proteins and transcripts in *Drosophila* tissues.

A) whole protein expression patterns. Coomassie blue staining of separated protein extracts from female and male flies.

B) Western blots of separated protein extracts probed with anti-DmPHGPx antibodies. Two distinct protein bands were labelled in both males and females.

Northern blots of total RNA from female (F) and male (M) flies.

C) Total RNA after separation on a 1.0% agarose gel under denaturing conditions.

D) Hybridisation on a Northern blot using the DmPHGPx (CG12013) cDNA as a probe. More PHGPx transcripts are found in male than in female flies.

E) Hybridisation of the same blot using ribosomal protein 49 (Rp49) as an internal loading control. Note that larger amount transcript was detected in male flies in spite of less loading of RNA in the male flies

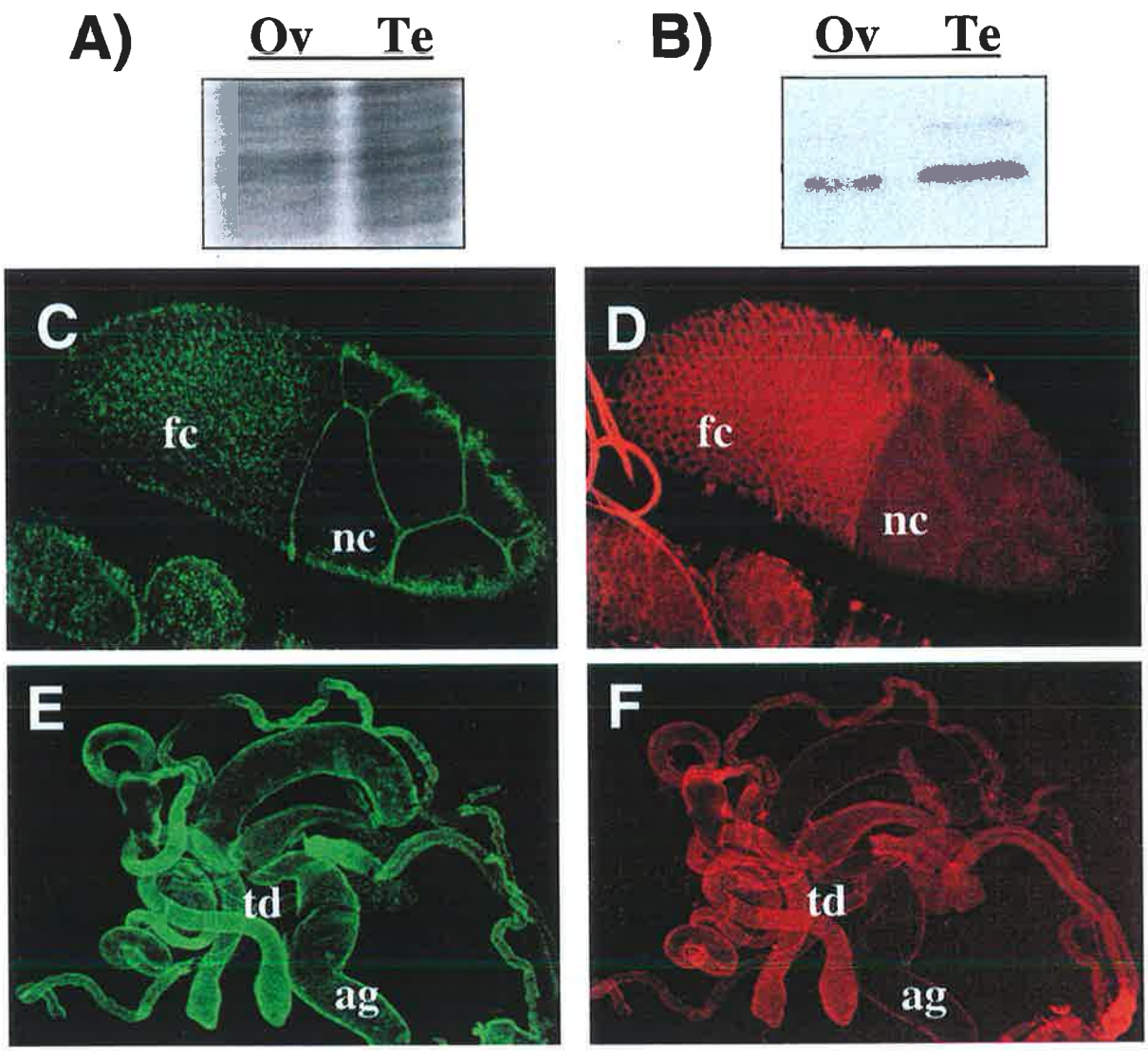


Fig. 14:

PHGPx expression in *Drosophila* female and male reproductive tracts.

A) Coomassie blue staining of protein extracts from *Drosophila* ovaries and testes.

B) Western blots stained with anti-DmPHGPx antibodies, showing larger expression of DmPHGPx in testes than in ovaries.

C) Confocal pictures of a *D. melanogaster* ovariole incubated with FITC-conjugated PNA, as an internal control to confirm intracellular staining of glycoproteins.

D) Confocal pictures of a *D. melanogaster* ovariole with anti-DmPHGPx antibodies.

E) *D. melanogaster* male reproductive tract incubated with HPL as an internal control.

F) *D. melanogaster* male reproductive tract incubated with anti-DmPHGPx antibodies.

nc, nurse cells; fc, follicle cells; td, testicular duct; ag, accessory gland.

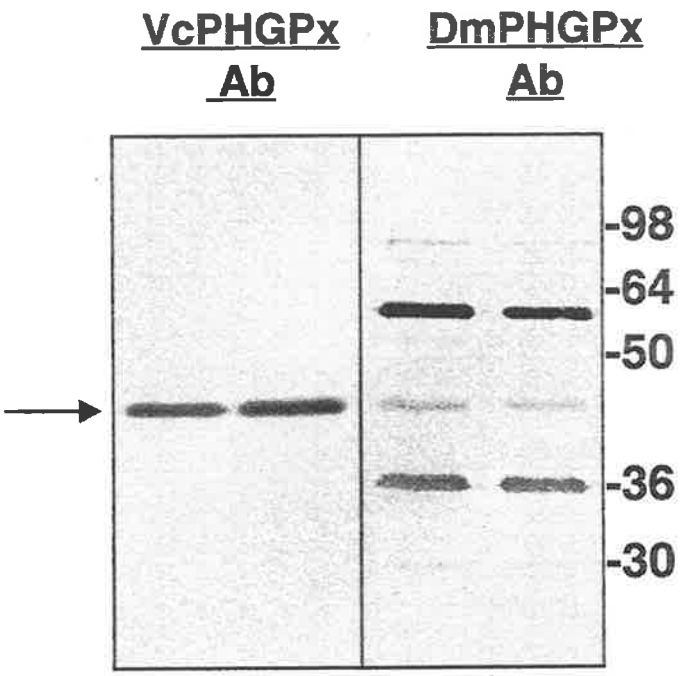


Fig 15:

Binding of anti-PHGPx antibodies to protein extracts from ovaries of *Venturia canescens*: Western blots of protein extracts of *V. canescens* ovaries using anti-VcPHGPxd (left) and anti-DmPHGPx (right) antibodies. Whereas, anti-VcPHGPxd (VLP1) antibodies only recognize VLP1, anti-DmPHGPx antibodies recognize VLP1 and additional bands in *V. canescens* ovaries.

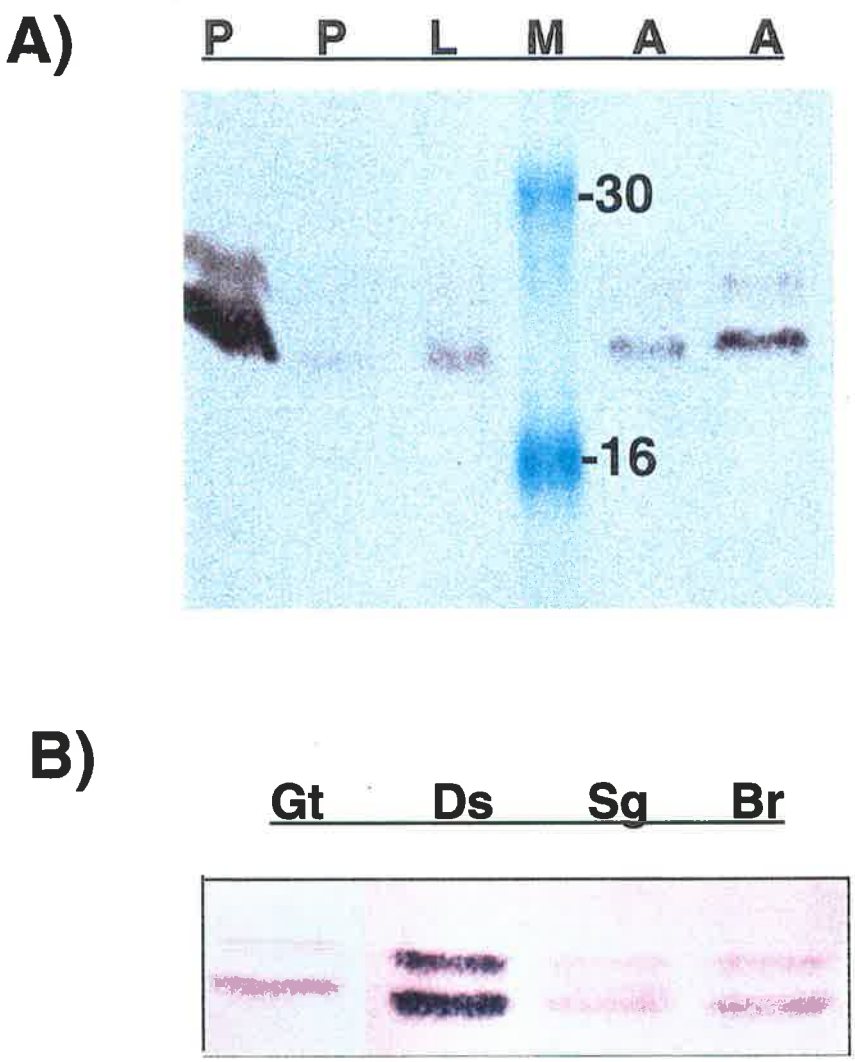


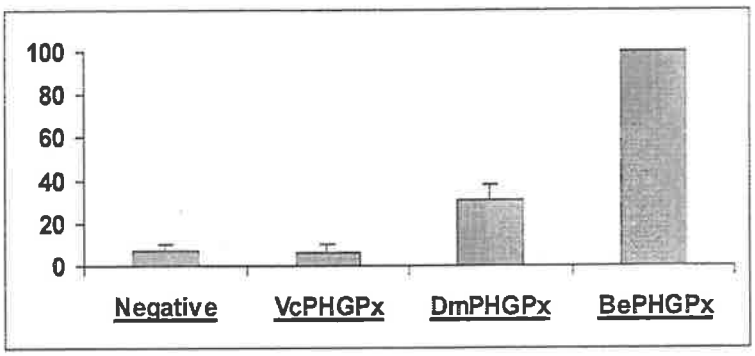
Fig. 16:

Western blot analysis of Drosophila tissues against anti-DmPHGPx antibodies.

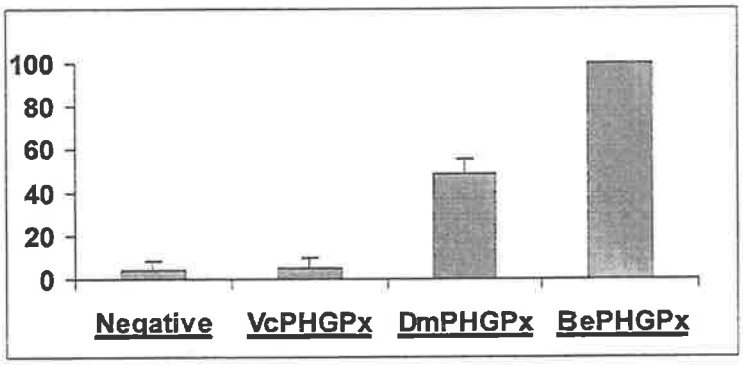
A) Western blots of protein extracts from different developmental stages of *Drosophila* against anti-DmPHGPx antibodies. P, pupal; L, larval; A, adult and M, molecular weight marker in kDa.

B) Western blots of protein extracts from different tissues of *D. melanogaster* larvae with anti-DmPHGPx antibodies. Gt, gut; Ds, imaginal disc; Sg, salivary glands; Br, Brain.

A)



B)



C)

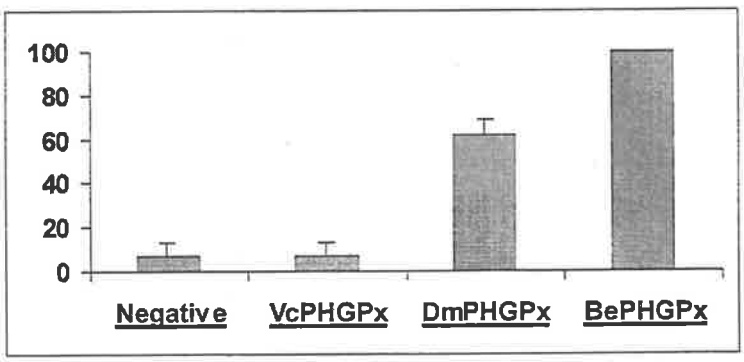


Fig. 17:

Enzymatic assay of native recombinant DmPHGPx and VcPHGPxd. Coupled assays were conducted with glutathione reductase and NADPH. Three substrates were employed in the assays. **A)** Hydrogen peroxide; **B)** t-butyl hydroperoxide; **C)** Cumene hydroperoxide.

3.2.1.6 Calyx tissues of allelic VLP1 strains

The VLP1 gene exists in two allelic forms in both laboratory and field populations (Hellers *et al.,* 1996; Beck *et al.,* 1999; Beck *et al.,* 2001). Although the VcPHGPxd is not directly affected by a partial deletion of the tandem repeat sequence adjacent to the VcPHGPxd, the structural differences between the two VLP1 alleles have been correlated with morphological differences in calyx tissues (Beck *et al.,* 2001; Chapter 3.1). To investigate the observed morphological differences in the context of possible membrane alterations, confocal and electron-microscopic analysis were performed using calyx tissues from the two allelic strains. VLPs released into the calyx lumen acquire an additional membrane from calyx cells as a result of the budding process. However, the ultra-structural features of the bilayer disappear by an unknown process in particles inside the calyx lumen (Stoltz and Vinson, 1979a). Two possible mechanisms for this are membrane lipid assimilation by individual particles or the fusion of particle-membranes to form large vesicles (Fig. 18B). In the normal strain, no visible membranes were found either on individual particles or around groups of particles (Fig. 18C). When VLPs from the mutant strain were analysed, an electron-dense membrane was detected, surrounding VLPs inside the calyx lumen (Fig. 18A, D). At the budding-site, the membrane was less electron-dense, resembling the cellular membrane of the calyx cell. This indicates that membrane fusion occurs in the mutant strain resulting in an electron-dense structure. Therefore, a possible function of a PHGPx in egg surface protection could be to reduce modified lipids on the egg surface. However, such a role could only be performed in the presence of reduced glutathione, which is probably not available in sufficient amount outside the extracellular calyx fluid. Since VcPHGPx is highly expressed in the calyx and distributed differently in the two strains, an alternative VcPHGPx function was indicated. Thus these results indicated that VcPHGPx may still be able to interact with modified phospholipids on the membrane to potentially protect damaging lipids from the surface of the parasitoid eggs.

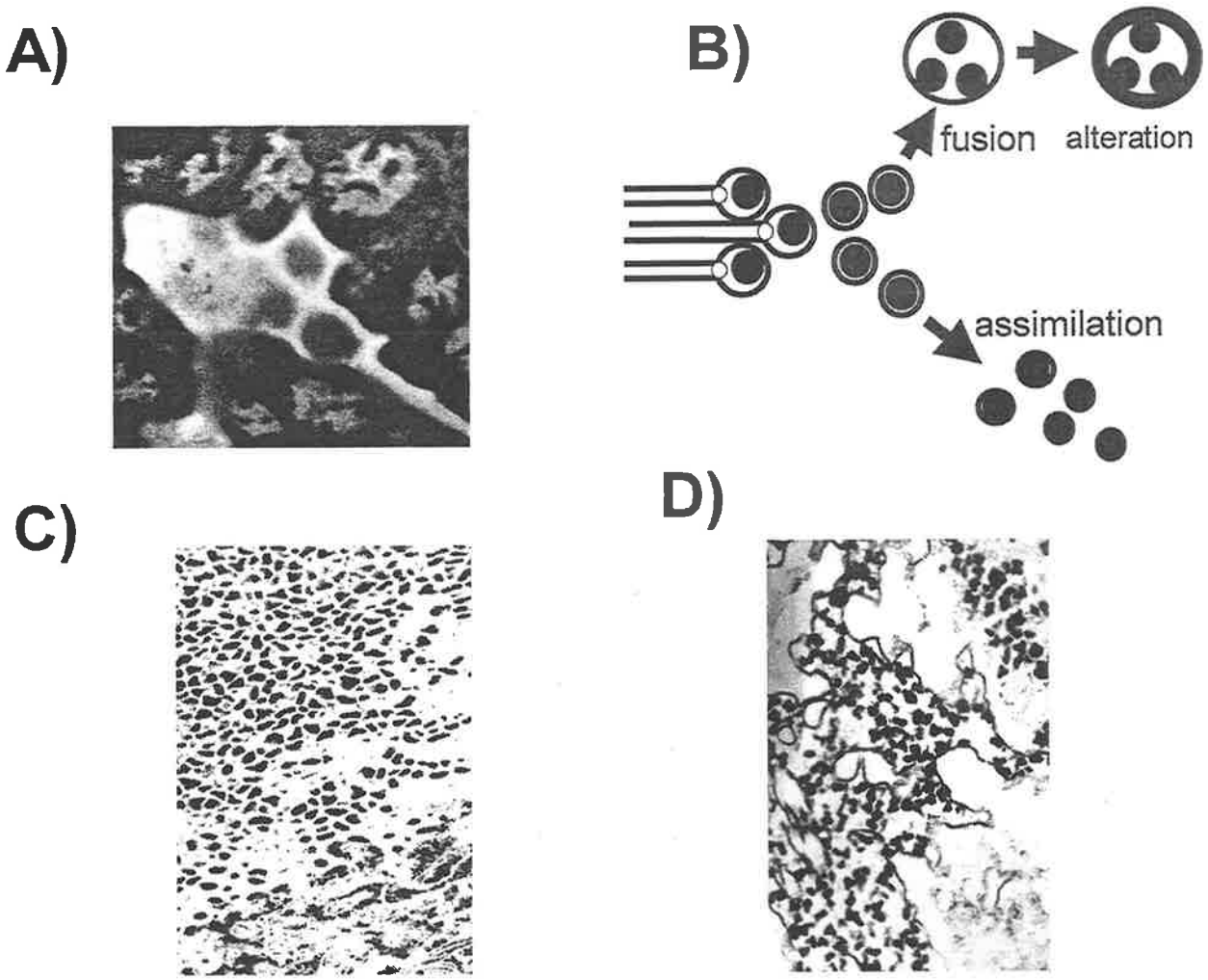


Fig. 18:

Confocal and electron-microscopic pictures of calyx tissues from *Venturia canescens*.

A) Confocal picture of calyx tissue from a mutant strain, stained with anti-VLP1 antibodies, showing the large nuclei and large membrane vesicles surrounding particles inside the calyx lumen.

B) Schematic drawing of possible changes of VLP membranes. Microvilli-like structures emerge from calyx cells containing electron-dense particles. Released particles are surrounded by membranes. These membranes may be assimilated on the particle surface by an unknown process or fused together to form large membrane-surrounded vesicles, which acquire an electrondense appearance.

C) Electronmicroscope image of particles inside the calyx lumen of a normal strain.

D) Particles in the calyx lumen of a mutant stain, showing electron-dense membranes surrounding many particles.

3.2.2 A possible role of localised coagulation reactions in *Venturia* egg surface

Previous studies (Schmidt and Schuchmann-Feddersen, 1989; Schmidt and Theopold, 1990; Schmidt *et al.,* 1990; Hellers *et al.,* 1996) have shown that VLPs protect the eggs against its host immune defence. The results in Chapter 3.2.1 about the VLP1 PHGPx domain showed that VLP1 has no enzymatic function but indicated a possible passive protection role through interacting with membrane phospholipids. Therefore, it was necessary to investigate other possible protective layers on the egg surface of the parasitoid wasps.

In addition to viruses and VLPs, mucinous components covering the egg surface have been implicated as factors protecting the egg from the host immune response in a number of systems (Rotheram, 1973b; Osman and Fuhrer, 1979; Davies and Vinson, 1986). To date none of the molecular components of this protective layer have been described. Hemomucin, a novel insect mucin, has been isolated from a *Drosophila* hemocyte line (Theopold *et al.,* 1996) and is specific to *Helix pomata* lectin (HPL). As well as being detected on hemocytes, hemomucin was also found on the gut lining and on freshly laid eggs (Theopold *et al.,* 1996). In the insect immune system, hemomucin is apparently involved in hemolymph coagulation by attracting the pro-coagulant, lipophorin (Theopold *et al.,* 1996; Theopold and Schmidt, 1997).

Hemomucin has also been found on subcellular structures, called insect microparticles due to their similarities to vertebrate microparticles. In vertebrates, microparticles are released from platelets during blood clotting. They show structural and functional similarities to insect microparticles (Yano *et al.,* 1994; Theopold and Schmidt, 1997). A protein with similar characteristics to *Drosophila* hemomucin was also identified in the lepidopteran *Galleria mellonella* Linnaeus (Lepidoptera: Pyralidae) (Theopold and Schmidt, 1997). To explore whether hemomucin is found on the egg surface, *Venturia* eggs were further analysed with HPL and anti-hemomucin antibodies.

3.2.2.1 A hemomucin homologue in *Venturia*

Drosophila hemomucin was originally isolated by affinity purification using a specific lectin (HPL). HPL recognised only hemomucin in hemocyte lysates and one additional protein in whole animal lysates (Theopold *et al.*, 1996). To search for a possible hemomucin homologue in *V. canescens*, HPL and a hemomucin-specific antiserum were used to analyse tissue sections and Western blots. The *Venturia* homologue showed a similar expression pattern to that seen for *Drosophila* hemomucin. Strong lectin binding was detected in hemocytes, on freshly laid eggs and on the surface of freshly emerged larvae (Kinuthia *et al.*, 1999). When stained with anti-hemomucin antiserum, one protein band with a similar molecular weight to *Drosophila* hemomucin was detected in *Venturia* ovaries (Fig. 19A, arrowhead). To further confirm that it was a homologue of the hemomucin protein, Western blots of *Venturia* ovaries with HPL and hemomucin antibodies were performed. Results demonstrated that a similar size protein reacted with HPL and anti-hemomucin antibodies (Fig 19B, arrowhead). When tissues from the host *E. kuehniella* were tested for the presence of hemomucin-related proteins, a protein band was detected in lysates from hemocytes, which was smaller in size than both *Drosophila* and *Venturia* hemomucin (Fig. 19C, arrowhead). Furthermore, the protein also cross-reacted with HPL and hemomucin-specific antibodies (Fig. 19C, arrowhead). These results suggest that hemomucin is a member of a conserved family of proteins that exists in several insect groups.

3.2.2.2 Hemomucin-lipophorin complex on the egg surface

Since *Drosophila* hemomucin binds lipophorin (Theopold and Schmidt, 1997), and the hemomucin homologue was enriched in *Venturia* ovaries, the question is whether dissected eggs from *Venturia* oviducts contained lipophorin on their surface or whether lipophorin was attached after incubation with hemolymph from the host *Ephestia*. It was previously shown that binding of lipophorin to hemomucin is likely to depend on the lipid moiety of lipophorin and would therefore be species-independent (Theopold and Schmidt, 1997). Attempts to purify *Venturia* hemomucin from the surface of

Venturia eggs failed (Kinuthia, 1996), and so hemomucin was purified from *Drosophila* cell line. A purified *Drosophila* hemomucin protein was obtained (Fig. 20A, Hmu1, Hmu2), and binding experiments with purified hemomucin were performed. When purified hemomucin was immobilised on Sepharose beads and incubated with *Ephestia* hemolymph, lipophorin was detected on hemomucin beads but not on control beads (Fig. 20B, Hmu, C, arrow).

Furthermore, binding experiments were performed using *Venturia* eggs that had been incubated with *Ephestia* hemolymph. Binding of lipophorin after extensive washing was detected using antibody against *Lucilia* lipophorin (Fig. 20C, E+, E, arrow). No signal in the size range of lipophorin was detected in lysates from eggs that had not been incubated with *Ephestia* hemolymph (Fig. 20C, E-). In a further experiment, deposited eggs and emerging larvae were dissected out of host caterpillars at different time points and tested on Western blots with anti-lipophorin antibodies. Lipophorin was detected on the surface of newly deposited eggs (Fig. 20D, arrow). Three days after oviposition, some higher molecular weight proteins were observed in addition to lipophorin (Fig. 20E, arrows). These results indicated that hemolymph proteins and hemomucin had formed a complex within one day of egg oviposition and the complex had cross-linked more hemolymph proteins to form a large complex after three days. In addition, *Ephestia* hemolymph proteins have been detected on the surface of larvae inside caterpillars (Kinuthia *et al.*, 1999). Thus the results show that proteins from the host hemolymph are enriched on the egg surface, in particular lipophorin, which apparently binds to the egg via hemomucin. This indicates that lipophorin and hemomucin form a complex that is normally part of an immune reaction and involved in hemolymph coagulation (Theopold and Schmidt, 1997). Therefore a localised coagulation reaction, involving egg surface hemomucin and host lipophorin will occur, which will potentially protect the eggs against further host cellular attacks.

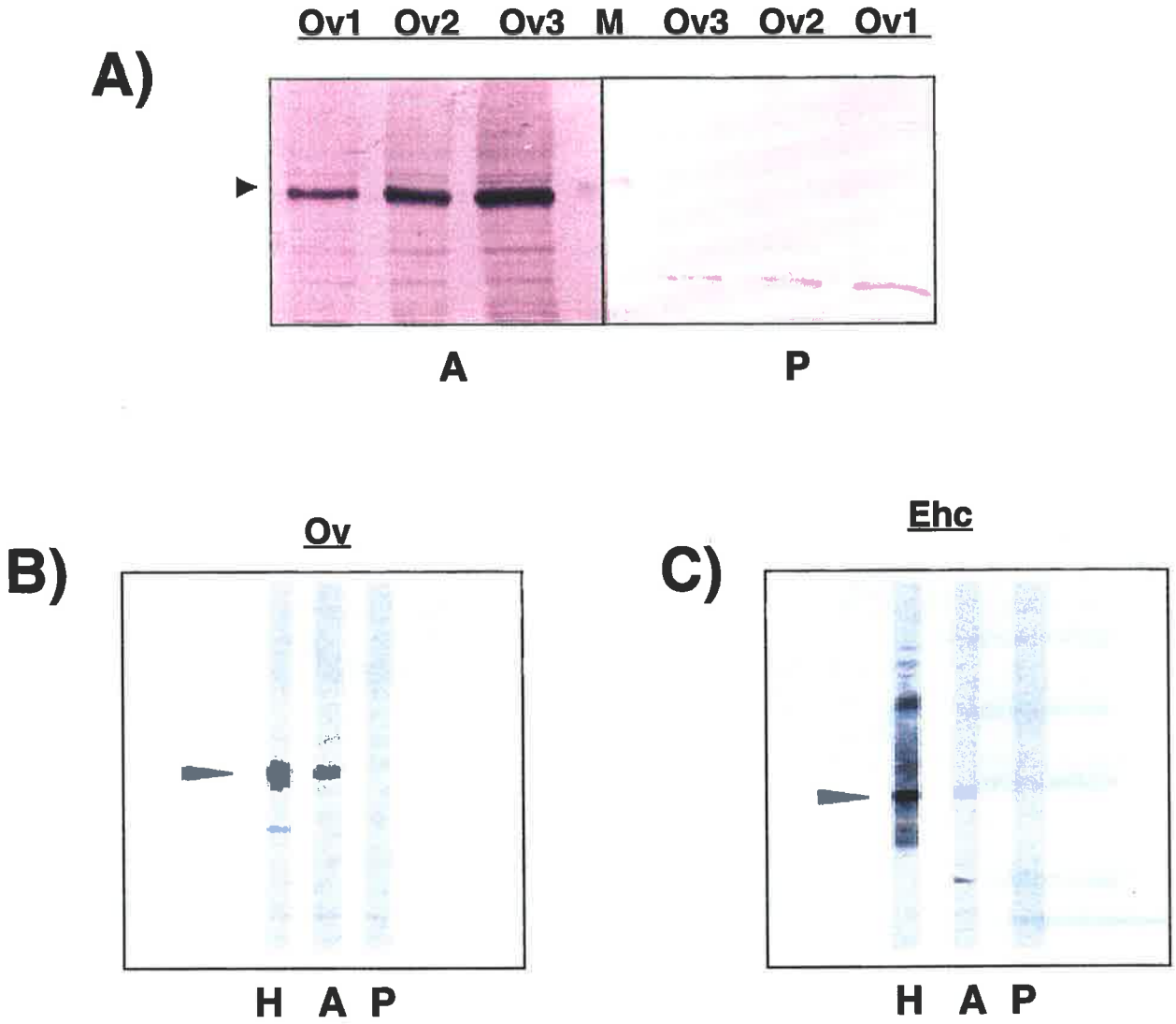


Fig. 19:

Hemomucin homologues in *Venturia* ovaries and *Ephestia* hemocytes.

A), B) Western blots of *V.* ovaries with anti-hemomucin antiserum (A), preserum (P) and HPL (H).

C) Western blots of *Ephestia* hemocytes with anti-hemomucin antiserum (A), preserum (P) and HPL (H).

The Western blots showed that hemomucin homologues exist in the *Venturia* ovaries as well as *Ephestia* hemocytes.

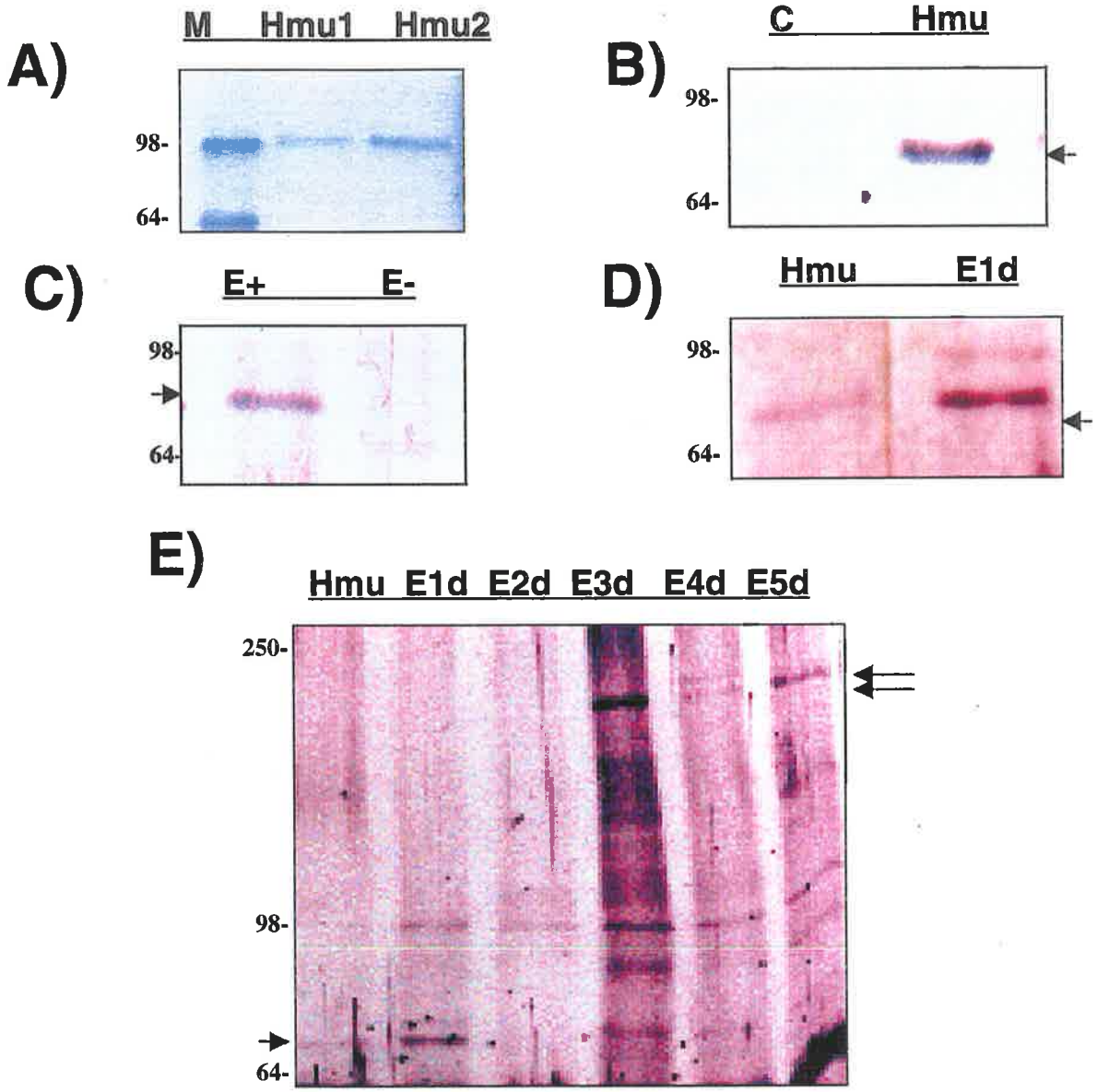


Fig. 20:

Purification of hemomucin and binding assays of hemolymph proteins

A) Hemomucin was purified from Sl2 cell line using HPL column. The purified proteins (Hmu1 and Hmu2) were running in the SDS-PAGE and staining with Coomassie blue.

B) Hemomucin was immobilised on Sephrose beads. The mucin-beads (Hmu) and control beads (C) were incubated with hemolymph and developed with lipophorin antibody.

C) The eggs from oviducts were dissected and incubated with *Ephestia* hemolymph (E+). Western blots were conducted with the eggs from oviducts (E-) and the eggs reacted with *Ephestia* hemolymph (E+) using anti-lipophorin antibody.

D), E) *Venturia* eggs were dissected from parasitised *Ephestia* caterpillars at different time points and developed with anti-lipophorin antibody. E1d, E2d, E3d, E4d and E5d were 1, 2, 3, 4, 5 days after parasitisation.

3.3 A study of insect coagulation reactions

In arthropods, such as insects, blood (hemolymph) circulates through large hemocoelic spaces that are in intimate contact with the cuticle. If the cuticle is wounded, the insect immediately loses hemolymph from its body. It is therefore essential that the wound can be closed immediately to prevent hemolymph leakage and microbial invasion. Wounding or injury to all animals, including insects, immediately induces coagulation. In mammals, platelets disintegrate and release thromboplastin, which converts prothrombin into thrombin. Thrombin thus catalyses the conversion of soluble fibrinogen into insoluble fibrin. In addition, thrombin also acts to attract more platelets that then adhere to fibrin and build up the clot (van Dam-Mieras and Muller, 1986).

In arthropods, such as lobster and crayfish, the granules inside hemocytes degranulate and release clotting factors. Subsequently, the proclotting enzyme is activated into clotting enzyme. Then the clottable protein coagulogen is converted into coagulin, and a clot forms (Iwanaga, 1993). However, the need for insects to seal their wounds immediately is even more urgent because of their open circulatory system. Coagulation in mammals and non-insect arthropods has been widely studied, however less is known about insects. Therefore, it is important to identify the coagulation factors involved and hence obtain a better understanding of the coagulation mechanisms in this class.

The results in the preceding sections show that lipophorin and hemomucin are involved in insect coagulation. However, other clotting factors remain unknown. In order to identify additional clotting factors, several procedures were employed, for example, hemomucin-beads were used to perform binding studies and separation on HPLC columns were conducted in order to obtain peptide sequences. However, none of these approaches were successful. Finally a new procedure was employed using *G. mellonella* as a model system, which led to the identification of a number of coagulation factors and their coding DNAs.

3.3.1 Coagulation of cell-free hemolymph

To identify putative coagulation molecules at a molecular level, an *in vitro* hemolymph coagulation reaction was conducted by mixing rabbit red blood cells with *G. mellonella* cell-free hemolymph (plasma). As soon as rabbit blood cells were added to the cell-free hemolymph, a clot formed (Fig. 21A). The clot was washed intensively and used directly to raise antibodies in rabbits. The antibodies were used to test the clot using confocal microscopy. It showed that the anti-clot antibodies reacted with components in the clot, which bound to the red blood cells (Fig. 21B). This indicated that the antibodies are specific to the hemolymph proteins that reacted with the rabbit red blood cells. To identify the staining pattern on hemolymph, protein extracts from the cell-free hemolymph were obtained and Western blots were conducted. When anti-clot antiserum was incubated with hemolymph proteins from *Galleria* and *Ephestia*, a small number of specific bands were detected in both larvae (Fig. 21C). These proteins included the hemolymph proteins, which show triple bands apparent molecular weights of 70-80 kDa (Fig. 21C) and some less abundant bands, including a 56 kDa protein (Fig. 21C, asterisk). In *Ephestia*, a similar binding pattern was observed except that an additional protein around 40 kDa was also detected (Fig. 21C, arrow). This indicates that the clotting factors are conserved among insects. When preserum was used as a negative control to eliminate unspecific binding of the antiserum, no signals were observed with the preserum (Fig. 21C, preserum). To compare the specificity of the anti-clot antiserum, Coomassie blue staining of hemolymph proteins was compared with Western blots stained with the anti-clot antibodies, which showed that only a small group of hemolymph proteins reacted with the antiserum (Fig. 22A, B). Further analysis of the identity of the triple bands around 70-80 kDa range was conducted using Western blots with different specific antibodies. More evidence was obtained and it showed that the top band is lipophorin, the middle one is proPO (Fig. 22C) and the lower band is probably arylphorin (hexamerin), although this needs further confirmation.

Previous results have shown that hemomucin is involved in coagulation reactions (Theopold and Schmidt, 1997). To further confirm binding of hemomucin with clot-forming components, hemomucin beads were incubated with hemolymph and tested with anti-clot antibodies. Strong binding was detected in the hemomucin beads, but not in the control beads (Fig. 23B, G+ vs. G-; E+ vs. E-). Several specific bands were detected with *Galleria* and *Ephestia* hemolymph. Therefore, hemomucin seems to react with hemolymph proteins that are involved in coagulation reactions.

3.3.2 Coagulation of hemocytes

In order to study the interaction between the soluble hemolymph proteins recognised by the anti-clot antiserum and hemocytes, cells were analysed at different time points after bleeding, using immunocytochemistry and anti-clot antiserum. Hemocyte immune staining was performed with non-permeabilised cells in order to detect binding of hemolymph proteins to the hemocyte surface. At all stages, there was much stronger binding to granular cells than to any other cell-type (Fig. 24, G), including plasmatocytes (Fig. 24, P), oenocytoids and spherulocytes (not shown). A similar staining pattern has previously been described for a number of Gal- and GalNAc-specific lectins, which also bind preferentially to granular cells (Theopold and Schmidt, 1997). After about 10 min membrane vesicles which were previously described as microparticles appeared around some of the granular cells (Theopold and Schmidt, 1997). These vesicles also stained with the antiserum (Fig. 25A, B). After 30 min, extracellular strands with attached microparticles appeared (Fig. 25C-F).

3.3.3 Identification of immune clot proteins

Preliminary attempts to separate proteins from clots were unsuccessful probably due to covalent cross-linking of clot proteins. Since the anti-clot antibodies showed specificity for a limited number of proteins and binding of these proteins to specific hemocyte classes, these observations indicated that the anti-clot antibodies were directed against the clot proteins. This provided the opportunity to use anti-clot

antibodies to screen expression libraries in order to isolate the genes coding for the clotting factors. *Galleria* hemocytes/fat body and whole larval libraries were constructed using λZAP library construction kits (Strategene). The size distribution of synthesised cDNA molecules was analysed on an alkaline agarose gel (Fig. 26). A range of high molecular weight cDNA molecules was produced. In addition, an expression library of *Galleria* epidermal tissues was provided by Dr Peter Wolbert (University of Wuerzburg). In order to identify clotting factors in insects, the three different *Galleria* expression libraries were screened with the anti-clot antibodies. Individual positive plaques were obtained after re-screening of original plaques. The positive clones were collected, and *in vivo* excision was performed in order to isolate the corresponding plasmid DNA. Each single plasmid DNA was purified to homogeneity and cut with the restriction enzymes SphI and KpnI (Fig. 27, 28). Afterwards, the plasmid DNAs were sequenced and further analysed and compared with known sequences in the GenBank. The results of the screenings for the three libraries used are summarised in Table 4. The average frequency of positive clones lies below 0.1%, indicating again that only a limited number of proteins are recognised by the clot-antiserum. When positive clones were sequenced and deduced protein sequences compared to other proteins, a significant number coded for major hemolymph proteins which were implicated in insect coagulation reactions (Bohn, 1986), including prophenoloxidase (Hall *et al.,* 1995; Kopacek *et al.,* 1995; Jiang *et al.,* 1998), lipophorin (Sundermeyer *et al.,* 1996) and a hexamerin (Memmel *et al.,* 1994). Several cDNA fragments that were frequently isolated from all libraries coded for a distant member of the α-crystallin/small heat shock protein (α-crys/SHSP) family (de Jong *et al.,* 1993; MacRae, 2000). In addition to the frequently isolated cDNAs, a number of cDNAs which apparently code for less abundant proteins were isolated as well. These include cDNAs coding for a potential mucin, a trypsin-like protease and a glutathione-S-transferase (GST).

Table 4 Results of library screenings

Used library	Hemocytes/fat body	Pupal epidermis	Larval
Prophenoloxidase	32		
Lipophorin	10	9	
Arylphorin			3
α-crystallin	17	5	2
Mucin-like		4	
Ser protease			1
Glutathione-S-transferase	1		
Screened clones	80,000	100,000	40,000
Positive (%)	0.08	0.02	0.1

Note that the names of the clones are listed on the left column and the numbers of the clones obtained are inside the boxes. The frequencies of the positives were calculated in percentage in the bottom row.

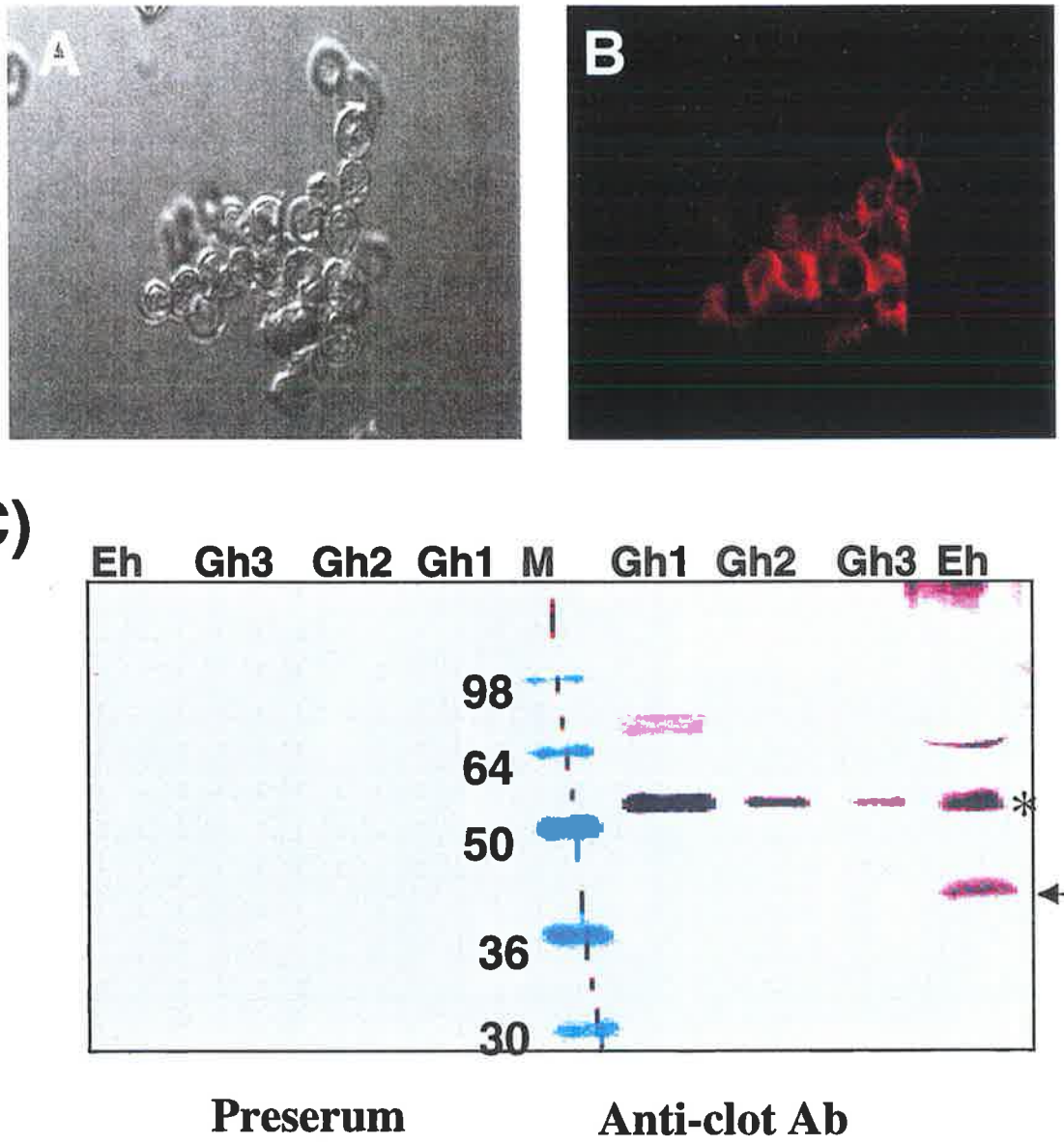


Fig. 21:

Clot formation and reaction of hemolymph proteins with anti-clot antibodies

A) Clot was formed with rabbit blood cells and components from *Galleria* cell-free hemolymph.

B) Clot incubated with a clot-specific antiserum, stained with TRITC-conjugated secondary antibody and inspected under confocal microscopy. Note that the proteins between the cells were labelled strongly.

C) Anti-clot antibodies reacted with hemolymph proteins. Western blots of protein extracts from cell-free hemolymph developed with preserum and anti-clot antiserum.

Gh1, Gh2, Gh3, different concentrations of *Galleria* hemolymph proteins; Eh, *Ephestia* hemolymph proteins; M, MW marker in kDa.

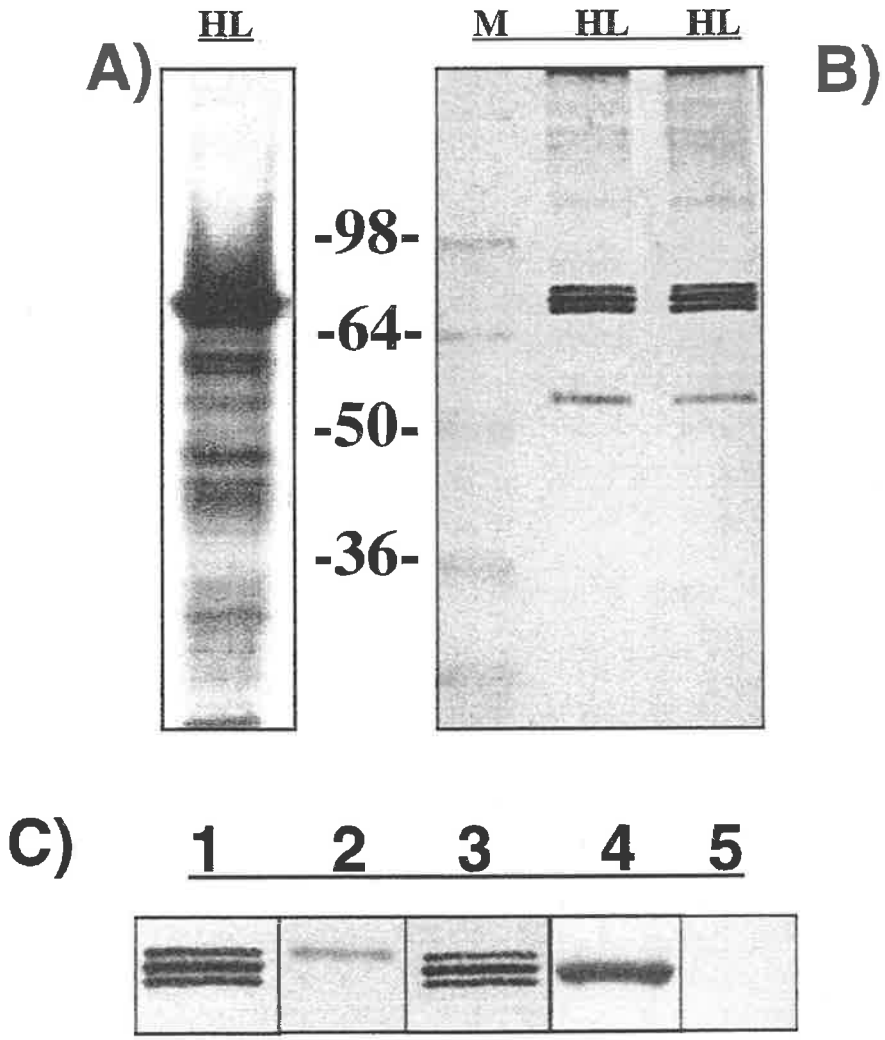


Fig. 22:

SDS-PAGE and Western blots of *Galleria* hemolymph proteins against clot antibodies

A) Protein profile of *Galleria* cell-free hemolymph. Note that quite a number of protein bands were detected in this Coomassie blue gel.

B) Western blots of *Galleria* cell-free hemolymph proteins against anti-clot antibodies. Note that only a limited number of protein bands were detected. HL, cell-free hemolymph protein extracts; M, molecular weight marker in kDa.

C) Characterisation of the three major hemolymph proteins, which were recognized by anti-clot antibodies. Western blots of hemolymph proteins were analysed with anti-clot serum and antisera against some of the major proteins: 1 and 3, anti-clot antibodies; 2, anti-Lipophorin antibody; 4, anti-proPO antibody; 5, pre-immune serum for the anti-proPO antibody.

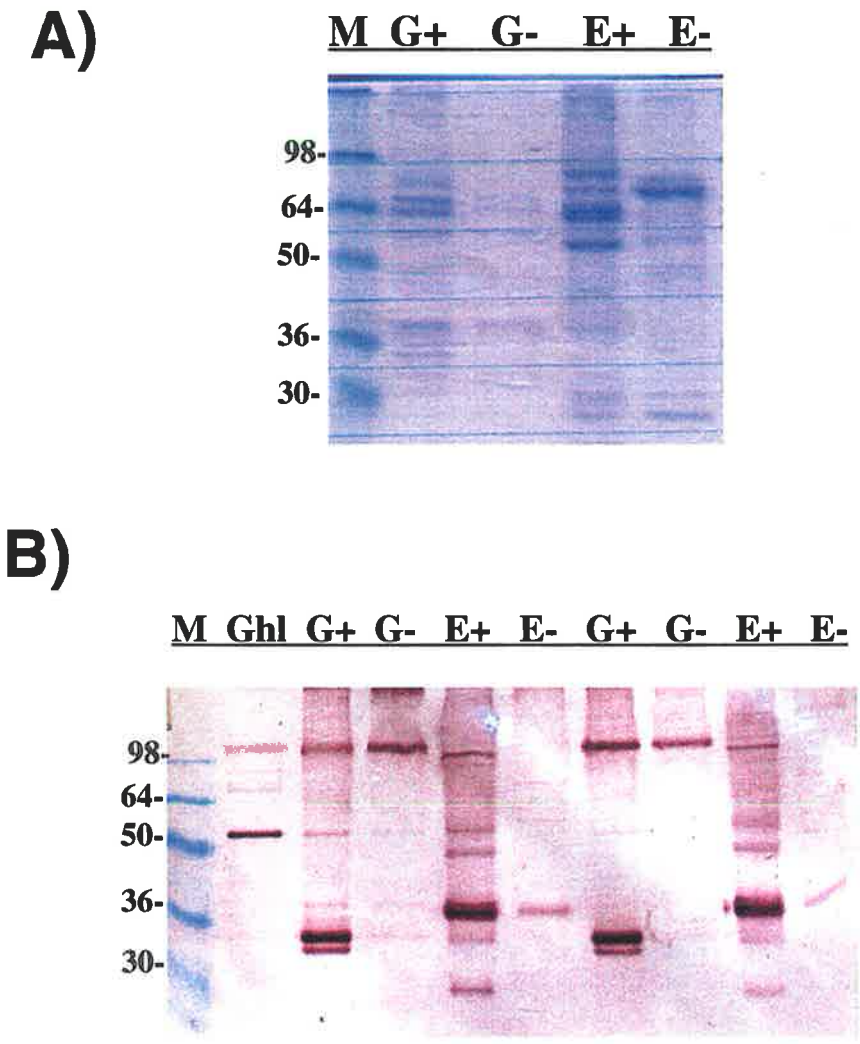


Fig. 23:

Hemomucin involved in the coagulation reactions.

A) SDS-PAGE of the incubations of hemomucin beads (+) and control beads (-) with cell-free hemolymph proteins from *Galleria* (G) and *Ephestia* (E).

B) Western blot with anti-clot antibodies. Note strong signals were detected on the hemomucin beads, but not in the control beads. Ghl, protein extracts of cell-free hemolymph from *Galleria*.

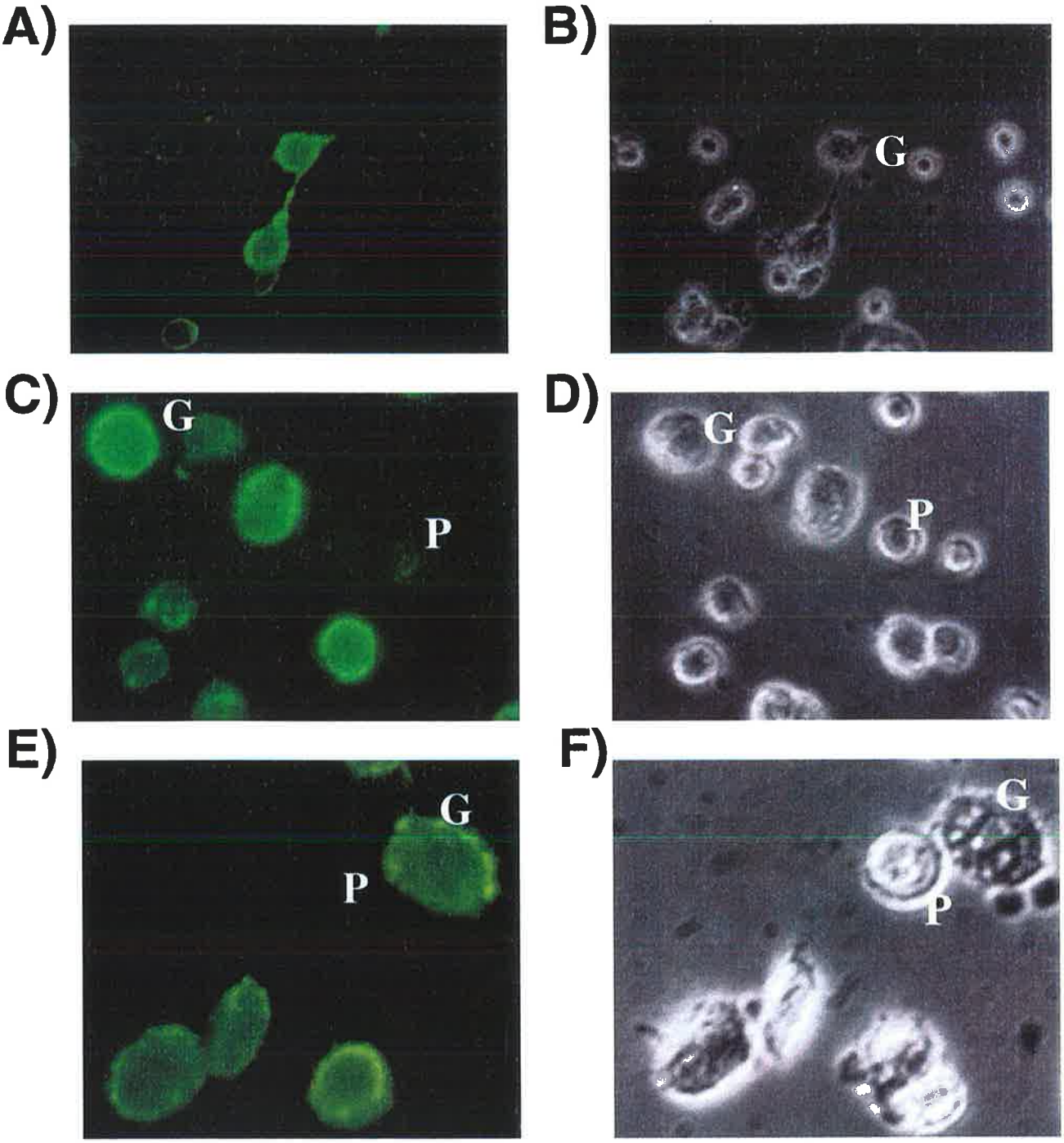


Fig. 24:

Hemocyte staining with anti-clot antibodies.

A), C) and **E)** are *Galleria* hemocytes stained with anti-clot antibodies, developed with FITC-conjugated secondary antibody and inspected under indirect UV-light.

B), D) and **F)** are phase contrast of A), C) and E) respectively. Granolocytes (G) showed strong staining while plasmatocytes (P) got no signal at all.

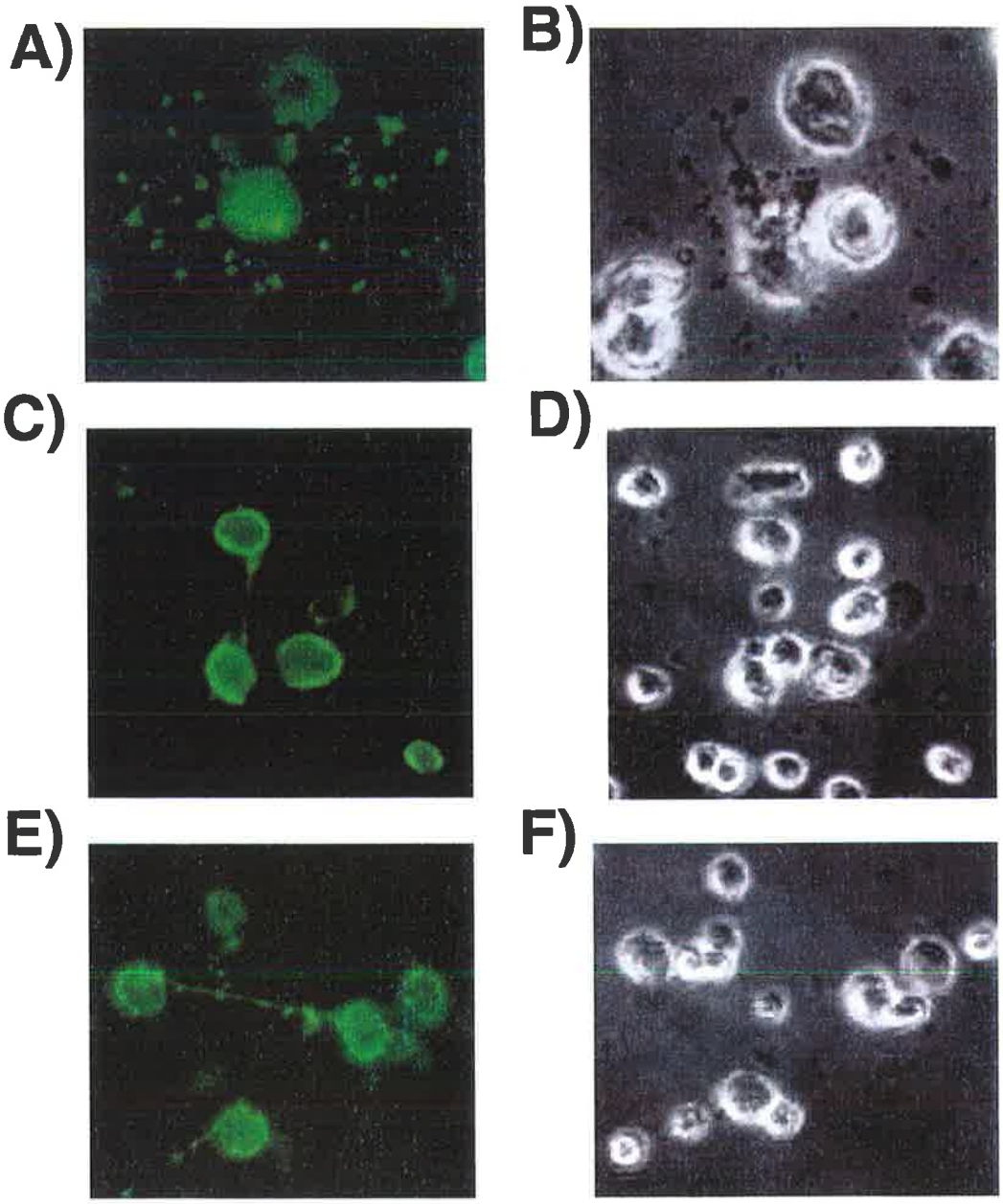


Fig. 25:

Microparticle formation. *Galleria* hemocytes were incubated with anti-clot antiserum, labelled with FITC-conjugated secondary antibody and observed under indirect UV light using microscopy.

A) and **B)** showed the microparticles formation after 10 min. Note the microparticles were stained heavily with anti-clot antiserum.

C)-F) showed the extracellular strands attached microparticles after 30 min.

B), D) and **F)** are the corresponding phase contrast of A), C) and E).

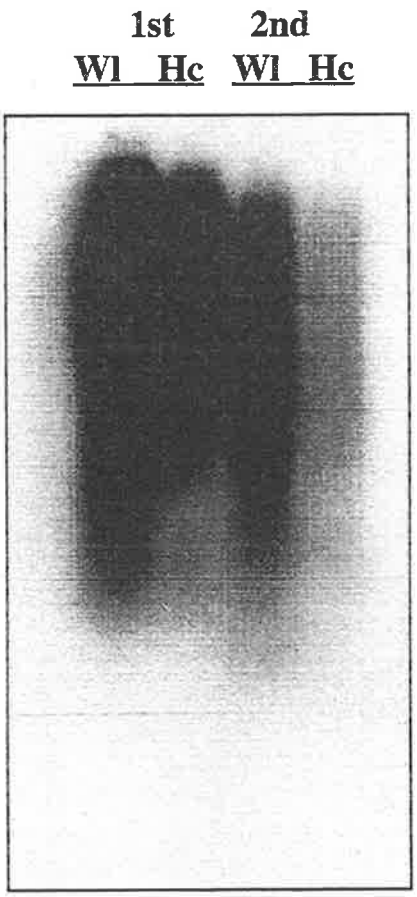


Fig. 26:

cDNA synthesis analysis. The first and second reactions of cDNA synthesis were run on an alkaline agarose gel, transferred onto a membrane and exposed to a diagnostic film. Larger amount of 1st strand cDNA than 2nd strand cDNA indicated that both cDNA are in good quality.

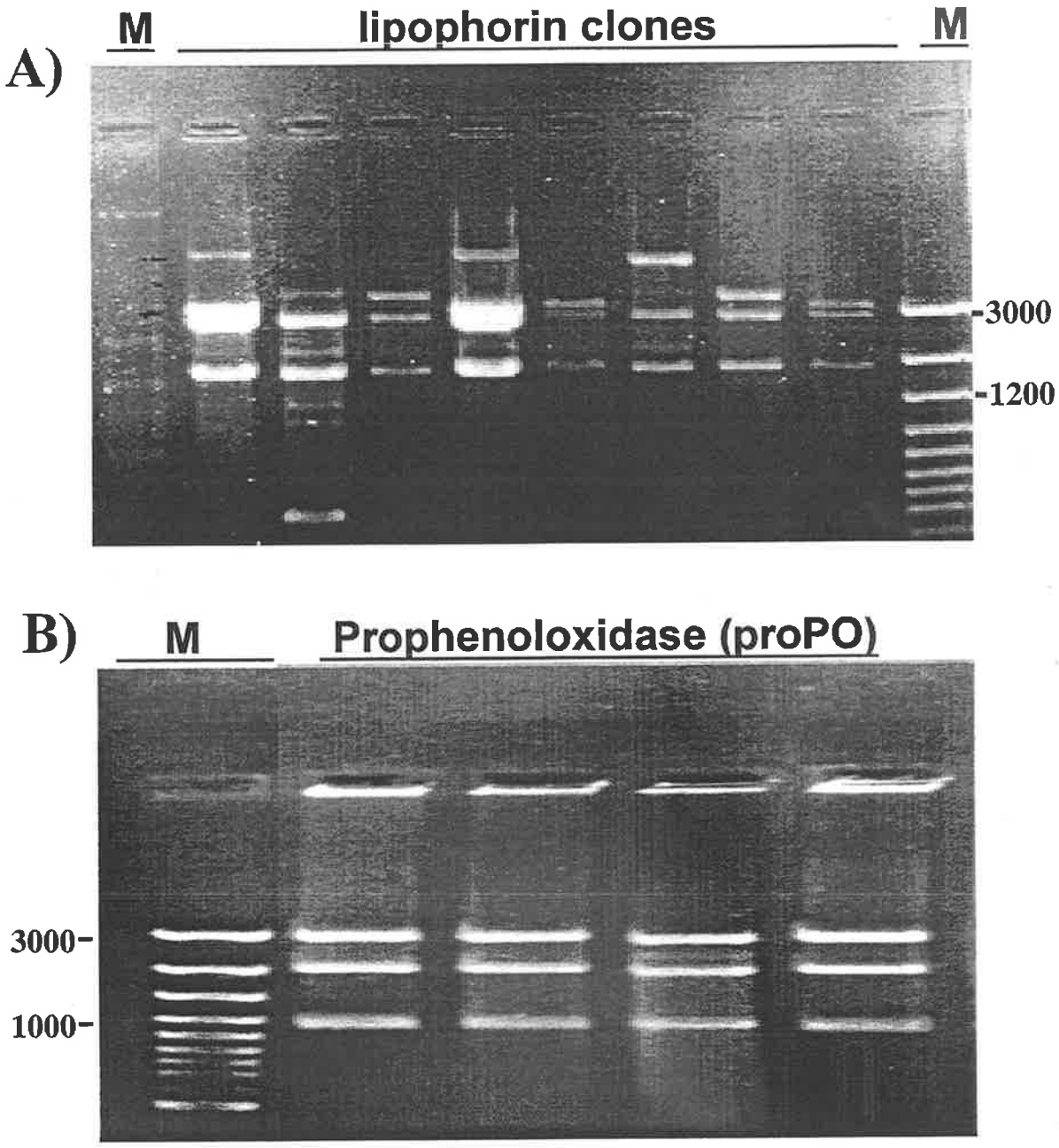


Fig. 27:

Some of the positive clones from library screening.

Selected clones were excised and corresponding plasmid DNAs were purified. The plasmid DNAs were digested with double restriction enzymes (SphI and KpnI).

A) Different sizes of lipophorin were obtained and sequenced. Sequence analysis showed that these clones are different fragments of *Galleria* lipophorin and are from the same gene.

B) Same proPO clones were isolated. The inserts were cut into two fragments because of a KpnI cutting site at 1080 bp from the 5'-end. Molecular weight markers are in bp.

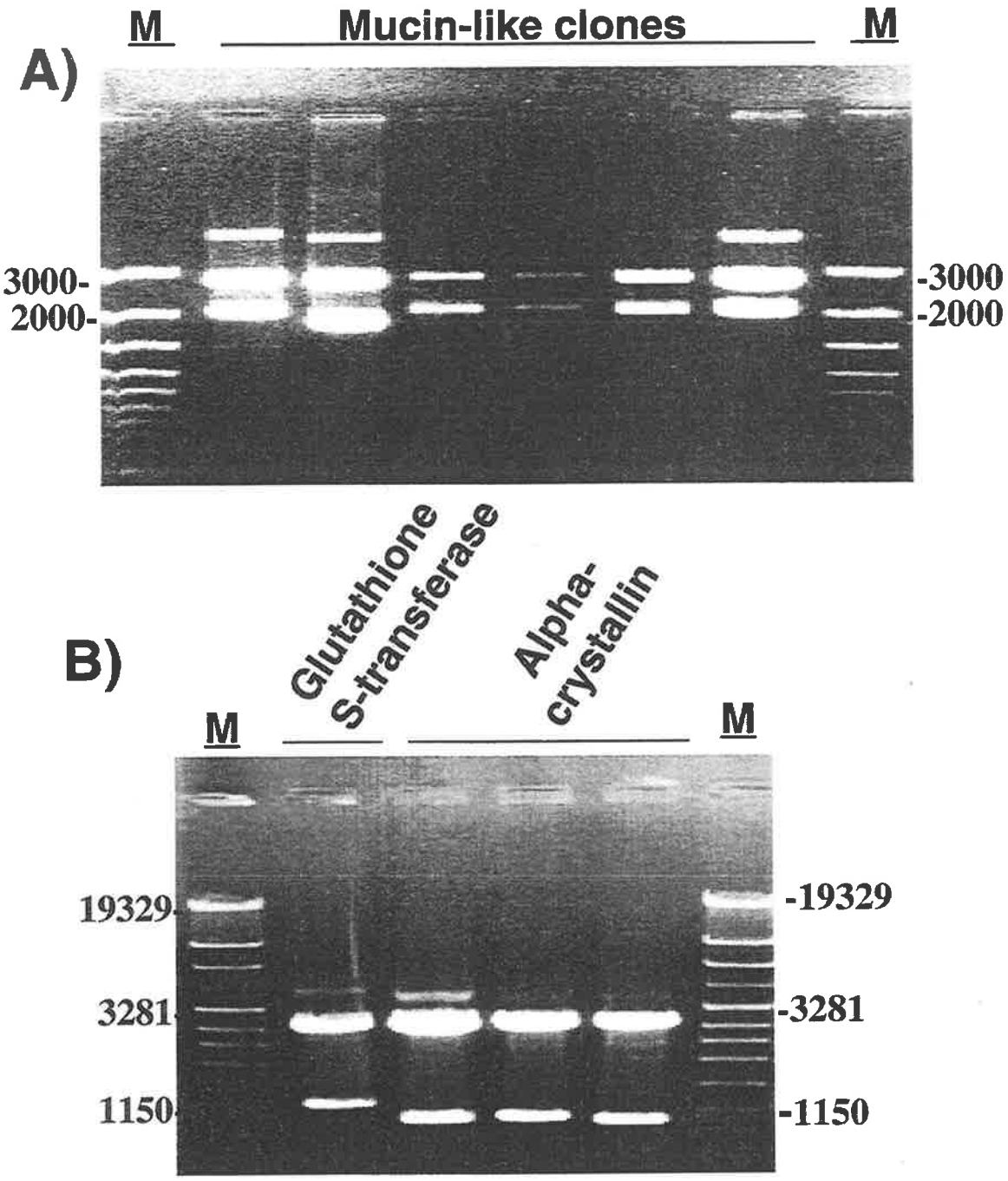


Fig. 28:

Some of the positive clones from library screening.

Selected clones were excised and corresponding plasmid DNAs were purified. The plasmid DNAs were digested with double restriction enzymes (Sph I and Kpn I).

A) Mucin-like clones. Note a few mucin-like clones were isolated, all of them show the same cDNA sequences.

B) Glutathione S-transferase (GST) clone and Alpha-crystallin clones.

3.3.4 Analysis of cDNA sequences

Lipophorin: A number of independent lipophorin clones were picked from the expression libraries. Due to the high molecular weight of lipophorin and the time limitation, *G. mellonella* lipophorin clones were only partially sequenced. The obtained clones showed high homology with *Manduca sexta* lipophorin cDNA, which is around 10 kb in length (Sundermeyer *et al.*, 1996). Sequence comparison of lipophorins was performed using partial sequences (Fig. 29) showing 43.4% similarity to *M. sexta* and 14.7 % homology to *Drosophila* lipophorin (Table 5). Parts of the *G. mellonella* lipophorin sequences were submitted to GenBank.

Table 5 Amino acid sequence similarities (%) of different proteins from different insects

G. mellonella	*M. sexta*	*D. melanogaster*
Lipophorin	43.4	14.7
ProPO	71.3	55.1 (ProPOA1)
Glutathione S-transferase (GST)	43.8 (GST1)	58.9 (GST1)
Trypsine-line protease (TP)	67.0	27.0 (*Easter*)

Prophenoloxidase: The proPO-like cDNA is 3069 nucleotides in length and has an open reading frame from nucleotide 78 extending to nucleotide 2126 (Fig. 30). The 3' untranslated sequence of 943 bp in length contains an AATAAA motif, as a polyadenylation signal near the 3'-end. The long 3'-non-coding region is similar to that found in the proPO1 cDNA from *B. mori* (Kawabata *et al.*, 1995), *H. cunea* (Park *et al.*, 1997) and *M. sexta* (Jiang *et al.*, 1997b) (Fig. 30, Table 6). A phylogenetic

comparison between several insect prophenoloxidases, including the one from *Galleria* is shown in Fig. 31, indicating a higher similarity of *Galleria* proPO to proPO1 compared to proPO2 of lepidopteran insects (Fig. 31, Table 6). This indicates that the novel clone codes for *Galleria* proPO1.

Table 6 cDNA sequence analysis of proPOs from *Galleria* and other insects

Name	5'-end noncoding region	3'-end noncoding region	No. of amino acids	Calculated molecular weight	References
Hc-proPO1	88	1032	681	78,192	Park *et al.*, 1997
Hc-proPO2	44	235	691	80,637	Park *et al.*, 1997
Bm-proPO1	20	n.d.	685	78.653	Kawabata *et al.*, 1995
Bm-proPO2	44	n.d.	693	80,003	Kawabata *et al.*, 1995
Ms-proPO1	169	1261	685	78,965	Jiang *et al.*, 1997
Ms-proPO2	68	308	695	80,020	Hall *et al.*, 1995
Gm-proPO1	77	943	683	78,511	Li *et al.*, 2002

Phenoloxidase is a copper-containing oxidase that hydroxylates monophenols to diphenols and oxidizes diphenols to quinines, which are further converted on the pathway leading to the black pigment melanisation. In insects melanin synthesis is involved in the processes of sclerotisation and wound healing of cuticle as well as in the defence reactions against non-self organisms (Kawabata *et al.*, 1995; Hughes, 1999; Lee *et al.*, 2000a). The target enzyme in the cascade is prophenoloxidase

(proPO), which is activated by a serine protease (Lee *et al.,* 2000a). The activation of the prophenoloxidase cascade can be initiated by bacteria and fungi or by their cell wall components, i.e. peptidoglucan from bacteria and ß-1, 3-D- glucan from fungi (Lee *et al.,* 2000b). Molecular cloning of proPO has been achieved in several arthropods, the insect *B. mori* (Kawabata *et al.,* 1995), *M. sexta* (Hall *et al.,* 1995, Jiang *et al.,* 1997), *D. melanogaster* (Fujimoto *et al.,* 1995) and freshwater crayfish *Pacifastacus leniusculus* (Aspen *et al.,* 1995). These proPOs have been found to be similar in amino acid sequence to arthropod hemocyanins in their putative copper binding sites (Kawabata *et al.,* 1995; Decker and Rimke 1998; van Holde *et al.,* 2001). Sequence comparison of the two conserved regions, the copper binding region A and B showed that the two very conserved regions for binding copper also exist in GmproPO1 (Fig. 32). Previously, *Galleria* proPOs have been purified and eleven peptide sequences were obtained (Kopacek *et al.,* 1995). From the 11 peptide sequences, 4 were present in the ORF predicted from the GmproPO1 cDNA sequence isolated (Fig. 30, boxed), indicating that the published sequences might be derived from a mixture of proteins (Kopacek *et al.,* 1995). Supporting this explanation, some of the other previously published sequences show a high degree of similarity with proPO2 from other species (not shown).

Hexamerin: cDNAs, which codes for a member of the hexamerin family, were isolated. The gene is *G. mellonella* hexamerin (Memmel *et al.,* 1994). Insect hexamerins belong to a growing protein family that includes arthropod hemocyanins and tyrosinases such as phenoloxidase (Beintema *et al.,* 1994). They are insect storage proteins but have been implied in immunity as well (Beresford *et al.,* 1997). Several clones of *Galleria* hexamerins were isolated and sequenced and the EST sequences were submitted to the GenBank.

Alpha-crystallin: The *Galleria* alpha-crys/SHSP cDNA is 1085 nucleotides long including a 45 nucleotide long 5'-untranslated region, a 717 nucleotide long open reading frame coding for 239 amino acids and a 323 nucleotide long 3'-untranslated region with a polyadenylation signal AATAAA and a poly A tail. The first 16 amino

acids of the deduced protein comprise a predicted signal peptide (Fig. 33, underlined). One potential N-glycolysation site (Fig. 33, dotted underlined) and a number of phosphorylation sites (Fig. 33, double underlined) were detected. Sequence comparison of *Galleria* alpha-crystallin with crystallins from other insects and a small heat shock protein did not show high similarity (Fig. 34A). Since members of this family tend to vary in their sequence but tend to retain structural similarities (de Jong *et al.,* 1993), comparison of hydropholicity patterns between the *Galleria*, a *Drosophila* and a prokaryotic member of the family was conducted (Fig. 34B). This analysis adds support to the sequence relatedness shown in Fig. 34A. Another common feature of alpha-crystallin and small heat shock proteins is their phosphorylation of specific serine residues (de Jong *et al.,* 1993). Several phosphorylation sites were identified in Gm-alpha crystallin amino acid sequences (Fig. 33, double underlined). For example, serines at position 16 and 83 in the N-terminal domain are phosphorylated and these are conserved in other crystallins (de Jong *et al.,* 1993). These features further classified this gene as Gm alpha-crystallin.

Glutathione S-transferase: Galleria glutathione S-transferase (GmGST) cDNA is 880 nucleotides long and has a 648 bp open reading frame coding for a deduced protein of 216 amino acids in length. A starting codon is localised at nucleotide 64 and a TGA stop codon at nucleotide 712 with a AATAAA signal and a poly A tail located at the 3'-UTR (Fig. 35). The estimated molecular mass of 216-amino acid residues was 24,352 Da with a deduced pI of 6.91. The deduced protein of Gm GST has over 40% similarity to *Manduca sexta* GST1 and 58.9% similarity with *D. melanogaster* GST1 (Table 5, Fig. 36A). GmGST has similarity to a particular subgroup of GST proteins (Tang and Tu, 1995; Franciosa and Berge, 1995; Pemble *et al.,* 1996; Singh *et al.,* 2000), which may allow identification of GSTs involved in defence-related processes (Fig. 36B).

Trypsin-like serine protease: One of the positive clones has sequence similarity with trypsin-like serine protease and was called *Galleria* trypsin-like protein (GmTP). The trypsin-like serine protease cDNA is 993 nucleotides long and includes a 17 bp 5'-

UTR, a 765 bp OPR and a 213 bp 3'-UTR (Fig. 37). The open reading frame codes for a 255 amino acids protein with a 27,342 Da molecular weight and an 8.92 pI value. The first 15 amino acids consist of a transmembrance signal peptide. An AATAAA polyadenylation signal was down-stream of the stop codon and a poly A tail at the end of the 3'-UTR. The ORF of the trypsin-like clone contains three conserved amino acids at the correct position characteristic for serine proteases (Gorman *et al.*, 2000; Bangyeekhun *et al.*, 2001), indicating that it codes for a functional enzyme (Fig. 37, boxed letters). Sequence alignment of GmTP protein with other trypsin-like proteins revealed that GmTP is more similar to *M. sexta* trypsin, alkaline A precursor and Indian meal moth, *Plodia interpunctella* trypsin (Fig. 38). In addition, GmTP also showed sequence similarity to *M. sexta* hemocyte proteinase HP3 and HP4 and with *D. melanogaster easter* (Table 5). In *Manduca* hemocytes, four serine proteases were classified in two groups (Jiang *et al.*, 1999). HP1 and HP2 contain an amino-terminal 'clip' domain; while HP3 and HP4 are smaller proteins with a short signal peptide followed by a serine protease domain similar to proteases from mammalian leucocytes. GmTP, showing similarity to HP3 and HP4, may indicate some immune functions in hemolymph coagulation.

Mucin-like proteins: Mucins are large, heterogeneous and highly glycosylated proteins in which the vast majority of the carbohydrate side chains are O-linked to Thr and Ser residues of the polypeptide backbone (Devine and McKenzie, 1992). Among the positive clones, a few clones had the same inserts (Fig. 28A) and the same sequences but were devoid of a stop codon. The open reading frames of these cDNA sequences were all connected directly with the poly A tail (Fig. 39). Therefore, the full length of these clones has not been obtained. In the deduced 610 amino acid sequence obtained, 89 amino acids are threonines, accounting for around 14.5% of the protein sequences obtained and 64 are serines, accounting for 10.5% (Fig. 39). Many of the threonines and serines constitute potential O-glycosylation sites, with 13 of them having a O-glycosylation potential above the threshold (Fig. 40). In addition, five putative N-linked glycosylation sites are present (Fig. 39, underlined). Therefore the clone was named *Galleria* mucin-like gene because of the high glycosylation sites. In order to

obtain the full-length sequences of the gene, 3'-race PCR were conducted but failed. The library was re-screened using the isolated cDNA sequences as a probe; unfortunately no full-length clones were isolated. Therefore, this part of work was not completed because time constraints.

3.3.5 Expression patterns of the clotting factors

After the cDNA clones were sequenced, further expression analysis was conducted. Different parts of tissues were dissected and Reverse Transcript (RT)-PCR was carried out with gene-specific primers. *Galleria* prophenoloxidase (proPO) showed specific expression in hemocytes at larval stages, which is consistent with the notion that proPO is produced in hemocytes only (Jiang *et al.,* 1997b). Results were confirmed using anti-proPO antibodies from *M. sexta.* Western blots containing protein extracts from hemocytes, larval, pupal and adult tissues were conducted, which showed that proPO is found mainly in the hemocytes and the larval stage, not in the pupal and adult stages (Fig. 41). This result further confirms larval hemocytes as the only source of proPO in lepidopteran insects (Jiang *et al.,* 1997b). When prophenoloxidase-specific antibodies were used in staining reactions of hemocytes (Fig. 42), a pattern similar to the one observed with the anti-clot serum (Fig. 24) was observed.

Alpha-crystallins constitute the major components of the eye lens fiber cell (de Jong *et al.,* 1993). They were also found to be expressed in a variety of extra-lenticular tissues and a general cellular function was indicated (Groenen *et al.,* 1994). The highest levels of alpha B-crystallin were observed in heart, striated muscle and kidney tissues (Bhat and Nagineni, 1989). Expression of the *Galleria* alpha-crys/SHSP mRNA was detected in the fat body, the hemocytes and the ovary but not in the gut (Fig. 43).

Lectins contain carbohydrate recognition domain (CRDs) and are known as insect recognition molecules. Mucins, which are lectin ligands, have been characterised in vertebrates and participate in the attachment of immune cells during inflammatory responses and allow their migration to the site of inflammation (Springer, 1994).

Lectins can bind to *Drosophila* hemocytes and are implicated in the existence of lectin receptors on these cells (Rizki and Rizki, 1983; Nappi and Silvers, 1984). One such receptor, hemomucin, was isolated from the *Drosophila* cell line (*mbn2*) using HPL (Theopold *et al.*, 1996). Hemomucin is expressed throughout the developmental stages of the fly, but it is strongly expressed in adult hemocytes. In addition, hemomucin is also present in cardia cells and follicle cells of the ovary (Theopold *et al.*, 1996). Expression studies of the *Galleria* mucin-like clone were conducted using RT-PCR. The mRNA for the mucin-like gene was expressed in all the developmental stages and several tissues tested (Fig. 44). This indicates that the *Galleria* mucin-like gene may have different functions in different developmental stages. In addition, the mucin-like gene was subcloned into a pQE expression vector. However, the protein could not be induced in this bacterial expression system. A new procedure of expressing the gene would be required to analyse the protein further.

A number of serine proteases have been isolated in insects (Finnerty *et al.*, 1999; Jiang *et al.*, 1999; Huang *et al.*, 2000; Jiang and Kanost, 2000; Zhu *et al.*, 2000; Bangyeekhun *et al.*, 2001). Some have functions in the digestive system (Zhu *et al.*, 2000); others are expressed in hemocytes and other tissues and have immune related functions (Finnerty *et al.*, 1999; Jiang *et al.*, 1999; Huang *et al.*, 2000). The results of this study showed that the GmTP was not only expressed in gut but also in hemocytes and other tissues examined (Fig. 45). This indicates that the GmTP gene might be a multifunctional protein.

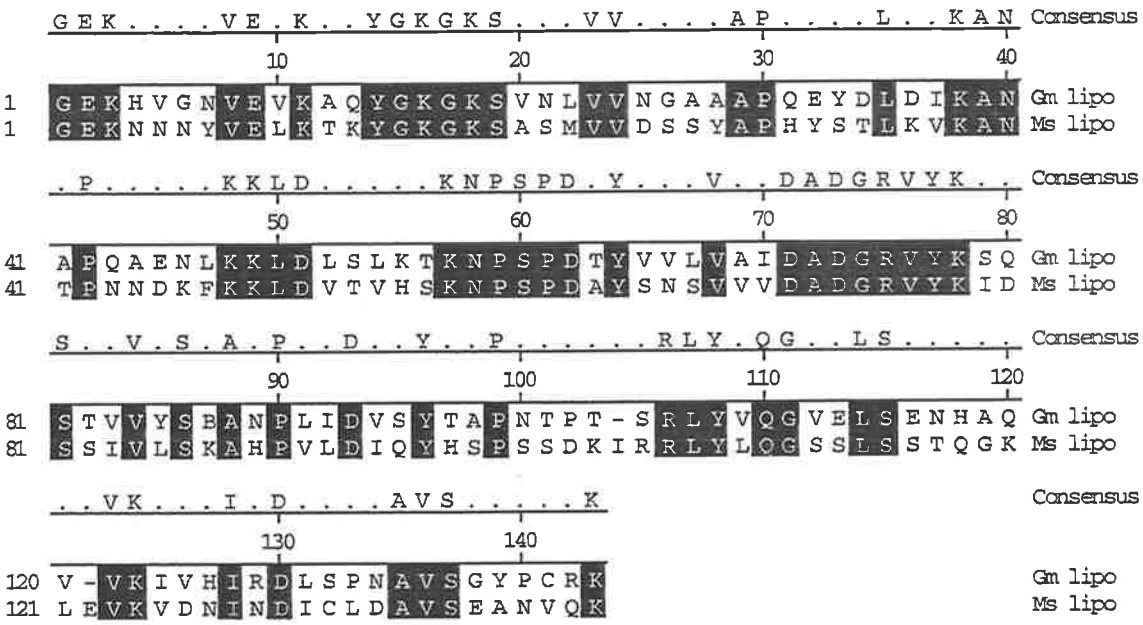


Fig. 29:

Amino acid sequence comparison of lipophorins from *Galleria* and *Manduca*. Partial lipophorin sequences of *Galleria* and *Manduca* were used to compare their similarities. Gm lipo, *Galleria mellonella* lipophorin, the *Galleria* lipophorin gene was submitted to gene bank as an EST and its accession number is BI076408. Ms lipo, *Manduca sexta*, apolipophorins precursor, amino acid sequence from 1788 to 1929 were used in the comparison and its accession number is Q25490.

CGGCTTGAAACACATACAAACATATTTAAATTAATTTGCTTTGAATCTGCCTG 53

M S D S K S R L L L F 11
AAGTCTTTTAACGGATACAACAAAATGAGTGATTCCAAGAGCAGACTGCTGCTGTTC 120

F D R P S E P C F L Q K G D D N V A F 30
TTCGACCGGCCGTCCGAGCCGTGTTTCCTGCAGAAAGGTGACGATAACGTCGCCTTC 167

E V P D H Y Y S D K Y K S L T N T L A 49
GAAGTGCCCGATCATTACTACTCGGATAAATACAAAAGTTTGACCAACACCCTGGCG 224

⇓

N R F G S G E V R T T P V K N I A L P 68
AACCGGTTCGGGAGCGGTGAGGTGCGCACCATTCCCGTGAAGAACATCGCCCTGCCC 281

D L S L P L Q L P Y N D Q F S L F V A 87
GACCTGTCCCTGCCACTCCAACTGCCTTACAATGACCAGTTCTCACTGTTCGTGGCC 338

K H R R M A G K L I D I F I N M R D V 106
AAGCACCGCCGGATGGCTGGCAAGCTTATCGACATCTTTATAAACATGCGTGACGTG 395

D D L I S L C S Y C Q M R V N P Y M F 125
GACGACCTGATCTCCCTGTGCTCGTATTGCCAAATGAGGGTCAACCCTTACATGTTC 452

N Y C L S V A I L H R P D T K G L Q V 144
AACTACTGTCTCTCAGTCGCCATATTGCACAGGCCGGACACGAAAGGCCTGCAGGTG 509

P P V V E T F P D K F M D P K V F R K 163
CCACCGGTGGTGGAGACGTTCCCTGACAAGTTCATGGATCCAAAGGTGTTCAGGAAG 566

A R E T T T V N A S G N R M P I T I P 182
GCCCGAGAAACCACCACCGTCAATGCATCTGGTAACAGGATGCCAATAACCATCCCA 623

T N Y T A S N S E P E Q R V A Y F R E 201
ACCAACTACACCGCGTCGAACTCAGAGCCGGAGCAGCGCGTGGCGTACTTCCGCGAA 680

D I G I N L **H** **H** W **H** W **H** L V Y P F E A 220
GACATCGGCATCAACCTTCACCACTGGCACTGGCACCTCGTGTACCCTTTCGAGGCC 737

A R E I V K K D R R G E L F Y Y M **H** Q 239
GCCCGCGAGATTGTCAAGAAGGACCGCAGGGGAGAACTGTTCTATTACATGCATCAA 794

Q I I A R Y N A E R L C N G L G R V T 258
CAGATCATCGCTAGATACAACGCGGAGCGACTGTGTAATGGTCTGGGCCGTGTTACC 851

R Y S D F R A P T G E G Y F P K L D S 277
CGGTACAGCGATTTCCGTGCGCCCATTGGCGAAGGCTACTTCCCCAAGCTCGACAGC 908

Q V A S R S W P P R F A N T V I R D I 296
CAGGTCGCCAGTCGCTCCTGGCCGCCAAGGTTTGCCAACACAGTGATCAGGGATATT 965

D R P V N E I K I D V F Q L E T W R D 315
GACCGTCCGGTGAATGAGATCAAGATCGACGTCTTCCAGCTGGAGACGTGGCGTGAC 1022

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R  F  L  Q  A  I  D  S  N  A  I  N  M  P  N  G  R  K  V          334
AGGTTCCTGCAGGCCATCGACAGCAATGCCATCAACATGCCAAACGGCAGGAAGGTA       1079

P  L  N  E  E  T  G  I  D  E  L  G  N  L  M  E  S  S  I          353
CCACTGAACGAGGAGACCGGCATCGACGAGCTCGGCAACCTGATGGAGTCCTCGATC       1136

L  S  L  N  R  G  Y  Y  G  D  L  H  N  M  G  H  V  F  I          372
CTGAGCTTGAACCGTGGCTACTACGGGGACCTGCACAACATGGGCCATGTCTTCATA       1193

A  Y  S  H  D  P  D  H  R  H  L  E  E  Y  G  V  M  G  D          391
GCATACTCGCATGACCCCGACCATCGCCATCTGGAAGAGTACGGCGTGATGGGCGAC       1250

S  A  T  A  M  R  D  P  V  F  Y  R  W  H  A  Y  I  D  D          410
TCGGCGACAGCGATGCGCGACCCGGTGTTCTACCGCTGGCACGCCTACATCGATGAC       1307

I  F  N  L  Y  K  S  K  L  T  P  Y  G  D  S  Q  L  D  Y          429
ATATTTAACCTTTACAAGAGCAAGCTCACGCCCTACGGAGACAGTCAGTTGGACTAC       1364

P  G  T  R  V  S  S  I  S  V  E  G  P  A  G  A  N  R  F          448
CCCGGCATACGGGTATCATCCATCAGCGTGGAGGGCCCCGCCGGCGCCAACCGGTTC       1421

A  T  Q  W  Q  Q  S  L  V  E  L  S  Q  G  L  D  F  T  P          467
GCGACCCAGTGGCAGCAGAGCCTGGTGGAACTGTCCCAGGGGCTGGACTTCACTCCT       1478

R  G  S  V  L  A  K  F  T  H  L  Q  H  E  E  F  T  Y  V          486
CGTGGCAGCGTGCTCGCCAAGTTCACGCACTTGCAGCACGAGGAATTCACTTATGTT       1535

I  E  V  N  N  T  S  G  Q  S  K  M  G  T  F  R  V  F  M          505
ATCGAGGTAAACAACACGAGCGGGCAGTCGAAGATGGGCACGTTCCGCGTGTTCATG       1592

A  P  K  T  D  E  R  G  Q  P  L  A  F  E  D  Q  R  R  L          524
GCGCCGAAGACCGACGAGCGCGGGCAGCCGCTCGCCTTCGAGGACCAGAGGCGGCTC       1649

M  I  E  L  D  K  F  T  R  G  L  K  P  G  N  N  T  I  R          543
ATGATCGAACTGGACAAGTTCACTAGGGGACTGAAACCTGGCAACAACACAATCCGC       1706

Q  R  S  L  D  S  S  V  T  I  P  F  E  R  T  F  R  N  Q          562
CAACGAAGTTTGGACTCCTCAGTGACTATTCCCTTCGAGAGGACATTCCGCAACCAG       1763

A  N  R  P  G  D  P  G  S  A  T  A  A  E  F  D  F  C  G          581
GCGAACCGGCCTGGAGACCCGGGCTCCGCCACCGCTGCAGAGTTTGACTTCTGCGGC       1820

C  G  W  P  H  H  M  L  I  P  K  G  T  E  Q  G  Y  P  V          600
TGCGGCTGGCCGCACCACATGCTGATACCCAAGGGAACGGAGCAGGGCTACCCTGTC       1877

V  L  Y  V  M  V  S  D  W  N  A  D  K  I  E  Q  D  T  V          619
GTTTTATACGTTATGGTTTCTGACTGGAATGCTGATAAAATCGAACAAGATACGGTC       1934

G  A  C  N  D  A  A  S  Y  C  G  L  R  D  R  K  Y  P  D          638
GGTGCGTGCAACGACGCCGCCTCGTACTGCGGTCTCCGGGACCGCAAGTACCCAGAC       1991

K  R  H  M  G  F  P  F  D  R  R  S  E  A  R  N  L  T  D          657
AAGAGGCACATGGGCTTCCCCTTCGACCGGCGCTCCGAGGCCCGTAACCTCACCGAC       2048
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F  L  K  P  N  M  A  T  R  D  C  T  I  K  F  T  D  A  I          676
TTCCTTAAACCCAACATGGCGACCAGGGACTGCACTATCAAGTTCACTGACGCTATC        2105

R  E  G  T  Q  R  Q  *                                             683
AGGGAGGGGACACAACGCCAATAAATGGAGACAGGGCTGACCGTAGCATAATCTACA        2162
GATTGGAATTAAAACCGTTAGCATCCTTTGAAAGAAGTGTTCTCCTAGCGAAACTGA        2219
ACTTTGTCACCAAAGAAACATGGCTCGAAAAGATGCAAACAAATTTGAGCCTCCCAT        2276
ACAAACATAAGATATGGAAATTTCGAAATTTTATTATCGTTTTGATTGTTTGATTCT        2333
CATAATTTTATTTTTACCATGTTTTATACCACAGATACATTCTTAGTCTTACGTTGC        2390
TAACGATTTTGATTCCACCCTATAAATTTTTTTATAGTGCCCTTGAAACTAACTTCC        2447
TGGATTTCCCCTTGAATTCCTTAGGAATGGCAACGCATGGGGGGTATAATGGGCGAA        2504
ACAGTATACTTTGTTACGTCCGTGGTACTGCTCATGTCTACAGGCGAAGGCTACCAC        2561
TTTCCATCAAGTGGGCCATCAGCTTGTTTGCCATTCTAAGTTGTATAAAAAAAAACT        2618
TTTTACTTAAGTATTAATTGATCTTTCTAAAATACGTGGCACCTGATGCCCTTTGTA        2675
TGCTCCGCTCCTTGAACTCGGTGCTGATCAAGAATGGCAACACGTGAGAGAATTACC        2732
ATGGATTATGGACACAGTGCATTCTATGACGTAGTGCTGTTCATGTCTACAGGCGAA        2789
GGTTACCACTTTCCATCAGGTGAACCATCAGTTTATTCACCATTCCAAGTTGACTAC        2846
GAACAATTTTTGGGTAAAGATTTTGGCTGTGTATAACGGACATTGATTTTACTATTA        2903
TTTGAATCCTGCTCATGTTAATTTTAATAGTTTTTAAGTTTAGTTATACAACAAATG        2960
GGTCAATTGTACGTAGCTACGACTATGTGCATATGTATGTATATATGTAAATATTGC        3017
TAAATTCATAAGACTGaataaaTATAGCCTTTCaaaaaaaaaaaaaaaaaaaa           3069
```

Fig. 30:

Nucleotide and deduced amino acid sequences of *G. mellonella* ProPO1. Amino acid residues, shown in one-letter code, are aligned with the first nucleotide of each codon. An asterisk (*) marks the stop codon. The predicted activation cleavage site is marked with an arrow. The putative copper-binding domains are underlined, and the copper-binding histidine residues at positions conserved in arthropod hemocyanins are in bold. AATAAA and poly A are in small letters. Peptides which are identical to the preliminary peptide sequences from *G. mellonella* (Kopacek *et al.*, 1995) are boxed. The sequence was submitted to GenBank under accession number AF336289.

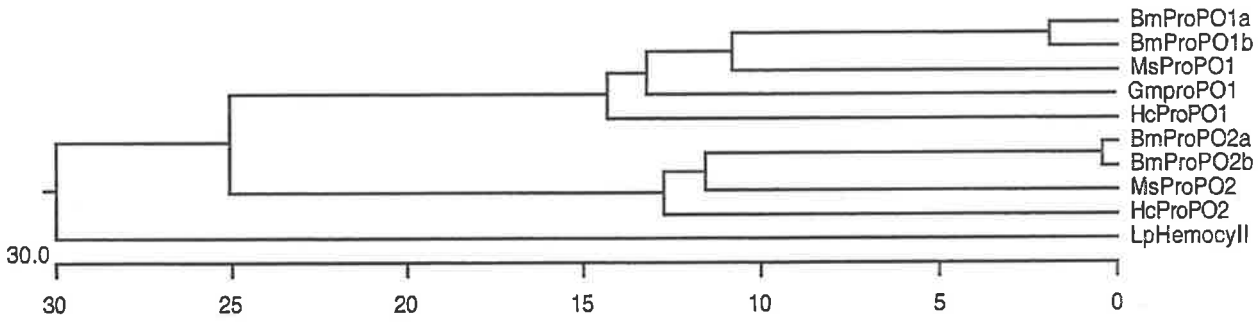

Fig. 31:

Phologenetic tree of prophenoloxidases 1 and 2 from several insect species. The phylogenetic tree was constructed by using ClustalW method PAM250 residue weight table. The sequences extracted from GenBanks and their accession numbers are as follow: BmProPO1a, *Bombyx mori* proPO1, D49370; BmProPO1b, *Bombyx mori,* AF178462; MsProPO1, *Manduca sexta* proPO1, AF003253; HcProPO1, *Hyphantria cunea* proPO1, U86875; BmProPO2a, *Bombyx mori* proPO2, D49371; BmProPO2b, *Bombyx mori* proPO2, AF178461; MsProPO2, *Manduca sexta* proPO2, L42556; HcProPO2, *Hyphantria cunea* proPO2, AF020391; LpHemocyII, *Limulus polyphemus* hemocyanin subunit II, BHHC2A.

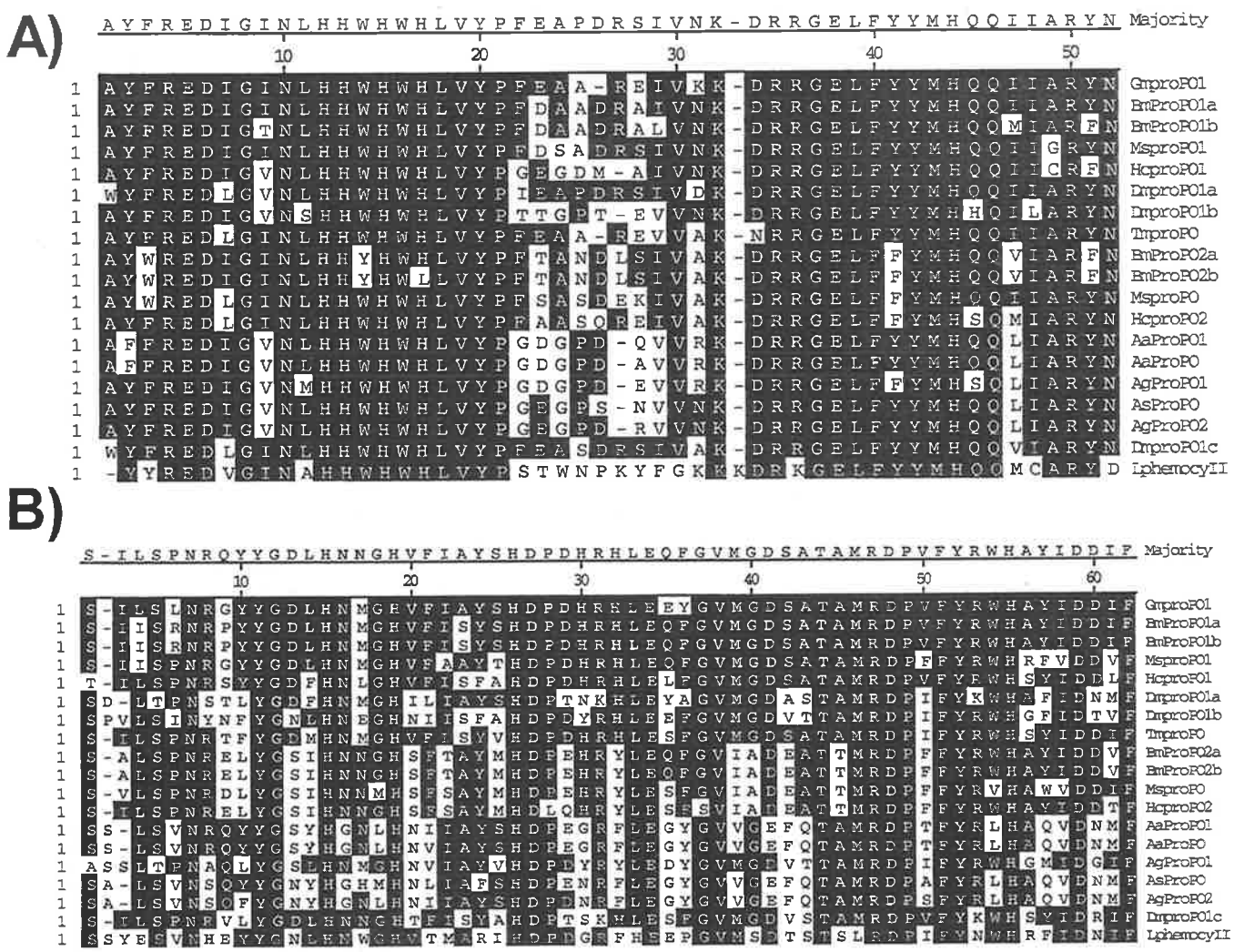

Fig. 32:

Phylogenetic comparison were made by using Clustal method with PAM250 residue weight table. GmproPO1 was compared with other proPOs from other insects and a hemocyanin from horseshoe crab. The sequences extracted from GenBank and their accession number are as follows: BmproPO1a, *Bombyx mori*, D49370; BmproPO1b, *B. mori,* AF178462; MsproPO1, *Manduca sexta*, AF003253; HcproPO1, *Hyphantria cunea*, U86875; *DmproPO1a, Drosophila melanogaster*, AE003459; DmproPO1b, *D. melanogaster*, AE003835; TmproPO, *Tenebrio molitor*, AB020738; BmproPO2a, *B. mori*, D49371; BmproPO2b, *B. mori*, AF178461; MsproPO2, *M. sexta*, L42556; HcproPO2, *H. cunea*, AF020391; AaproPO, *Aedes aegypti*, AF292114; AaproPO1, *Aedes aegypti*, AF292113; AgproPO1, *Anopheles gambiae*, AF004915; AgproPO2, *Anopheles gambiae*, AF004916; AsproPO, *Anopheles stephensi*, AF062034; DmproPO1c, *D. melangaster*, D45835; LpHemocyII, Atlantic horseshoe crab *Limulus polyphemus* hemocyanin subunit II, BHHC2A.

A) CuA region of GmproPO1 sequence. **B)** CuB region of proPO1 sequences.

```
                                                        M  Y                2
      CAGTCAGTCATCAGACAACGCAGTTAATACAACATCGCAATCAAAATGTAC                  51

R  L  I  I  L  L  T  L  L  I  S  I  E  S  H  R  H  C  G                  21

CGTCTTATAATTCTTCTAACTTTACTAATATCTATCGAGTCACATCGCCACTGCGGG              108

R  H  T  S  L  C  H  R  R  H  H  D  R  H  M  R  N  H  E                  40
AGGCACACATCGCTATGCCACAGGAGACACCATGACCGTCACATGAGAAACCATGAG              165

R  S  F  D  S  L  A  R  S  V  I  S  L  D  R  S  L  N  E                  59
AGATCCTTCGACAGTTTAGCAAGAAGCGTCATCTCCCTGGATCGGAGTCTAAACGAG              222

L  C  T  D  N  N  N  N  R  S  K  E  I  F  K  T  D  E  Y                  78
TTATGTACCGACAACAACAATAACAGAAGCAAAGAGATATTCAAAACAGACGAGTAT              279

T  I  Q  V  S  L  E  D  Y  A  K  E  S  V  V  V  K  I  K                  97
ACTATACAAGTGTCACTGGAAGATTACGCAAAAGAAAGCGTTGTGGTCAAAATTAAA              336

Y  R  V  M  Y  I  Y  A  E  K  K  D  E  S  K  S  N  Y  F                 116
TATCGCGTTATGTACATATACGCAGAGAAAAAGGACGAGTCGAAATCCAATTATTTC              393

E  L  R  V  L  P  E  I  V  D  V  H  K  A  T  W  N  Y  N                 135
GAGCTTAGAGTTTTGCCGGAAATTGTTGATGTGCATAAAGCCACTTGGAATTACAAC              450

D  G  D  L  E  I  I  I  Q  Y  K  M  N  R  V  D  E  Y  I                 154
GATGGTGACTTGGAAATAATAATTCAGTACAAAATGAATAGAGTTGATGAATATATA              507

R  N  C  E  Q  D  I  D  S  S  V  I  V  V  E  N  Y  E  P                 173
AGAAATTGTGAGCAAGATATTGATAGCTCGGTGATTGTAGTTGAAAATTATGAACCG              564

V  I  D  L  R  K  G  I  D  R  E  D  T  T  E  V  Y  P  D                 192
GTTATCGATTTAAGAAAAGGAATCGATCGAGAAGACACAACTGAAGTGTACCCAGAT              621

R  V  R  E  E  S  D  S  H  T  D  G  N  D  D  G  I  N  N                 211
CGTGTTCGTGAGGAATCTGATTCACACACAGATGGTAATGACGATGGTATAAATAAT              678

I  N  N  V  P  E  D  K  N  K  F  V  K  T  A  S  E  T  V                 230
ATTAATAATGTGCCTGAAGATAAGAATAAATTTGTTAAAACTGCGAGCGAGACAGTT              735

Y  R  R  V  T  I  N  L  V  *                                            249
TACAGACGTGTTACTATTAATTTGGTATAATGCTTTTACTTCATTAGTGATAACAAT              792
GCTAACGTAATTATTTTGTTTCaataaaTACTTAAAATATGTAAATTAATTACAGT               849
TTCTTTGTTTGTTACTGTCTGTGGAATGAAAGTAGTCTGAAATGAATATCCTATTGT              906
CCTATTAATATACTAAAATGTGAAAATTTGTAACGATAGATGATGGATGAGGATGCT              963
GTCCATCCAGGACAGGTGGATGAACGGATGGATGTTTTATACTCTTTCACGCAAAAA              1020
CTATAAAACAGATTAAAAATATATACAATAATTTAGaaaaaaaaaaaaaaaaaaaaa              1077
aaaaaa                                                                 1083
```

Fig. 33:

Galleria α-crystallin cDNA and deduced amino acid sequences. An asterisk marks the stop codon and the start and stop codon are indicated in bold letters. Signal peptides are underlined. AATAAA and poly A are in small letters. The potential N-glycolysation site is dotted underlined. The Casein kinase phosphorylation sites are double underlined. The sequence was submitted to GenBank under accession number AY040539.

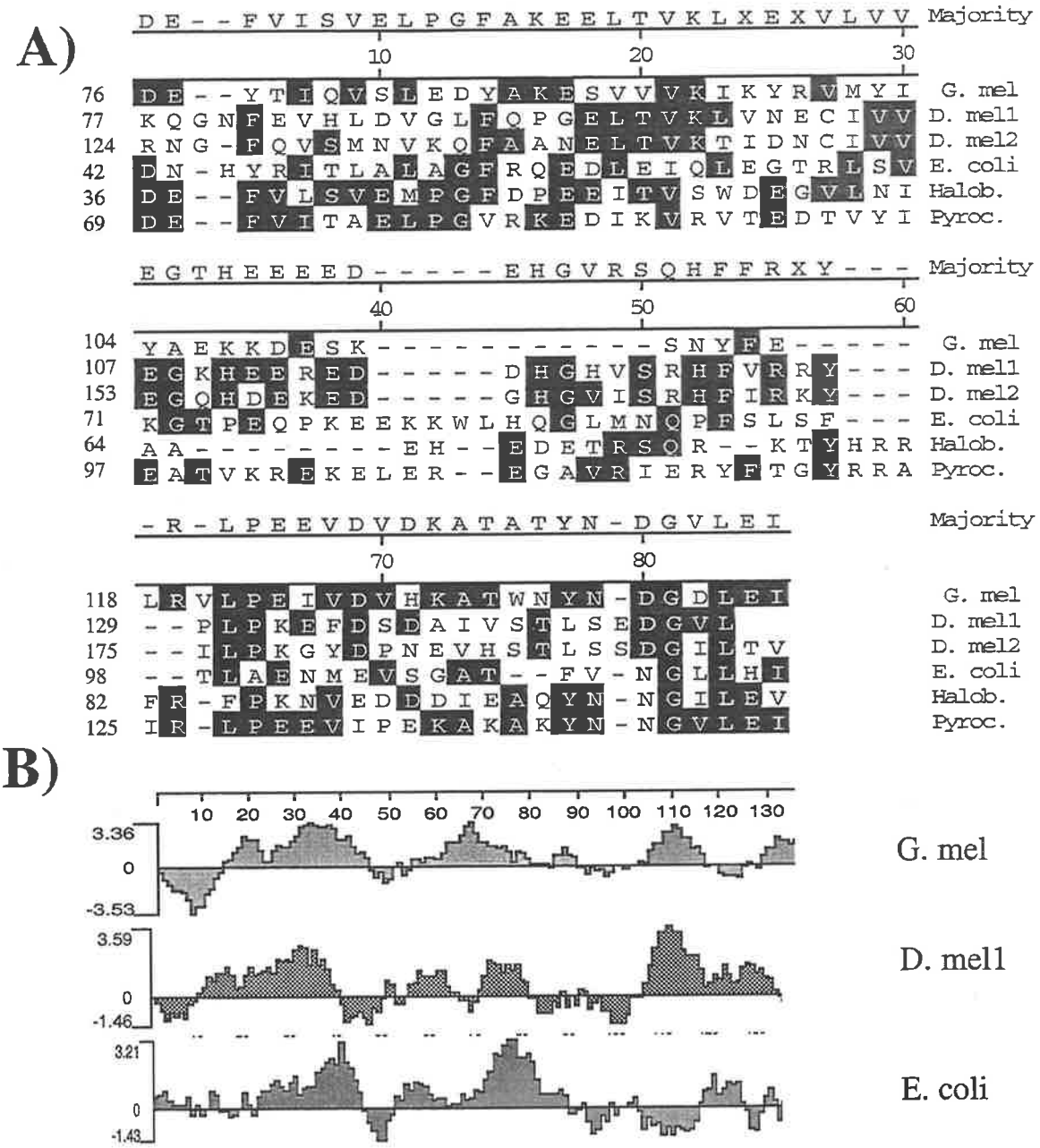

Fig. 34:

A distant *Galleria* member of the α-crys/SHSP family.

A) The sequence of the *Galleria* member of the family is aligned with two *Drosophila* members, two archaea and one bacterial sequence. Sequences are extracted from GenBank and their accession number are: G. mel, *G. mellonella*, AY040539; D. mel1 and D. mel2, *D. melanogaster*, annotated genes CG4190 and CG 4167; Halob., *Halobacterium sp.*, AE005154; Pyroc., *Pyrococcus furiosus*, AF256212 and E. coli, *Escherichia coli*, M94104.

B) hydrophilicity plot of the *Galleria*, one *Drosophila* and the *E.coli* member of the family. The first 135 amino acids from each of the open reading frames were used for comparison. Positive values indicate high hydrophilicity.

```
                ATCCACACTCGTGCATACGCCTCACTGTACCTTAGTTTCTAAATTTAAAAC      51

                    M  P  I  D  L  Y  Y  V  P  G  S  A  P  C  R       15
ATCAAATCCACAATGCCCATCGATCTCTACTACGTGCCTGGCTCCGCGCCGTGCAGG           108

A  V  L  L  T  A  R  A  L  N  L  N  L  N  L  K  L  V  D             34
GCTGTCTTGCTCACAGCGAGGGCTCTCAACCTAAATCTTAACTTGAAACTGGTTGAC           165

L  H  H  G  E  H  L  K  P  E  Y  L  K  I  N  P  Q  H  T             53
TTGCATCACGGCGAGCATTTGAAGCCTGAGTATCTGAAGATCAACCCCCAGCACACA           222

V  P  T  L  V  D  D  G  F  S  L  W  E  S  R  A  I  I  T             72
GTCCCCACACTGGTTGACGATGGATTCTCCCTATGGGAATCCCGTGCTATCATAACC           279

Y  L  V  N  K  Y  G  K  G  S  T  L  Y  P  E  D  P  K  A             91
TACCTGGTCAACAAATATGGCAAGGGCTCCACGCTGTACCCTGAGGACCCGAAGGCG           336

R  A  L  V  D  Q  R  L  Y  F  D  I  G  T  L  Y  Q  R  F            110
CGTGCGCTCGTTGACCAGAGGCTGTACTTCGATATTGGCACCCTGTACCAGAGATTT           393

A  D  Y  F  Y  P  Q  V  F  G  G  A  P  A  D  K  E  K  A            129
GCTGATTATTTCTATCCACAAGTTTTCGGCGGTGCCCCGGCCGACAAAGAGAAGGCC           450

S  K  I  E  D  S  L  K  L  L  D  T  F  L  E  G  Q  K  Y            148
TCCAAAATCGAAGACTCTCTTAAGCTCCTGGACACATTCCTTGAGGGCCAGAAATAC           507

V  A  G  P  N  L  T  V  A  D  L  S  L  I  A  S  V  S  S            167
GTGGCAGGCCCCAACCTCACTGTCGCCGATCTGAGTCTCATCGCGAGCGTATCCAGC           564

F  E  A  S  D  I  D  F  K  K  Y  P  N  V  K  R  W  Y  E            186
TTCGAGGCATCCGATATTGACTTCAAGAAATACCCTAATGTTAAGAGATGGTACGAG           621

T  V  R  S  T  A  P  G  Y  Q  E  A  N  E  K  G  L  E  A            205
ACAGTGAGGAGCACCGCACCAGGATACCAAGAGGCTAACGAGAAAGGACTGGAGGCG           678

F  K  G  F  V  N  S  M  L  K  K  *                                  216
TTCAAGGGGTTCGTCAACAGCATGCTCAAGAAGTGAACGAGGGCTGAGAAGCTTTTA           735
AAATTTTGTTACGAAATTTTACACTTTACGAATAAGTAAAGCCACCGTATTTTCAAA           792
TTTAACACCGTTTTCCATGTTACTTATTAATATAAGTTTAGTGATAAGGCACAaata           849
aaTTATATTGTATTaaaaaaaaaaaaaaaa                                      880
```

Fig. 35:

Nucleotide and deduced amino acid sequences of *G. mellonella* GST. Amino acid residues, shown in one letter codon, are aligned with the first nucleotide of each codon. An asterisk (*) marks the stop codon. The start codon ATG and the stop codon TGA are boxed. AATAAA and poly A are in small letters. The sequence was submitted into GenBank under accession number AF336288.

A)

```
GmGST     MPIDLYYVPGSAPCRAVLLTARALNLNLNLKLVDLHHGEHLKPEYLKINPQH
          M +D YY+PGS+PCR+V++TA+A+ + LN KL++L  GEHLKP +LKINPQH
DmGST     MV DFYYLPGSSPCRSVIMTAKAVGVELNKKLLNLQAGEHLKPEFLKINPQH

GmGST     TVPTLVDDGFSLWESRAIITYLVNKYGKGSTLYPEDPKARALVDQRLYFDIG
          T+ TLVD+GF LWESRAI  YLV KYGK + LYP+ PK RA+++QRLYFD G
DmGST     TIPTLVDNGFALWESRAIQVYLVEKYGKTDSLYPKCPKKRAVINQRLYFDMG

GmGST     TLYQRFADYFYPQVFGGAPADKEKASKIEDSLKLLDTFLEGQKYVAGPNLTV
          TLYQ+FA+Y+YPQVF+ APAD E   KIE+++ L+ TFLEGQ Y AG  LTV
DmGST     TLYQSFANYYYPQVFAKAPADPEAFKKIEAAFEFLNTFLEGQDYAAGDSLTV

GmGST     ADLSLIASVSSFEASDIDFKKYPNVKRWYETVRSTAPGYQEANEKGLEAFK
          AD +L A+VS+FE + + + KY NV RWYE +   PG++E N  G   FK
DmGST     ADIALVATVSTFEVAKFEISKYANVNRWYENAKKVTPGW EENWAGCLEFK
```

B)

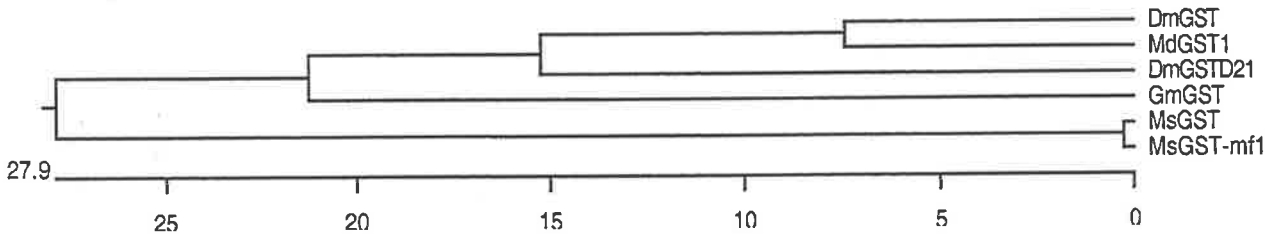

Fig. 36:

A) Sequence alignment between *G. mellonella* GST(GmGST) and a GST from *D. melanogaster* (DmGST). Their accession number are GmGST, AF336288; DmGST, P20432. The sequence alignment covers the complete length of both protein sequences.

B) Phylogenentic tree of GSTs. MdGST1, *Musca domestica* GST, P28338; DmGSTD21, *D. melanogaster* GST, Q9VG98; MsGST, *M. sexta* GST, AAF16718; MsGST-mf1, *M. sexta* GST-msolf1, AAD28279.

```
                                  M  R  F  V  F  V  W  L  A  I  I  A          12
      GAAGTGACGAGGTCAAAATGCGTTTCGTATTTGTGTGGCTAGCTATCATCGCT                  53

A  V  A  A  V  P  R  N  P  Q  R  I  V  G  G  S  V  T  N                     31
GCTGTTGCAGCCGTACCTAGGAACCCGCAGCGTATCGTAGGTGGTTCAGTTACGAAC                 110

I  N  S  Y  P  E  I  A  A  L  L  Y  S  W  N  G  A  T  Y                     50
ATCAACAGCTACCCAGAGATCGCTGCACTCTTGTACTCATGGAATGGTGCCACCTAC                 167

S  Q  A  C  G  G  S  I  L  N  N  R  S  I  L  S  A  A  H                     69
AGCCAAGCCTGCGGTGGATCTATCCTCAACAACAGATCCATCCTGTCTGCTGCTCAC                 224

C  F  V  G  D  P  A  N  R  W  R  V  R  V  G  S  T  N  A                     88
TGCTTTGTAGGTGACCCAGCCAACAGATGGCGCGTTCGCGTTGGCTCTACCAACGCT                 281

N  S  G  G  V  V  Y  N  V  A  S  I  I  N  H  G  S  Y  N                    107
AACAGCGGCGGTGTTGTTTACAATGTCGCTTCCATCATCAACCACGGCAGCTACAAC                 338

S  W  T  N  D  N  D  V  A  I  L  R  L  S  S  N  L  A  Y                    126
AGCTGGACCAACGACAATGACGTCGCCATCTTGCGTCTCTCCTCCAACCTTGCGTAC                 395

T  N  V  I  Q  P  A  S  I  A  G  S  N  Y  N  L  A  D  N                    145
ACCAATGTCATTCAGCCTGCCAGCATTGCTGGGTCCAATTACAACCTCGCTGACAAC                 452

Q  V  V  Y  A  A  G  W  G  A  I  S  V  G  G  P  G  S  E                    164
CAAGTCGTTTATGCCGCTGGATGGGGTGCCATATCTGTTGGCGGTCCAGGGTCAGAA                 509

Q  L  R  H  V  Q  I  W  T  V  N  Q  A  T  C  R  N  R  Y                    183
CAGCTCCGTCACGTACAAATCTGGACTGTAAACCAAGCGACATGCAGAAACCGTTAC                 566

A  E  L  G  L  T  V  T  D  N  M  L  C  S  G  W  L  D  V                    202
GCTGAGCTCGGACTTACTGTGACCGACAACATGTTGTGCTCCGGTTGGTTGGATGTC                 623

G  G  R  D  Q  C  Q  G  D  S  G  G  P  L  Y  H  N  R  I                    221
GGCGGTCGCGACCAGTGCCAGGGTGACTCTGGCGGTCCCCTCTACCACAACAGAATC                 680

V  V  G  I  C  S  W  G  T  Q  C  A  L  A  R  Y  P  G  V                    240
GTCGTCGGTATTTGCTCTTGGGGTACACAGTGCGCTCTCGCTCGCTACCCTGGTGTC                 737

N  A  R  V  S  R  F  T  T  W  I  Q  A  N  A  *                            255
AACGCTCGTGTATCTCGGTTCACAACCTGGATCCAGGCCAATGCTTAAATGGTGCCA                 794
TTGAAATATCTGCCCGGTCTATGTTCATTGTGATAACaataaaTATAGACTAAACAG                 851
ATTACTTATTATTCAATACTTTTTGGAATTATTAATTAATTAGTCATTAATTAATTA                 908
ATAATTAATTATTATTTTACAATGTTTTTTGTAAGAACAATTAAATATACAGATATT                 965
CTGAATTATaaaaaaaaaaaaaaaaaaa                                              993
```

Fig. 37:

Nucleotide and deduced amino acid sequences of *G. mellonella* trypsin-like clone. ATG start and TAA stop codon are in bold. A signal peptide is underlined and AATAAA and poly A are in small letters. The conserved catalytic residues are boxed. The sequence was submitted to GenBank under accession number AY040819.

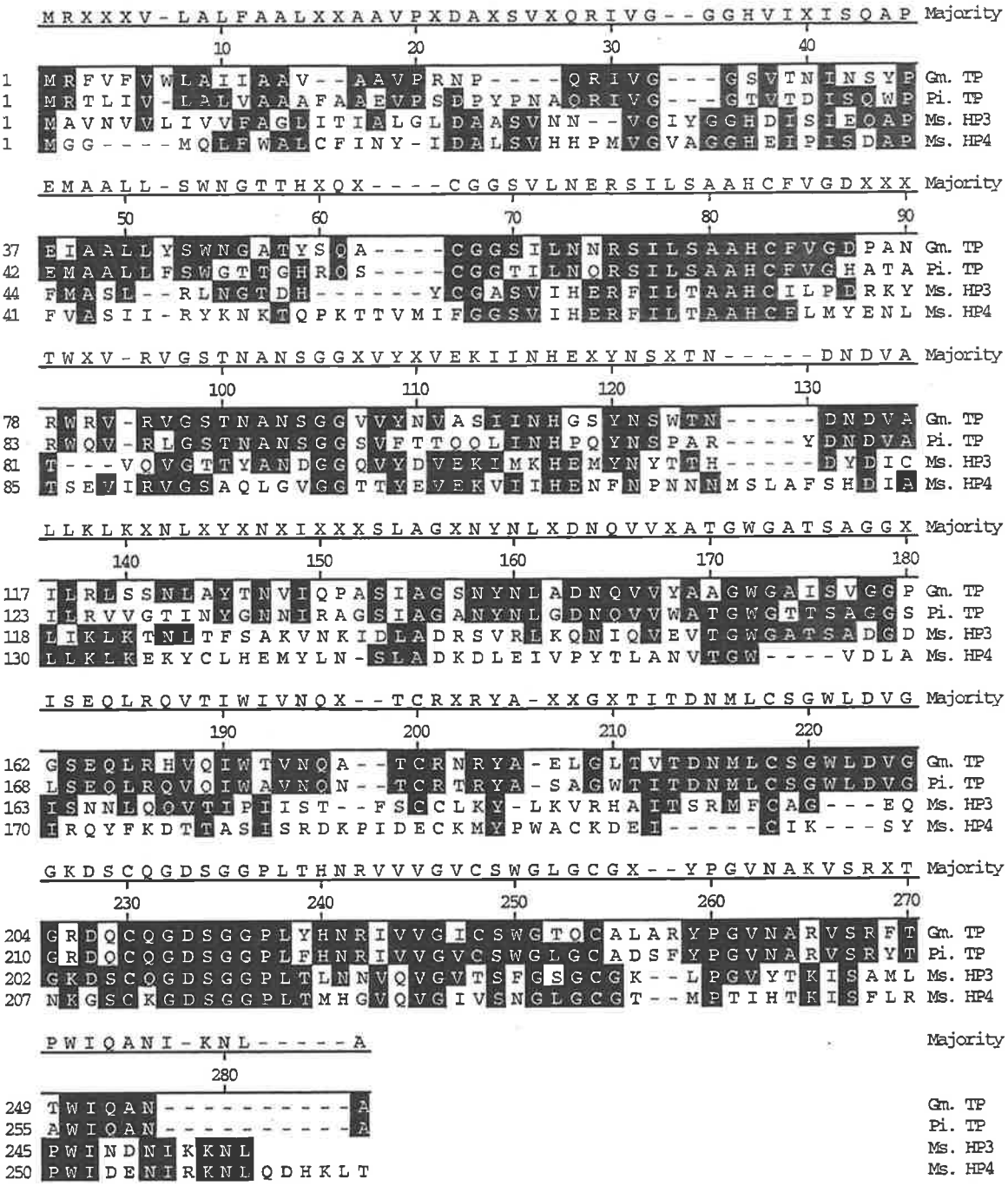

Fig. 38:

Amino acid sequence comparison of trypsin-like serine proteases. Sequences were extracted from genBank and their accession numbers are as following: Gm TP, *Galleria* trypsin-like protein, AY040819; Pi. TP, *Plodia interpunctella* trypsin, Af064525; Ms. HP3, *M. sexta* hemocytes proteinases, AF017665; Ms. HP4, AF017666.

```
      GTTGCACGAGATAGTTTGACCCAGAACGTTAGTACATGGTAGTAGTTCTGAA     52

                 M  S  L  E  E  K  Y  N  Y  E  S  T  E  K        14
GATATTTCAAGAGAAATGAGCTTAGAAGAAAAATACAATTATGAAAGTACTGAAAAA    109

V  D  K  K  P  I  T  K  D  L  K  E  E  K  P  P  V  P  F      33
GTAGATAAAAAACCTATTACGAAAGACTTGAAGGAAGAAAAGCCTCCTGTGCCTTTT    166

D  N  F  S  S  C  L  D  S  M  T  N  I  S  S  Q  E  R  N      52
GATAATTTTAGCAGTTGCCTTGATTCTATGACTAATATAAGTAGTCAAGAAAGAAAT    223

G  Y  E  S  E  C  S  R  G  D  L  S  I  H  S  T  N  T  V      71
GGATATGAAAGCGAATGTAGTAGAGGCGATCTCTCTATTCATTCAACAAATACAGTA    280

D  V  S  K  F  S  T  N  D  K  H  K  N  I  V  S  S  K  E      90
GATGTATCTAAATTTTCGACAAATGATAAACATAAAAATATAGTGTCATCCAAAGAA    337

V  L  L  S  E  P  L  D  S  V  Y  L  D  T  E  A  N  S  S      109
GTATTATTATCTGAACCACTAGATTCTGTTTATTTAGATACTGAGGCAAATAGTTCC    394

C  S  D  E  C  S  S  S  P  K  A  K  D  S  A  V  R  L  L      128
TGTTCAGATGAATGCTCTTCTAGCCCAAAGGCCAAAGATAGTGCAGTCAGGCTATTA    451

E  S  I  H  K  A  F  N  E  E  P  V  Q  Q  G  I  T  T  E      147
GAATCAATACACAAAGCATTTAATGAAGAACCGGTTCAACAAGGCATTACAACTGAA    508

N  L  E  A  I  G  A  R  S  E  F  D  R  V  S  T  P  P  T      166
AATCTAGAAGCAATAGGCGCACGATCAGAATTTGATAGAGTTTCAACACCACCGACA    565

V  P  V  S  P  L  P  K  T  P  G  S  F  Q  D  V  K  I  S      185
GTTCCTGTTAGTCCCTTACCAAAAACACCTGGGAGCTTTCAGGATGTAAAAATAAGT    622

E  G  V  Q  S  E  V  T  Y  D  K  S  D  G  C  E  E  T  I      204
GAAGGAGTACAAAGTGAAGTAACATATGATAAATCAGACGGATGTGAAGAAACTATT    679

T  K  V  V  H  V  G  E  D  V  L  T  Q  K  I  S  T  S  T      223
ACTAAAGTCGTACATGTCGGAGAAGATGTATTAACACAAAAAATTTCAACATCTACA    736

E  K  V  T  K  P  I  K  T  M  T  D  I  N  G  D  Q  D  L      242
GAAAAAGTAACTAAACCTATTAAAACAATGACTGATATTAATGGTGACCAGGATTTA    793

I  G  L  M  Q  T  M  G  K  I  K  T  E  T  D  T  I  T  K      261
ATAGGTCTTATGCAGACAATGGGGAAAATAAAAACCGAAACGGACACTATAACAAAA    850

I  I  K  E  G  E  N  V  V  T  Q  T  I  T  T  V  T  T  K      280
ATAATTAAAGAAGGTGAAAATGTAGTTACTCAAACTATAACAACAGTTACAACAAAA    907

E  V  I  S  R  D  D  G  T  P  Q  N  I  K  T  T  I  E  T      299
GAAGTA TATCAAGGGATGATGGCACACCACAAAATATTAAGACAACCATTGAAACT    964

T  T  L  S  K  G  P  D  G  S  T  T  T  T  K  D  T  Q  T      318
ACTACTTTAAGTAAAGGTCCAGATGGTTCTACTACTACCACTAAAGATACTCAAACC    1021

L  L  S  E  C  S  S  S  L  R  S  T  S  Q  M  D  L  Y  S      337
TTGTTATCAGAATGCTCTTCTAGTCTGCGATCTACCTCACAAATGGATTTATATAGC    1078

K  D  T  R  L  D  K  D  V  F  E  S  S  E  T  K  S  D  T      356
AAGGATACTAGATTAGATAAAGACGTTTTTGAATCAAGTGAAACAAAATCAGATACT    1135
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Q V S D A T D K T N E Q E S T I D T H          375
CAAGTTAGTGATGCCACTGATAAAACAAATGAGCAAGAGTCTACCATTGATACTCAC     1192

S K I A Y V T E M D D S D V E D A I I          394
AGCAAAATTGCATATGTAACAGAAATGGATGATTCAGACGTAGAGGATGCAATAATA     1247

D T D V S K R I I K G N N I D I I E T          413
GATACAGATGTTAGTAAGAGAATAATTAAAGGAAATAATATTGATATAATAGAAACT     1306

V T T I T K K E T L N V S D D K K I I          432
GTAACTACCATAACAAAAAAAGAAACATTGAACGTAAGTGACGATAAAAAAATTATA     1363

R T T I E T N T V K E Y P D G S K D V          451
AGAACTACTATCGAAACAAATACTGTTAAAGAATATCCGGATGGTTCTAAAGATGTT     1420

Q K N V E V K T E E L T V D Y C S N L          470
CAAAAGAATGTTGAAGTCAAAACCGAAGAATTAACAGTTGACTACTGTAGTAATCTT     1477

E N I L S N F T V Y G E P E E I V T T          489
GAAAATATATTAAGTAATTTCACAGTCTACGGTGAACCAGAAGAAATCGTCACGACA     1534

K T E D I L Q E N I L I K R T I L T E          508
AAAACGGAAGATATTTTACAAGAAAATATTTTAATTAAACGAACTATTTTAACTGAA     1591

I V K T K Y A D A N Q R P T K I K I V          527
ATTGTGAAAACTAAGTATGCTGACGCAAACCAAAGACCTACGAAAATCAAAATAGTT     1648

T T I T T T D N Y P D G S S S T K V D          546
ACTACTATAACCACCACCGATAATTATCCAGATGGTTCATCTAGTACTAAAGTTGAT     1705

T S T S L M D I D V E N E K E T S E L          565
ACTAGTACTTCACTAATGGATATTGATGTAGAAAACGAGAAAGAAACATCGGAACTA     1762

I D F T E I E D K S T K V D K Y E K N          584
ATAGACTTTACAGAAATTGAAGATAAATCAACGAAAGTAGATAAATATGAGAAAAAT     1819

I F I D G K S V L Q I I T I T T I K E          603
ATTTTTATCGATGGTAAAAGTGTATTGCAAATTATAACAATAACAACGATAAAAGAA     1876

I L T T K D R
ATATTGACAACTAAAGATCGGAAAAAAAAAAAAAAAAAAAAAAAAAAAA             1925
```

Fig. 39:

Nucleotide and deducted amino acid partial sequences of *Galleri*a mucin-like gene. ATG start codon is located in position 68 (bold). This sequence goes straight to the poly A tail and no stop codon was found. The residues, shown high O-glycosylation potential, are in bold. Potential N-glycosylation sites are underlined. Cell attachment sequence, RGD, is double underlined. The sequence was submitted to GenBank under accession number AY040818.

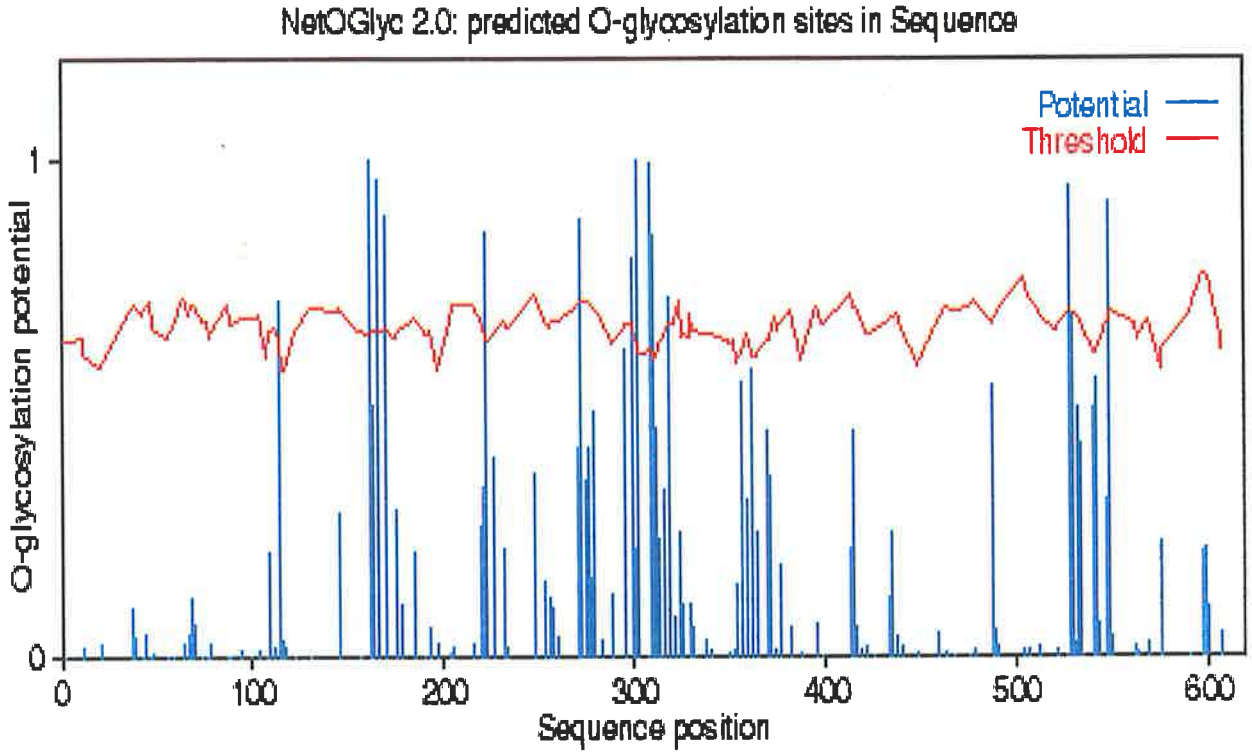

Fig. 40:

O-glycosylation prediction of mucin-like clone.

The O-glycosylation potential of some amino acids are higher than the threshold. A number of Thr and Ser are close to the threshold as well. This figure shows that this gene is highly O-glycosylated and belongs to mucin family. Therefore, it was named *Galleria* mucin-like protein.

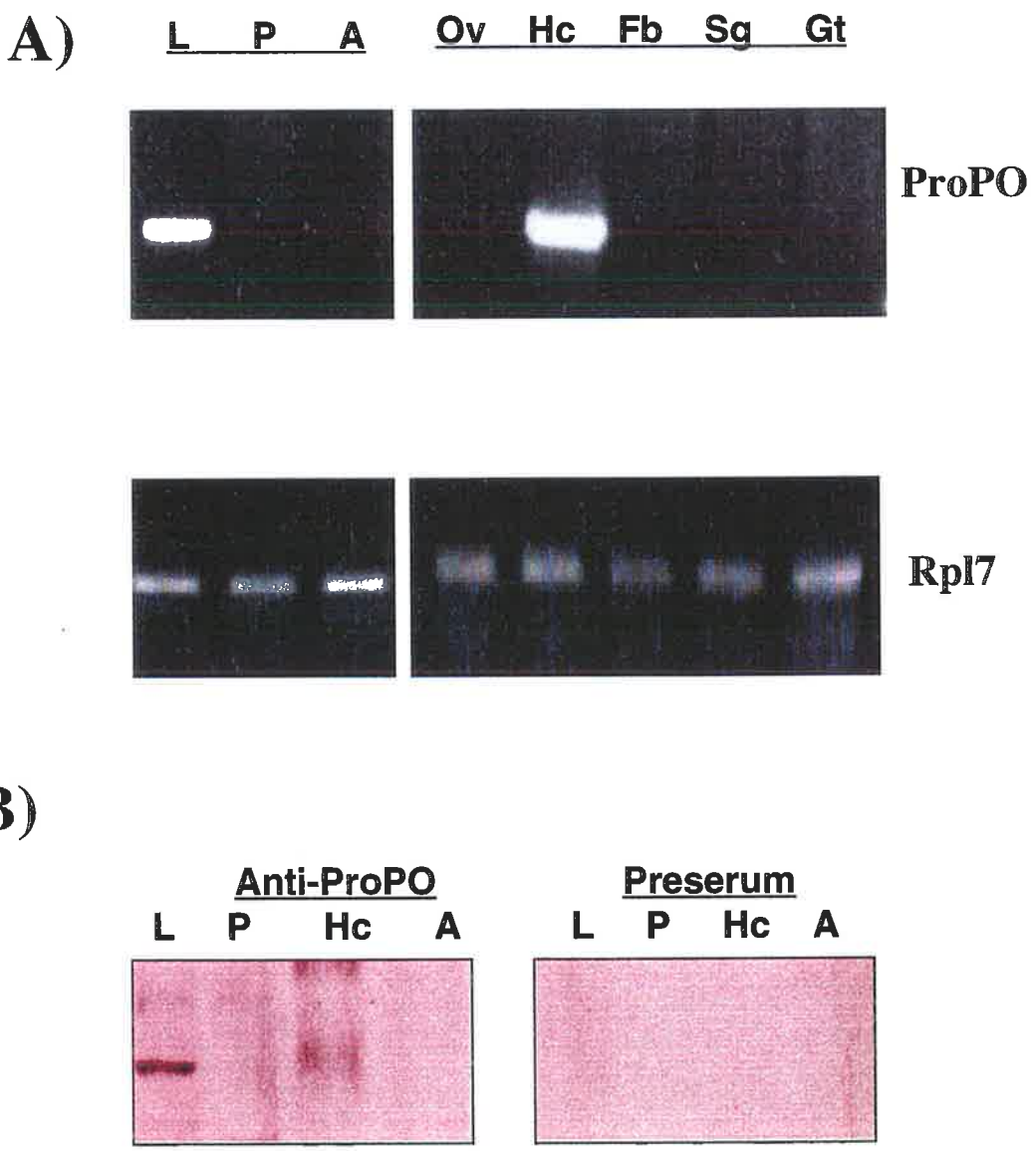

Fig. 41:

Expression of prophenoloxidase (ProPO) in larval hemocytes.

A) Total RNA were extracted from different developmental stages and different tissues, RT-PCR were performed using GmproPO1 specific primers and ribosomal protein L7 as an internal control.

B) Western blots were conducted with preserum and anti-ProPO antisera.

L, larvae; P, pupae; A, adults; Ov, ovaries; Hc, hemocytes; Fb, fat bodies; Sg, silk glands; Gt, guts.

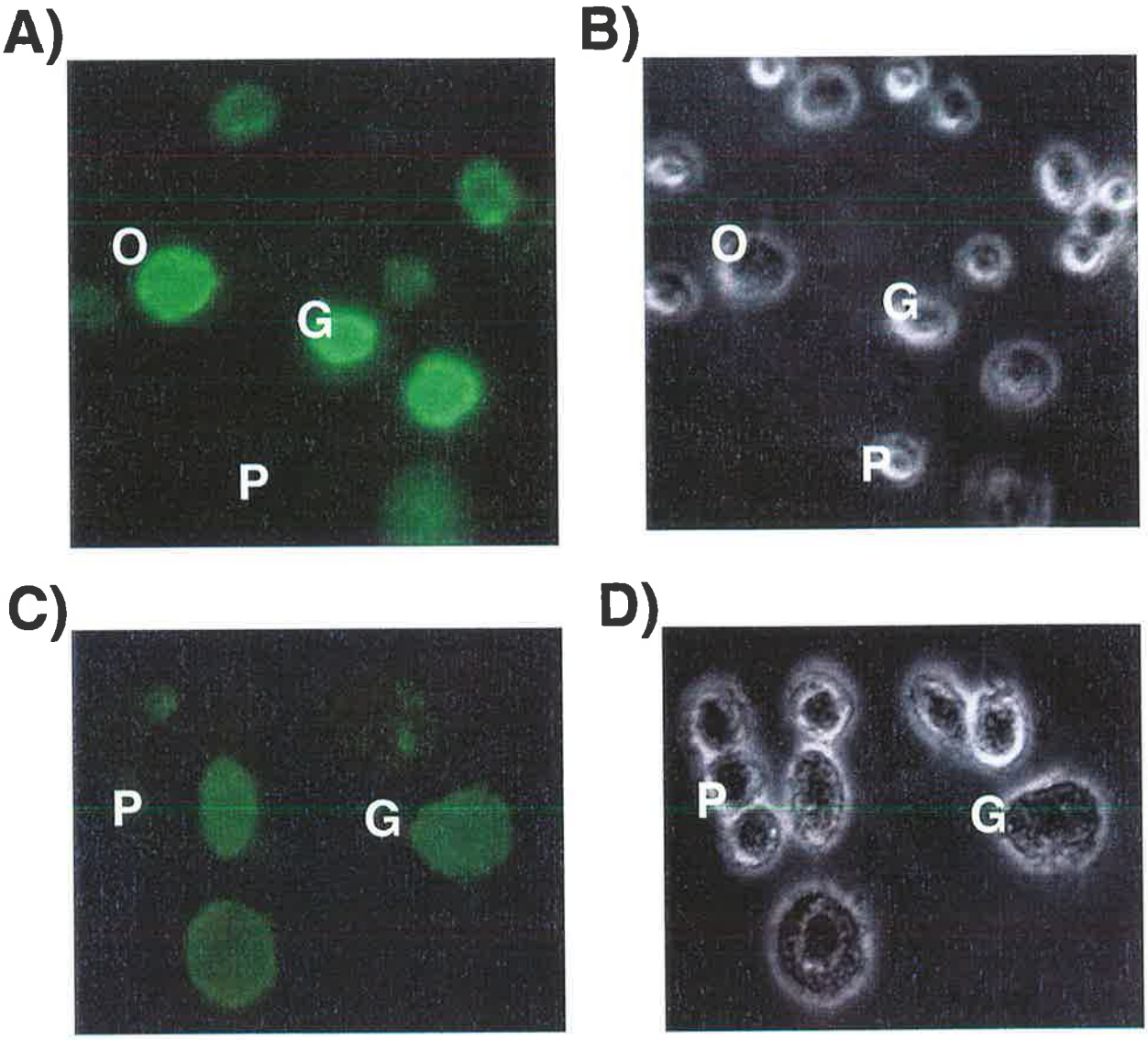

Fig. 42:

Prophenoloxidase binds to granolocytes.

A), **C**) Hemocytes from *Galleria* were attached to glass surface, treated with anti-ProPO antibody (Ma and Kanost, 2000) labelled with FITC-conjugated secondary antibody and inspected under indirect UV-light. Granolocytes (G) and oenicytoids (O) were labelled with anti-proPO antibody while plasmotocytes (P) were not.

B) and **D**) are phase contrast of A) and C).

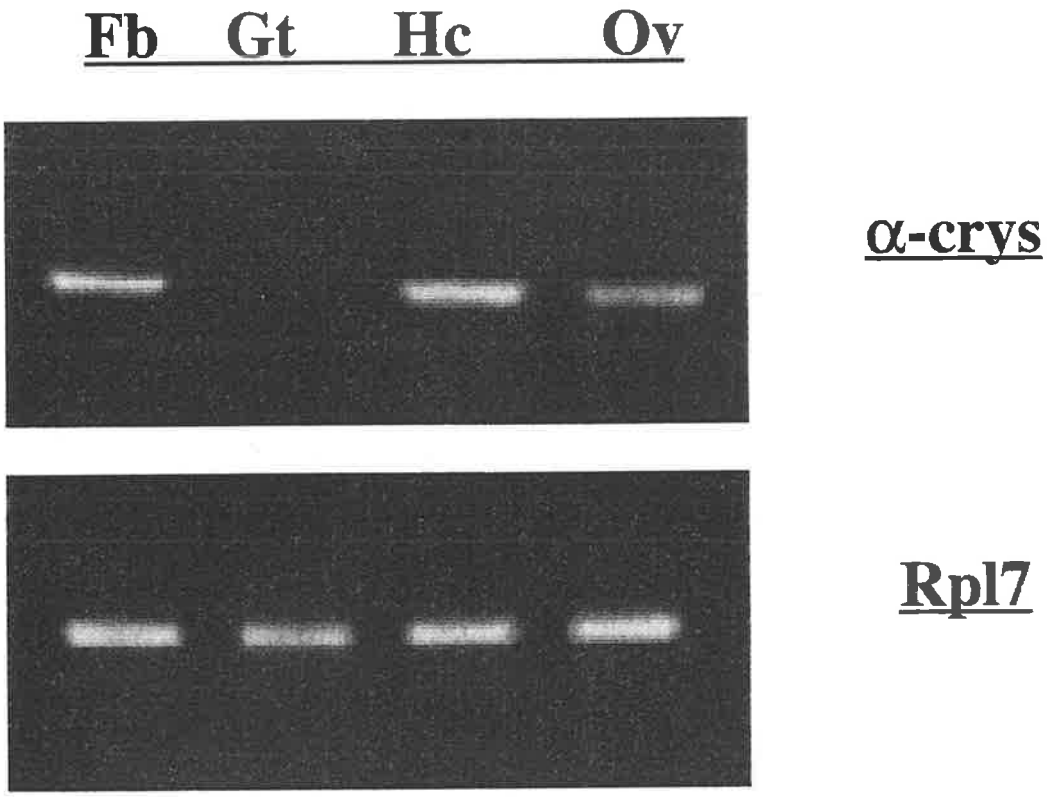

Fig. 43:

Expression studies of *Galleria* α-crys/SHSP (Gm. α-crys) was analysed using RT-PCR. Ribosomal protein L7 (RpL7) was used as an internal loading control. Fb, fat bodies; Gt, guts; Hc, hemocytes; Ov, ovaries.

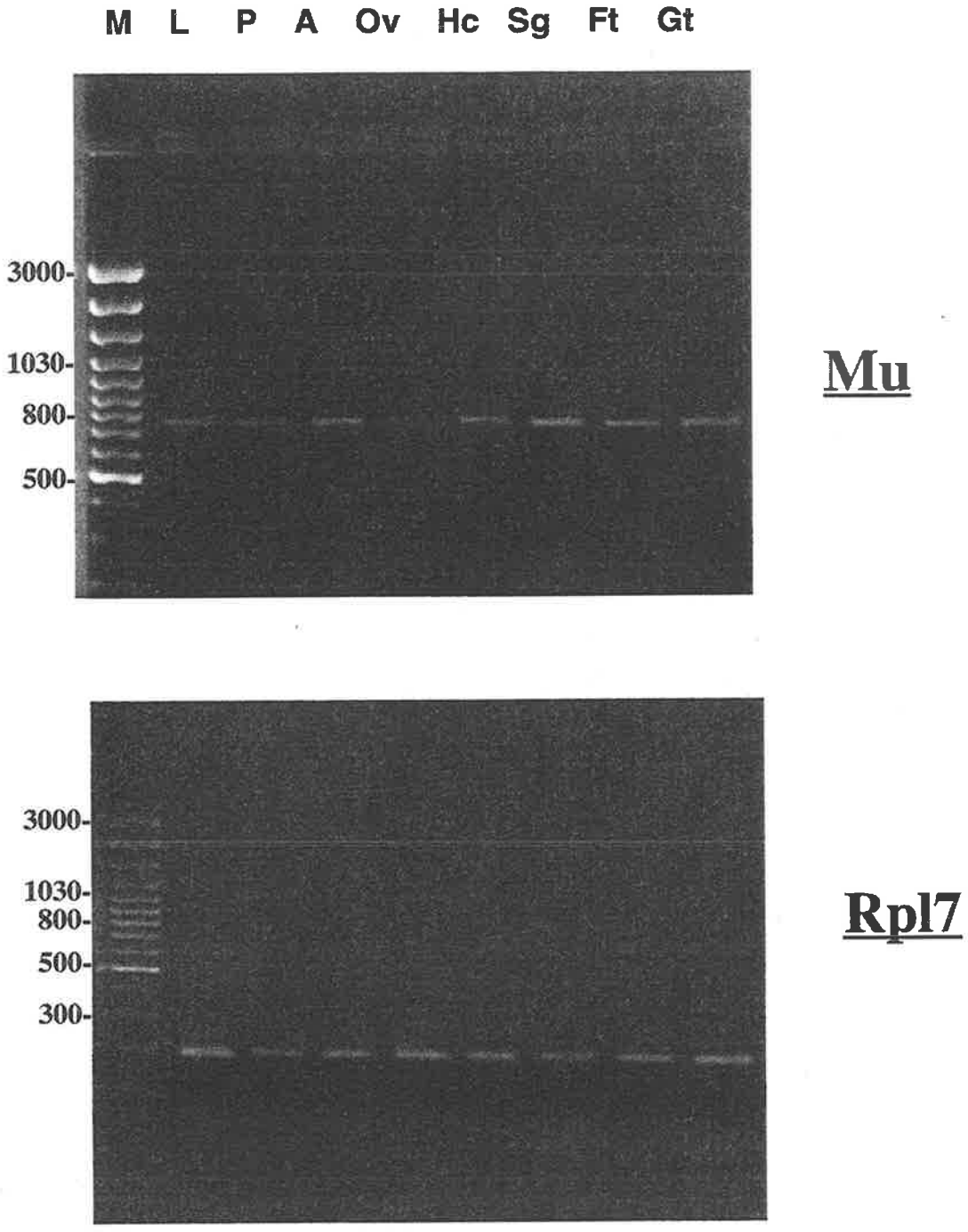

Fig. 44:

Expression of mucin-like gene. RT-PCR were conducted using mucin-like (Mu) and ribosomal protein L7 (Rpl7)-specific primers respectively. M, molecular weight marker; L, larvae; P, pupae; A, adults; Ov, ovaries; Hc, hemocytes; Fb, fat bodies; Sg, silk glands; Gt, guts.

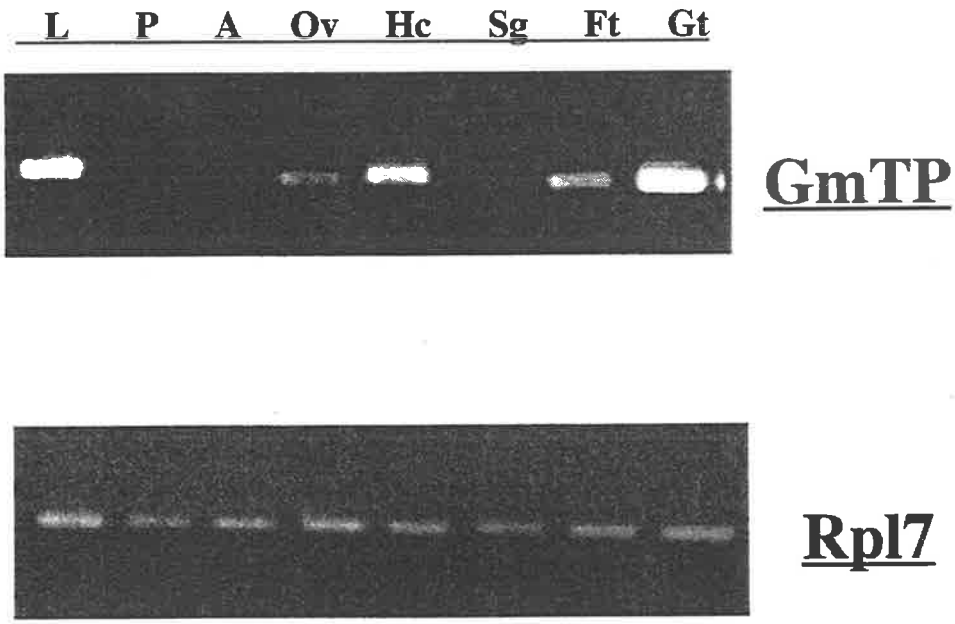

Fig. 45:

Expression studies of *Galleria* trypsin-like gene (GmTP). RT-PCR were carried out using GmTP and ribosomal protein L7 (Rpl7) specific primers respectively. L, larvae; P, Pupae; A, adults; Ov, ovaries; Hc, hemocytes; Fb, fat bodies; Sg, silk glands; Gt, guts.

3.4 Immune-related proteins in ovarian tissues

As shown by Kinuthia *et al.* (1999), a homologue of the *Drosophila* hemomucin is the major glycoprotein on the surface of *V. canescens* eggs. Hemomucin belongs to a group of Ser/Thr-rich proteins and is produced in hemocytes and other tissues as well, such as ovaries, midgut, salivary glands and silk glands (Theopold *et al.,* 1996; Fabbri *et al.,* Pers Comm.). Inside the hemocoel, mucin-like glycoproteins are visible when hemocytes undergo microparticle formation or degranulation (Theopold and Schmidt, 1997). Since it is known that a mucin-like glycoprotein is the major ovarian glycoprotein stained with hemomucin-specific antibodies and Gal/GalNAc-specific lectins in *Drosophila* (Theopold and Schmidt, 1997), FITC-conjugated lectins were used to further explore the localisation and possible functions of mucins in other insects.

Immune recognition can activate a number of processes including the phenoloxidase activating system (Söderhäll *et al.,* 1998). One of the great puzzles in arthropod immunity is the structure and function of the proPO-activating system (Ashida and Brey, 1995; Söderhäll and Cerenius, 1998). Although proPO and other tyrosine modifying enzymes are without a transmembrane signal peptide, these enzymes can be transferred into insect hemolymph. The prophenoloxidase activating cascade plays a role in insect humoral defence reactions (Söderhäll and Cerenius, 1998; Muller *et al.,* 1999; Ma and Kanost, 2000; Lee *et al.,* 2000a). One of the proPO-specific cDNA fragments was isolated from *Galleria* cDNA expression library with anti-clot antibodies. RT-PCR expression experiments with *Galleria* (Fig. 41) and *Pieris rapae* (not shown) also showed that proPO1 is expressed in larval hemocytes, but not in the ovaries. Surprisingly, proPO antibody staining in *Galleria* and *P. rapae* ovaries was observed (Fig. 51). However, only the proPO1-gene in *G. mellonella* has been cloned and proPO2 is implicated in a hetero-dimeric complex with additional proPO genes identified in other insect species (Jiang *et al.,* 1997a; Muller *et al.,* 1999). Since other phenoloxidase-like proteins exist and may cross-react with the antibodies used, cell-

and tissue-specific localisation experiments were performed to identify immune-related proPO-containing protein complexes without referring to any specific phenoloxidase-related proteins. Previous observations indicated that the proPO activity is correlated with immune-related processes (Gillespie *et al.*, 1997). Therefore, anti-proPO antibodies were also used in combination with lectins in the following experiments to identify immune-related proteins in ovaries.

3.4.1 Expression of glycoprotiens and proPO in *Venturia* ovaries

After being oviposited into the host caterpillar, the eggs of some endoparasitoid wasps take up liquid from host hemolymph increasing their volume several-fold. In such hydropic eggs some of the cross-linking of chorion proteins occurs after egg deposition. If there is deposition of cross-linking immune proteins into the eggshell, it may be possible to detect its presence in oocytes and eggs of parasitoids producing hydropic eggs. When *Venturia* ovaries were incubated in L-DOPA solutions, tissue-specific staining was observed due to oxidisation of the compound and formation of specific precursor molecules leading to melanin (Fig. 46A). These observations indicate the involvement of prophenoloxidase activities in egg maturation. Localised oxidisation of L-DOPA was observed in ovarioles during vitellogenesis and on the surface of stored mature oocytes inside the oviduct (Fig. 46A). To examine the presence of proPO inside the ovaries, whole tissues were incubated with anti-proPO antibodies, stained with TRITC-conjugated secondary antibody and inspected under confocal microscopy. A staining pattern (Fig. 46C) was observed that resembled the DOPA-staining (Fig. 46A), which indicates that antibodies used in this experiments are directed against active prophenoloxidase. It was known that glycoproteins exist in ovaries (Theopold *et al.*, 2001a), FITC-conjugated PNA was incubated with *Venturia* ovary whole mounts. The results also showed strong signals in the ovariloes (Fig. 46B) as the proPO staining (Fig. 46C).

To explore the protein expression further, calyx tissues were investigated in detail by using anti-proPO antibodies and lectins. Staining with lectins was observed on the

surface and inside of the calyx (Fig. 47A, C, E). In contrast, incubation with proPO antibodies only weak staining was observed on the surface and no signal was detected inside the calyx (Fig. 47B, D, F). Strong staining with PNA and HPL as observed in calyx tissues (Fig. 47C, E), which are the major sources of immune-protective proteins in wasps (Salt, 1965; Stoltz and Vinson, 1979b; Beckage, 1997). This may suggest that the lectin-binding glycoproteins are major immune proteins and have function in immunity, whereas proPO may have a developmental function in *V. canescens* ovaries and immune function in the hemocytes.

PNA-stained glycoproteins were detected on the *Drosophila* egg surface, they may be produced by the follicle cells and probably deposited onto egg surface as part of chorion (Theopold *et al.,* 2001). To determine whether PNA-stained glycoproteins are on *Venturia* egg surface, the ovariloe cells were inspected. When the ovaries were incubated with FITC-conjugated PNA, it revealed that PNA-stained glycoproteins are found in large amounts in follicle cells at all the stage of oogenesis and may deposit onto the surface of the oocytes (Fig. 48 A, C). In addition to PNA, HPL-form of glycoprotein was also observed inside the follicle cells closed to the oocyte surface (Fig. 48E). These observations indicate that glycoproteins may play a developmental role in *V. canescens* ovaries.

Incubation of ovarioles with anti-prophenoloxidase antibodies (Ma and Kanost, 2000) revealed a staining pattern that was similar to lectin-staining (Fig. 48B, D). Little or no phenoloxidase staining was observed in control tissues with preserum (Fig. 48F). To investigate further, lectin and proPO staining can be observed in the cytoplasm of the follicle cells, nurse cells and oocytes in the early oogenesis (Fig. 49A, B), however, the staining patterns were different between proPO and lectins in the later oogenesis (Fig. 49C-F). Strong signals were mainly observed in the follicle cells in lectin-staining (Fig. 49C, E). In comparison, two groups of proPO staining was observed, one is localised in the follicle cells, which are correlated with some of the lectin staining (Fig. 49A, C, E, arrowheads), but less dense. Another group of proPO staining was observed

in the extracellular space of nurse cells (Fig. 49B, D, arrows), where no lectin staining was detected (Fig. 49C, E).

3.4.2 Expression of glycoproteins and proPO in lepidopteran ovaries

To get further insight into the uptake and secretion of some immune-related proteins in ovary tissues, lectins and proPO staining pattern was examined in lepidopteran ovaries. *P. rapae* was chosen because previous studies showed that CrV1, an immune suppressor, could inactivate larval hemocytes (Asgari *et al.*, 1997). However, attempts to inactivate adult hemocytes have not been successful so far and proPO and lectin staining patterns were similar in ovaries from CrV1-injected and control animals. When ovaries from *P. rapae* were analysed using confocal microscopy, lectin-specific binding was located on the basement membrane and on a subpopulation of ovarian cells. For example a group of cells were identified to contain relatively high PNA-staining in the germarium (Fig. 50A). At this developmental stage two staining patterns could be detected, one group of cells showing a honeycomb-like staining indicative of lectin-binding to glycoconjugants on cell membranes and in intercellular space. Another group of morphologically similar cells stained intracellular vesicles presumably due to endocytosis of material located in intercellular space (Fig. 50A, arrows) where weak proPO staining was observed as well (Fig. 50B, arrow).

When these cells organize into ovarioles, endocytotic cells also show higher proPO-staining (Fig. 51A, arrowhead). At the start of vitellogenesis these cells become detached from the merging follicle epithelium and accumulate as a small group of adepithelial cells adjacent to follicle cells (Fig. 51C). Initially these cells are found evenly distributed across the space between ovarioles (Fig. 50E, arrow). In later stages groups of adepithelial cells are frequently found aggregated on one side of the anterior-posterior axis (Fig. 51E, F, arrowheads). Lectin staining is initially restricted to basement membranes and adepithelial cells, where it is detected on the cell surface and in cytoplasmic vesicles. The cellular staining is more intense towards the basement membrane (Fig. 51B). Strong signals with proPO were also detected inside the follicle

cells and nurse cells (Fig. 51D, F, H). Take together these data, which showed that lectins and proPO staining revealed streaks of staining visible from the outer extracellular space into the perivitellogenic space (Fig. 52A-D, arrowheads). This may indicate that the stained glycoproteins are derived from the hemolymph and move to the oolemma of the oocyte but more evidence is required to confirm this assumption.

At later stages lectin-staining patterns differ from proPO-staining patterns. Whereas, lectin staining is found in early stages predominantly in extracellular space (Fig. 51B, arrowhead), it is enriched inside the perivitellin space and possibly in the oocyte (Fig. 52A, C, oo). In contrast, proPO-staining is mainly detected in the cytoplasm of follicle cells (Fig. 52B, D, fc). Compared to proPO-staining in follicle cells, nurse cells are stained less intensively (Fig. 52B, nc). The lectin staining was visible as dot-like structures clustered around cell borders, some located in extracellular space, some in the cytoplasm. The proPO-staining co-localised with some of the major lectin-stained dots visible at the same location (Fig. 52, arrows). In *Galleria*, the same staining patterns were frequently observed with both lectin and proPO antibodies (Fig. 53).

Together these observations show that two immune-related proteins are found inside the ovary, where they are localised in the cytoplasm and on the cell surface of follicle and nurse cells. The lectin-stained glycoproteins and proPO accumulate in the perivitelline space, an indication that they are incorporated into the eggshell or ooplasm of the growing oocyte. However, proPO could not be detected in the calyx tissues where immune-protective glycoproteins are produced. These results in combination with previous observations suggest that both immune related proteins might involve in both immunity and development.

Since prophenoloxidase and glycoproteins show similar tissue-specific distribution in *V. canescens* ovarioles (Fig. 48, 49), the question is whether the two molecules are co-localised at the sub-cellular level. In co-stained ovarioles prophenoloxidase and PNA-staining only coincided in the perivitellogenic space between the oolemma and follicle epithelium (Fig. 48). Prophenoloxidase staining was observed in the cytoplasm of

nurse cells, follicle cells and oocytes as dot-like aggregations in extracellular space (Fig. 48, 49). No proPO staining was observed in the control tissues (Fig. 46 F). PNA-staining was visible mainly in follicle cells as large irregular vesicles, which correspond to the cytoplasmic location. The observation that the Gal/GalNac-forms of hemomucin are not only produced in hemocytes, but also in follicle cells, cardia cells and on the surface of sclerotised structures, suggests that the PNA-glycoform is regulated in a tissue-specific fashion (Theopold *et al.*, 1996; Theopold and Schmidt 1997).

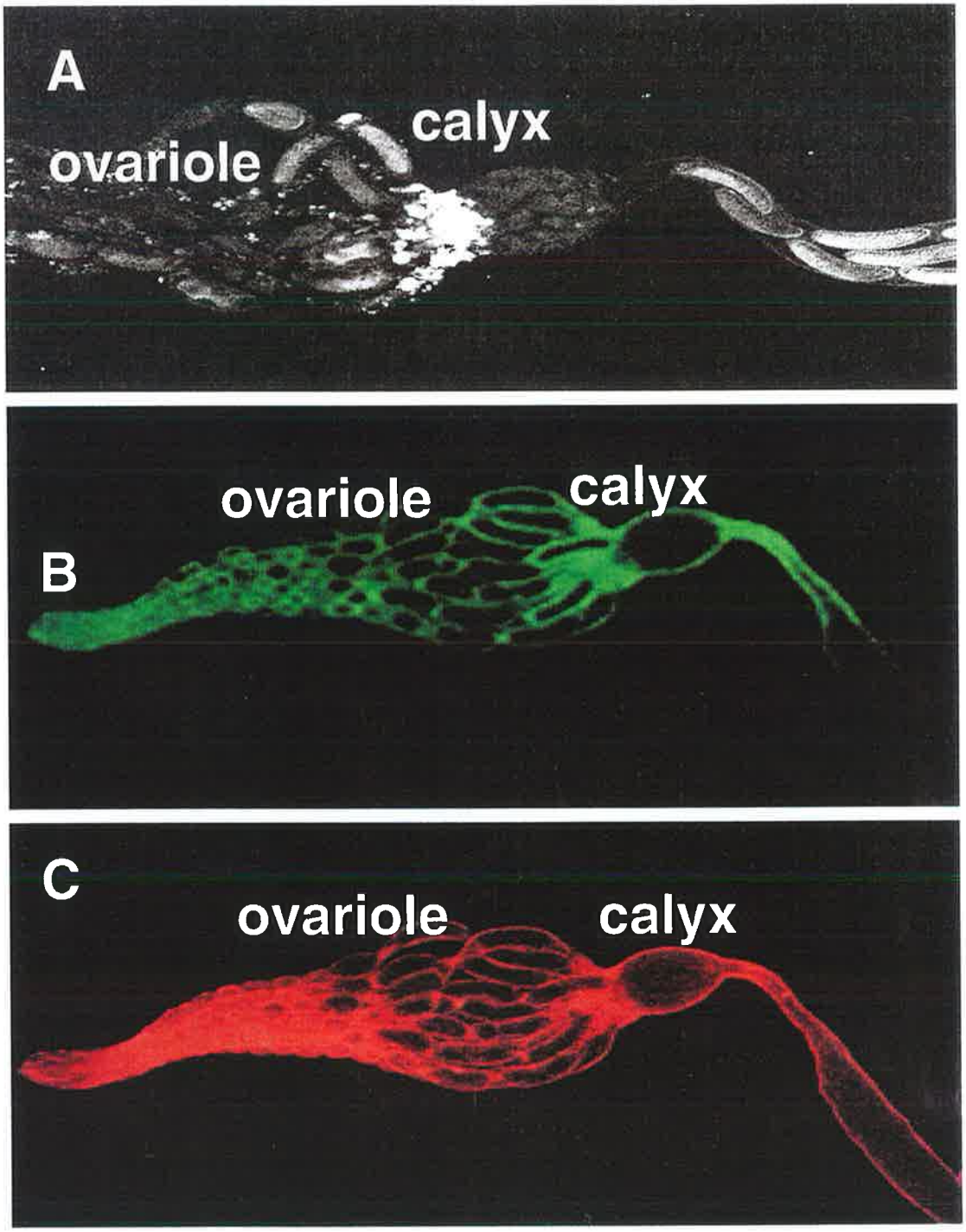

Fig. 46:

Immune staining of *Venturia canescens* ovarian tissues.

A) Confocal microscopy of *V. canescens* ovary whole mount incubated with L-DOPA.

B) Confocal microscopy picture of ovary tissue incubated with FITC-conjugated peanut agglutinin (PNA).

C) Confocal picture of *V. canescens* ovary whole mount incubated with anti-proPO antibodies and stained with TRITC-conjugated secondary antibody.

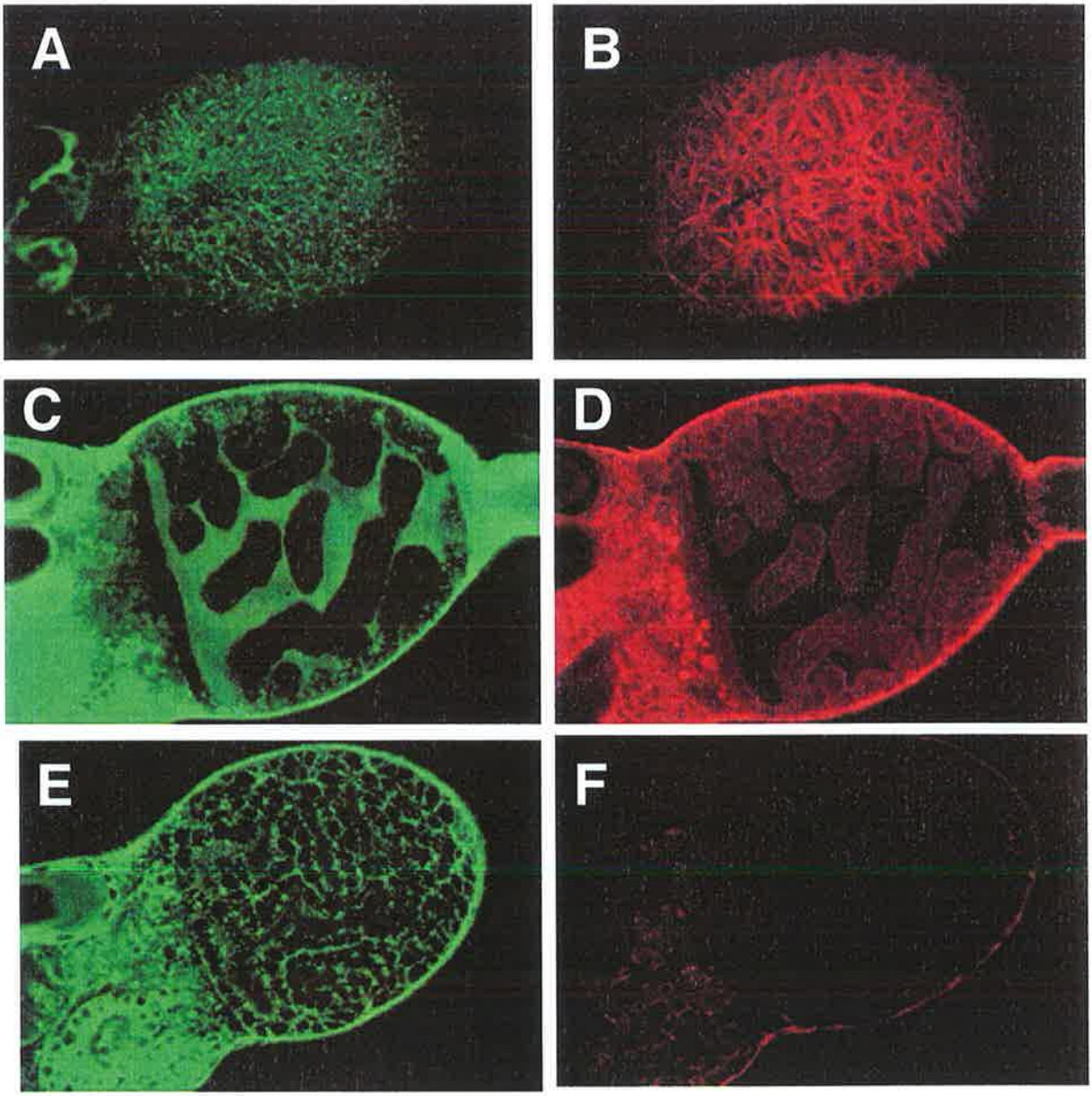

Fig. 47:

Immune staining of *Venturia canescens* calyx tissues.

A) Surface of the calyx tissue stained with FITC-conjugated PNA.

B) Co-staining of A) with anti-proPO antibodies and developed with TRITC-conjugated secondary antibody .

C), D) Section of A) and B) showed inside staining. Note that strong staining with PNA can be detected while no proPO staining visible inside the calyx tissues.

E) Section of calyx tissues incubated with FITC-conjugated HPL.

F) Same section as E) incubated with preserum.

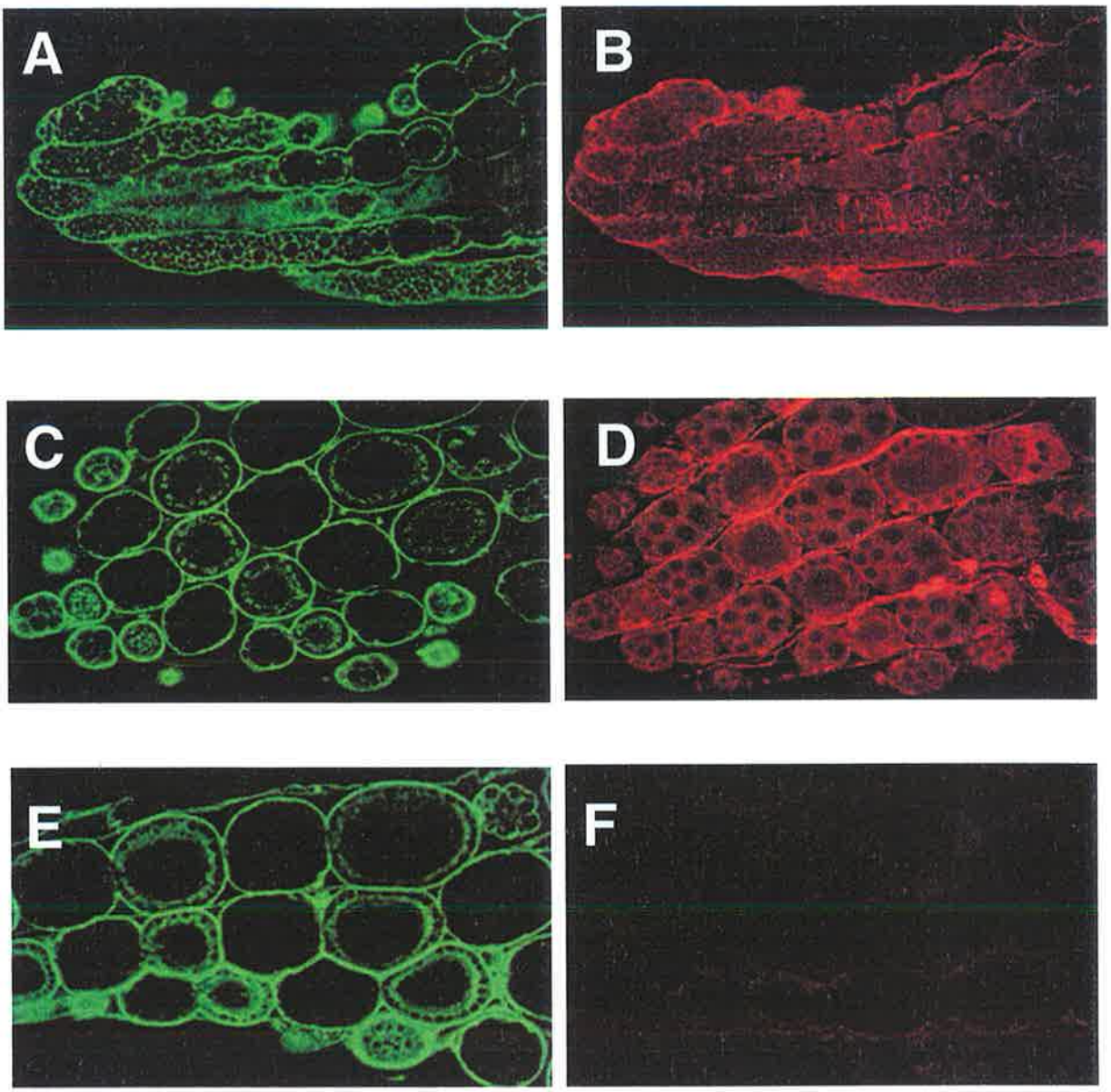

Fig. 48:

HPL and PNA-specific glycoconjugants and proPO-like proteins inside ovarian tissues. Incubation of *Venturia canescens* ovaries with FITC-conjugated lectins and anti-proPO antibodies with TRITC-conjugated secondary antibody. Inspection under indirect UV-light using confocal microscopy.

A), C) PNA staining of ovaries showing focal sections of early stages of oogenesis.

B), D) Same focal sections showing proPO staining.

E) HPL- staining of focal plane section of early oogenesis. Similar staining patterns were observed with HPL as with PNA.

F) Incubated of *V. canescens* ovary with TRTIC-conjugated secondary antibody only. Note the absence of staining was visible.

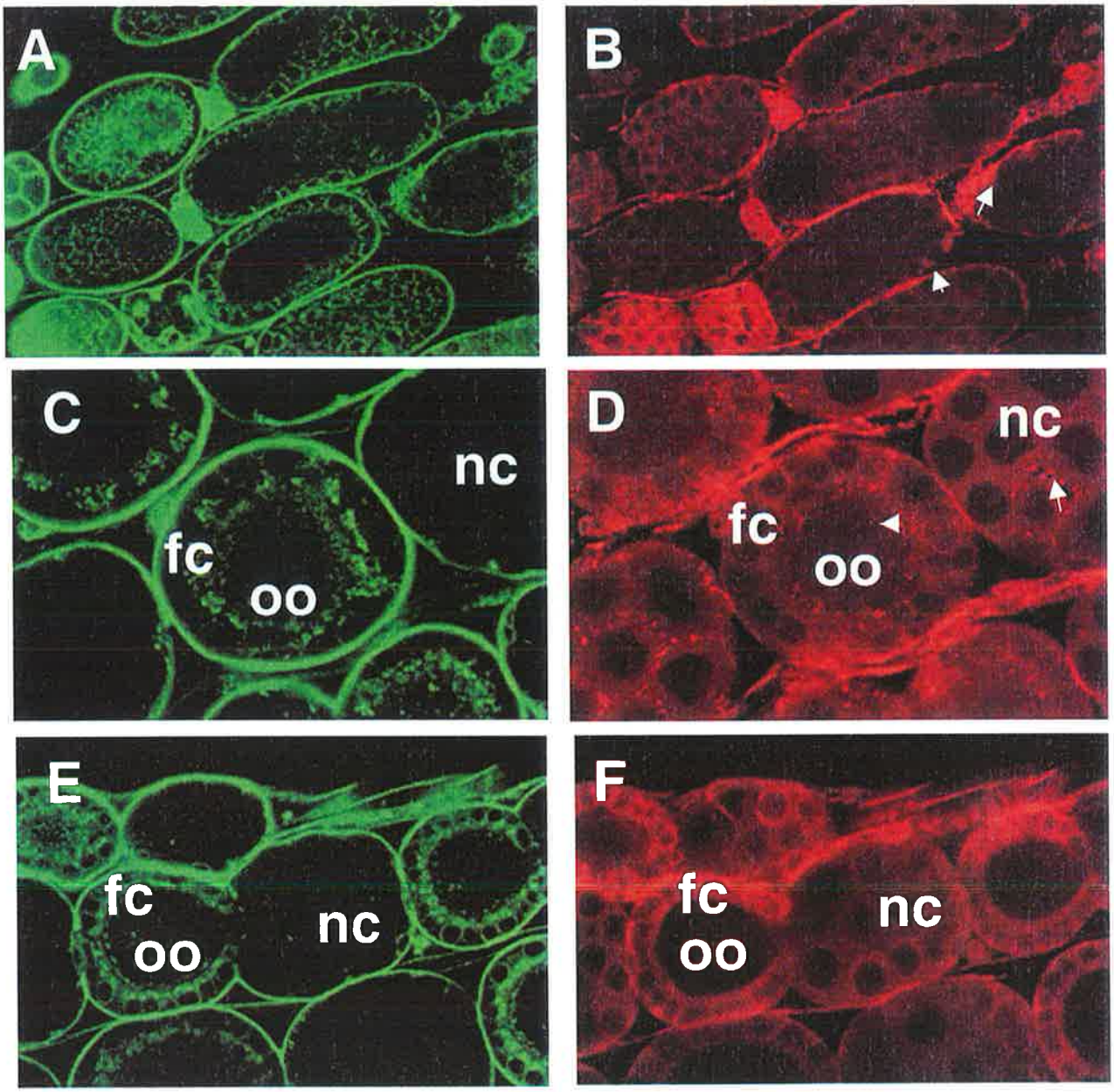

Fig. 49:

Lectins and proPO staining of middle and later oogenesis of *Venturia canescens* ovaries.

A), C) FITC-conjugated PNA staining pictures showed sections of middle and later oogenesis of ovaries respectively. °

E) HPL-staining of section of later oogenesis, showing the similar pattern as PNA-staining.

B), D), F) are the corresponding sections stained with anti-proPO antibodies. Staining is even distributed in the cytoplasm of nurse cells (nc), follicle cells (fc) and oocytes (oo), but it is absent in the nucleus.

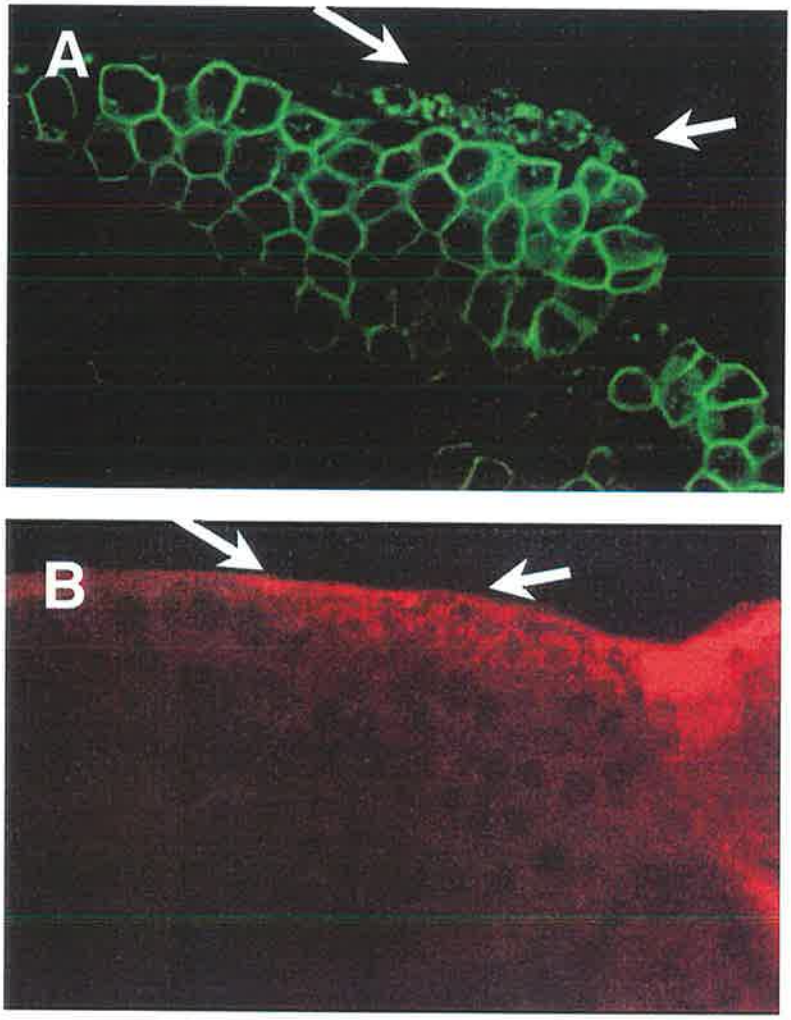

Fig. 50:

Lectin- staining in the germarium of *Pieria rapae* ovaries.

A) staining pattern in the germarium after incubation with FITC-conjugated PNA and inspection with confocal microscopy. Note the intensive staining in the area around the cellular membranes in some cells, whereas other cells show intracellular vesicle-staining (arrow).

B) Same focal plane showing low level of staining with proPO-antibodies visualised with TRITC-conjugated secondary antibodies.

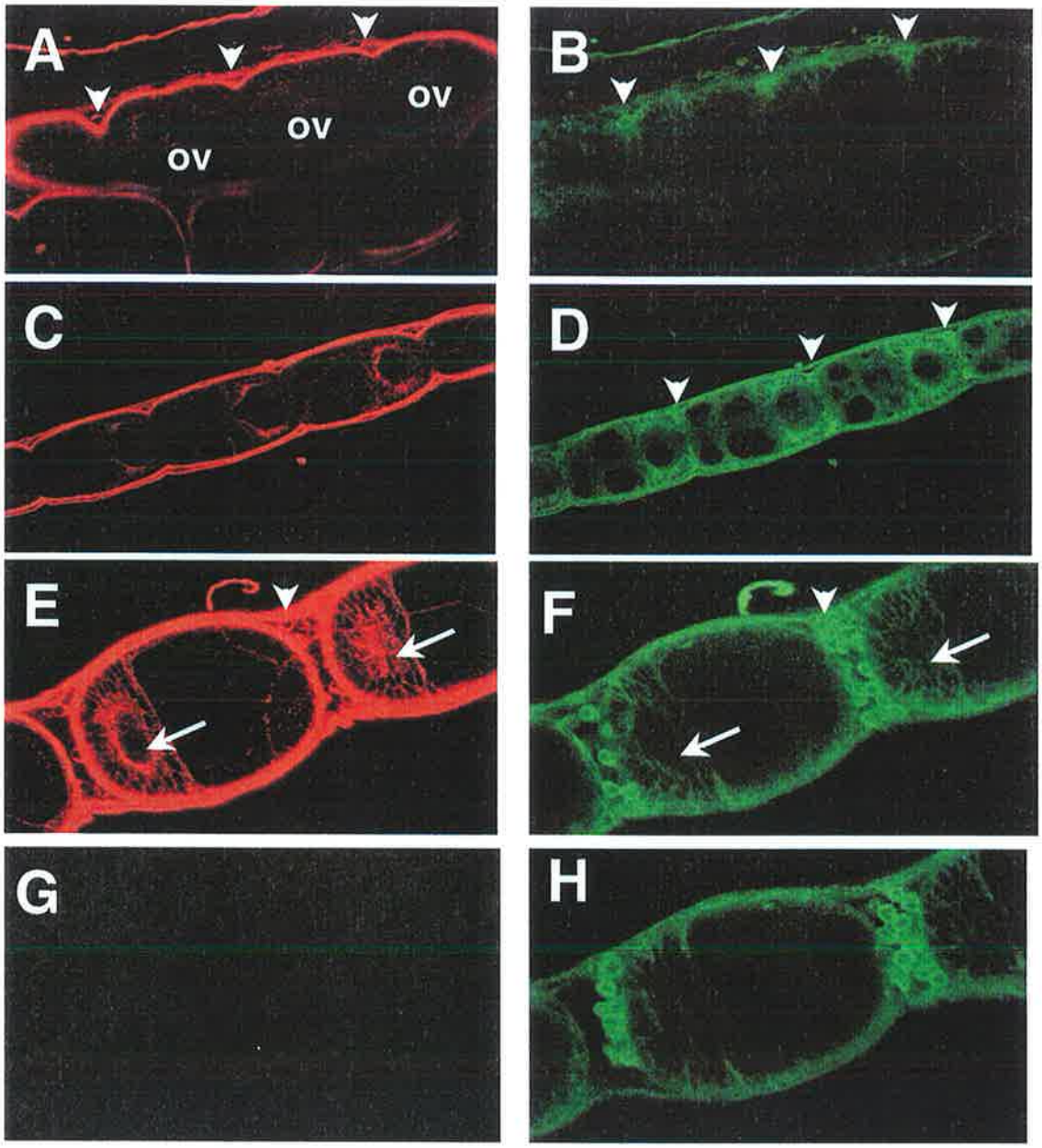

Fig. 51:

Tissue location of proPO and glycoproteins in ovarian tissues of *Pieris. rapae*.

A) Treatment of ovary whole mount with anti-proPO antibodies, staining with TRITC-conjugated secondary antibody and inspection under indirect UV-light using confocal microscopy. **B)** Same section stained with FITC-conjugated HPL.

C) Ovarioles at an undefined previtellogenesis stage in oogenesis with anti-proPO antibodies.

D) Same section stained with HPL.

E) Ovarioles of an undefined previtellogenesis stage in oogenesis stained with anti-proPO antibodies. **F)** Same section stained with HPL

G) Ovarioles with TRITC-conjugated secondary antibody only. **H)** proPO-staining ovaries.

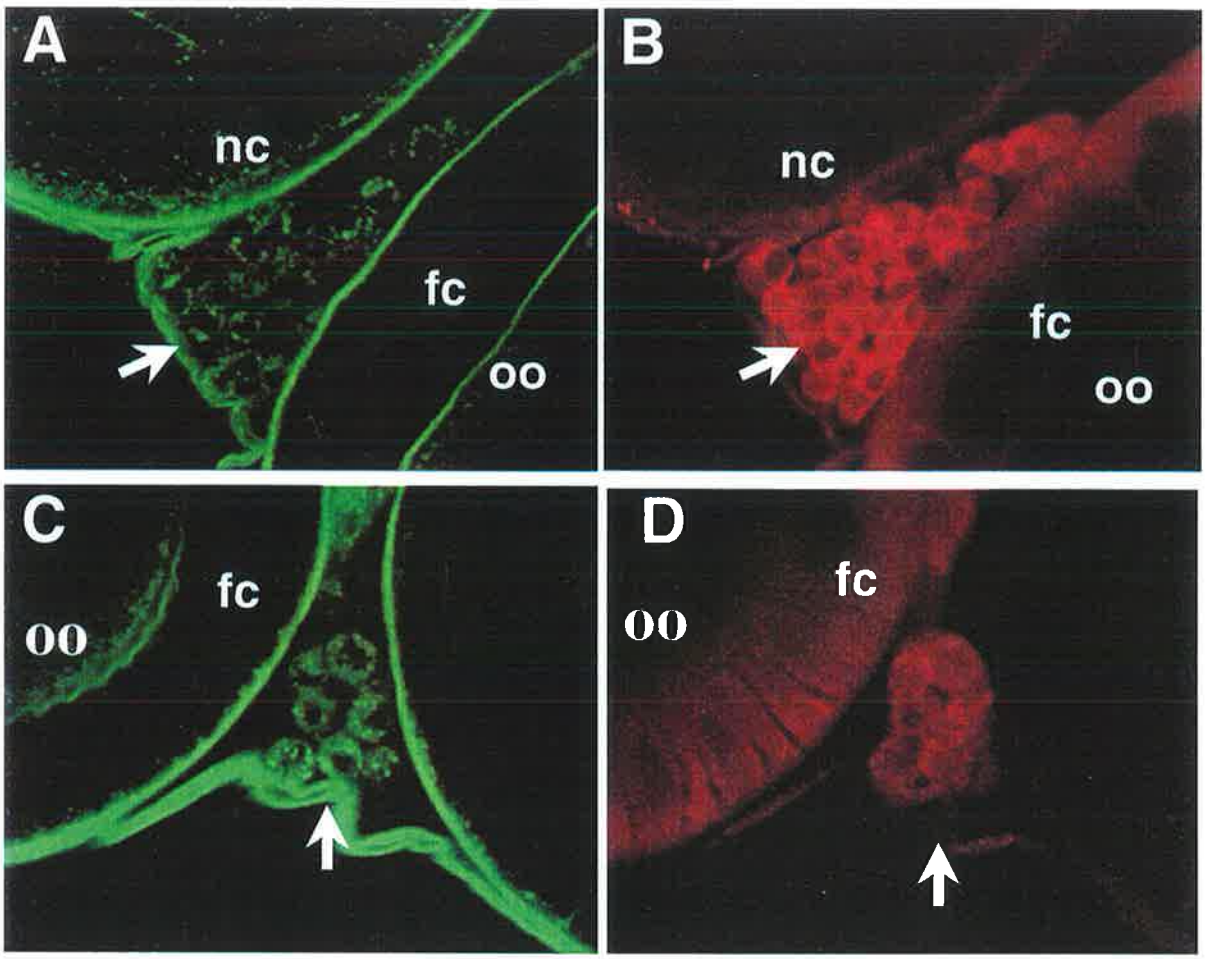

Fig. 52:

Localised proPO- and HPL-staining in ovarioles of *Pieris rapae*.

A), C) Ovarioles of an undefined previtellogenesis stage in oogenesis incubated with FITC-conjugated HPL. Adepithelial cells (arrow) between ovarioles are intensively stained.

B), D) Same focal section of A) and C°) with anti-proPO antibodies , stained with TRITC-conjugated secondary antibodies. fc, follicle cells; oo, oocytes.

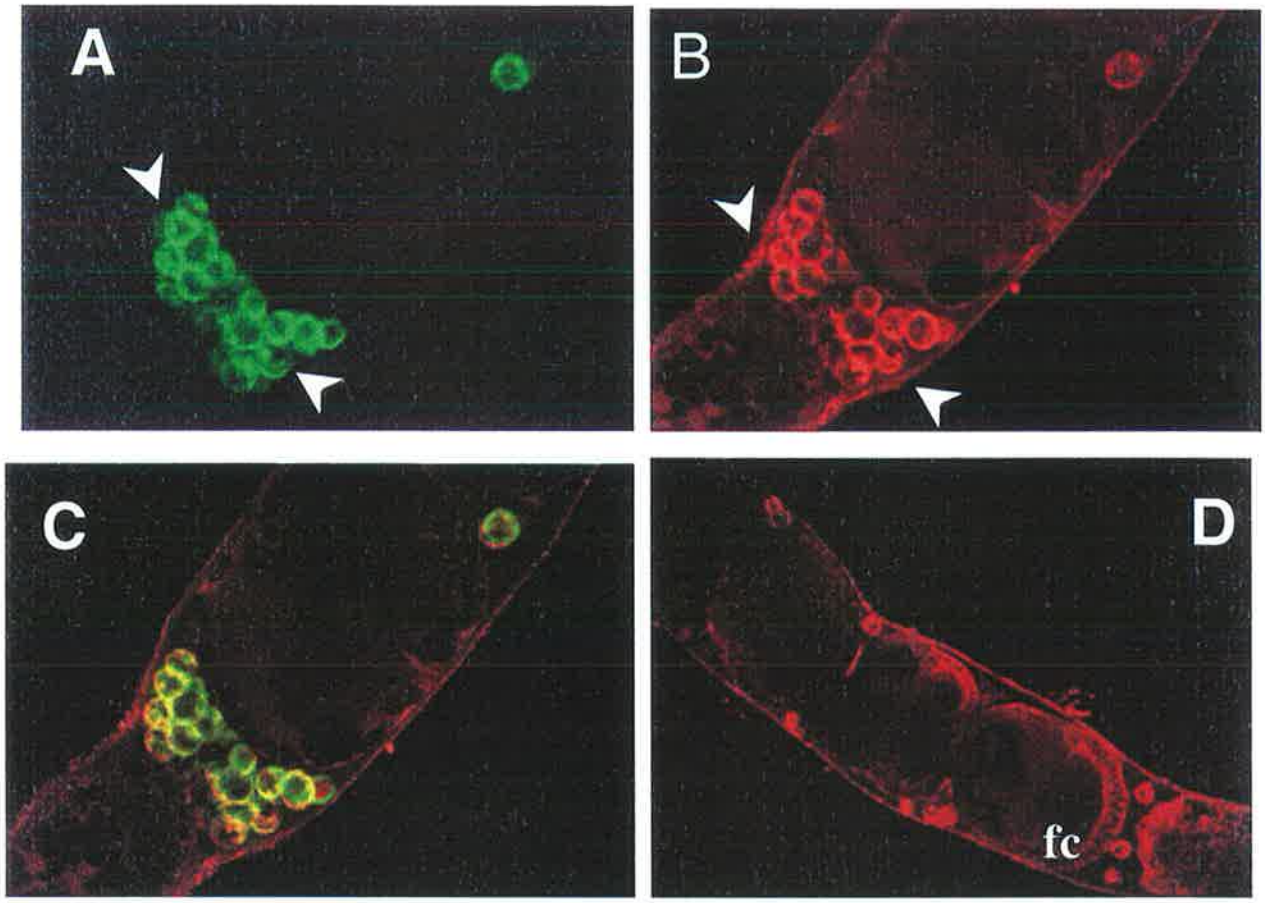

Fig. 53:

Tissue location of proPO in ovarian tissues of *Galleria mellonella*.

A) Treatment of ovary whole mount with FITC-conjugated HPL and inspection under indirect UV-light using confocal microscopy. Note the intensive staining of cells located between ovarioles (arrowheads) and in intercellular spaces of adjacent epithelial cells.

B) Same section stained with TRITC-conjugated secondary antibody. The localised distribution of HPL-staining coincides with the proPO-staining.

C) Combined picture of **A)** and **B)**. Yellow staining is the co-localised parts of proPO and glycoprtoeins.

D) ProPO-staining *G. mellonella* ovarioles. Follicle cells are stained with anti-proPO antibodies. The heavily stained cells between ovariloes are completely separated from epithelial cells. Note that similar staining patterns were observed in *G. mellonella* and *P. rapae*.

Chapter 4: Discussion

4.1 Genetic and molecular characterisation of VLP1 in parasitoid wasps

With the exception of a single species, a hallmark of host manipulation by hymenopteran parasitoids from the families of braconidae and ichneumonidae is the presence of polydnaviruses (PDVs), which are produced in the female reproductive organs. The PDVs are encoded by proviral DNA that is integrated into the wasp genome (Stoltz and Vinson, 1979). They are expressed in host cells within a few hours following parasitisation, producing specific viral products that are able to actively suppress the host's defence system. To date, two types of PDVs have been characterised, ichnoviruses and bracoviruses. One example is the *Campoletis sonorensis* ichnovirus (CsV), which is involved in the suppression the host's cellular defence (Edson *et al.*, 1981). Another is the *Cotesia rubecula* bracovirus (CrV1), the protein products of which suppress the immune system of its host, *Pieris rapae,* by binding to hemocytes and inactivating the endocytoskeleton by an unknown mechanism (Asgari *et al.*, 1997).

The exception involves the ichneumonid *Venturia canescens*. Females of this species do not produce PDVs but instead a similar product described as a virus-like particle. Virus-like particles were detected in *V. canescens* and are produced by calyx tissues of female reproductive organs (Schmidt and Schuchmann-Feddersen, 1989). While VLPs resemble ichnoviruses in a number of key aspects, including particle morphology, particle assembly in the nuclei of the calyx tissues and in their mode of secretion, they differ in that they completely lack nucleic acids (Rotheram, 1967; Bedwin, 1979; Feddersen *et al.*, 1986). Although early observations showed that VLPs did not inactivate host hemocytes (Salt 1976; Salt 1980), virus-like particles were later shown to be essential in immune protection of wasp eggs (Feddersen *et al.*, 1986; Schmidt and Theopold, 1990; Schmidt *et al.*, 1990). Further research found antigenic similarities between VLP proteins and a host component (Schmidt and Schuchmann-

Feddersen, 1989), indicating that VLPs passively protect the wasp egg by "molecular mimicry". In order to clarify the protection mechanism, molecular characterisation of VLP proteins was conducted (Theopold *et al.,* 1994; Hellers *et al.,* 1996). Among VLP proteins, VLP1 was the first to be cloned, sequenced and characterised (Hellers *et al.,* 1996). Sequence analysis of the deduced VLP1 proteins revealed three domains: an amino-terminal hydrophobic region, a tandem repeat and a carboxyl-terminal phospholipid hydroperoxide glutathione peroxidase (PHGPx) domain (Hellers *et al.,* 1996). Subsequent research found that two allelic variants of the VLP1 protein exist, involving the presence or absence of a tandem repeat region. Interestingly, all asexual females tested were found to be homozygous for one of the two alleles (Beck *et al.,* 1998; Beck *et al.,* 1999). Accordingly, two lines of the asexual laboratory strain (RP and RM) were established based on the two VLP alleles. Following this, the function of the VLP1 protein was further investigated, both in terms of the phenotypic differences between the asexual and sexual lines among the VLP1 allelic variants, and in terms of the function of the common carboxyl-terminal PHGPx domain at the molecular level.

4.1.1 Genetic analysis of two distinct VLP1 strains

An asexual laboratory stock of *V. canescens* was previously separated into two genetically strains based on the variant forms of the VLP1 gene. The two laboratory strains were also found to differ in their ovarian morphology and reproductive strategies (Beck *et al.,* 1998; Beck *et al.,* 1999). The recent discovery of both asexual and sexual *V. canescens* populations in several locations of southern France (Malmaberg *et al.,* 2000) provided the opportunity to test the genetic basis of the observed differences between the two lines. The two questions of particular interest were, (1) whether the allelic VLP1 gene is linked to the phenotypic differences described for the laboratory strains, and (2) whether these phenotypic differences are also observed in field populations.

The offspring of RP and RM lines showed distinct ovarian phenotypes (Beck *et al.*, 1999; Beck *et al.*, 2001), the segregation of homozygous VLP1 offspring correlated with the ovarian phenotype, indicating that the VLP1 gene is genetically linked to the phenotype. However, genetic linkage does not constitute proof that the allelic VLP1 gene is the cause of the phenotype. Further experiments including molecular analysis of the VLP function and genetic transformation of the VLP1-gene, involving phenotype rescue, would be required to provide evidence for the genetic cause of the phenotype. Nevertheless, the genetic linkage of the VLP1-gene to the observed phenotype adds to the following indirect evidence that the VLP1-gene may be functionally related to the phenotype:

The VLP1 gene is highly expressed in the calyx tissues, which also show distinct morphological differences between the two phenotypes (Beck *et al.*, 1999). Further, the amount of VLP1-protein produced differs between the two phenotypes (Fig. 4).

- The putative VLP1-protein predicts a VLP-membrane association through a lipophilic and a phospholipid hydroxyperoxide glutathione peroxidase (PHGPx) domain (Hellers *et al.*, 1996). Further observation showed that conspicuous VLP-derived membranes exist in the calyx lumen of RM-females, which preventing the VLP1 proteins from moving freely within the calyx lumen (Fig. 4B, D). This results in the transfer of eggs from the ovarioles into the oviduct being impeded, causing the observed differences in egg distribution (Beck *et al.*, 2001; Fig. 5, 6). The evidence indicates that the subcellular location of the VLP1-protein coincides with the cytological phenotype.

- Although the predicted PHGPx-domain in the VLP1-protein is probably not involved in reducing oxidized lipids (Chapter 3.2.1), the VLP1-protein may be involved in facilitating the binding and removal of modified lipid moieties from the VLP-membrane (see below).

- Obligate asexual individuals emerge occasionally from sexual populations (D. Li, pers. obser.).

- Most importantly, RP and RM wasps from three geographic locations, including both sexual and asexual strains, all displayed similar patterns of distinct ovarian phenotypes and reproductive strategies, which correlated to the VLP1 genotypes.

Since allelic VLP1-genes are always found in conjunction with the phenotype in asexual populations ranging from laboratory strains to field populations and from Australia to southern France, the apparent linkage in asexual populations (Beck *et al.*, 2001), together with the genetic linkage in the asexual and sexual strains (Fig. 5), suggest that the VLP1-gene may contribute to the phenotype.

However, the VLP1-gene may not be the only gene contributing to the phenotypic effects. Although the two VLP1-alleles segregate with the relative differences in the distribution of eggs, the absolute egg numbers differ in the corresponding asexual and sexual genotypes (Fig. 6). This suggests that an additional unlinked gene may be involved in the transfer of eggs from the ovarioles into the oviduct. Further research would be required to determine whether the phenotype observed in asexual strains is due to a genotype fixation of a combination of two or more unlinked allelic genes.

Large egg numbers in the calyx are associated with small egg numbers (egg load) in the oviduct (Fig. 7). An outcome of this phenotypic difference may be two distinct reproductive strategies, where one line (RP) is effective in exploiting large clusters of hosts, whereas the other line (RM) is more effective in searching for few and widely distributed hosts. Other differences in reproductive strategies are also possible and may not be mutually exclusive, such as differences in host specificity and compatibility, and differences in inter-larval competition in superparasitised hosts. It has previously been found that the two asexual laboratory lines differ in reproductive success under inter- and intra-line competition (Beck *et al.*, 1999). The result that a similar difference

with the asexual strains was observed in the two corresponding genotypes of the field-collected sexual strains (Fig. 8), suggests that the VLP1 genotypes correlate with complex physiological traits that may be the basis for distinct reproductive strategies in the field.

Two types of larval competition are known to exist; one is physiological suppression of younger larvae by older larvae due to oxygen deprivation (Fisher, 1961; Fisher, 1963), or nutrient depletion (Vinson and Hegazi, 1998) and the other is physical fighting (Salt, 1961). A unique feature of larval fighting is that the dominant larvae tends to be the younger ones, probably due to their having greater mobility and stronger mandibles (Marris and Casperd, 1996). The two levels of competition provide opportunity, where the age difference among competing larvae may determine the outcome of fights for host dominance. Under conditions that favour superparasitism, the period and timing of egg deposition may be crucial factors. For example, if RM-females lay fewer eggs at a time but spread egg laying over longer periods, the chance of being the youngest larvae inside superparasitised caterpillars may be increased (Beck *et al.*, 2001). This assumption was supported by data for field-collected sexual strains (Fig. 8B), which suggests that conclusions based on laboratory strains can, in general, be applied to other strains. However, the actual competition events that occur inside superparasitised caterpillars are not known and competing physical and physiological reactions among larvae and host may be more complicated. For example, the number of emerging wasps in highly superparasitised caterpillars is small compared to that in the caterpillars with shorter egg deposition periods and fewer eggs (Fig. 8). The number of emergent wasps is inversely correlated with the number of eggs deposited and probably due to perished hosts being unable to produce neither wasps nor moths. This indicates that the fight for host dominance in superparasitised hosts may frequently lead to failures and is probably more complicated than previously envisioned.

In summary, the observed morphological and functional differences in two laboratory lines of the asexual parasitoid wasps (Beck *et al.*, 1999; Beck *et al.*, 2001) also exist in

the field-collected asexual VLP1 lines and in homozygous VLP1 genotypes of sexual strains, indicating that the VLP1 gene alteration is genetically closely linked and probably the cause of the phenotype.

4.1.2 Non-enzymatic function of VLP1

PHGPx is an anti-oxidant enzyme that can directly reduce peroxidased phospholipids and cholesterols within membranes (Ursini *et al.*, 1985; Thomas *et al.*, 1990). Sequence analysis showed that a PHGPx domain is present in the VLP1 protein (Heller *et al.*, 1996). The question is whether VLP1 has an enzymatic function. In continuing previous work, one of my objectives was to examine a possible enzymatic activity of VLP1 and compare it to related PHGPx proteins, which may provide some new insights into immune protective mechanisms of *V. canescens.*

PHGPx is the only known intracellular antioxidant enzyme that can directly reduce lipid hydroperoxides in membranes. It is expressed at high levels in rat and mouse testes (Imai *et al.*, 1995; Nam *et al.*, 1998). An extraordinarily high rate of transcription of PHGPx gene was detected in late spermatocytes and round spermatids in mice (Nam *et al.*, 1998) and rats (Maiorino *et al.*, 1996). The abundance of PHGPx in spermatozoa suggests that this unique antioxidant enzyme might be involved in the development of spermatozoa. Recently a report by Imai *et al.* (2001) indicated that insufficient expression of PHGPx in spermatozoa might lead to serious impairment of fertilization in human.

Compared with vertebrate PHGPXs much less is known about the members of this family from insects. Studies of the expression patterns and enzymatic activities of VcPHGPxd and DmPHGPx showed that DmPHGPx is expressed more strongly in male flies than females, which is most likely due to strong expression in testes (Fig. 14A, B). DmPHGPx shows peroxidase activity, whereas no such activity was observed in VcPHGPxd in assays using three standard PHGPx substrates. This indicates that

with the exception of the inactive VcPHGPxd, insect PHGPxs have selenium-independent activity similar to the plant members of the family.

The enzymatic data are in agreement with the protein sequence data. In PHGPx enzymes, catalytic residues X (selenocysteine), Q and W form part of three conserved domains: NVASQXG, ILAFPCNQF and KWNF(S/T)KFL respectively (Epp *et al.,* 1983). Sequence comparison of DmPHGPx (CG12013), VcPHGPxd and other PHGPx genes from human, animal and plant was carried out. The comparison showed that the three conserved domains were all present in DmPHGPx and other PHGPx proteins, except that selenocysteine (encoded by a stop codon, UGA) (Brigeliusflohe *et al.,* 1994; Maiorino *et al.,* 1998) is replaced by cysteine (encoded by UGC in DmPHGPx, UGU for others). This indicates that DmPHGPx may acquire selenium or other heavy metal ions by a post-translational process. In contrast, only the second conserved domain that contains the catalytic residue Gln (Q) is conserved in the VcPHGPxd sequence. Although two out of three conserved amino acids are absent in the VcPHGPxd, the hydrophobic pattern is very similar to other PHGPx proteins, including vertebrates (Hellers *et al.,* 1996). Therefore, it is concluded that the VcPHGPx domain of VLP1 does not have a peroxidase function but may be involved in binding of phospholipids (see below). This is supported by the observations that three PHGPx-like proteins were detected in the *V canescens* ovaries using the antibodies against DmPHGPx (Fig. 15). This finding reveals the possibility that these PHGPx proteins may have peroxidase function in *Venturia* ovary, whereas the VcPHGPxd is involved in other functions.

Further comparisons of VcPHGPxd with the DmPHGPx protein suggest that VLP1 is unique. The VLP1-gene is expressed in the calyx tissue in very high amounts, where it is secreted into the calyx lumen as one of the particulate protein secretions that resemble virus-like particles. The particle is a component of the calyx fluid that is co-injected together with the egg into the host caterpillar, where it protects the egg surface against the host's defence reactions. It is known that modified phospholipids play a major role in immune induction (Lennartz, 1999). Therefore, a possible functional role

of PHGPx in egg-surface protection may exist in masking or removing modified phospholipids from the egg surface. A binding role can be performed in the absence of reduced glutathione, which is probably not in sufficient amounts in the calyx lumen to sustain PHGPx activity. Instead it is highly likely that the calyx lumen is exposed to oxidative influences from passing eggs. The chorion of mature oocytes is not completely cross-linked and a low but significant phenoloxidase activity can be detected on the surface of oocytes in ovarioles and inside the oviduct (Fig. 45, 47, 48).

Given the high levels of VLP1 expression in the calyx tissue and the membrane-alterations observed in the mutant, an alternative function of the VcPHGPxd is indicated based on the unique ability of PHGPx enzymes to bind modified phospholipids inside the membrane bilayer. The highly conserved domain PCNQF, a glutathione peroxidase signature (Depege *et al.*, 1998) is present in VcPHGPxd as well as all the other PHGPx protiens (Fig. 9). Therefore, in spite of the absence of a reactive centre, the VcPHGPxd may still be able to recognise and bind modified phospholipids, thereby masking or otherwise removing their immune-elicitor properties. Since each VLP1 molecule would only be able to react with one modified phospholipid, the concentration required to achieve a protective effect would need to be much higher than that required by an active enzyme. Furthermore, the phenotypic differences seen in the two allelic wasp strains confirm a possible role of VLP1 in membrane-protection against detrimental effects on VLPs.

- The allelic VLP1 proteins differ in the structural organisation of a large hydrophobic domain and the VcPHGPxd (Hellers *et al.*, 1996). Since the tandem-repeat region between the hydrophobic and the VcPHGPxd is deleted in the 'mutant' strain (RM), this could change the configuration of the VLP1 structure and thus affect the membrane interaction of the two variant VLP1 proteins. Confocal microscopy revealed that both variants are able to attach to the membrane (Beck *et al.*, 2001).

- VLP-membranes in the mutant strain (RM) are different from the normal strain (RP). Whereas in normal VLPs, the bilayer structure is less visible probably because it becomes more firmly attached to the particle, the membranes in the mutant strain fuse together forming large membranes enclosing secreted particles inside the calyx lumen (Fig. 18A, D).

- These membranes prevent calyx fluid from moving freely inside the calyx lumen and form a barrier to the passage of eggs from ovarioles into the oviduct (Beck *et al.,* 2001). The membranes surrounding multiple particles in the mutant strain have a conspicuous electron-dense appearance (Fig. 18D), which was not seen in membranes on cells or on the surface of VLPs in the normal strain (Fig. 18C). This indicates that there is sufficient free moving VLP1 in the calyx of the normal (RP) strain to adequately bind modified phospholipids.

- The analysis of genetic crosses between the two strains suggests that the VLP1-gene is genetically linked to the phenotype (Chapter 3.1).

In summary, the VcPHGPxd is not enzymatically active but is probably still able to interact with modified phospholipids on the membrane, thereby masking or removing potentially damaging lipids from the surface of the parasitoid's eggs. It is possible that accumulations of large amounts of PHGPx molecules on structures that are deficient of glutathione recycling mechanisms may have similar functions. For example, high amounts of PHGPx on the sperm surface have been observed (Ursini *et al.,* 1999). Rather than a structural role, PHGPx may also be involved in the non-enzymatic protection of sperm surface against detrimental effects caused by accumulation of modified lipids.

4.1.3 Surface protection of *Venturia* eggs by immune disguise

As mentioned earlier, viruses and virus-like particles are involved in the protection of eggs against the host defence (Salt, 1973; Bedwin, 1979; Feddersen *et al.,* 1986). In

addition to viruses and VLPs, mucinous components from ovarian glands covering the egg surface, have been implicated in protecting the egg from the host immune response in a number of systems (Rotheram, 1973; Osman and Fuhrer, 1979; Davies and Vinson, 1986). Recently, hemomucin, a novel insect mucin was isolated from a *Drosophila* hemocyte line (Theopold *et al.,* 1996). It was also found on the gut lining and on freshly laid eggs. In the insect immune system, the protein is apparently involved in hemolymph coagulation by attracting lipophorin (Theopold and Schmidt, 1997). In accordance with an involvement in hemolymph coagulation, hemomucin was found on subcellular structures, insect microparticles. In vertebrates, microparticles are released from platelets during blood clotting (Yano *et al.,* 1994). A protein with similar characteristics as *Drosophila* hemomucin was identified in the lepidopteran *Galleria mellonella* Linnaeus, (Theopold and Schmidt, 1997). A hemomucin homologue is also found in *Venturia* and it might interact with host hemolymph components to constitute a protective mucinous layer on the surface of the parasitoid egg (Kinuthia *et al.,* 1999).

Like its *Drosophila* counterpart, *Venturia* hemomucin is expressed on hemocytes and on the surface of eggs (Kinuthia *et al.,* 1999). Microparticle formation was also observed in *Venturia* hemocytes. Like vertebrate microparticles, *Venturia* microparticles seem to be produced by the formation and fragmentation of filopodia (Kinuthia *et al.,* 1999). Whereas, hemomucin is easily recovered from hemocytes and cell lines, it is not readily isolated from the egg surface. The most likely explanation for this observation is that covalent cross-linking occurs between the surface components on the eggshell.

It is likely that a hemomucin homologue constitutes one of the glycoproteins that form the protective mucinous layer on the egg surface of *Venturia* and other ichneumonid endoparasitoids (Rotheram, 1973; Osman and Fuhrer, 1979; Davies and Vinson, 1986). This layer was previously implicated in binding the VLPs (Rotheram, 1973). Several attempts were made to bind VLPs to purified *Drosophila* hemomucin but failed (Kinuthia *et al.,* 1999), indicating that the hemomucin component is not likely to

interact with VLPs. Alternatively, this interaction might be more species-specific and may require *Venturia* hemomucin, which were not able to be purified in sufficient quantities.

The binding of lipophorin, and possibly additional hemolymph proteins, to the egg surface (Fig. 20), may protect the parasitoid egg by camouflaging the egg surface with host proteins during the initial contact with hemolymph. Theopold and Schmidt (1997) reported that the complex formation between hemomucin and lipophorin participates in hemolymph coagulation, which provided the possibility that a localised complex of hemolymph coagulation components on the surface of the egg further adds to immune protection. Thus the question was how the freshly deposited parasitoid eggs use part of its host's humoral immune components to avoid the cellular response. A limited coagulation reaction, which involves the parasitoid's hemomucin and host lipophorin, seems to protect the egg from any further cellular attacks. The important difference between a normal coagulation reaction and the reaction on the egg surface may be the limited nature of the latter. The data indicate that two independent layers protect the egg surface, one consisting of VLPs, the other of hemomucin and hemolymph components by immune disguise. A surface protection, independent of the VLPs, would also solve the long-standing problem of how the larval cuticle is protected. It was observed that the hemomucin homologues on the larval surface during late embryogenesis and the influx of hemolymph components through the egg chorion (Kinuthia *et al.*, 1999), thus a similar complex might form on the larval surface inside eggshell. Therefore, the same mechanisms could explain how the larva is protected once it emerges from the eggshell (Theopold *et al.*, 2000). Previous reports showed that lipophorin inhibits hemocyte attachment (Coodin and Caveney, 1992; Mandato *et al.*, 1996) and more recently, that the lipophorin complex derived from a coagulation reaction has inhibitory activity on hemocyte phenoloxidase and the regulatory protease which activates phenoloxidase (Duvic and Brehelin, 1998). All these findings support the idea that lipophorin can provide protection to eggs and emerging larvae by avoiding and inhibiting host cellular encapsulation. Therefore, it can be concluded that

lipophorin outside the egg surface may provide protection against the host immune responses, however, direct experiments are required to prove a protective mechanism.

The expression of hemomucin on the egg surface might constitute a pre-adaptation for an endoparasitic lifestyle since proteins that are phylogenetically conserved and, like hemomucin, exposed to the hemolymph, are not expected to induce an immune response and thus would confer some protection to the egg surface. The fact that hemomucin was also detected on the surface of *Venturia* larvae opens up the possibility that it contributes to immune protection at later stages as well (Schmidt *et al.*, 2001). Therefore, the hemomucin homologue and its complex with lipophorin and other proteins can protect the parasitoid wasps from host immune defence reactions.

4.2 New aspect of insect hemolymph coagulation reactions

Since a limited coagulation reaction was indicated in the parasitoid wasp system, it would be necessary to investigate insect hemolymph coagulation cascades further. Insect hemolymph coagulation reactions consist of an interaction between the humoral components and the cellular coagulogens. Lipophorin was widely accepted as a humoral clotting factor in several species (Bohn, 1986). In addition, previous results (Chapter 3.2.2) also showed that hemomucin was involved in coagulation reactions (Theopold and Schmidt, 1997). In the cellular component, insect microparticles were implicated as having clotting function. Research suggests that more clotting factors may exist, which might shed light on the immune disguise protection of hemomucin on the eggs or larval surfaces. Using *G. mellonella* as a model system, additional insect hemolymph coagulation components were identified and characterised.

Since having an open blood circulation system, insects need wounds to be sealed quickly, even more urgently than aquatic animals. This will lead to an even faster formation of a cross-linked extracellular matrix during wound healing. This matrix is difficult to resolubilise and it is even harder to isolate the individual components. Isolation of the clotting components was attempted by various methods but without

success (not shown). For example, hemomucin was used as a matrix to bind to hemolymph proteins, but, although some binding was observed, it was not possible to get a sufficient quantity for purifying and sequencing. Therefore, a novel method, which avoids solubilising the clotting components, was employed in order to isolate the molecular components of insect hemolymph coagulation. Using rabbit blood cells mixed with *Galleria* cell-free hemolymph, an *in vitro* coagulation reaction was conducted. The *in vitro* clots were directly injected into rabbits to produce an antiserum with specificity for the *in vitro* induced clot. When the anti-clot antibodies were tested with *Galleria* hemolymph protein extracts, a limited number of protein bands were detected (Fig. 21, 22). Furthermore, the anti-clot antiserum nevertheless reacted exclusively with granulocytes (Fig. 24, 25). This observation suggests that clotting components bind to granulocytes only. Following this, immune screenings of bacterial expression libraries were carried out using the anti-clot antiserum. The resulting collection of clones revealed a number of protein sequences with similarity to known insect proteins involved in coagulation reactions and prophenoloxidase activating cascades, such as lipophorin, prophenoloxidase, hexamerin, trypsin-like serine protease and a mucin-like protein. Two new candidates, a glutathione S-transferase (GST) and a member of alpha-crystallin/small heat shock protein family were identified as well (Chapter 3.3).

Insect hemolymph coagulation is regulated by the so-called "prophenoloxidase-activating system" consisting of a regulatory cascade of proteolytic activation reactions which can be initiated by microbial antigens, such as lipopolysaccharide (LPS), peptidoglucan and ß-1, 3-glucan (Cerenius *et al.*, 1994; Ma and Kanost, 2000). Lipophorin is an abundant, multi-functional lipoprotein found in insect plasma (Kanost *et al.*, 1990), which has been regarded as an insect humoral coagulogen (Bohn, 1986). It was previously reported that lipophorin I as well as three other plasma proteins form a complex during the prophenoloxidase activating process (Ashida and Yoshida, 1988). In this study, lipophorin cDNA fragments were frequently found in the cDNA library screenings, which further confirmed its function in coagulation reactions.

In addition to lipophorin, prophenoloxidase has previously been implicated as being involved in coagulation reactions. Only one proPO gene (proPO1) was obtained using the anti-clot antiserum. Since at least two proPO genes are known to exist in *Galleria* (Kaepack *et al.,* 1995 and Chapter 3.3), this might indicate that only proPO1 is involved in clot formation or alternatively that rabbit blood cells can only activate the proPO1 gene. Nevertheless, these results can be regarded as evidence for an interaction between both systems during hemostatic reactions (Theopold *et al.,* 2001b; Li *et al.,* 2002). A phylogenetic link between the prophenoloxidase cascade and the clotting reaction has recently been proposed, which indicates that hemolymph coagulation and prophenoloxidase activation cascades have evolved from a common ancestral protease cascade (Nagai and Kawabata, 2000).

Galleria hexamerin (Memmel *et al.,* 1994) was isolated from the immune screenings. This protein belongs to the arylphorin subfamily of arthropod hexamerins. Arylphorin is a member of a large family of insect hemolymph proteins and are known as insect storage proteins. They have a molecular weight of about 500 kDa and are composed of six subunits of approximately 72-80 kDa (Telfer, 1991). However, GalIysin-1, a *Galleria* arylphorin, was identified as an effector protein in the protective response of *G. mellonella* (Beresford *et al.,* 1997). Thus arylphorin may also have an insect immune function (Beresford *et al.,* 1997). In addition, a cellular immunosuppressive factor was purified from the larval cell-free hemolymph of armyworm, *Pseudaletia separata*, parasitised with the endoparasitoid wasp, *Cotesia kariyai.* This factor was fund to be a 470 kDa hexameric insect protein (Hayakawa, 1994). This hexamerin exists in the unparasitised larvae, but in a higher amount in the parasitised larvae. Functional analysis of the hexamerin showed that it suppressed hemocyte degranulation and subsequent immune reactions such as encapsulation and nodule formation in the armyworm larvae (Hayakawa, 1994). There is evidence (Hayakawa, 1994; Beresford *et al.,* 1997) that hexamerins have immune functions. The result that hexamerin was isolated from immune screenings using the anti-clot antibodies provides more evidence that hexamerin is involved in insect immune reactions.

Therefore, it can be concluded that *Galleria* hexamerin may be one of the insect hemolymph coagulation components.

The predicted amino acid sequence of one of the positive clones identified with anti-clot antibodies was similar to a trypsin-like serine protease. The encoded protein included the amino acid sequence motif of serine proteinase active sites, conserved cysteine residues and a signal peptide sequence. Thus an enzymatic proteolytic function is indicated. Since it is known that serine proteases are part of the proPO activating cascade, GmTP is potentially a candidate for hemolymph coagulation and proPO cascades.

Another component that was isolated from the library screenings is a mucin-like protein. The protein exists a number of glycolysation sites, which indicates that the mucin-like protein may interact with other proteins. For example, hemomucin, an insect mucin, can attract other hemolymph proteins such as lipophorin, forming a complex involved in hemolymph coagulation reactions (Theopold and Schmidt, 1997). Since mucin can attract other hemolymph proteins, all these morphological and functional characteristics of the protein made it a likely candidate for being a member of the clotting cascades.

Two other new candidates, glutathione S-transferase (GST) and alpha-crystallin were isolated. The two cDNAs have not previously been implicated in proteolytic cascades. Given the specificity of the anti-clot serum, both during immunocytochemistry and screening, these proteins are likely candidates for components of the insect immune response, although their functional role remains to be determined. GST is multi-functional protein family involved in detoxification of chemical compounds. The mammalian GSTs can be divided into eight classes with different cytological localisations and functional properties (Meyer *et al.*, 1991; Meyer and Thomas, 1995; Pemble *et al.*, 1996; Board *et al.*, 1997). Although not much is known about insect GSTs, evidence shows that insect GSTs belong to two classes (Franciosa and Berge, 1995). However the functional role of insect GSTs are largely unknown at the

molecular level. In support for a role for GST during blood clotting, GST was shown to be released from platelets (Kura *et al.,* 1996) and to be present on mucosal surfaces in vertebrates (Samiec *et al.,* 2000). Furthermore, it has been reported that 5-S-GAD (formed from glutathione and β-alanyl-dopa) shows antibacterial activity in insects (Hijikata *et al.,* 1997; Akiyama *et al.,* 2000).

A significant result of our immune screening is the identification of a member of the alpha-crys/SHSP family as a candidate coagulogen. Proteins in this family show a number of characteristics, which make them likely candidates for clotting reactions. These features include: first, their tendency to aggregate easily, leading to the formation of crystalline structures in the eye with other components - hence the name crystallins (de Jong *et al.,* 1993). And second, the fact that they are substrates for transglutaminase, one of the key enzymes of clotting reactions in crustaceans and vertebrates (Groenen *et al.,* 1993; Lorand, 1998; Shridas *et al.,* 2001).

The ORF of the *Galleria* alpha-crystallin cDNA displays hydrophobic sequences in the amino-terminal, which is predicted to be a signal sequence, since the protein was isolated from cell-free hemolymph. It is possible that the *Galleria* alpha-crystallin might act as a substrate for hemolymph transglutaminases during the production of a hemolymph clot.

Although the antiserum was raised against a mixture of proteins, it turned out to be specific for a limited number of proteins indicated by the following observations:

- The antiserum can be used to characterise the binding of hemolymph proteins to specific subsets of hemocytes, granulocytes, the cell type which had been previously implied in clotting reactions (Ratcliffe and Rowley, 1979; Gupta, 1991).

- The antiserum reacts specifically with a sub-fraction of hemolymph proteins. When using the antiserum in the immune screening of expression libraries,

only a limited number of cDNAs (under 0.1% positives, Table 4, Fig. 21, 22) were detected.

- The clot formation was shown to be dependent on calcium (Fig. 24, 25 and Li *et al.*, 2002).

- A number of the cDNAs code for proteins that have been shown to be involved or been implied in the clotting reactions (Bohn, 1986; Ashida and Yoshida, 1988). This includes lipophorin and prophenoloxidase (Chapter 3.3).

- Further analysis of the coagulation candidates has been performed by Dr Ulrich Theopold and Dr Ahmed Korayem. In their experiments, double staining using a transglutaminase (TG) substrate and the anti-clot antibodies was conducted. Labeling with the TG substrate was observed for a protein which co-migrates with the 56 kDa protein recognised by the anti-clot antiserum (Fig. 54, Li *et al.*, 2002). Additional proteins were also observed with TG substrate and showed migration behaviour expected for the products of the cDNAs, in particular GST and the alpha-crystallin. Thus these data confirmed the specificity of the anti-clot antibodies.

In conclusion, a novel method was developed in the isolation of hemolymph components involved in coagulation reactions in the moth *G. mellonella*. A number of proteins, which had been implicated in immunity in other species, were isolated as well as novel candidates for immune proteins. Further analysis of these proteins will provide great insights into insect hemolymph coagulation reactions.

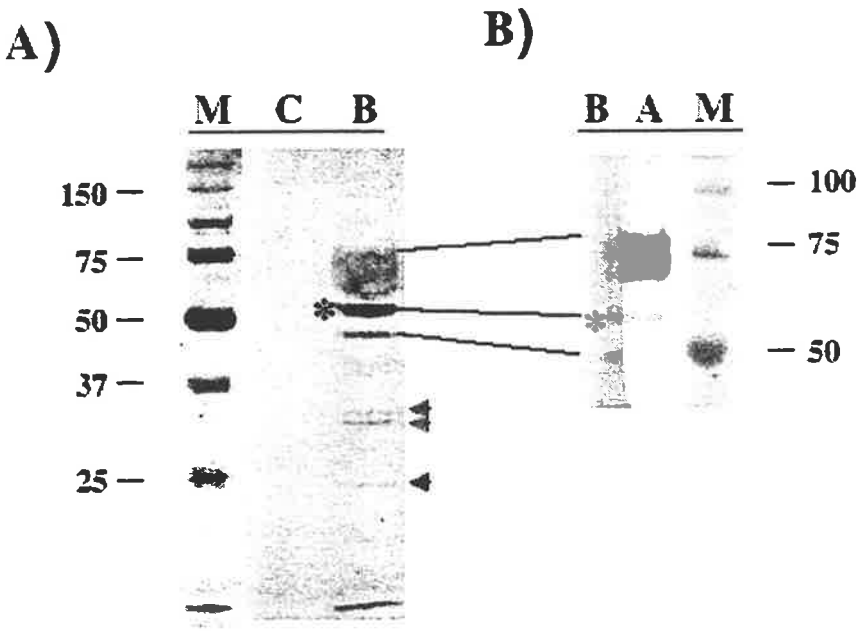

Fig. 54:

Double staining of hemolymph proteins

A) 10% SDS-PAGE gel and the blots were stained with peroxidase-conjugated streptavidin which specific recognize transglutaminase substrates. Labelling of hemolymph proteins using a biotin substrate for transglutaminase. Hemolymph proteins were analysed before (C) and after (B) transglutaminase-dependent labelling reaction. Bands, which show a migration behaviour expected for some of the cDNAs cloned during the immune screening (GST, 24 kDa and crystallin, 28 kDa, arrows).

B) 6% SDS-PAGE gel to allow optimal separation in the 70-80 kDa range. The blots were developed with peroxidase-conjugated streptavidin (B) and anti-clot antibodies (A). The 56 kDa band marked by an asterisk in **A)** shows the same migration behaviour as the band labelled with the anti-clot antibodies in Fig. 19C, 20B.

4.3 Developmental and immune function of glycoproteins and prophenoloxidases

What is the significance of mucin-like glycoproteins on the egg surface and in the hemocoel? Hemomucin, an insect mucin, which can be specifically recognised by *Helix pomatia* lectin was previously identified in parasitoid eggs and emerging larvae (Theopold and Schmidt, 1997). In addition, the results of this study also showed that hemomucin may provide protective function for the parasitoid wasp (Chapter 3.2.2). Similar to vertebrates, O-linked glycosylation in insects involves the covalent linkage of N-acetyl-galactosamine (GalNAc) to serine or threonine residues on the protein backbone forming the Tn-determinant recognised by HPL. Further addition of galactose (Gal) by beta-1-3-galactosyltransferase forms the T-determinant or core I (Maerz *et al.*, 1995) recognised by peanut agglutinin (PNA). Hemomucin exists in two glycoforms, the HPL-form and the PNA-form (Theopold *et al.*, 2001a) and both forms of hemomucin can be detected on the hemocytes (Theopold *et al.*, 1996; Theopold *et al.*, 2001a). However, significant amounts of PNA-form glycoproteins were also detected in *Drosophila* ovaries (Theopold *et al.*, 2001a). It was also demonstrated that this PNA-form glycoprotein is produced by the follicle cells and is probably deposited onto the egg surface as part of the egg chorion, which indicates a developmental as well as an immune function (Theopold *et al.*, 2001a).

Insect parasitoid-host (*Venturia/Ephestia*) interactions, where one species lays its eggs inside another species, are ideal experimental systems to explore immune recognition models (Schmidt *et al.*, 2001). Results presented in chapter 3.2.2 showed that HPL-form hemomucin homologues are present in the *Venturia* ovaries and thus potentially protect the eggs against its host defence reactions. Therefore, to further investigate the expression patterns and functions of these glycoproteins, PNA and HPL were used as indicators to detect the presence of mucin-like glycoproteins in ovaries of *V. canescens, G. mellonella* and *Pieris rapae*.

Incubation of ovaries with lectins revealed a strong staining pattern in *Venturia* ovaries (Fig. 48C, E). Both the follicle cells and the chorion surface show HPL- and PNA-staining (Fig. 48, 49). Expression pattern of hemomucin in ovaries was detected in *Drosophila* (Theopold *et al.*, 2001a, D. Li pers. obser.). Whether there is any role played by immune-related molecules in *V. canescens* oogenesis is not known, but the presence of the PNA-form glycoprotein on the egg shell surface in other insects, including non-parasitic species such as *Drosophila*, indicates that mucin-like glycoproteins may contribute developmentally in eggshell production and immune-related functions in hemocytes. Since hemocytes are confined to the hemocoel by a basement membrane lining, the two functions would be precluded from interfering with each other by spatial separation.

Another immune-related protein found in the ovary of *V. canescens* is prophenoloxidase. Since hymenopteran parasitoids such as *V. canescens* produce hydropic eggs, where eggshell hardening is delayed until after egg deposition, allowing detection of proPO-like activity in the ovary using DOPA solutions. In this system, tissue-specific DOPA-staining correlates with antibody staining (Fig. 46A, C). ProPO is a key enzyme involved in melanisation (Söderhäll and Aspan, 1993), and other insect defence reactions (Gillespie *et al.*, 1997), including production of toxic (Nappi and Ottaviani, 2000) and antibacterial compounds (Leem *et al.*, 1996). In some lepidopteran species proPO-genes are only expressed in larval oenocytoids (Jiang *et al.*, 1997), but the protein is also present in larval and adult granulocytes (Schmit *et al.*, 1977). In *Venturia* ovaries, proPO can be detected in two spaces as dot-like vesicles. One is present in the inter-space between the follicle cells and oocytes and the other is localised in the extracellular parts of the nurse cells (Fig. 49B, D).

Since phenoloxidase and hemomucin homologues have similar tissue-specific distribution in *V. canescens* ovarioles (Fig. 48, 49), the question is whether the two molecules are co-localised at the sub-cellular level. In co-stained ovarioles phenoloxidase and PNA-staining only coincided in the perivitellogenic space between the oolemma and follicle epithelium (Fig. 49C, D). Prophenoloxidase staining was

observed in the cytoplasm of nurse cells, follicle cells and oocytes as dot-like aggregations in extracellular space (Fig. 48, 49). Little or no proPO staining was observed in calyx tissues (Fig. 47). PNA-staining was visible mainly in follicle cells as large irregular vesicles, which corresponding to the cytoplasmic location (Fig. 49C, E).

Roles of specific glycoform proteins in development and immunity have also been implied by other researchers (Acosta-Serrano *et al.,* 2001; Theopold *et al.,* 2001a). For example, mucin-like glycoproteins of *Trypanosoma cruzi*, TcMUC form a protective coat that covers the entire parasite surface, and are important for parasite development and growth in the insect vector (Acosta-Serrano *et al.,* 2001). Hemomucin may be one part of the eggshell components in *Drosophila* and a developmental function has been suggested (Theopold *et al.,* 2001a). In addition, LPS-binding proteins have been identified as lectins (Jomori and Natori, 1991; Jomori and Natori, 1992) with opsonin functions and C-type lectins have been shown to be involved in leg regeneration (Arai *et al.,* 1998). Furthermore a C-type lectin from *D. melanogaster* was purified and its analysis showed that this lectin had functions in defence and development as well (Haq *et al.,* 1996). Therefore, the expression of lectin-form glycoproteins in *Venturia* ovaries may indicate their dual functions in immunity and development.

The observation that proPO-containing protein complexes are localised in *P. rapae* and *G. mellonella* ovarian tissues is unexpected since proPO is only expressed in larval hemocytes in all lepidopteran species studied so far (Jiang *et al.,* 1998). However, the expression of dipteran prophenoxidase genes in adult ovarian (Cui *et al.,* 2000) and gut tissues (Dimopoulos *et al.,* 1996), suggest that proPO-expression in non-hemocyte tissues are possible and should not be discounted. Although it is known that proPO is transported from the hemolymph through epidermal cells where it is involved in cuticle formation (Terwilliger, 1999), it is not known how the enzyme is transported and regulated in cell-free environments, such as epithelial secretions (Sass *et al.,* 1994). After proPO-activation, the production of cross-linking compounds (Marmaras *et al.,* 1996) requires several tyrosine-metabolizing enzymes (Sugumaran *et al.,* 2000), suggesting a highly structured protein complex in the cuticle (Ashida and Brey, 1995).

The observation of proPO-like proteins present in the nurse cells and follicle cells indicates that proPO may have a function in oogensis. However, the two lepidopteran ovaries analysed in this study were not stained after incubation in L-DOPA (Schmidt, pers. comm.). Therefore, it is possible that in lepidopteran oocytes proPO molecules are not deposited inside and onto the eggshell as seen in *V. canescens*. Alternatively, proPO may be incorporated but in small amounts, it may not be visible due to masking by other components or it may have any unknown function in the ovaries.

At this stage, the possible function of immune-related proteins inside the ovariole can only be a matter for speculation. One possibility is that immune molecules are stored inside the embryo and the eggshell for defence-related functions as occurs in epidermal cuticle (Brey *et al.*, 1995; Asling *et al.*, 1995). Another is a role in developmental processes, such as the formation of the eggshell (Theopold *et al.*, 2001a) and the establishment of dorsal-ventral polarity in the *Drosophila* embryo (Anderson *et al.*, 1985; Lemaitre *et al.*, 1996; Hoffmann *et al.*, 1999). Further experiments, such as transfection of cell lines and analysis of mutant strains are needed to elucidate the functions of glycoproteins and proPOs.

Appendix 1 Vectors, markers and proteins

Glutathione peroxidase from	Sigma Chemical Co., St. Louis, MI, USA
DNA markers IV & VI	Boehringer Mannheim, Mannheim, Germany
DNA marker (GeneRuler 100bp)	MBI Fermentas, Vilnius, Lithuania
Glutathione reductase type IV from bakers yeast	Sigma Chemical Co., St. Louis, MI, USA
pGEM-T easy vector system	Promega Corp., Madison, WI, USA
pQE vectors	QIAGEN Inc., Chatsworth, CA, USA
Protein markers, SeeBlue & Mark12	Novex, San Diego, CA, USA
Proteinase K	Sigma Chemical Co., St. Louis, MI, USA
Restriction endonucleases	Promega Corp., Madison, WI, USA
RNase A	Boehringer Mannheim, Mannheim, Germany
Taq DNA polymerase (5 U/µl)	Promega Corp., Madison, WI, USA
T4 DNA ligase (3 U/µl)	Promega Corp., Madison, WI, USA
pQE vectors	QIAGEN Inc., Chatsworth, CA, USA

Appendix 2 Solutions and media

Common used solution and media are listed below, some solutions were also described in the corresponding method chapter.

Antifade solution	2% (v/v) 1,4-diazabicyclo-(2,2,2)-octane in 9 parts glycerol: 1 part 1M Tris-HCl pH 7.5
Denhardt's reagent (50x)	Per litre: 10 g Ficoll (type 400), 10 g poly-vinylpyrrolidone, 10 g BSA. Store at -20°C
DNA extraction buffer	10 mM Tris-Cl pH 8, 10 mM EDTA pH 8, 1% SDS
DNA loading buffer (6x)	40% (w/v) sucrose in water, 0.25% BPB
LB medium	Per litre: 10 g tryptone, 5 g yeast extract, 5 g NaCl; adjusted to pH 7 with 0.2 ml NaOH
LB plates	To 1 litre LB, 15 g bacto-agar is added. Autoclaved, allow to cool down to 50°C, add antibiotics
NZCYM broth	Sigma Chemical Co., St. Louis, MI, USA
PBS	1.47 mM KH_2PO_4, 7.3 mM NaH_2PO_4, 138 mM NaCl, 2.7 mM KCl; pH 7.5
RNA extraction buffer	100 mM NaCl, 50 mM EDTA, 1% SDS

SDS gel-loading buffer (3x)	100 mM Tris-HCl pH 6.8, 20% glycerol, 10% (v/v) β-Me, 4% SDS, 0.2% BPB
SM buffer	Per litre: 5.8 g NaCl, 2 g $MgSO_4 \cdot 7H_2O$, 50 ml 1 M Tris-HCl pH 7.5, 5 ml 2% gelatin solution; autoclaved for 20 min at 15 lb/sq. in fluids cycle
SSC (20x)	Per litre: 175.3 g NaCl, 88.2 g sodium citrate. Adjusted to pH 7 with NaOH. Autoclaved
Sonication buffer	50 mM NaH_2PO_4, 300 mM NaCl; Adjust to pH 8 with NaOH
TAE	40 mM Tris-acetate, 1 mM EDTA
TBE	45 mM Tris-borate, 1 mM EDTA
TBST	10 mM Tris-HCl pH 8, 150 mM NaCl, 0.05% Tween 20
TE	10 mM Tris-HCl pH 8, 1 mM EDTA pH
TFB1	100 mM RbCl, 50 mM $MnCl_2$, 30 mM KAc, 10 mM $CaCl_2$, 15 % glycerol; pH 5.8 Sterile-filtered and adjusted with diluted acetic acid

TFB2	10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2$, 15 % glycerol; pH 8.0 Autoclaved and adjusted with diluted NaOH
Top-agar/agarose	To 1 litre of LB medium, 7 g bacto-agar or alternatively agarose was added. Autoclaved
Wash buffer	50 mM NaH_2PO_4, 300 mM NaCl, 10% glycerol; pH adjusted to 6.0 with NaOH

Appendix 3 Genomic DNA preparation

1. *Venturia* abdomens were put into an eppendorf tube containing 395 µl of freshly prepared DNA extraction buffer and homogenised thoroughly by using matching pestles.

2. After adding 5 µl of proteinase K (20 µg/ml), the sample was incubated at 40°C overnight. (The phenol was taken out off the fridge and allowed to warm up to RT overnight.)

3. To remove RNA, 0.8 µl of RNase A (10 mg/ml) was added, and the tube was incubated at 37°C for 30 min.

4. After 400 µl of phenol was added, the sample was gently mixed by inversion and left at RT for 5 min.

5. Then the tube was centrifuged at 12500 ***g*** for 5 min, the upper phase was collected carefully avoiding the interphase, and transferred to a fresh tube.

6. To remove phenol residues, 400 µl of chloroform was added, mixed gently and then left at RT for 5 min.

7. The mixture was centrifuged again at 12500 ***g*** for 5 min. The upper phase was removed and transferred to a new tube. To 350 µl of the upper phase 730 µl of 100% ethanol and 15 µl of 5 M NaCl were added. The tube was inverted carefully several times and placed on ice for 20 min.

8. To precipitate the DNA, the sample was spun at 12500 ***g*** for 20 min at RT. Afterwards the supernatant was discarded and the pellet dried at 37°C for about 5 min in a heating block, with the lid open.

9. Finally, the DNA pellet was redissolved in 15 µl of sterile double distilled water or TE buffer and stored at 4°C for up to several weeks.

10. OD_{260} and OD_{280} were measured in the spectrophotometer to determine the quality and quantity of the DNA.

Appendix 4 Total RNA extraction (Hot phenol)

1. About 100 mg of the tissue was ground in liquid nitrogen using a mortar and pestle and then transferred to Eppendeff tube.

2. After adding 0.5 ml of RNA extraction buffer (preheated to 80°C), the tissues were mixed thoroughly by pipetting up and down in 80°C heat block.

3. Then 0.5 ml of phenol (preheated to 80°C) was added and pipetted up and down again. The mixture was centrifuged at RT full speed for 10 min.

4. The water phase was collected and re-extracted with 0.5 ml of phenol once and then 2 times with phenol/ chloroform.

5. The total RNA was precipitate with 0.5 ml of isopropoanol for 60 minutes at RT. Followed by 10 min centrifugation.

6. 100 µl TE was used to dissolve the pellet and 33 µl of 10 M LiCl was added and kept at –20°C overnight.

7. The solution was centrifuged in cold room at full speed for 10 min and then the pellet was dissolved in 100 µl TE.

Appendix 5 RNA blots

Briefly, total RNA was extracted as described in Appendix 4 and run in 1% denatured agarose gel. Afterwards, it was blotted into nylon membrane.

1. In advance: Gel former and all chambers, combs and so on were cleaned thoroughly with water and dried.

2. Preparing Gel: 0.5 g agarose, 5 ml 10x MOPS buffer and 42.5 ml water were mixed and microwaved to melt the agarose. Let it cool down to 50°C, and then 2.7 ml formaldehyde, 1 µl of ethidium bromide were added and the gel was poured.

3. Preparing samples and running gel: 10-15 µg RNA in 20µl loading buffer (if the total volume is more than 5 µl must dry it first) was heated in 95°C for 2 min and cooled down on ice immediately. The RNAs were then running in 1x MOPS buffer until the loading dye reached the ¾ of the gel.

4. Blotting gel:
    a. The RNA gel was fixed under the UV light for 10 min.

    b. The gel was rinsed for 20 min each in 2 changes of 10x SSC.

    c. At the mean time, 500 ml 10x SSC was added into a tray, 200 x 300 mm glass plate was placed over the tray and 230 x 500 mm Whatman was put on the top of the glass plate, with both ends hanging into buffer to act as wick.

    d. The gel was placed upside down over wick. The nylon membrane was soaked with water and placed on the top of gel. Two pieces of

Whatman (the same size with the nylon membrane) were soaked with water and laid on the top of the nylon membrane.

e. Six paper towels were flated over Whatman paper. 4 piles of folded paper (200-300 mm thick) and a small weight were put on the top. RNAs were allowed transferred to nylon membrane by capillary action to proceed for at least 12 h at RT.

f. The paper towels were gently flipped over the filter and gel. Pinholes were made through gel and membrane to mark the electrophoretic origin and determine filter orientation.

g. The membrane was dried and cross-linked under UV light. The blot was then ready for hybridisation.

Appendix 6 GenBank accession numbers

The sequences described in the thesis have been submitted to GenBank under the accession numbers:

Galleria mellonella prophenoloxidase, AF336289

Galleria mellonella α-crystallin-like protein, AY040539

Galleria mellonella mucin-like protein, AY040818

Galleria mellonella trypsin-like protein, AY040819

Galleria mellonella glutathione S-transferase, AF336288

Galleria mellonella ESTs coding for lipophorin: BI076403; BI076404; BI076408; BI076409; BI076410.

Galleria mellonella ESTs coding for hexamerin (arylphorin): BI076405; BI076406; BI076407.

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